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Depletion of the mitochondrial electron transport abrogates the cytotoxic and gene-inductive effects of TNF

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Tumor necrosis factor (TNF) has cytotoxic and gene-inductive activities on several cell types. Previous studies on L929 fibrosarcoma cells have revealed that the mitochondrial electron transport system plays a key role in inducing TNF cytotoxicity, presumably by the formation of reactive oxygen intermediates (ROI). Here we report that mitochondria-derived intermediates are not only cytotoxic but, in addition, function as signal transducers of TNF-induced gene expression. The activation of NFκB, which fulfills an important role in TNF-induced gene transcription, could be blocked by interference with the mitochondrial electron transport system. Furthermore, antimycin A, a mitochondrial inhibitor that increases the generation of ROI, potentiated TNF-triggered NFκB activation. The dual role of mitochondria-derived intermediates in cytotoxicity and immediate-early gene induction of TNF was further substantiated by isolating L929 subclones which lacked a functional respiratory chain. This depletion of the mitochondrial oxidative metabolism resulted in resistance towards TNF cytotoxicity, as well as in inhibition of NFκB activation and interleukin-6 gene induction by TNF. These findings suggest that mitochondria are the source of second messenger molecules and serve as common mediators of the TNF-cytotoxic and gene-regulatory signaling pathways.

Key words: cytotoxicity/interleukin-6/NFκB/respiratory chain/tumor necrosis factor

Introduction

The cytokine tumor necrosis factor (TNF) elicits on a variety of cells two major types of responses (reviewed in Beutler and Cerami, 1989; Fiers, 1991; Beutler, 1992). First, TNF exerts a rather selective cytotoxic activity on several tumor and transformed cells, whereas normal cells are generally resistant towards the cytokine. The mechanism of TNF cytotoxicity is currently rather incompletely understood. Depending on the cell type, different cellular events and processes may contribute to TNF-induced cell killing, such as generation of reactive oxygen intermediates (Zimmerman *et al.*, 1989; Yamauchi *et al.*, 1989, 1990; Schulze-Osthoff *et al.*, 1992), activation of phospholipases (Suffys *et al.*, 1991) or proteases (Ruggiero *et al.*, 1987; Suffys *et al.*, 1988) or DNA damage (Dealtry *et al.*, 1987). Recently, we have provided evidence that in the classical TNF-sensitive fibrosarcoma cell lines L929 and WEHI 164 clone 13 a functional mitochondrial respiratory chain is crucial to the events leading to TNF cytotoxicity (Schulze-Osthoff *et al.*, 1992).

While the cytotoxic activity of TNF seems to be rather restricted to tumor cells, nearly every cell type responds to TNF by the activation of a wide range of different genes. Owing to this broad gene-activating effect, TNF is suggested as a cardinal mediator of several differentiation and immunoregulatory processes. Among others, TNF has been shown to induce expression of other cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6) or GM-CSF, and surface-bound proteins such as cytokine receptors and cell adhesion molecules (reviewed in Fiers, 1991). Furthermore, transcriptional virus replication of HIV-1 is also inducible by TNF (Duh *et al.*, 1989; Osborn *et al.*, 1989).

In the last few years, it has been found that TNF gene-regulatory activities are mediated by certain transcriptional activators, which include the transcription factors AP-1 (Brenner *et al.*, 1989) and, probably most importantly, NFκB (Osborn *et al.*, 1989). The latter was initially identified as a nuclear factor of mature B cells, that specifically interacts with an enhancer element of the immunoglobulin kappa light chain gene (reviewed in Lenardo and Baltimore, 1988; Baeuerle, 1991; Blank *et al.*, 1992). NFκB consists of a heterodimeric complex, which in its classical form comprises the two proteins p50 and p65. It now appears that NFκB or structurally related proteins belonging to the Rel family are ubiquitously present in other cell types and are inducible by a wide variety of extracellular stimuli. NFκB controls the inducible expression of various genes that are involved in immune responses, as well as inflammatory and cellular defense mechanisms. Target genes of NFκB include cytokines, cytokine receptors, MHC antigens, acute-phase proteins and several viral enhancers.

A characteristic feature of immediate-early NFκB activation is that it occurs without new protein synthesis. In the uninduced cell, NFκB resides cytoplasmically as a latent form complexed to the inhibitory protein IκB (Baeuerle and Baltimore, 1988). Upon cell stimulation, NFκB is rapidly activated by its release from IκB, which allows migration of the p50/p65 complex into the nucleus and subsequent DNA binding. The dissociation of IκB, which seems to be the key event of NFκB activation, is still rather elusive. *In vitro*, it has been shown that protein kinase C or heme-regulated eIF-2 kinase can activate the transcription factor (Ghosh and Baltimore, 1990). However, it seems unlikely that one of these kinases or protein kinase A is involved in NFκB activation *in vivo* (Meichle *et al.*, 1990; Bromsztyk *et al.*, 1991; Hohmann *et al.*, 1991). Quite recently, it was proposed that activation of NFκB may be

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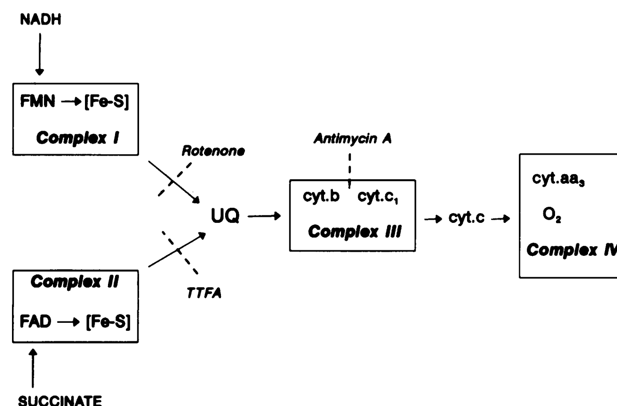

Fig. 1. Schematic diagram of the electron carriers of the mitochondrial respiratory chain. The sites of inhibition by the respiratory inhibitors rotenone, TTFA and antimycin A are indicated.

generally controlled by reactive oxygen intermediates (ROI) and the cellular redox state (Staal *et al.*, 1990; Mihm *et al.*, 1991; Schreck *et al.*, 1991, 1992). Firstly, at least in some cell types the transcription factor could be activated by exogenously applied hydrogen peroxide. Secondly, activation of NFκB by different stimuli, such as TNF, IL-1, phorbol esters, viral transactivator proteins, or cycloheximide, was commonly inhibited by antioxidant thiols or iron chelators. Thus, these observations suggest that oxygen radicals acting as second messengers may integrate the diverse variety of NFκB-inducing signals.

In our previous studies, we have shown that mitochondrial intermediates, possibly oxygen radicals generated from the respiratory chain, are causally involved in the cytotoxic effects of TNF (Schulze-Osthoff *et al.*, 1992). Presumably, TNF causes a short circuit of the electron flow, resulting in direct production of radicals from the ubiquinone site of the respiratory chain. Here we report that mitochondria are not only implicated in cytotoxicity, but may also participate in the gene-regulatory effects of TNF. Our data suggest that gene induction and cytotoxicity by TNF share an at least partially common pathway. We show that elimination of the mitochondrial oxidative metabolism not only inhibits TNF cytotoxicity, but also considerably reduces TNF-mediated activation of NFκB and expression of IL-6 as an example of a TNF-inducible, NFκB-regulated gene. Oxygen intermediates may therefore act as signal transducers and have a more widespread role than currently appreciated.

Results

Mitochondrial inhibitors modulate TNF-induced NFκB activation

The mitochondrial electron transport can be inhibited by specific inhibitors which prevent electron flow between certain complexes of the respiratory chain (Figure 1). Previously, we have reported that inhibition of the electron entry from complex I to ubiquinone by the complex I inhibitors amytal and rotenone results in a strong reduction of TNF cytotoxicity (Schulze-Osthoff *et al.*, 1992). However, when electron flow in L929 cells was inhibited distal to the ubiquinone pool, by the addition of antimycin A, a marked potentiation of TNF-induced killing was observed. As an explanation for these differential drug effects, it was proposed that TNF-induced cell killing is largely mediated by the generation of ROI at the ubiquinone site of the mitochondrial chain. Early investigations with isolated mitochondria have repeatedly shown that ubiquinone is the major radical source of ROI derived from the mitochondrial chain (Boveris and Chance, 1973; Turrens and

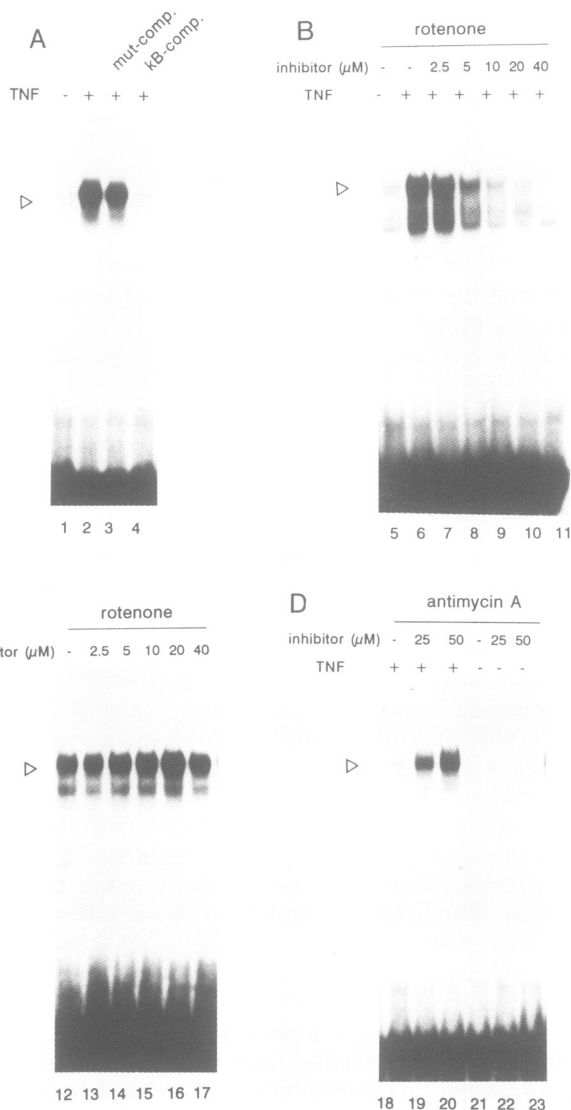

Fig. 2. Differential effects of mitochondrial inhibitors on NFκB binding activity in L929 cells. **(A)** Competition analysis showing the specificity of NFκB binding: 3 μg of nuclear extract obtained from L929 cells treated without (lane 1) or with TNF (1000 IU/ml; lanes 2–4) were analyzed in electrophoretic mobility shift assays with a ^{32}P-labeled probe encompassing the κB-binding site of the mouse kappa light chain gene. To the nuclear extracts a 100-fold excess of unlabeled mutated (lane 3) or wild-type oligonucleotide (lane 4) were mixed with the radioactive κB enhancer probe. **(B)** The effect of rotenone on NFκB activation in L929 cells. Cells were treated for 1 h with the indicated concentrations of rotenone prior to TNF addition (1000 IU/ml). Incubation was continued for 45 min before extraction of the cells. **(C)** The effect of rotenone on DNA binding of nuclear extracts *in vitro*. Identical nuclear extracts from cells treated with TNF (1000 IU/ml, 45 min) were incubated for 30 min with the indicated concentrations of rotenone, followed by the addition of the ^{32}P-labeled κB probe. **(D)** The effect of antimycin A on NFκB activation in L929 cells. Cells were pretreated for 1 h with the indicated concentrations of antimycin A, followed by the addition of a suboptimal dose (10 IU/ml) of TNF (lanes 18–20) or medium (lanes 21–23). After 45 min of further incubation, nuclear extracts were prepared and analyzed by electrophoretic mobility shift assays.

Boveris, 1980; Turrens *et al.*, 1985). ROI generation in isolated mitochondria can be inhibited by amytal and rotenone, whereas antimycin A leads to an increase in ROI formation (Boveris *et al.*, 1976; Cadenas and Boveris, 1980; Konstantinov *et al.*, 1987; Cino and Del Maestro, 1989).

We were interested in analyzing the effects of mitochondrial inhibitors on early TNF effects such as activation of the transcription factor NFκB, which has been reported, at least in some cell types, to be additionally inducible by hydrogen peroxide (Schreck *et al.*, 1991, 1992). L929 cells were preincubated for 1 h with different concentrations of mitochondrial inhibitors; 45 min after TNF stimulation cells were lysed, and cellular and nuclear lysates were analyzed in electrophoretic mobility shift assays (EMSA). TNF induced a κB binding activity in L929 cells which was not detectable in unstimulated cells (Figure 2A). Competition with a 100-fold molar excess of unlabeled oligonucleotide led to an inhibition of κB binding, thereby confirming the specificity of the activated NFκB. No reduction of DNA binding, however, was observed with an excess of mutated oligonucleotide.

When cells were pretreated with non-toxic concentrations of the complex I inhibitor rotenone, a strong inhibition of NFκB activation was observed (Figure 2B). Even a concentration of 5 μM led to an inhibition of $\sim$50% of NFκB binding. There remained the possibility that decrease of κB binding in EMSA was either due to a reduction of NFκB activation in the cytoplasm or to a reduced DNA-binding affinity of the released NFκB complex. To answer this question, rotenone was added directly to the lysates. However, no interference of the inhibitor could be detected (Figure 2C). Therefore, reduction of κB binding by rotenone was most probably caused by inhibition of TNF-induced NFκB activation in the cytoplasm.

When we analyzed the complex III inhibitor antimycin A, which potentiates TNF cytotoxicity, contrasting effects on NFκB activation became evident (Figure 2D). When a suboptimal TNF dose (10 IU/ml) was applied, antimycin A triggered a strong potentiation of NFκB activation. Sometimes antimycin A itself induced a faint κB band at the highest concentration. A synergistic effect of antimycin A was only observed with suboptimal TNF doses, which may be indicative for a similar action mechanism of both agents.

In accordance with our previously published data regarding TNF-mediated cytotoxic effects on mitochondria (Schulze-Osthoff *et al.*, 1992), the present observations indicate an involvement of mitochondria-derived intermediates, possibly ROI, also in the activation process of NFκB. The presumptive activation of NFκB by ROI was further supported by experiments using butylated hydroxyanisole (BHA) or nordihydroguaiaretic acid (NDGA), two potent antioxidants (Niki, 1987). Figure 3 shows that both agents reduced NFκB binding. We also analyzed the effect of two antioxidants which have been recently described as efficient inhibitors of NFκB activation (Schreck *et al.*, 1991, 1992). However, in our cell system, inhibition was only observed at rather high concentrations of *N*-acetylcysteine (30 mM), a radical scavenger and precursor of reduced glutathione, and of pyrrolidine dithiocarbamate (PDTC, 100 μM), an iron chelator (data not shown).

As ROI may be generated by different enzymes in several cellular compartments, we also analyzed the contribution of other potential ROI sources for the induction of NFκB.

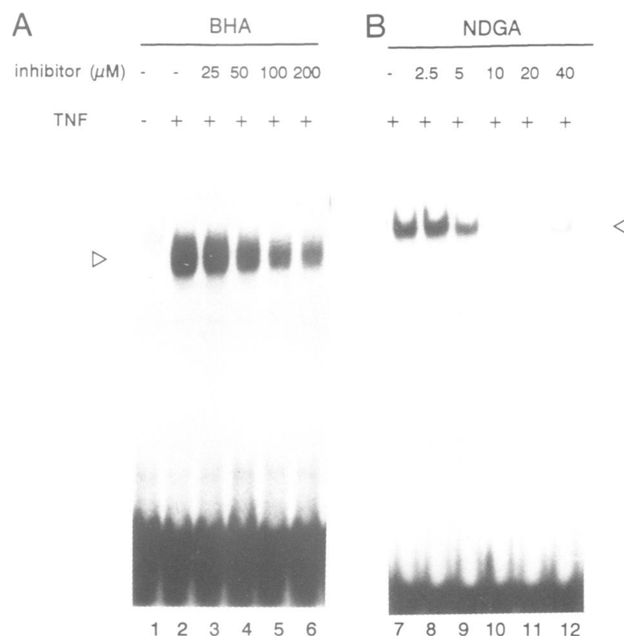

Fig. 3. Oxygen radical scavengers reduce activation of NFκB in TNF-stimulated L929 cells. Cells were pretreated for 1 h with various concentrations of BHA (**A**) or NDGA (**B**) prior to the addition of 1000 IU/ml TNF and further 45 min of incubation. Equal amounts of total cell lysates (10 μg) were analyzed by electrophoretic mobility shift assays.

Neither allopurinol, an inhibitor of cytoplasmic xanthine oxidase, nor diphenylene iodonium (DPI), an inhibitor of the plasma membrane-bound NADPH oxidase, interfered with TNF-induced NFκB activation (data not shown). Therefore, NFκB activation in the TNF-sensitive cell line L929 seems to be largely and specifically controlled by intermediates derived from the respiratory chain.

The specific involvement of mitochondrial ROI for the activation of NFκB was tested by assaying nuclear extracts for the DNA-binding activity of other transcription factors. DNA-binding activities of AP-1, Oct-1 and NF-IL6 are constitutively present in L929 cells and barely inducible upon TNF stimulation. Figure 4 shows that pretreatment of L929 cells with rotenone selectively inhibited TNF activation of NFκB, whereas the DNA-binding activities of AP-1, Oct-1 and NF-IL6 remained unchanged in the same nuclear extracts. This suggests that inhibition of mitochondrial ROI formation specifically modulates the activation of NFκB.

Depletion of the mitochondrial respiratory chain results in acquired TNF resistance

In order to substantiate further the dual involvement of mitochondria for both TNF effects, cytotoxicity and NFκB activation, we isolated subclones of L929 which were deficient in a functional respiratory chain. Respiration deficiency was generated by long-term culture of L929 cells in ethidium bromide (EB)- or chloramphenicol (CA)-containing medium, procedures which have been repeatedly used to deplete cells from a functional mitochondrial electron transport system (Nass, 1972; Wiseman and Attardi, 1978; Desjardins *et al.*, 1985; Morais *et al.*, 1988; King and Attardi, 1989). Ethidium bromide has been described as a specific inhibitor of mitochondrial DNA (mtDNA) replication (Wiseman and Attardi, 1978; Desjardins *et al.*, 1985). Chloramphenicol is known to block mitochondrial

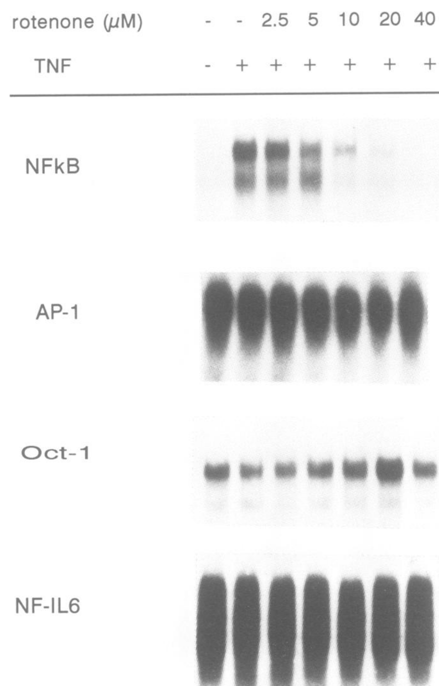

Fig. 4. The effect of rotenone on different DNA-binding activities. L929 cells were pretreated for 1 h with the indicated concentrations of rotenone, followed by a stimulation with TNF (1000 IU/ml) for 90 min. Nuclear extracts were analyzed in EMSA with ^{32}P-labeled DNA probes detecting binding activities of NFκB, AP-1, octamer-binding proteins (Oct-1) and NF-IL6. Only the shifted bands are shown.

Table I. Cytochrome oxidase activity and lactate production in L929 cells and EB- and CA-treated subclones

	Cytochrome oxidase[a]	Lactate (mM)[b]
L929	65 ± 16	2.26
EB-2	21 ± 6	3.61
EB-12	12 ± 3	3.89
EB-21	5 ± 3	4.62
CA-22	29 ± 8	3.92
CA-34	16 ± 3	4.43

[a]nmol of cytochrome *c* oxidized/min/mg protein.
[b]10^6 cells were seeded in 1 ml in a 6 well dish. After 24 h, cells were changed to fresh medium containing dialyzed FCS. After 12 h, lactate production was determined in the supernatant.

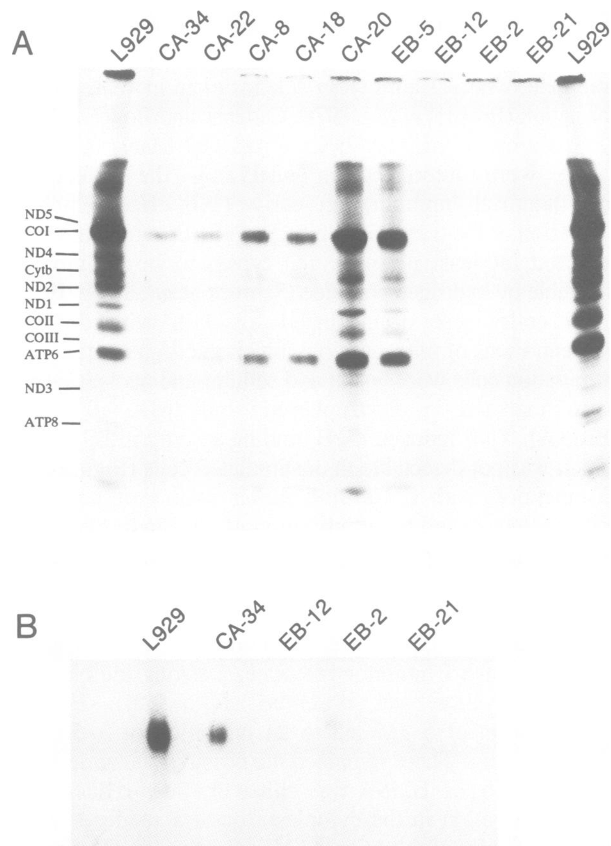

Fig. 5. Analysis of mtDNA-encoded protein synthesis (**A**) and mtDNA levels (**B**) in ethidium bromide- and chloramphenicol-treated L929 subclones. (A) Cells were grown in 6 well dishes until confluent. Fresh medium containing 100 μg/ml emetine was applied and after 30 min 100 μCi/ml [^{35}S]methionine were added and the incubation continued for 3 h. Cells were washed and lysed in 25 mM Tris−HCl (pH 6.8), 2% SDS and 8 M urea. Extracts were analyzed on 12.5% SDS−urea−PAGE. The radioactive bands on the fluorogram were identified according to their molecular size (Chomyn *et al.*, 1985). Cyt *b*: cytochrome *b*; COI, COII, COIII: subunits 1, 2, 3 of cytochrome oxidase; ND1, ND2, ND3, ND4, ND5: subunits 1, 2, 3, 4, 5 of NADH dehydrogenase, ATP8: subunit 8 of the H$^+$-ATPase. CA and EB denote different subclones which were isolated by limiting dilution in chloramphenicol- or ethidium bromide-containing selection medium, respectively. (B) Southern blot analysis of restriction endonuclease fragments obtained from DNA of the wild-type L929 cells and subclones. DNA (10 μg) was digested with *Hin*dIII and electrophoresed on a 0.7% agarose gel. The gel was transferred to a nylon membrane and the separated DNA hybridized with a ^{32}P-labeled fragment of the rat mitochondrial 12S rRNA gene.

protein synthesis by binding to the 70S ribosome, while leaving cytoplasmic protein synthesis intact (Spolsky and Eisenstadt, 1972). From the EB- and CA-resistant cell populations, several subclones were isolated by limiting dilution and analyzed in detail. Measurements of lactate production and cytochrome *c* oxidase revealed that most cell clones recruited most of their energy from anaerobic glycolysis (Table I). To analyze further the presence of mtDNA-encoded protein subunits in the subclones, cells were labeled with [^{35}S]methionine in the presence of emetine, an inhibitor of cytoplasmic protein synthesis. By this procedure, synthesis of the different mtDNA-encoded proteins could be visualized in SDS−urea gels. Figure 5A shows that most of the cell clones had a reduced mitochondrial protein synthesis. In three of the EB-resistant clones (EB-2, EB-12 and EB-21), no mtDNA translation products could be detected. A reduction or total depletion of mtDNA was further confirmed by Southern blottings with a probe coding for the mitochondrial 12S rRNA. In all subclones tested, the amount of mtDNA was considerably reduced (Figure 5B). In accordance with Figure 5A, mtDNA was undetectable in EB-12 and EB-21 clones under conventional hybridization conditions, whereas subclone EB-2 gave a weak hybridization signal.

We first analyzed in cytotoxicity assays the TNF sensitivity of the subclones. In the absence of the transcription inhibitor actinomycin D (Act D), all subclones were largely TNF resistant in comparision with the sensitive wild-type cells (Figure 6A). In some clones, no detrimental effects on cell viability could be detected even at high TNF concentrations. It is noteworthy that the simple addition of EB or CA to untreated wild-type L929 cells did not affect TNF cytotoxic

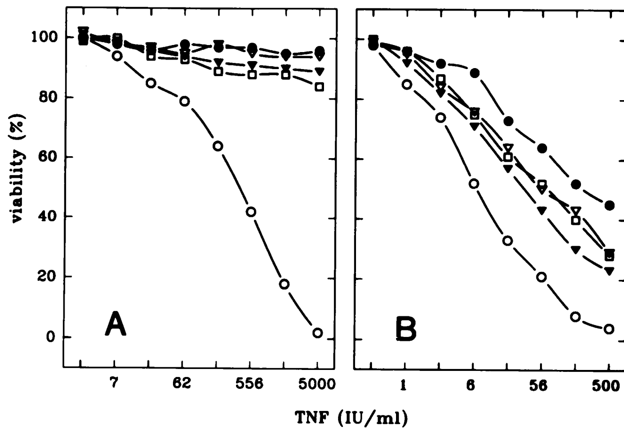

Fig. 6. Comparison of the TNF sensitivity of the respiration-deficient subclones and wild-type L929 cells in the absence (**A**) and presence (**B**) of actinomycin D. Cells were seeded in 96 well plates at a density of 2 × 10^4 cells/well. After 16 h, serial concentrations of TNF ranging from 7 to 5000 IU/ml (A) or 0.7 to 500 IU/ml (B) were applied and incubated for 18 h. Survival was assayed by reduction of MTT and calculated as the percentage of the staining value of untreated cultures. ○, L929 cells; ▼, EB-12; ●, EB-21; □, CA-22; ▽, CA-34 subclone.

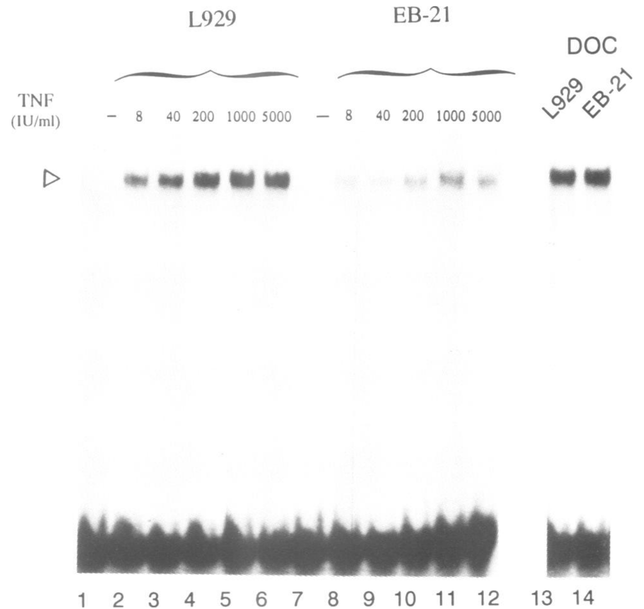

Fig. 7. NFκB activation by TNF is reduced in respiration-deficient L929 subclones. Wild-type L929 cells (lanes 1–6) and EB-21 cells, as a representative respiration-deficient subclone (lanes 7–12), were incubated for 45 min with the indicated concentrations of TNF. Nuclear extracts were prepared and tested for the presence of activated NFκB in electrophoretic mobility shift assays. In lanes 13–14, cytoplasmic extracts of both cell types were prepared and latent NFκB–IκB complex activated by treatment with deoxycholate (DOC) as described in Materials and methods. The EMSA reveals that reduction of NFκB activation after TNF stimulation in EB-21 cells is not due to diminished amounts of the inactive complex.

activity. We next analyzed the TNF sensitivity in the presence of Act D, which generally enhances TNF killing and additionally sensitizes some cell types which are resistant in the absence of the inhibitor. In the presence of Act D, TNF resistance of the cell clones was partially overcome, although cells were still less sensitive than their wild-type homologues (Figure 6B).

NFκB activation by TNF is strongly repressed in respiration-deficient cell clones

Next to the cytotoxic effects, the respiration-deficient subclones of L929 were analyzed for the activation of NFκB in response to TNF. Cells were treated with serial dilutions of TNF. In the wild-type cells the addition of 200 IU/ml TNF already resulted in maximal activation of the transcription factor (Figure 7). The respiration-deficient clones, however, only weakly responded to TNF, which was obvious by a strongly reduced NFκB activation. Even at the highest TNF concentrations used, only light κB bands were observed.

To analyze whether the reduction of κB binding was really due to a diminished cytoplasmic NFκB activation or just the effect of decreased NFκB protein expression, we further compared levels of latent NFκB–IκB complex in the respiration-deficient clones and wild-type L929 cells. When cytoplasmic extracts of both cell types were activated with deoxycholate in order to dissociate the IκB subunit, no differences could be observed in the EMSA (Figure 7). These results indicate that depletion of a functional mitochondrial electron transport system largely abrogates TNF-triggered NFκB activation.

Inhibition of IL-6 gene expression in the respiration-deficient subclones

The effects of depletion of the mitochondrial electron transport on TNF-induced NFκB activation prompted us to further analyze TNF-induced gene expression in the L929 subclones. IL-6 represents a cytokine which is rapidly induced by TNF in many cell types (Defilippi *et al.*, 1987). Although several other transcriptional control elements have been identified in the IL-6 promoter, such as a cAMP-responsive element and an AP-1 site, induction of IL-6 by TNF has been reported to be largely controlled by a κB element (Libermann *et al.*, 1990; Shimizu *et al.*, 1990; Zhang *et al.*, 1990).

When wild-type and EB- and CA-treated cells were stimulated with TNF, respiration-deficient cell clones produced remarkably less IL-6 than wild-type cells (Figure 8A). In some cell clones, IL-6 expression was reduced even up to 500- to 600-fold. To analyze whether reduction of IL-6 expression was specific for the TNF stimulus, both cell types were further treated with dibutyryl cyclic AMP (db-cAMP), which induces IL-6 expression by the elevation of cAMP levels. Indeed, in these experiments no significant differences could be observed between L929 cells and the subclones, suggesting that the IL-6 gene could still be induced by other signaling pathways in the respiration-deficient cell clones. The same differences in TNF-induced IL-6 expression were also observed at the transcriptional level (Figure 8B). In fact, in the respiration-deficient clones IL-6 mRNA could hardly be detected after TNF stimulation, although comparable amounts of IL-6 mRNA were found after cell activation by a combination of db-cAMP, cycloheximide and staurosporine, which superinduces IL-6 gene expression. Therefore, these results indicate that inhibition of the mitochondrial oxidative metabolism also affects gene-regulatory effects of TNF.

Discussion

ROI, which comprise hydrogen peroxide, hydroxyl radicals and superoxide anions, are essential compounds of an

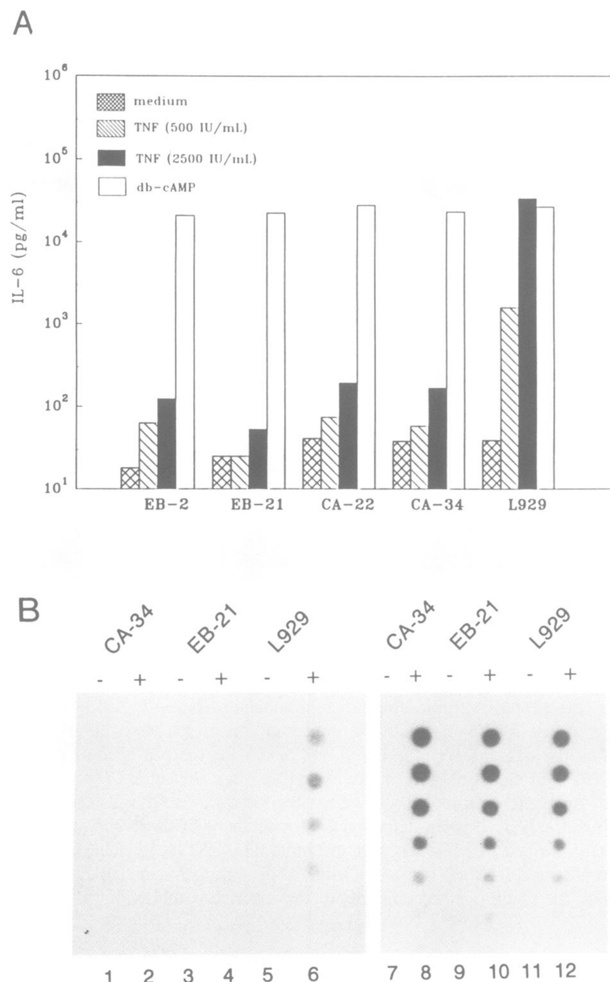

Fig. 8. TNF induction of IL-6 is defective in respiration-deficient L929 subclones. **(A)** IL-6 bioassay: 10^6 cells were seeded in 6 well dishes. After 12 h, medium was refreshed and cells were stimulated with TNF (500 IU/ml, 2500 IU/ml) or db-cAMP (1 mM); 18 h later supernatants were harvested and analyzed for IL-6 bioactivity. **(B)** Analysis of IL-6 mRNA levels in L929 subclones. Confluent cell monolayers were either untreated (lanes 1, 3, 5) or treated with 5600 IU/ml TNF (lanes 2, 4, 6). In a second experiment, the cells were untreated (lanes 7, 9, 11) or stimulated with a combination of db-cAMP (2 mM), cycloheximide (50 μg/ml) and staurosporine (6 nM, added at −2 h). After each stimulation, total cytoplasmic RNA was extracted and immobilized onto a nylon membrane in a dot-blot device. Consecutive lanes correspond to 20 μg RNA and serial 1:3 dilutions, respectively. The membrane was hybridized with a ^{32}P-labeled DNA fragment derived from mIL-6 cDNA and exposed to an X-ray film.

oxidative metabolism (reviewed in Halliwell and Gutteridge, 1990). Generally, ROI are regarded as toxic and harmful metabolites. In phagocytes, large amounts of ROI are generated by the membrane-bound NADPH oxidase, providing an antimicrobial defense mechanism against pathogens (reviewed in Rossi, 1986). Nonetheless, when formation goes uncontrolled, ROI may be implicated in several diseases and autoimmune disorders, leading to cellular damage by peroxidizing lipids and disrupting structural proteins, enzymes and nucleic acids. An important source of ROI are mitochondria (Boveris and Chance, 1973; Turrens and Boveris, 1980; Turrens *et al.*, 1985). Overproduction by these organelles may be toxic and harmful for the producing cell. Several cytostatic or antitumor drugs, such as adriamycin or menadione, act as redox-cycling agents that continuously produce deleterious oxygen species.

Accumulating evidence now exists that short-lived oxygen radicals may provide a versatile cellular control mechanism and additionally play important roles central to gene regulation. Pro-oxidant conditions can induce expression of immediate-early genes (reviewed in Holbrook and Fornance, 1991), in particular c-*fos* and c-*jun* (Crawford *et al.*, 1988; Shibanuma *et al.*, 1988; Stein *et al.*, 1989; Amstad *et al.*, 1992). Indeed, some gene-inductive effects of phorbol esters may also be independent of protein kinase C activation, since phorbol esters can generate a pro-oxidant cellular environment (Cerutti, 1985). Recently, activation and DNA binding of several transcription factors have been shown to depend on the cytoplasmic or nuclear redox state, including steroid receptors (Silva and Cidlowski, 1989), ISGF3 (Levy *et al.*, 1989), TFIIIC (Cromlish and Roeder, 1989), c-myb protein (Guehmann *et al.*, 1992) and the transcriptional activators AP-1 (Abate *et al.*, 1990; Devary *et al.*, 1991; Xanthoudakis *et al.*, 1992) and NFκB (Mihm *et al.*, 1991; Schreck *et al.*, 1991, 1992). Although the mechanism of redox regulation in transcriptional activation is still rather unclear, a substantial body of evidence now exists that ROI may act as second messengers and, in addition to their harmful effects, may also exert signaling functions.

In this study, we show that two diverse effects of TNF, i.e. cytotoxicity and transcriptional activation, require functional mitochondria, presumably as an intracellular source of ROI.

TNF cytotoxicity and mitochondrial oxygen radicals

In our previous studies (Schulze-Osthoff *et al.*, 1992), we have reported that mitochondria play a crucial role in causing TNF-induced cytotoxicity in TNF-sensitive cells. Presumably, upon TNF stimulation mitochondrial radicals are formed at the ubiquinone site of the respiratory chain, where electrons are directly transferred from ubisemiquinone to molecular oxygen. This one-electron transfer results in the formation of superoxide anions as the primary reactive oxygen species of mitochondria (Loschen *et al.*, 1974). Consequently, inhibition of electron entry to the ubiquinone pool of the respiratory chain, by complex I inhibitors (rotenone, amytal) or complex II inhibitors (thenoyltrifluoroacetone), greatly reduced TNF cytotoxicity (Schulze-Osthoff *et al.*, 1992). On the other hand, when the electron flow was inhibited distal to the ubiquinone site by the addition of complex III inhibitors (antimycin A), increased amounts of ROI may be generated, resulting in a marked potentiation of TNF cytotoxicity.

This study extends the former findings and provides further supportive evidence for the involvement of the mitochondrial electron transport in TNF cytotoxicity. Subclones of L929 cells which were depleted of mitochondrial respiration revealed a clear-cut resistance towards TNF cytotoxicity. Depletion of mitochondrial respiration was achieved by long-term treatment of the cells with either ethidium bromide or chloramphenicol which block mitochondrial DNA replication and protein synthesis, respectively (Nass, 1972; Wiseman and Attardi, 1978; Desjardins *et al.*, 1985; Morais *et al.*, 1988; King and Attardi, 1989). The isolated cell clones did not reveal any significant morphological changes. However, the growth rate of the cells was reduced since the energy was mostly derived from anaerobic glycolysis. Most

importantly, receptor binding assays with iodinated TNF did not reveal alterations of TNF receptor numbers, thus ruling out reduced TNF binding as a possible explanation of the acquired resistance (data not shown). Surprisingly, when the respiration-deficient cells were incubated with TNF in the presence of the transcriptional inhibitor actinomycin D, which generally increases sensitivity, TNF resistance was again partially overcome. An explanation for this sensitization may be the existence of an alternative TNF-activated pathway that is less prominent in the absence of transcriptional inhibition. Indicative of a second cytotoxic pathway may be the observation that mitochondrial inhibitors or radical scavengers which protect parental L929 cells were not or only marginally effective in the respiration-deficient L929 clones, when tested in the presence of actinomycin D. Furthermore, overexpression of manganous superoxide dismutase (MnSOD), which efficiently reduces TNF cytotoxicity at least in some cell types, is rather inefficient at providing protection against a combination of TNF and metabolic inhibitors (Wong *et al.*, 1989).

Redox regulation of transcriptional activators

This study shows that mitochondrial intermediates are not only implicated in TNF cytotoxicity, but may also be involved in TNF-mediated activation of the transcription factor NFκB. Firstly, the respiration-deficient L929 subclones revealed a strongly reduced NFκB activation by TNF. Secondly, in the parental L929 cells mitochondrial inhibitors, that interfered with TNF cytotoxicity, apparently affected NFκB activation in similar ways. Inhibitors, which were protective by blocking an early step in the mitochondrial electron transfer, strongly reduced NFκB activation by TNF. Vice versa, the complex III inhibitor antimycin A, which confers potentiation to TNF cytotoxicity, exerted a synergistic effect on NFκB activation by TNF. These observations suggest an at least partially common pathway for both the cytotoxic and NFκB-inducing effects of TNF. In accordance with this, it has been reported recently that activation of NFκB by different agents such as TNF, IL-1 or phorbol myristate acetate could be prevented by antioxidants and chelation of iron ions (which is required to convert oxygen intermediates to the highly reactive hydroxyl radical). In another study, it has been shown that ionizing irradiation, by which ROI are continuously generated, can induce NFκB activation (Brach *et al.*, 1991). With respect to TNF signal transduction, our results also implicate a role of the mitochondrial oxidative metabolism in NFκB activation and suggest that ROI formed by perversion of the electron transfer are responsible in this process. Moreover, when we tested a contribution of other potential radical sources, such as NADPH oxidase or xanthine oxidase, no involvement of these enzymes in TNF-induced NFκB activation was detectable. We also analyzed the influence of ATP synthesis on the activation of NFκB. However, like TNF cytotoxicity, early NFκB activation was not influenced by uncouplers of oxidative phosphorylation or a direct inhibition of ATPase by oligomycin (Schulze-Osthoff *et al.*, 1992, and our unpublished results).

Our results do not allow the molecular mechanism by which NFκB becomes activated to be identified. Intracellular radicals could regulate early transcriptional regulation by NFκB at several points in the pathway of signal transduction, e.g. the release of the inhibitory subunit IκB, translocation of the p55/p65 complex into the nucleus, or DNA-binding activity at the target sequences. It is remarkable that, contrary to activation of NFκB in the cytoplasm, binding of the purified p50/p65 NFκB complex to its target sequences *in vitro* is even promoted under reducing conditions (Toledano and Leonard, 1991; Xanthoudakis *et al.*, 1992). In the cytoplasm, the target of the latent NFκB-complex seems to be IκB which is released by a poorly defined mechanism. One can envision a direct effect of ROI by influencing protein folding or damaging the IκB protein. However, purified NFκB−IκB cannot be simply activated *in vitro* by exogenous hydrogen peroxide, although there may be a selective induction of NFκB by specific oxygen intermediates (Schreck *et al.*, 1992). Previously, it has been reported that the bacterial transcription factor oxy R is inducible following exposure to H_2O_2, whereas the bacterial sox R regulon has been defined as being responsible for the positive control of proteins induced by O_2^- and distinct from H_2O_2 (Storz *et al.*, 1990; Demple and Amabile-Cuevas, 1991).

However, it seems to be more likely that radicals participate indirectly in NFκB activation. An indirect role of radicals in NFκB activation by TNF might be exerted through the activation of specific proteases or kinases. In this respect, it should be noted that under *in vitro* conditions protein kinase C can activate the NFκB complex (Ghosh and Baltimore, 1990). A physiological role of protein kinase C or A, however, seems to be rather unlikely as TNF-induced NFκB activation is not abolished by PKC or PKA inhibitors (Hohmann *et al.*, 1991; Meichle *et al.*, 1991). However, several novel kinases have been described in recent times, such as a redox-activated tyrosine kinase (Bauskin *et al.*, 1991), the possible role of which in NFκB activation should be examined. Other candidate proteins controlling redox regulation may be specific factors acting similarly to the recently cloned REF-1 protein, which mediates the redox control of AP-1 activity and other transcriptional activators in the nuclear compartment (Xanthoudakis *et al.*, 1992). Furthermore, it may be possible that NFκB activation is particularly influenced by thiol-containing molecules, which may become oxidized upon TNF stimulation. Although it has recently been shown in a T cell line that TNF did not change the total cellular thiol and glutathione levels (Israël *et al.*, 1992), specific antioxidant systems may be modified and affect NFκB activation. In this context, it has to be pointed out that cells contain a number of different redox systems which are not necessarily linked to each other.

Recently, it has been also suggested that TNF stimulation of NFκB involves the activation of an acidic sphingomyelinase (Schütze *et al.*, 1992), which is a compartmentalized enzyme with a pH optimum of pH 4.5. At present it is unclear how ceramide, the reaction product of sphingomyelinase, might control NFκB activation. It should be noted that these results were obtained with cell lines which, unlike L929, were not or only weakly susceptible to the cytotoxic action of TNF; it is still an open question whether sphingomyelinase plays a role in the TNF-mediated NFκB activation in L929 cells and, if so, whether ceramide influences ROI formation.

IL-6 as an oxidative stress-responsive cytokine

Another remarkable difference between the respiration-deficient L929 subclones and their parental counterparts was observed by studying the TNF induction of IL-6 gene

expression. Expression of IL-6 in the subclones was considerably reduced at the transcriptional level and 200- to 600-fold when the biological activity was measured. This observation indicates that not only TNF cytotoxicity, but also IL-6 induction, may be mediated by reactive oxygen intermediates. The reduction of IL-6 expression may be explained by a decreased activation of NFκB binding to a κB element (−73 to −64) which has been suggested by several authors to be indispensible for TNF- or IL-1-induced IL-6 expression (Libermann *et al.*, 1990; Shimizu *et al.*, 1990; Zhang *et al.*, 1990). However, the importance of NFκB in determining TNF-induced IL-6 expression should not be overemphasized. We have recently described the potentiating effect of lithium ions in TNF-induced IL-6 expression (Beyaert *et al.*, 1991; Vandevoorde *et al.*, 1991), although we could not observe any contribution of NFκB in these phenomena. Furthermore, it should be taken into account that NFκB activation, though seriously reduced, was still detectable to a significant extent in the subclones. Thus, it is conceivable that a cooperative reduction of additional transcriptional activators accounts for the sharp decrease of IL-6 expression. Ray *et al.* (1989) have defined a 26 bp region of the IL-6 promoter, called multiple responsive element (MRE, −173 to −151), which is important in TNF-induced transcription of the IL-6 gene. In human fibroblasts, it has been further suggested that TNF induction of IL-6 may be mediated by a cellular increase of cAMP (Zhang *et al.*, 1988, 1990) which may be additionally involved in the activation of NFκB (Shirakawa and Mizel, 1989). Although a cAMP-responsive element (CRE) is contained in the IL-6 promoter (−163 to −158), it seems to be dispensible for TNF induction of IL-6 expression. In L929 cells, cAMP and TNF induce gene expression in a synergistic manner, suggesting separate pathways of IL-6 induction (Beyaert *et al.*, 1991). Supportive for a distinct regulation of gene expression by TNF and cAMP is the finding that in the respiration-deficient L929 subclones IL-6 could still be induced by cAMP, whereas TNF was ineffective.

The dual role of mitochondrial intermediates in both the induction of IL-6 and cytotoxicity may explain earlier findings that IL-6 expression generally parallels the outcome of TNF cytotoxicity. In studies with several activators or inhibitors, it has been shown that under conditions where cytotoxicity was either enhanced or decreased, IL-6 expression was equally modulated (Vandevoorde *et al.*, 1991, 1992). Thus, IL-6 expression may be determined at least in part by the same cytoplasmic reactions and metabolites responsible for cellular toxicity. That ROI may be the common denominator in these processes is further suggested by the fact that some radical scavengers can inhibit IL-6 expression (our unpublished results).

Oxygen radicals as putative signal transducers of a mitochondria – nucleus pathway

The data presented here define a previously unexpected role for mitochondria in the control of nuclear gene expression. Apparently, mitochondrial intermediates can control both the cytotoxic and gene-regulatory effects of TNF, thus providing a basis for a mitochondria–nucleus signaling pathway. Up to now, there is only limited information regarding a proposed retrograde signaling pathway (reviewed in Grivell, 1989), although there are several reported instances of alterations in gene expression of nuclear genes in response to changed mitochondrial functions. Early investigations in *Drosophila* have shown that application of mitochondrial inhibitors can influence the puff expression pattern in polytene chromosomes (Leenders *et al.*, 1974, and references therein). Yeast cells respond to the complex III inhibitor antimycin A by induction of cytoplasmic citrate synthase expression (Liao *et al.*, 1991). Moreover, in yeast the level of expression of nuclear genes, not all of them directly related to mitochondrial functions, was found to change in response to the loss of the mitochondrial genome (Parikh *et al.*, 1987). These observations therefore indeed provide additional evidence for the existence of a signal communication between the mitochondrial and nuclear compartment. Oxygen radicals may provide a versatile cellular control mechanism in this signaling system since they comprise very short-lived molecules which can be tightly regulated by a machinery of different enzymes.

A major question that has to be addressed in future experiments is the mechanism of how TNF interferes with the mitochondrial electron transport. There has been the suggestion that alterations in mitochondrial calcium homeostasis affect the mitochondrial respiratory chain (Richter and Frei, 1988). However, it is not clear whether changes in calcium metabolism precede the oxidative stress or are just a consequence of it. Another interesting issue will be whether phosphorylation events, induced by TNF, may regulate the electron transport. Up to now, there have been no studies concerning phosphorylation of respiratory chain proteins. One could speculate that an increase of radical production at the ubiquinone site of the respiratory chain results in a reduced electron transfer from the ubiquinone cycle to the cytochrome chain. Thus, a target protein between these components could be a ubiquinone-binding protein, which would regulate the electron flow to complex III, or certain subunits of cytochrome *b* of the respiratory chain (Yu *et al.*, 1985). Interestingly, in plant photosynthesis it has been shown that the redox-regulated LHC kinase can regulate the electron flow by phosphorylation (Gal *et al.*, 1992). Future studies may therefore reveal whether these or similar mechanisms transmit the TNF signal into the mitochondria and thereby regulate mitochondrial radical generation.

Materials and methods

TNF and reagents

TNF preparation. Recombinant murine TNF was produced in *Escherichia coli* and purified to at least 99% homogeneity (Fransen *et al.*, 1985). The preparation had a specific activity of 3.7×10^7 IU/mg protein and contained <4 ng endotoxin/mg protein. TNF activity was determined as described by Ostrove and Gifford (1979) using an international standard TNF preparation (code no. 88/532, obtained from the Institute for Biological Standards and Control, Potters Bar, UK) as a reference.

Reagents. The following reagents were purchased from Sigma Chemical Co. (Deisenhofen, FRG): *N*-acetyl-L-cysteine, actinomycin D, amytal, butylated hydroxyanisole (BHA), dibutyryl cyclic AMP (db-cAMP), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), nordihydroguaiaretic acid (NDGA), oligomycin, pyrrolidine dithiocarbamate (PDTC), rotenone and thenoyltrifluoroactetone (TTFA). Antimycin A was from Serva (Heidelberg, FRG). Stock solutions of the reagents were routinely prepared in medium, dimethylsulfoxide or ethanol as appropriate.

Cell culture

The standard medium for L929 cells was Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum, 100 U penicillin/ml and 0.1 mg streptomycin/ml. Isolation of respiration-deficient cells was carried out by long-term culture in medium containing 50 μg

chloramphenicol/ml or 400 ng ethidium bromide/ml. The selection medium was further supplemented with 4.5 mg glucose/ml and 5 μg uridine/ml, since respiration-deficient cells have been reported to become pyrimidine auxotrophs (King and Attardi, 1989). Addition of these agents did not, however, influence any of the assays.

TNF cytotoxicity assay

Cells were seeded in 96 microwell plates at 3×10^4 cells in 100 μl medium. Twelve to 16 h later, 50 μl of a drug solution were given. TNF with or without actinomycin D (1 μg/ml) was added 2 h later in a 50 μl volume and a concentration range of 0.7–5000 IU/ml. After 18–24 h of further incubation, cell viability was routinely determined via MTT staining (Tada *et al.*, 1986). Similar results were obtained with crystal violet staining of attached cells.

Interleukin-6 assay

Cells were seeded in 6 well plates at 10^6 cells/well; 16–24 h later cells were washed twice and treated in 1 ml medium/well containing various TNF concentrations. Following another 16–24 h of incubation, the medium was taken off and assayed in serial dilutions for IL-6. IL-6 was determined on the basis of the proliferative response of the mouse plasmocytoma cell line 7TD1 (Van Snick *et al.*, 1986).

Nuclear and whole cell extracts

Subconfluent cell cultures in a 6 well plate or 60 cm^2 Petri dishes were treated with TNF for 45 min. Inhibitors were applied 1 h before the onset of TNF addition. After incubation cells were rinsed in ice-cold phosphate-buffered saline, scraped off using a rubber policeman and collected by centrifugation. Nuclear extracts were prepared essentially as described by Dignam *et al.* (1983). Cells were resuspended in 1 ml of a hypotonic lysis buffer (buffer A, Dignam *et al.*, 1983). After 20 min, cells were homogenized by 20 strokes with a loose-fitting Dounce. Nuclei were collected in a microcentrifuge and proteins extracted in a high-ionic-strength buffer (buffer C). After 30 min, nuclear debris was removed by centrifugation. The extracts were diluted with 4 vol of low-salt buffer (pH 7.5) (buffer D; Dignam *et al.*, 1983) containing 1% NP-40, and kept frozen until use.

For whole-cell extracts, cells were lysed in a high-salt buffer (20 mM HEPES, pH 7.5, 400 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 20% glycerol, 1% NP-40, 0.5 mM DTT, 10 μg/ml leupeptin, 1 mM PMSF, 0.001% aprotinin). After 10 min of incubation, extracts were cleared by centrifugation and kept frozen.

Electrophoretic mobility shift assays

Equal amounts of the extracts (~3 μg nuclear protein or ~10 μg crude protein; determined by the Bio-Rad assay kit) were incubated with an NFκB-specific ^{32}P-labeled oligonucleotide. The sequence of the double-stranded 30 bp probe encompassing the NFκB site and flanking sequences from the mouse kappa light chain enhancer is shown in Zabel *et al.* (1991). Binding reactions were performed in a 20 μl volume containing 2–4 μl of extract, 4 μl 5 × binding buffer (20 mM HEPES pH 7.5, 50 mM KCl, 1 mM DTT, 2.5 mM $MgCl_2$, Ficoll), 2 μg poly(dI-dC) as non-specific competitor DNA, 2 μg bovine serum albumin (BSA) and 3000–6000 c.p.m. Cerenkov of the random primer-labeled oligonucleotide. After 30 min binding reaction at room temperature, samples were loaded on a 4% non-denaturing polyacrylamide gel and run in 0.5 × TBE buffer (pH 8.0). As a negative control, an oligonucleotide mutated in the NFκB binding site by two point mutations, but otherwise identical with the wild-type fragment, was used. In all cases, DNA–NFκB complexes were only observed using the wild-type oligonucleotide. In some experiments, the DNA-binding activity of the extracts was quantified by scintillation counting of the ^{32}P-labeled NFκB–DNA complexes excised from the dried gels. In the case of cytosols, NFκB was activated by incubating 2 μl of the cytosolic extract with 3 μl 6.5% sodium deoxycholate for 10 min before addition to a mixture of probe.

For the assay of the DNA-binding activities of AP-1 and octamer binding proteins (Oct), the oligonucleotides used in this study have been described previously (AP-1, Angel *et al.*, 1987; Oct, Wirth *et al.*, 1987). The NF-IL6 specific oligonucleotide containing the binding motif from the human IL-6 promoter (5′-GTACACATTGCACAATCTTA-3′) was synthesized on an Applied Biosystems DNA Synthesizer.

Anaerobic glycolysis

Exponentially growing cells were washed twice with phosphate-buffered saline (PBS) and incubated at time zero in DMEM with 10% dialyzed fetal calf serum (FCS). After 12 h, supernatants were assayed for lactate content with a commercial kit (Sigma) using lactate dehydrogenase. Cellular cytochrome oxidase was measured by a spectrophotometric method analyzing the rate of oxidation of ferrocytochrome *c* (Smith and Conrad, 1956). Cells were scraped off and lysed with 0.3% digitonin in 10 mM potassium phosphate (pH 7.0). Enzyme reactions were started by the addition of 50 μM reduced ferrocytochrome *c* and recorded at 25°C every 15 s at 550 nM. Ferrocytochrome *c* in the blank cuvette was fully oxidized with 1 mM ferricyanide. Rates of cytochrome oxidation were determined from the initial linear rates using an extinction coefficient of 21/mM/cm.

Mitochondrial protein synthesis

Cells were seeded in 6 well plates at 1×10^6 cells/well; 1 h before the addition of 100 μCi/ml [^{35}S]methionine, cytoplasmic protein synthesis was inhibited with emetine at a concentration of 100 μg/ml. After further incubation for 3 h, the adherent cells were scraped off and washed three times in PBS. Lysates were prepared in 20 mM Tris–HCl (pH 7.4) containing 8 M urea and 2% SDS. Samples were loaded without prior heating on 12.5% SDS–polyacrylamide gels containing 6 M urea (Chomyn *et al.*, 1985). Gels were dried and exposed to X-ray films.

Mitochondrial DNA analysis

Enriched mtDNA was prepared from the cells by the method of Wallace *et al.* (1988). *Hin*dIII restriction fragments of the DNA were electrophoresed in 0.7% agarose in TAE buffer. After depurination and denaturation according to standard procedures, gels were blotted by capillarity in 20 × SSC onto Hybond N^+ membranes (Amersham). DNA was fixed by UV irradiation and prehybridized in 5 × SSPE, 0.5% SDS and 1 × Denhardt's solution. For hybridization, an *Acc*I insert, cloned in a Bluescript vector and coding for the 12S rRNA of rat mtDNA (Gadaleta *et al.*, 1989), was labeled by extension of random hexanucleotide primers. After hybridization at 45°C for 18 h, blots were washed in 0.5 × SSC, 0.1% SDS and exposed to X-ray films.

RNA manipulations

Total cytoplasmic RNA was isolated essentially as described previously (White and Bancroft, 1982) using an NP-40 lysis buffer. IL-6-specific mRNA was quantitated in dot-blot analyses using a restriction fragment of the murine IL-6 cDNA containing plasmid pUC8MIL6 (Van Snick *et al.*, 1986). Appropriate amounts of the isolated RNA were denatured with formaldehyde and applied onto a nylon membrane (Pall Biodyne A, Pall Biosupport, East Hills, NY) in the presence of 5 × SSC and immobilized by heat treatment (1 h at 80°C). Prehybridization (1 h at 42°C) and hybridization (overnight at 42°C) were carried out in a mixture containing 5 × Denhardt's solution, 5 × SSC, 50 mM sodium phosphate (pH 6.5), 0.1% SDS, 250 μg/ml non-homologous DNA and 50% formamide. The washing steps were carried out in 2 × SSC/0.1% SDS (4 × 5 min) and finally 0.1 × SSC/0.1% SDS.

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