

Daunorubicin Activates NF κ B and Induces κ B-dependent Gene Expression in HL-60 Promyelocytic and Jurkat T Lymphoma Cells*

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The anthracycline antibiotic, daunorubicin, can induce programmed cell death (apoptosis) in cells. Recent work suggests that this event is mediated by ceramide via enhanced ceramide synthase activity. Since the generation of ceramide has been directly linked with the activation of the transcription factor, NF κ B, this was investigated as a novel target for the action of daunorubicin. Here we describe how treatment of HL-60 promyelocytes and Jurkat T lymphoma cells with daunorubicin results in the activation of the transcription factor NF κ B. The effect of daunorubicin was evident following 1–2 h treatment, which was in contrast to the time course of activation obtained with the cytokine, tumor necrosis factor, where NF κ B activation was detected within minutes of cellular stimulation. Activated complexes were shown to contain predominantly p50 and p65/RelA subunit components. Daunorubicin also induced I κ B degradation and increased the expression of an NF κ B-linked reporter gene. In addition, the drug was found to strongly potentiate the ability of tumor necrosis factor to induce an NF κ B-linked reporter gene, suggesting a synergy between these two agents in this response. These events were sensitive to the iron chelator, deferoxamine mesylate (desferal), and the anti-oxidant and metal chelator pyrrolidine dithiocarbamate. A structurally related compound, mitoxantrone, which, unlike daunorubicin, is unable to undergo redox cycling in cells, also activated NF κ B in a pyrrolidine dithiocarbamate-sensitive manner. A specific inhibitor of ceramide synthase, fumonisins B1, had no effect on daunorubicin induced NF κ B activation at a range of concentrations previously reported to block apoptosis induced by this drug. However, this agent could inhibit increases in ceramide induced by daunorubicin, in addition to blocking ceramide synthase activity from HL-60 cells which was activated in response to daunorubicin treatment. These data therefore suggest that the effect of daunorubicin on NF κ B is unlikely to involve ceramide, but may involve reactive oxygen species generated as a result of endogenous cellular processes rather than reductive metabolism of the drug. As NF κ B may be involved in apoptosis, this effect may be an important aspect of the cellular responses to this agent.

The anthracycline antibiotic, daunorubicin, is widely used in cancer chemotherapy with proven therapeutic benefit in the treatment of a variety of neoplasia (1). Although its mechanism of anti-tumor action is uncertain, DNA is believed to be a primary target (2). Its ability to cause strand scission may be mediated by stabilizing a cleavable complex between DNA and the enzyme, topoisomerase II, and/or oxygen radicals arising from redox cycling following its bioreduction. Additionally, bioreduction products and reactive oxygen species have been associated with anthracycline induced alkylation of cellular macromolecules, DNA intercalation and cross-linking, lipid peroxidation, and cell membrane damage (2). Irrespective of the initial insult, anthracyclines, along with a variety of agonists, ultimately activate the event of programmed cell death or apoptosis in cells (3). Their ability to induce this pathway may be a mechanism underlying their therapeutic efficacy in certain tumor types.

The development of the apoptotic morphology is well defined; however, signaling pathways that may act as primary mediators of apoptosis and growth suppression are poorly characterized (4). Some of those relevant to the cytotoxic action of chemotherapeutic drugs include the triggering of CD95/CD95-L interaction resulting in a type of autocrine suicide (5), and the activation of effector molecules such as interleukin-1 converting enzyme-like proteases (6). Recent studies suggest that the neutral lipid ceramide may also play a role in mediating drug-induced apoptosis (7, 8). Ceramide is a putative second messenger which can also be generated following the activation of distinct sphingomyelinase activities in response to a range of extracellular agents including TNF- α ,¹ interleukin-1 β , γ -interferon, nerve growth factor, Fas ligand, and 1,25-dihydroxyvitamin D₃ (9). A general role in growth arrest and suppression is suggested by its ability to induce cell differentiation, cell-cycle arrest, apoptosis, or cell senescence (9), although a mitogenic role has been demonstrated in certain cell types (10). In a recent study, daunorubicin was shown to increase ceramide levels in cells following induction of the enzyme ceramide synthase (7). Furthermore, inhibition of this enzyme by the mycotoxin, fumonisins B1, blocked apoptosis induced by daunorubicin. The regulated biosynthesis of ceramide may represent a signaling mechanism by which apoptotic events are induced by this drug.

The generation of ceramide following activation of lysosomal acid sphingomyelinase has not only been linked with TNF-induced apoptosis, but also with the activation of NF κ B (11). This inducible transcription factor has been implicated in the regulation of many genes which code for mediators of the immune, acute phase, and inflammatory responses (12). The

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¹ The abbreviations used are: TNF, tumor necrosis factor; PDTC, pyrrolidine dithiocarbamate; ROS, reactive oxygen species; NF κ B, nuclear factor κ B; CAT, chloramphenicol acetyltransferase.

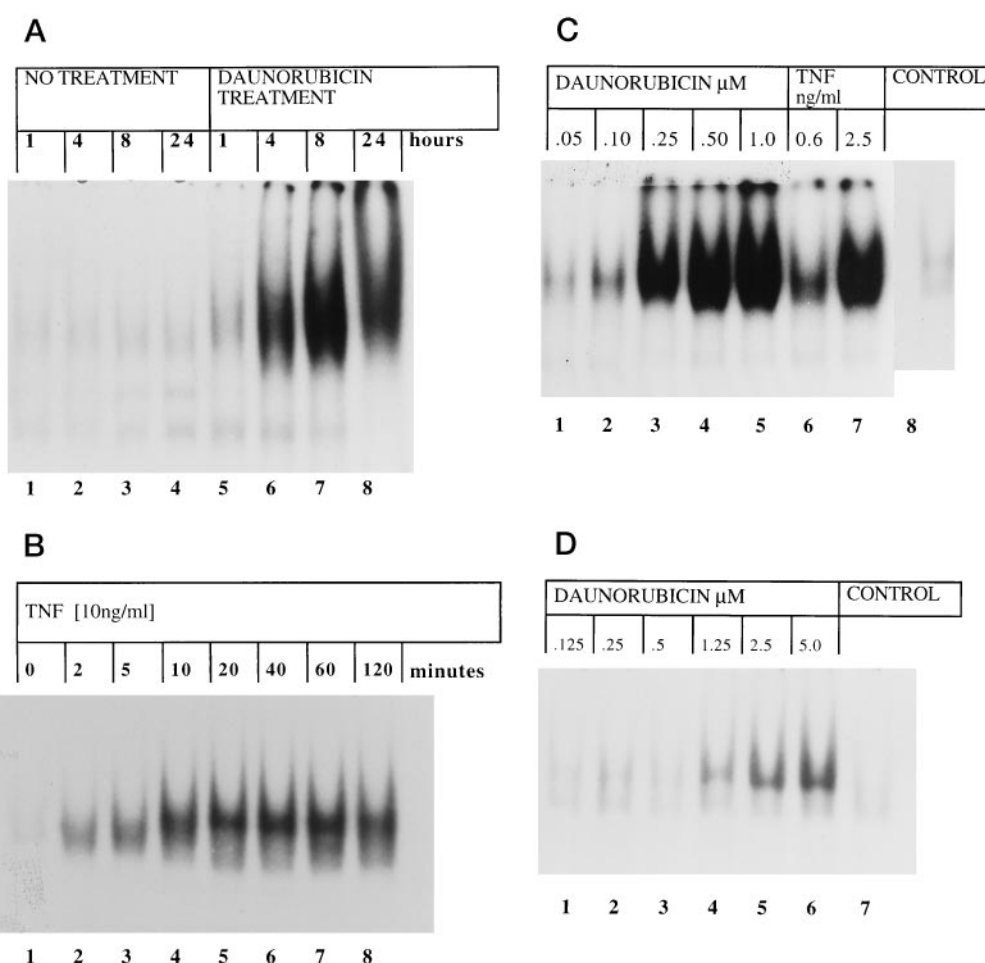


FIG. 1. **Daunorubicin activates NF κ B in HL-60 promyelocytic leukemia and Jurkat T lymphoma cells.** **A**, HL-60 cells (1×10^6 /ml) were treated with either daunorubicin (10μ M) (lanes 5–8) or an equivalent volume of vehicle control (medium) (lanes 1–4) for various times. **B**, HL-60 cells (5×10^6 /ml) were stimulated with TNF (10 ng/ml) for increasing time points (lanes 1–8). **C**, HL-60 cells (1×10^6 /ml) were exposed to increasing concentrations of daunorubicin (lanes 1–5) or TNF (lanes 6 and 7) or vehicle control (lane 8) for 4 h. **D**, Jurkat T cells (1×10^6 /ml) were treated with either daunorubicin (lanes 1–6) or an equivalent volume of vehicle control (medium) (lane 7) for 4 h. In all experiments, nuclear extracts were prepared following stimulation and analyzed for NF κ B binding activity as described under “Experimental Procedures.” NF κ B-DNA complexes are shown. Results are representative of two to three separate experiments.

DNA-binding protein complex recognizes a discrete nucleotide sequence (5'-GGGACTTCC-3') in the upstream regions of a variety of responsive genes. Subunits belonging to the NF κ B family comprise five members in mammals: p50, p65 (RelA), c-Rel, p52, and RelB. These proteins share a conserved 300-amino acid sequence in the N-terminal portion, termed the Rel homology domain, which mediates DNA binding, protein dimerization, nuclear localization, and binding of the inhibitor protein I κ B (either α or β) (12). Various dimer combinations of these proteins have distinct DNA binding specificities and may serve to activate specific sets of genes (13). In resting cells, the NF κ B dimer is sequestered in the cytosol by associating with I κ B, and can be “liberated” from this complex by a variety of inducers. A simple model for NF κ B activation is as follows. Phosphorylation of I κ B by specific activated protein kinase(s) tags it for proteolytic degradation (14). This facilitates the nuclear translocation of activated NF κ B complexes, whereupon binding to cognate sequences, gene expression is activated.

The signaling pathways linking receptor stimulation to NF κ B activation are poorly defined. A number of kinases have been implicated in the phosphorylation of I κ B, the most notable being a recently identified ubiquitination-dependent multisubunit protein kinase (14). Phosphorylation at two specific residues, serine 32 and 36, on I κ B α , is thought to lead to its ubiquitination and subsequent degradation by the proteasome,

facilitating NF κ B release and translocation into the nucleus (15). Mutants lacking these residues cannot undergo phosphorylation (and subsequent proteolytic degradation) in response to a variety of stimuli (16) suggesting that many signal transduction pathways converge on the putative I κ B kinase(s).

In addition to ceramide being implicated as an upstream regulator of I κ B α phosphorylation, a model has been proposed whereby reactive oxygen species (ROS) act as second messengers in this event (17). Evidence to support this model is based on the ability of H_2O_2 to activate NF κ B (18) and the inhibitory effects of antioxidants such as *N*-acetylcysteine (a thiol antioxidant and glutathione precursor) and pyrrolidine dithiocarbamate (PDTC), which is also a metal chelator, on NF κ B activation (18–21). The link between ceramide and ROS in signaling either transcriptional activation or apoptotic events is unclear (22, 23). In a model for NF κ B proposed by Baeuerle and Henkel (12), agonist-stimulated ceramide generation lies upstream of an event which triggers H_2O_2 production, leading to the activation of this transcription factor.

Because previous work demonstrated the production of ROS and ceramide in response to daunorubicin (2, 7), we investigated the effect of this agent on NF κ B activation. We have found that daunorubicin signals NF κ B activation in HL-60 promyelocytes and Jurkat T cells by a PDTC-sensitive mecha-

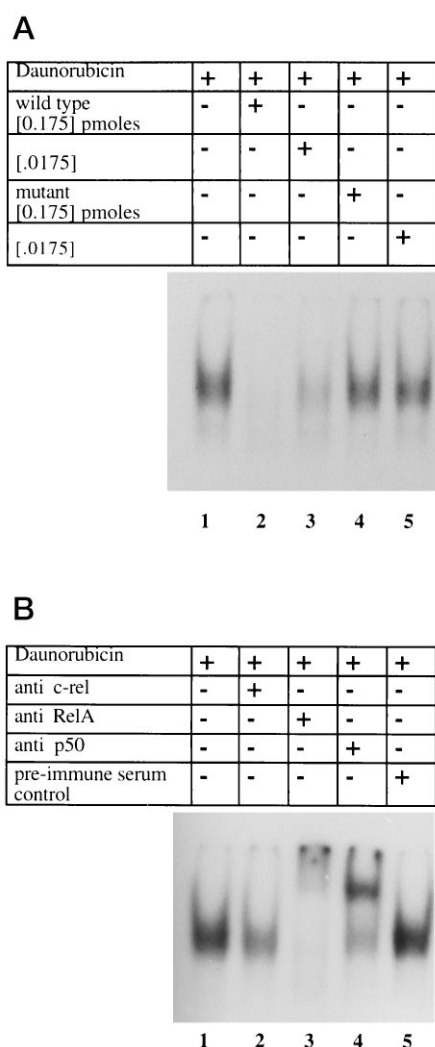


FIG. 2. Activated NF κ B complexes from daunorubicin-treated HL-60 cells contain p50 and RelA subunit components and are specific for binding to κ B-motif. A, nuclear extracts from daunorubicin (0.25 μ M)-treated HL-60 cells (lane 1) were incubated with non-radioactive wild type (lanes 2 and 3) and mutant probes (lanes 4 and 5) for the κ B consensus motif (concentrations indicated) at room temperature for 30 min prior to the addition of labeled probe. NF κ B-DNA complexes are shown. B, nuclear extracts from daunorubicin (0.25 μ M)-treated HL-60 cells (lane 1) were incubated with antibodies to c-Rel (lane 2), p65/Rel A (lane 3), and p50 (lane 4) subunit components for 30 min on ice prior to the addition of labeled NF κ B probe. Preimmune serum was employed as control (lane 5). Complexes were resolved as described under "Experimental Procedures." The position of supershifted complexes is indicated. Results are representative of two experiments.

nism, suggesting the involvement of ROS, and not activated ceramide synthase. The importance of this signal for daunorubicin-mediated apoptosis is discussed.

EXPERIMENTAL PROCEDURES

Materials—HL-60 and Jurkat T cells (both obtained from the European Collection of Animal Cell Culture (ECACC, Salisbury, United Kingdom) were grown in suspension culture in RPMI 1640 supplemented with 10% fetal calf serum, penicillin/streptomycin (100 units/ml and 100 mg/ml, respectively), and L-glutamate (2 mM final concentration), all obtained from Life Technologies, Inc. (Paisley, United Kingdom). Recombinant human TNF α was a gift from Zeneca Pharmaceuticals Ltd., Macclesfield, United Kingdom. Mitoxantrone was also a generous gift from Wyeth-Ayerst Research (United Kingdom). Poly(dI-dC) was from Pharmacia Biosystems (Milton Keynes, United Kingdom), T4 polynucleotide kinase and oligonucleotide containing the consensus sequence (5'-GGGACTTTC-3'), corresponding to the κ -light chain enhancer motif, were purchased from Promega (Southampton,

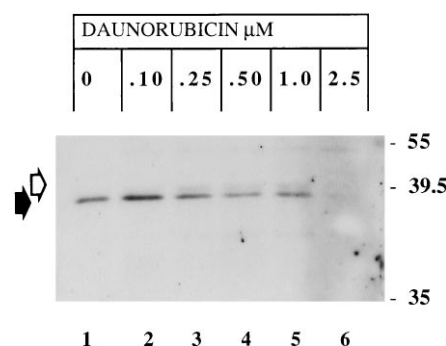


FIG. 3. Daunorubicin induces I κ B degradation in HL-60 cells. HL-60 cells (1×10^6 /ml) were treated with either daunorubicin (lanes 2–5), or an equivalent volume of vehicle control (medium) (lane 1) for 4 h. Cell lysates were prepared following stimulation and analyzed for I κ B degradation as described under "Experimental Procedures." Closed and open arrowheads indicate the position of the I κ B inhibitor protein and its phosphorylated form, respectively. Molecular mass markers are shown in kilodaltons. Results are representative of three separate experiments.

United Kingdom). [γ - 32 P]ATP (3000 Ci/mmol), [14 C]chloramphenicol (56 mCi/mmol), [14 C]palmitoyl-coenzyme A (55 mCi/mmol), and ECL reagent were from Amersham (Aylesbury, United Kingdom). Diacylglycerol kinase was from Calbiochem (United Kingdom). Rabbit polyclonal antibody preparations to the DNA-binding subunits of NF κ B (c-Rel and RelA) and the inhibitor protein I κ B α were from Santa Cruz Biotechnology Inc. Mutant NF κ B oligonucleotide was also from Santa Cruz. An antiserum to the p50 subunit of NF κ B was a generous gift from Dr. Jean Imbert (INSERM, Marseille). All other reagents were purchased from Sigma (Poole, Dorset, United Kingdom) unless otherwise stated.

Cell Culture—For treatments, cells in late log phase of growth were resuspended in fresh medium at a concentration of 1×10^6 /ml and incubated at 37 $^{\circ}$ C in a humidified atmosphere of 5% CO $_2$, 95% air. Where required, cells were preincubated with inhibitors (fumonisin B1 and PDTC for 60 min, and desferal for 16 h) prior to the addition of drug (4 h). Following stimulation, incubations were discontinued by the addition of ice-cold phosphate-buffered saline, and either nuclear or whole cell extracts were prepared as described previously (24). Protein determinations were made using the Bradford assay with bovine albumin as standard.

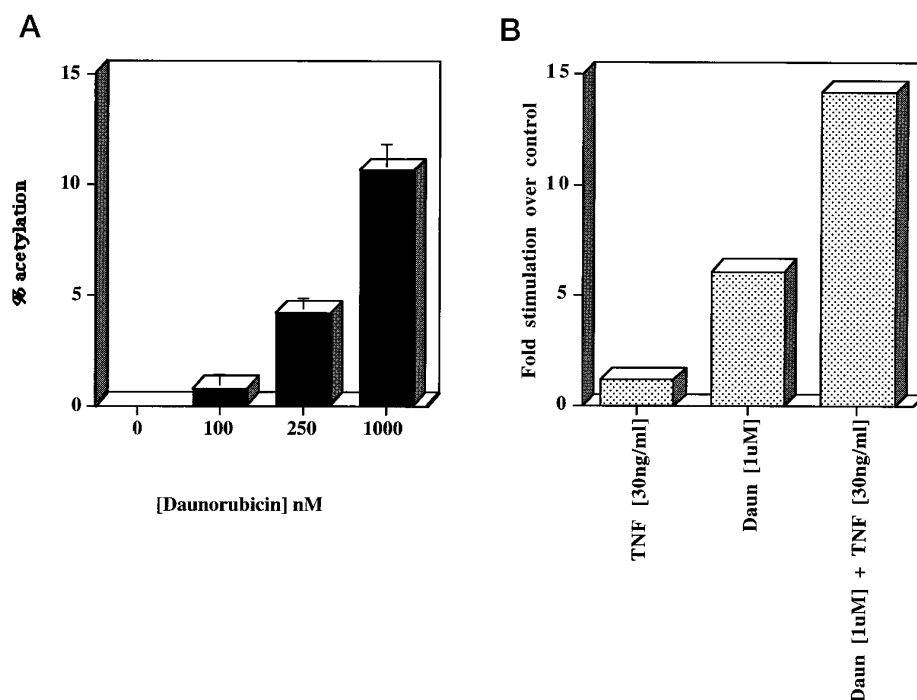
Transfection Studies—The transactivating potential of activated NF κ B complexes was assessed following transfection of cells (25) with a plasmid containing five NF κ B consensus sequences upstream of a chloramphenicol acetyltransferase reporter gene (pCATTM-Promoter plasmid, a gift from Dr. Tim Bird, Immunex Corp., Seattle, WA). Following treatment (indicated in legends), extracts prepared from harvested cells were assayed for CAT activity as described previously (26). Statistical significance was evaluated by employing Student's *t* test for unpaired data.

Electrophoretic Mobility Shift Assays—Nuclear NF κ B was assessed by the electrophoretic mobility shift assay using a 22-base pair oligonucleotide containing the human κ -light chain enhancer motif, which had previously been end-labeled with [γ - 32 P]ATP as described (24). Typically, 4 μ g of nuclear extract protein was incubated with radiolabeled oligonucleotide (10,000 cpm) at room temperature for 30 min using conditions as described previously (24). NF κ B complexes were resolved on 5% acrylamide gels and identified following autoradiography. To identify the subunit components of activated NF κ B complexes, supershift analysis was carried out where extracts from treated cells were preincubated with antibody preparations to p50, RelA (p65), and c-Rel subunit components on ice for 30 min prior to the addition of labeled probe. A similar protocol was employed in competition studies (incubations were at room temperature), where mutant and wild type NF κ B consensus sequence were assessed for their ability to block binding of activated complexes to labeled wild type NF κ B probe.

Western Blot Analysis—Equal amounts of whole cell lysate protein (as indicated) were resolved by SDS-polyacrylamide gel electrophoresis, transferred onto nitrocellulose, and I κ B α immunoblot analysis was performed as described previously (24). Secondary antibody was used at a dilution of 1:400. The blots were developed by ECL according to the manufacturers recommendations.

Lipid Studies—Ceramide was quantified by the diacylglycerol kinase assay as described (7), with some modifications. In brief, following

FIG. 4. A, daunorubicin stimulates expression of CAT activity in Jurkat T cells transfected with an NF κ B-linked reporter plasmid. Transfected Jurkat T cells (1×10^6 /ml) were incubated with daunorubicin for 24 h (concentrations indicated). Cell lysates were prepared and assayed for CAT activity as described under "Experimental Procedures." Results are the mean $\pm$ S.D. for a single experiment (triplicate samples), which is representative of three separate experiments. B, daunorubicin and TNF synergize in stimulating CAT activity in Jurkat T cells transfected with an NF κ B-linked reporter plasmid. Transfected Jurkat T cells (1×10^6 /ml) were incubated with daunorubicin (1.0 μ M) for 30 min prior to the addition of TNF (30 ng/ml). After 24 h, cell lysates were prepared and assayed for CAT activity as described under "Experimental Procedures." Results are represented as fold stimulation over control levels and are from an experiment representative of three separate experiments.



stimulation, cell pellets were extracted with 600 μ l of chloroform, methanol, 1 N HCl (100:100:1, v/v/v). Following alkaline hydrolysis (1 h at 37 $^{\circ}$ C), re-extracted samples were dried down and redissolved in 50 μ l of reaction buffer (7). The reaction was started by the addition of 40 μ g/ml (4 milliunits/ml) *Escherichia coli* diacylglycerol kinase followed closely by 10 μ Ci of [γ - 32 P]ATP. Reaction termination was as described following incubation at room temperature for 90 min. The level of ceramide was determined by comparison with a standard curve generated with known amounts of ceramide (ceramide type III; Sigma).

Ceramide Synthase Assay—This activity was measured in HL-60 microsomal membranes as described previously (7). In general, 50×10^6 cells were pelleted following drug treatment and disrupted in 300 μ l of homogenization buffer by repeatedly passing through a 26-gauge needle. Microsomal membrane protein (37.5 μ g) was incubated in a 250- μ l reaction volume/mixture as described (7) with dihydrosphingosine as substrate. The reaction was started by the addition of 3.6 μ M (0.2 μ Ci) [14 C]palmitoyl-coenzyme A, the incubation was allowed to proceed for 1 h at 37 $^{\circ}$ C, and stopped by extraction with an equal volume of chloroform/methanol (2:1, v/v). The substrate concentrations chosen were based on those reported to allow maximal enzyme activity to be monitored (7), with 100 μ M dihydrosphingosine being optimal. Following TLC as described (7), radioactivity corresponding to synthesized dihydroceramide was determined using an InstantImagerTM (Packard Instrument Co., Meriden, CT).

RESULTS

Daunorubicin Activates NF κ B in HL-60 Promyelocytic Leukemia and Jurkat T Lymphoma Cells—Treatment of both HL-60 and Jurkat T cells with the anthracycline antibiotic, daunorubicin, resulted in the activation of NF κ B, which was dose-dependent and time-responsive (Fig. 1A, C, and D). Fig. 1A illustrates data obtained with HL-60 cells, where activation of NF κ B is demonstrated by the appearance of DNA-protein complexes. The activation was time-dependent occurring from 1 to 4 h and was sustained up to 24 h (Fig. 1A). This was in contrast to that seen with TNF, where the activation of NF κ B was rapid occurring within minutes of cellular stimulation (Fig. 1B). Concentrations of daunorubicin employed were similar to those previously reported to induce apoptosis in this cell line (7), with activation being apparent at 0.05 μ M, and peaking at 0.5 μ M (Fig. 1C). These concentrations also paralleled that reported for ceramide elevation induced by this drug (7). Data for TNF (0.6 and 2.5 ng/ml) are shown in Fig. 1C for comparison purposes. A less potent induction was observed in Jurkat T

cells (Fig. 1D) where weak activation could be detected at 0.125 μ M and a strong signal observed at 2.5 μ M daunorubicin.

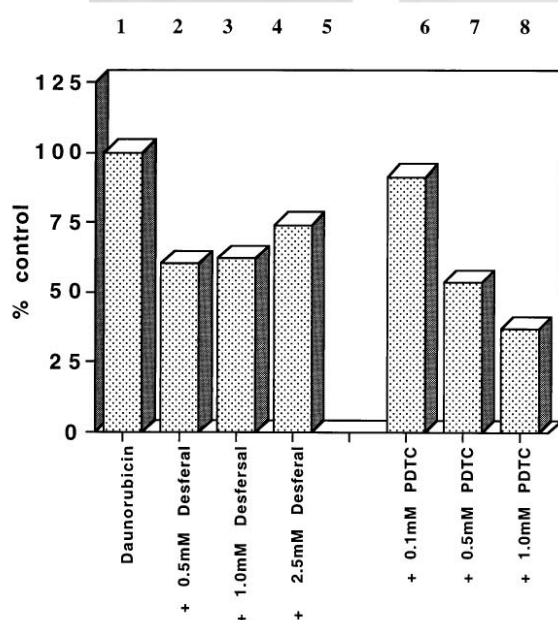
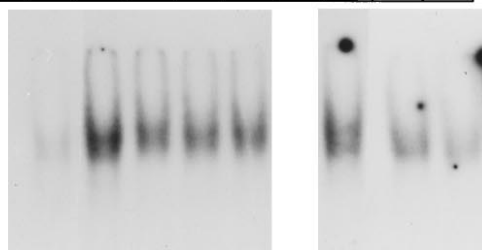
The binding specificity of activated complexes was demonstrated by competition studies in which unlabeled oligonucleotide containing NF κ B consensus sequence inhibited the appearance of retarded complexes, whereas a mutant oligonucleotide had no effect at equivalent concentrations (Fig. 2A). Analysis of specific subunit components in activated NF κ B complexes revealed the presence of p50 and to a lesser extent p65/RelA as indicated by enhanced retardation of labeled complexes following gel electrophoresis (Fig. 2B). Although supershifted complexes were not seen with anti-c-Rel antibodies, a weaker signal when compared with control lanes suggested the presence of this subunit component in the complex (Fig. 2B, lane 2).

Daunorubicin Induces I κ B α Degradation—HL-60 cells treated with daunorubicin at doses which resulted in NF κ B activation were examined for degradation of the inhibitor protein, I κ B α , a critical event in the activation of this transcription factor (12). A marked degradation of this inhibitor protein was observed which was dose-responsive (Fig. 3). In support of the proposed model for I κ B α degradation, a doublet was observed prior to degradation (*open arrow*), most probably corresponding to the phosphorylated form of this protein, which is a signal for its degradation (15). At the highest concentrations of daunorubicin employed (2.5 μ M), I κ B α degradation was complete (Fig. 3, lane 6).

Daunorubicin Stimulates κ B-driven Gene Expression—Following transfection of Jurkat T cells with a CAT reporter gene construct containing five NF κ B sites upstream of a chloramphenicol acetyltransferase (CAT) reporter gene, the effects of daunorubicin on κ B-dependent gene expression were investigated. Daunorubicin induced expression of CAT activity in a dose dependent fashion (Fig. 4A). At 0.25 μ M daunorubicin, a concentration previously shown to activate NF κ B, CAT activity was increased 4-fold over control values (unstimulated cells), indicating that induced complexes were transcriptionally active. In addition, daunorubicin and TNF were found to synergize in this response (Fig. 4B). Combining a concentration of TNF which was marginally inducing (1.2-fold over control) with

A

Daunorubicin	-	+	+	+	-	-	+	+	+
Desferal [mM]	-	-	0.5	1.0	2.5	-	-	-	-
PDTC [mM]	-	-	-	-	-	-	0.1	0.5	1.0



B

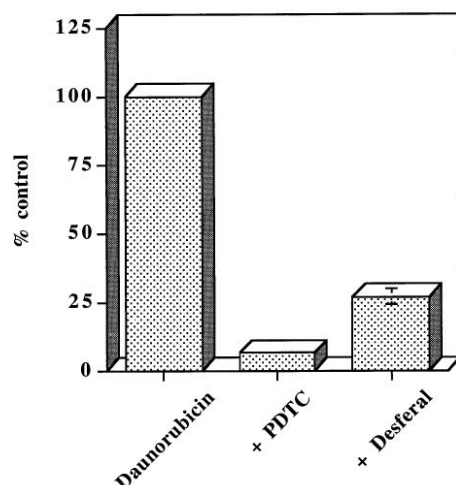


FIG. 5. PDTC and desferal inhibit daunorubicin-mediated NF κ B activation and κ B-linked gene expression. A, HL-60 cells (1×10^6 /ml) were preincubated with either desferal for 16 h (lanes 3–5) or PDTC for 1 h (lanes 6–8) (concentrations indicated), prior to the addition of daunorubicin (0.25 μ M). Nuclear extracts were prepared and assayed for NF κ B as described under “Experimental Procedures.” NF κ B-DNA complexes are shown. Densitometric analysis was performed on autoradiograms corresponding to the experiment shown by UVP transillumination scanning. Quantitation was by Gelworks 1D Advanced™ software where inhibition is represented as % of control value (daunorubicin only). B, Jurkat T cells (1×10^6 /ml) transfected with an NF κ B-linked reporter plasmid were incubated with either PDTC (1 mM) or desferal (2.5 mM) for 30 min prior to the addition of

a concentration of daunorubicin inducing a 6-fold increase in CAT activity, resulted in a 14-fold induction. This synergy suggests that TNF and daunorubicin activated NF κ B by different pathways, as was previously suggested from the different time courses of activation observed for these two agonists (Fig. 1, A and B).

Activation of NF κ B by Daunorubicin Is Inhibited by PDTC and Desferal—We next investigated the mechanism by which daunorubicin activates NF κ B. In cells pretreated with the metal chelators desferal and PDTC (which also has anti-oxidant properties), activation was inhibited (Fig. 5A), as indicated by a diminished signal corresponding to activated complexes. Desferal (1 mM) inhibited the response by 38% with no further inhibition being observed at higher doses (lanes 3–5). PDTC was the more potent inhibitor of the two at equivalent concentrations employed, inhibiting the response by 63% at 1 mM (lane 8). PDTC and desferal were also found to inhibit the daunorubicin-mediated increase in κ B-dependent CAT expression (Fig. 5B).

Mitoxantrone Activates NF κ B in HL-60 in a PDTC-sensitive Manner—We next determined whether the mechanism of NF κ B activation involved redox cycling of daunorubicin. For this purpose, we used a closely related anthraquinone, mitoxantrone, which does not undergo redox cycling (27, 28). Mitoxantrone was found to be as potent an activator of NF κ B in HL-60 as daunorubicin (Fig. 6A), with an effect being evident from 0.05 μ M. In addition, PDTC was found to inhibit this activation, with 1 mM completely blocking the response induced by 1.0 and 0.2 μ M mitoxantrone (Fig. 6B). These results suggested that activation of NF κ B by daunorubicin involved the generation of ROS via endogenous cellular processes rather than through redox cycling of the drug *per se*.

The Ceramide Synthase Inhibitor Fumonisin B1 Does Not Block NF κ B Activation by Daunorubicin—Finally, we tested the effect of a specific ceramide synthase inhibitor, fumonisin B1, for its ability to block daunorubicin-induced NF κ B activation at a range of concentrations previously reported to block apoptosis induced by this drug (7). Fumonisin B1 failed to inhibit NF κ B activation at all concentrations tested (Fig. 7A) and, furthermore, no inhibition of daunorubicin-mediated CAT activation was observed (data not shown). However, treatment of HL-60 cells with daunorubicin (0.5 and 10 μ M) for 4 h increased ceramide levels, as shown in Fig. 7B, with 10 μ M causing a 2.7-fold increase over controls. This effect was inhibited by fumonisin B1 (300 μ M), with ceramide levels being reduced to that in unstimulated cells. Furthermore, treatment of HL-60 cells with daunorubicin (10 μ M) for 4 h was found to increase microsomal ceramide synthase activity more than 2-fold over controls. 300 μ M fumonisin B1 inhibited this activity, reducing it to below control levels, which was consistent with its ability to act as a competitive inhibitor toward dihydrosphingosine (Fig. 7B). This confirmed a previous report where induction of ceramide synthase was found to mediate increases in ceramide levels in cells in response to daunorubicin (7). However, our data indicated that this process was not involved in NF κ B activation here.

We therefore concluded that increases in ROS, generated by endogenous cellular processes, but not ceramide synthase induction, were mediating the effect of daunorubicin on NF κ B activation and κ B-driven gene expression.

daunorubicin (24 h). Cell lysates were prepared and assayed for CAT activity as described under “Experimental Procedures.” Results are representative of three separate experiments (triplicate samples), and inhibition is expressed as percentage (mean $\pm$ S.D.) of control (daunorubicin only).

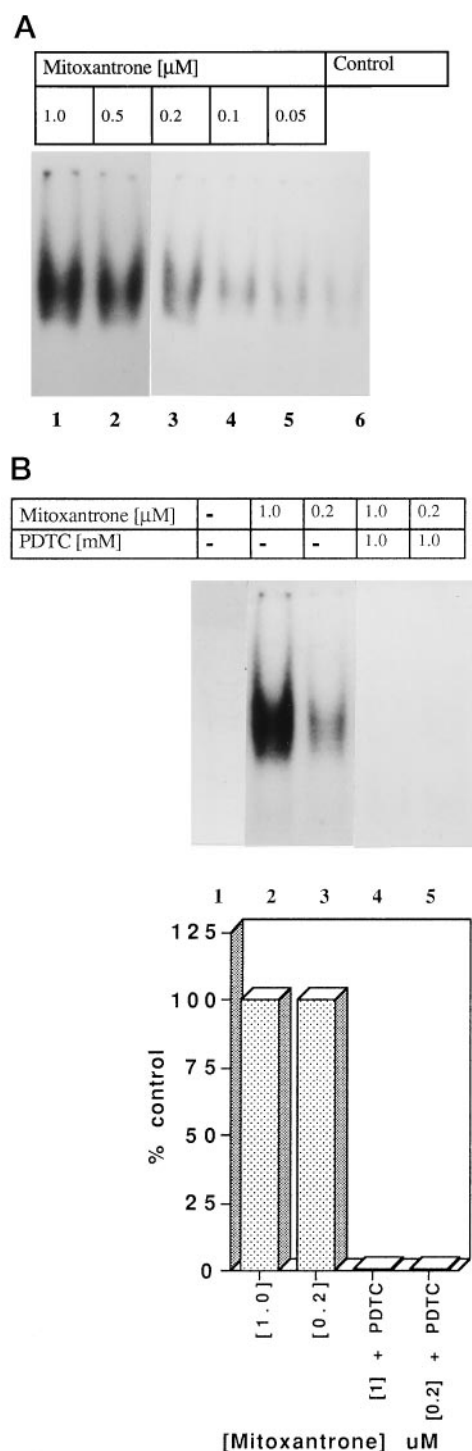


FIG. 6. Mitoxantrone activates NF κ B in HL-60 promyelocytic leukemia cells by a PDTC-sensitive mechanism. A, HL-60 cells (1×10^6 /ml) were treated with mitoxantrone (concentrations indicated, lanes 1–5) or an equivalent volume of vehicle control (medium) (lane 6) for 4 h. B, HL-60 cells (1×10^6 /ml) were preincubated with PDTC (1.0 mM) for 1 h (lanes 4 and 5) prior to the addition of mitoxantrone (1.0 and 0.2 μ M) for 4 h (lanes 2 and 3 represent drug treatments in the absence of PDTC). Nuclear extracts were prepared and assessed for NF κ B as described under “Experimental Procedures.” NF κ B-DNA complexes are shown. Densitometric analysis was performed on autoradiograms corresponding to the experiment shown by UVP transillumination scanning. Quantitation was by Gelworks 1D Advanced™ software where inhibition is represented as % of control value (mitoxantrone only).

DISCUSSION

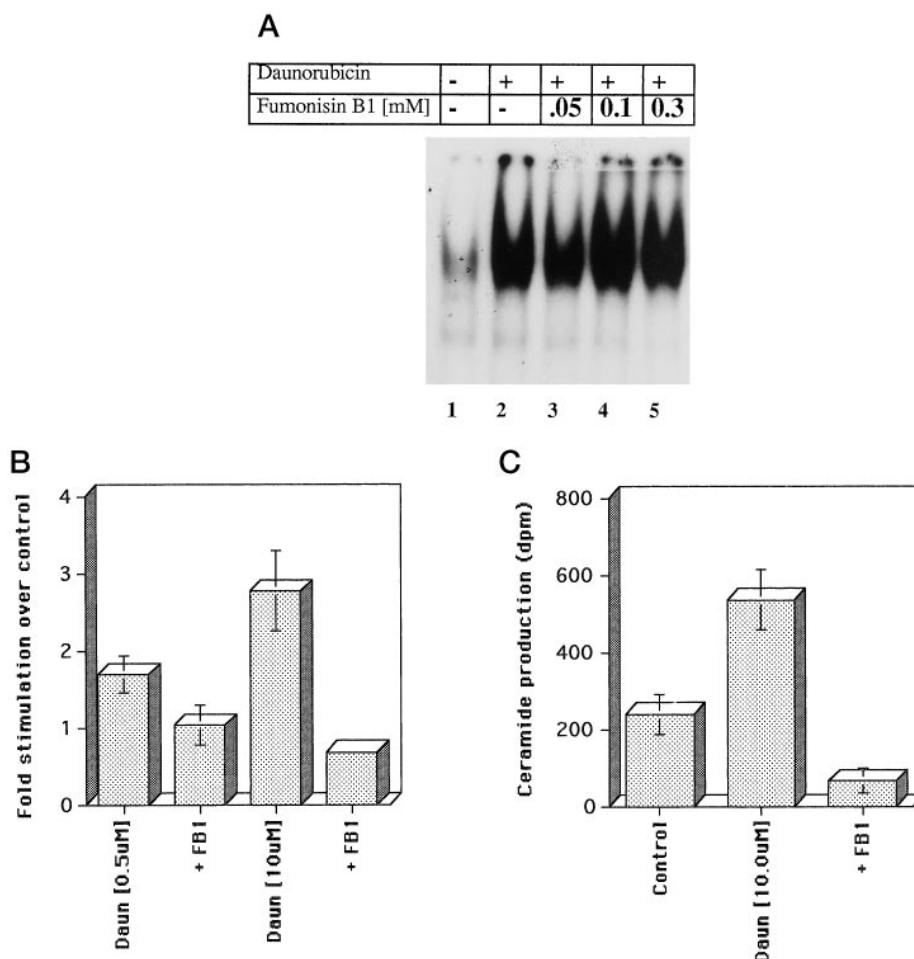
In this study, we present the report that daunorubicin, an anthracycline antibiotic, activates NF κ B. The significance of

this effect was first examined in terms of its transcriptional potential. In this regard, it is established that the dimer composition of the NF κ B complex determines its fine DNA-binding specificity (13), giving rise to selective transcriptional activation or attenuation, the latter of which has been observed with non-transactivating p50 homodimeric forms (29). Transcriptional activation of specific sets of genes will primarily depend on various dimer combinations being activated distinctly, or whether their relative amounts in cell types and tissues are subject to regulation. p50, RelA (p65), and c-Rel are the major components of NF κ B complexes (12), binding to most of the identified cis-acting κ B sites. Analysis of subunits present in activated complexes from daunorubicin-treated cells indicated the presence of both RelA and p50 components, which together constitute the predominant transcriptionally active NF κ B dimer combination. The ability of these activated complexes to promote transactivation was confirmed in a reporter gene assay, where treatment of cells with daunorubicin stimulated activity from a transfected NF κ B-linked reporter plasmid in a dose-responsive manner. Furthermore, in line with the classical model of NF κ B activation (12), I κ B phosphorylation and degradation was demonstrated at concentrations which paralleled those shown to activate NF κ B and stimulate transcription.

We considered the role of oxidative stress and ROS as second messengers in daunorubicin-mediated NF κ B activation. In this regard, NF κ B is considered to be an oxidative stress-responsive transcription factor (17) and interestingly, daunorubicin itself can be reductively metabolized to a semi-quinone radical intermediate (2) which further participates in reactions which give rise to ROS. The single-electron reduction of daunorubicin is catalyzed by a number of cellular enzymes, including cytochrome P450, the flavin NADPH-cytochrome p450 reductases, NADH-cytochrome b_5 reductase, and mitochondrial NADH dehydrogenase (2). The resulting semiquinone is highly reactive and in the presence of O_2 rapidly autoxidizes to the parent quinone with concomitant production of superoxide anion radical. Furthermore, ROS are generated through its participation in futile/redox cycling. Reactive oxygen species have previously been implicated in the mechanism of NF κ B activation in response to cytokines, phorbol esters, and bacterial toxins (17, 18). Conclusions have been drawn from studies using modulators of signaling events with antioxidant and metal chelating properties (18, 19, 21), and the overexpression of enzymes such as catalase and superoxide dismutase (20). To investigate the mechanism of NF κ B activation by daunorubicin, compounds were employed which included PDTC which has both antioxidant and metal chelating properties, and previously has been shown to inhibit the activation of NF κ B mediated by H_2O_2 (19). Another inhibitor utilized, deferoxamine (desferal), can interfere with the production of oxygen radicals, in particular OH radicals generated in the presence of catalytic amounts of transition metals (the Fenton reaction), by preferentially chelating iron ions (30). Both compounds inhibited daunorubicin-induced NF κ B activation and κ B-linked gene expression. The more potent inhibition observed with PDTC at equivalent concentrations may be due to additional antioxidant properties. Interestingly, desferal has previously been shown to reduce the growth inhibitory effects of daunorubicin to cells (2), possibly suggesting that NF κ B-mediated transcriptional regulation might in some way participate in the growth inhibitory effects of this compound.

Further evidence for ROS involvement in the effect of daunorubicin came from time course studies where NF κ B activation was significantly slower when compared with that obtained

FIG. 7. Effect of fumonisins B1 (FB1) on daunorubicin-mediated NF κ B activation and ceramide generation. A, HL-60 cells (1×10^6 /ml) were preincubated with fumonisins B1 for 1 h (lanes 3–5) (concentrations indicated), prior to the addition of daunorubicin ($0.25 \mu\text{M}$) for 4 h. Nuclear extracts were prepared and assessed for NF κ B as described under "Experimental Procedures." NF κ B-DNA complexes are shown. Results are representative of three separate experiments. B, HL-60 cells (1×10^6 /ml) were treated with daunorubicin for 4 h in the presence and absence of fumonisins B1 ($300 \mu\text{M}$, 1-h preincubation). Extracts were prepared and ceramide levels measured by the DAG kinase procedure as described under "Experimental Procedures." Labeled ceramide was quantitated following TLC, where data is representative of two to three separate experiments (mean $\pm$ S.E.). Baseline ceramide levels in non-stimulated cells were determined to be $89.9 \pm 15.5 \text{ pmol}/10^6$ cells. Cells were treated with TNF (100 ng/ml) for 1 h as positive control, where ceramide levels were stimulated 2.64 ± 0.29 -fold over control values. C, [^{14}C]dihydroceramide was resolved by TLC, following assay of ceramide synthase activity in microsomal membrane preparations of HL-60 cells treated with daunorubicin ($10 \mu\text{M}$), as described under "Experimental Procedures." Activity was quantitated by InstantImagerTM (Packard Instrument Co., Meriden, CT) scanning. Inhibition by fumonisins B1 was assessed by its inclusion ($300 \mu\text{M}$) in the *in vitro* assay. Data (mean $\pm$ S.D.) are representative of three separate experiments.



with the cytokine, TNF, with enhanced nuclear complexes only being detected 1–2 h post-treatment. However, this time course for NF κ B activation is similar to that exhibited by H_2O_2 in endothelial and epithelial cells (31, 32), suggesting that a similar signaling pathway for NF κ B activation might be mediated by agents which directly generate ROS. Furthermore, we found that daunorubicin and TNF could synergize in the induction of a κ B-linked reporter gene. The basis for this is unclear, but suggests that both agents activate NF κ B by different mechanisms, as was also indicated from time course studies.

Interestingly, the photosensitizer, proflavine, can activate NF κ B in a time course which mirrors that previously evinced by H_2O_2 (32) and it has been suggested that DNA oxidative damage might initiate a signaling event (distinct from that initiated by cytokines) which promotes translocation of NF κ B complexes resident in the cytoplasm into the nucleus. A similar effect may be occurring with daunorubicin-mediated NF κ B activation. A number of agonists which have also been shown to activate NF κ B directly, for example, ionizing radiation, TNF, and oxidative stress, can also induce DNA strand breakage in treated cells (33). It is possible that this damage may be a signal for the later activation of specific NF κ B complexes, as has been previously suggested (32).

Although enzyme-mediated daunorubicin free-radical formation may have been the source of ROS which activated NF κ B, it was also possible that endogenous cellular processes were responsible for ROS generation. To test this, we examined a closely related compound, mitoxantrone, which, although structurally similar to daunorubicin, does not undergo redox cycling (27, 28). It was developed with the intention of maintaining the DNA-complexing ability of doxorubicin, but reduc-

ing systemic side effects such as cardiotoxicity, the cause of which is purported to be ROS generation via redox cycling (1). Mitoxantrone was found to be as potent at activating NF κ B as daunorubicin, and was even more susceptible to inhibition by PDTC. This suggested that the mechanism of NF κ B activation by daunorubicin (and mitoxantrone) involved generation of ROS, not through redox cycling of the drug, but through cellular events activated by the compounds leading to oxidative stress. The precise source of the ROS awaits determination, as is the case for several other activators of NF κ B which are sensitive to antioxidant inhibition.

Other studies have questioned the role of redox cycling in daunorubicin's cytotoxic effects, with mitoxantrone being more potent than the daunorubicin analogue, doxorubicin, in this regard (27). It therefore appears that redox cycling may not be critical for either cytotoxicity or NF κ B activation induced by such anthraquinones. Mitoxantrone is a potent inducer of DNA strand breakage (27). While this has been implicated in mediating its cytotoxic effects (27), it may also be a signaling event leading to NF κ B activation, analogous to that proposed above for daunorubicin.

The time course of NF κ B activation by daunorubicin paralleled that reported in a previous study for ceramide elevation induced by this drug (7). Bose *et al.* (7), employed a mycotoxin, fumonisins B1, which is a specific inhibitor of ceramide synthase, to demonstrate that this enzymic activity was responsible for daunorubicin-induced ceramide elevation. However, in our studies, fumonisins B1 did not block daunorubicin-mediated NF κ B activation. This result suggested that ceramide reportedly generated during apoptotic induction by this drug was not responsible for NF κ B activation, although it is an established

inducer of this event (11). We were, however, able to demonstrate that under conditions where fumonisins B1 failed to inhibit NF κ B activation, increases in ceramide induced by daunorubicin in HL-60 were blocked. In addition, fumonisins B1 inhibited ceramide synthase activity in microsomal extracts from daunorubicin-treated HL-60 cells. This confirmed results from a previous study which demonstrated that daunorubicin increases ceramide in cells through an induction of ceramide synthase activity (7). In another study, it has been shown that daunorubicin activates neutral sphingomyelinase activity and that this is responsible for the ceramide increase in response to clinically relevant doses of daunorubicin (8). Furthermore, in direct contradiction to the report by Bose *et al.* (7), they failed to demonstrate inhibition by fumonisins B1 of apoptosis induced by daunorubicin. The basis for these inconsistencies is unclear. In our study, any increases in ceramide were abolished by fumonisins B1, implying that ceramide synthase is the enzyme responsible for such increases. As it was only with higher doses of daunorubicin that we observed increases in ceramide and ceramide synthase activity, it was possible, although unlikely, that the NF κ B activation evident at lower doses of daunorubicin was mediated via an undetectable rise in ceramide occurring as a result of sphingomyelinase activation. We have concluded from our findings that ceramide was unlikely to be important in the effect of daunorubicin on NF κ B. This conclusion is consistent with observations indicating that ceramide is unlikely to be an important signal for other activators of NF κ B such as TNF (34).

Another recent study questions the importance of ceramide synthase in the apoptotic effect of daunorubicin. Doses of this agent used to induce the enzyme are suggested to be above therapeutic concentrations (5) and in the same paper, the investigators show that a closely related analogue, doxorubicin, induced apoptosis via FAS ligand (5) which like daunorubicin has been shown to activate sphingomyelinase (35). The effective concentration range of daunorubicin employed in our study was in agreement with that reported to induce apoptosis (7) and the suggested therapeutic plasma concentrations for the closely related analogue, doxorubicin (5), underlining the potential clinical relevance of our observations. The precise role of ceramide synthase in the induction of apoptosis by daunorubicin therefore awaits clarification, although as stated our study indicates that it is not involved in NF κ B activation.

It is tempting to speculate that increased expression of genes regulated by NF κ B in response to daunorubicin may be involved in daunorubicin-mediated apoptosis. One candidate gene would be *c-myc* which plays a pivotal role in the induction of apoptosis (36). Its expression has been shown to be regulated in response to different hetero- and homodimeric NF κ B complexes (29) and studies are consistent with the possibility that its overexpression might be related to apoptotic induction (37). It has also been proposed that c-Rel, which is present in the NF κ B complex, may function in the activation of a set of death genes where its elevated expression was shown to coincide with the onset of apoptosis (38).

Other chemotherapeutic agents have been shown to activate NF κ B. For example, the deoxycytidine analogue, ara-C, has been reported to activate NF κ B, via neutral sphingomyelinase (39). Similar to daunorubicin, it was also found to induce NF κ B-linked gene expression independently at a concentration which correlated with its ability to activate this transcription factor. The DNA alkylating agents, mitomycin C, has recently been shown to activate NF κ B (40, 41) by a novel mechanism involving enhanced nuclear processing of p105 in Epstein-Barr Virus-immortalized B cells (40). NF κ B activation may, therefore, be a common mechanism for apoptosis-inducing anti-neo-

plastic agents. It is also possible, however, that NF κ B activation represents an anti-apoptotic response. This has been convincingly demonstrated in three recent reports. Studies in cells from transgenic mice deficient in p65/RelA, or in cells where NF κ B is inhibited demonstrated enhanced apoptosis in response to a range of agents including daunorubicin (42, 43, 44). In addition, the p65/RelA-deficient mice exhibited massive liver degeneration by apoptosis (45). Significantly, other mouse tissues did not show enhanced apoptosis. Drug resistance is frequently associated with altered expression of certain xenobiotic-metabolizing enzymes in the liver and NF κ B may play a role in regulating the expression of such proteins, as has been suggested (46). A shift in the balance between apoptosis and NF κ B could therefore determine whether cells survive or die and so the study of a functional link between NF κ B-mediated transcriptional activation and apoptotic induction or inhibition may provide important information on anthracycline antitumor efficacy.

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