American Journal of Pathology, Vol. 145, No. 4, October 1994

Dexamethasone Inhibits Induction of Liver Tumor Necrosis Factor-α mRNA and Liver Growth Induced by Lead Nitrate and Ethylene Dibromide

G.M. Ledda-Columbano,* A. Columbano,* A. Cannas,* G. Simbula,* K. Okita,† K. Kayano,‡ Y. Kubo,‡ S.L. Katyal,‡ and H. Shinozuka‡

From the Institute of Experimental Pathology, University of Cagliari, Cagliari, Italy; First Department of Internal Medicine,† Yamaguchi University School of Medicine, Ube, Japan; and Department of Pathology,‡ School of Medicine, Pittsburgh, Pennsylvania*

We have recently demonstrated that a single injection of the mitogen lead nitrate to rats induced a rapid increase of tumor necrosis factor-α (TNF-α) mRNA in the liver and suggested that this cytokine may be involved in triggering hepatocyte proliferation in this model of direct hyperplasia. In this study, we examined whether a similar induction of liver TNF-α mRNA could be observed preceding the onset of hepatocyte proliferation induced by ethylene dibromide, another hepatocyte mitogen. In addition, we used dexamethasone, a well known inhibitor of TNF-α production, to determine whether its administration could suppress hepatocyte proliferation induced by lead nitrate and ethylene dibromide. A single intragastric administration of ethylene dibromide (100 mg/kg) to male Wistar rats enhanced liver TNF-α mRNA after 4 and 7 hours, which then returned to control levels by 24 hours. TNF-α mRNA was detectable only in a nonparenchymal cell fraction of the liver. Pretreatment of rats with a single dose of dexamethasone (2 mg/kg) 60 minutes before lead nitrate (100 μmol/kg) or ethylene dibromide completely abolished the increased levels of liver TNF-α mRNA induced by these agents. Inhibition by dexamethasone of TNF-α mRNA was associated with an inhibition of liver cell proliferation induced by these mitogens, as measured by [³H]thymidine incorporation into hepatic DNA, mitotic index, and DNA content. These results further support the hypothesis that TNF-α may be involved in triggering hepatocyte proliferation induced by primary mitogens. (Am J Pathol 1994, 145:951–958)

Hepatocyte growth factor (HGF), transforming growth factor-α (TGF-α), and TGF-β are the three most extensively studied growth factors in the control mechanisms of liver cell regeneration.[1–3] The former two are potent mitogens for hepatocytes in culture and the latter an inhibitor of hepatocyte proliferation.[4–7] Studies *in vivo* have provided further evidence for the role of these growth factors in the control of liver cell proliferation. In fact, the time course of appearance of HGF, TGF-α, and TGF-β in the blood and the enhanced expression of their genes in the liver coincided with the time course of initiation and termination of hepatocyte proliferation in several models of compensatory cell proliferation, including surgical partial hepatectomy or regeneration after administration of necrogenic agents.[8,9]

Recently, however, it has been shown that another type of cell proliferation, ie, direct hyperplasia induced by primary mitogens, is not associated with any significant changes in the hepatic levels of HGF or TGF-α mRNAs.[10,11] Instead, direct liver hyperplasia induced by a single administration of lead nitrate (LN) resulted in a rapid and transient increase in the hepatic expression of TNF-α mRNA during a period

Supported by grants from CNR (PF ACRO), Associazione Italiana Ricerca sul Cancro, MURST, Assessorato Programmazione, Sardegna, Italy, National Institutes of Health (CA 26556 and CA 53453), and Pathology Education Research Foundation, Pittsburgh, Pennsylvania.

Accepted for publication June 29, 1994.

Address reprint requests to Dr. G.M. Ledda-Columbano, Instituto di Patologia Sperimentale, Universita di Cagliari, via Porcell 4, 09124, Cagliari, Italy.

preceding the onset of hepatocyte DNA synthesis.[11] Based on this observation, we suggested that TNF-α may be involved in triggering hepatocyte proliferation induced by primary mitogens.

TNF-α is a monokine thought to be a major regulatory cytokine in several pathophysiological processes and is known to stimulate induction of a number of genes including lymphokines and growth factors,[12,13] which may act as modulators of liver cell proliferation. Indeed, increasing evidence suggests that TNF-α may play an important role in triggering cell proliferation. As far as hepatocytes are concerned, it was shown that TNF-α induces DNA synthesis of mouse and rat hepatocytes in primary cultures.[14,15] In addition, administration *in vivo* of nonnecrogenic dosages of TNF-α increased thymidine incorporation into hepatic DNA and mitotic activity of liver cells in intact adult rats, although some controversy exists as to the nature of the primary target cells in the liver stimulated to proliferate by TNF-α.[16–18] Furthermore, there are reports suggesting that TNF-α, in addition to the known growth factors, may also participate in the triggering of compensatory liver regeneration such as after partial hepatectomy.[19,20]

To clarify further possible roles of TNF-α on hepatocyte proliferation induced by primary mitogens, we extended our earlier study to determine whether ethylene dibromide (EDB), another known liver mitogen, induces an induction of TNF-α mRNA before the onset of hepatocyte proliferation. In addition, we examined whether an inhibition of the mitogen-induced TNF-α production could lead to suppression of liver cell growth. For this purpose, we selected the glucocorticoid dexamethasone (DEX), which is known to inhibit TNF-α production at a level of transcription both *in vitro* and *in vivo*.[21,22] The results indicate that: 1) EDB induced increase in liver TNF-α mRNA similar to LN, 2) pretreatment of rats with DEX completely prevented the induction of TNF-α expression by EDB and LN, and 3) this effect was associated with inhibition of the mitogen-induced hepatocyte proliferation. The details of these experiments are the subject of this report.

Materials and Methods

Treatment of the Animals

Eight-week-old male Wistar rats (200 g) were purchased from Charles River (Milan, Italy) or from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). The animals were fed a laboratory chow diet provided by Ditta Piccioni (Brescia, Italy) or Ralston Purina (St. Louis, MO) and had free access to food and water. All treatments were performed in the morning between 9 and 12 a.m. LN (Carlo Erba, Milan, Italy, or Sigma Chemical Co., St. Louis, MO; 100 μmol/kg) dissolved in distilled water was injected intravenously through the saphenous vein. EDB (Sigma Chemical Co.; 100 mg/kg) dissolved in corn oil was administered intragastrically. DEX (Sigma Chemical Co.; 2 mg/kg) was dissolved in ethanol/distilled water and injected intraperitoneally 60 to 90 minutes before LN or EDB.

Experimental Design

To determine the effect of EDB on TNF-α mRNA, male Wistar rats were given a single intragastric intubation of EDB or corn oil (control) and rats were killed 2, 4, 7, and 24 hours thereafter. Approximately 1-g samples of the liver were immediately frozen in liquid nitrogen and stored at −70 C for future studies. In a separate experiment, hepatocytes and unfractionated nonparenchymal cells (NPC) were isolated from rats given EDB or corn oil and killed 4 hours later.

To determine the effect of DEX pretreatment on EDB or LN-induced increase in TNF-α mRNA levels, Wistar rats were treated with DEX (2 mg/kg). Sixty minutes after DEX treatment, LN or EDB was given and rats were sacrificed 3 and 6 hours (LN) or 4 and 7 hours (EDB) thereafter.

Because the time of maximal DNA synthesis is different between LN and EDB,[23,24] different experimental protocols were used to determine the effect of DEX on LN or EDB-induced cell proliferation. As for LN, cell proliferation was induced by a single dose of LN 90 minutes after treatment with DEX or an equivalent volume of the vehicle. Twenty-six, 30, and 36 hours after LN, [^{3}H]thymidine (Amersham, UK; specific activity 25 Ci/mmol) was injected intraperitoneally at a dose of 10 μCi/100 g body weight to 16 rats and the animals were sacrificed 12 hours after the last injection (48 hours after LN). The remaining rats (not receiving thymidine) were sacrificed 72 hours after treatment with LN. Hepatic DNA content and mitotic index were determined in all groups. As for EDB, liver cell proliferation was induced 90 minutes after DEX or the vehicle. Rats were then given [^{3}H]thymidine (10 μCi/100 g) at 20, 22, and 24 hours and were sacrificed 2 hours later.

Determination of Specific Activity of DNA

Tissue samples were homogenized in 6 volumes of 0.075 M NaCl-0.025 M EDTA, pH 7.6, and precipitated in ice-cold 1 N perchloric acid (PCA). The pel-

lets were washed three times with ice-cold 0.5 N PCA and extracted with 0.5 N PCA at 70 C for 1 hour. Suitable hydrolysate aliquots were used for measurement of radioactivity in a Beckman LS 1801 liquid scintillation spectrophotometer using Biofluor (New England Nuclear, Boston, MA) as solvent.

Determination of Hepatic DNA Content

After sacrifice the livers were frozen at −70 C. Total hepatic DNA was quantitatively assayed by Burton's diphenylamine method.[25]

Histological Examination and Measurement of Liver Mitoses

Immediately after the rats were killed, liver sections were fixed in 10% formalin and embedded in paraffin. Deparaffinized sections were then stained with hematoxylin and eosin. For the mitotic index, expressed as number of mitotic figures per 100 nuclei, at least 2000 nuclei were scored for each rat.

Hybridization Probes

The rat TNF-α probe was a 254-bp, *Kpn*I-*Hin*cII fragment of rat TNF-α gene cloned in a bluescript vector.[26] Glyceraldehyde-3-phosphate dehydrogenase, a 780-bp *Pst*I-*Xba*I fragment excised from the pHcGAP clone,[27] was purchased from American Tissue Culture Collection (Rockville, MD).

Northern Blot Analysis

Total RNA was isolated from frozen liver by extraction in guanidine isothiocyanate and ultracentrifugation in cesium chloride.[28] Poly(A)$^+$ RNA was then isolated by two cycles of oligo(dT) cellulose affinity chromatography (Collaborative Research, Waltham, MA). Poly(A)$^+$ RNA was isolated from isolated hepatocyte and NPC fractions by the QD rapid poly(A)$^+$ mRNA Isolation System (5 prime-3 prime Inc., Boulder, CO), according to the manufacturer's specification. Northern blot analyses were performed as previously described.[10]

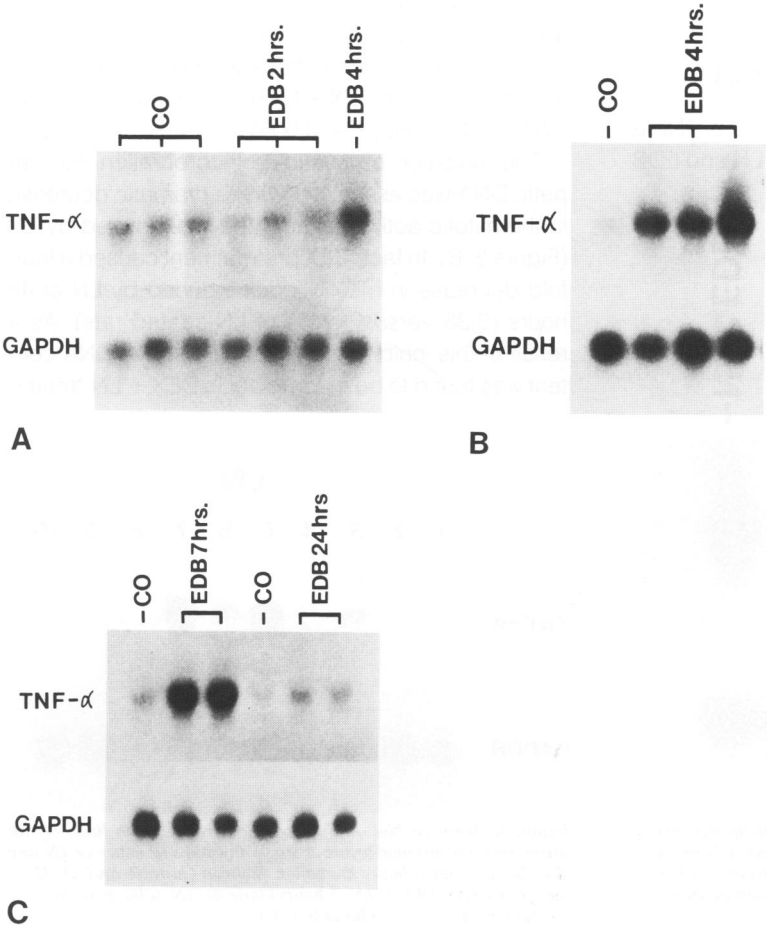

Figure 1. *Northern blot of TNF-α mRNA from the liver of rats treated with EDB or oil (CO) and sacrificed 2 and 4 hours* (a, b) *or 24 hours* (c) *thereafter.*

Separation of Parenchymal Cells and NPC

The livers were perfused with Ca^{2+} Mg^{2+}-free Hanks' balanced salt solution followed by the same solution containing 0.015% collagenase (Collagenase H; Boehringer Mannheim, Mannheim, Germany).[29] Hepatocytes and unfractionated NPC were separated by repeated centrifugation at 45 × *g* for 2 minutes (3×) and 50 × *g* for 2.5 minutes (2×). The pellets were frozen in liquid nitrogen and stored at −70 C.

Results

Effect of EDB on Liver TNF-α mRNA

Figure 1 shows Northern blot analysis of steady-state levels of liver TNF-α mRNA 2, 4, 7, and 24 hours after a single intragastric administration of EDB. As shown, the 1.8-kb transcript increased 4 hours after EDB treatment (Figure 1a, 1b); it remained elevated at 7 hours but returned to control levels by 24 hours (Figure 1, c). Liver TNF-α mRNA was apparent only in nonparenchymal fractions of the liver (Figure 2) and its levels were elevated 4 hours after EDB treatment.

Effect of DEX on Liver TNF-α mRNA

Next, we investigated the effect of DEX on liver TNF-α mRNA after treatment with the mitogens LN and EDB (Figure 3). In agreement with our previous studies,[11] LN induced an increase in TNF-α mRNA levels 3 (Figure 3, lane 3) and 6 hours (Figure 3, lanes 5 to 7) after treatment. The induction of TNF-α mRNA by LN was totally prevented by pretreatment with DEX both at 3 (Figure 3, lane 4) and 6 hours (Figure 3, lanes 8 to 10). A similar effect of DEX was observed when TNF-α mRNA was stimulated by EDB. As shown in Figure 4, the increase of TNF-α mRNA caused by EDB at 4 (lanes 3 and 4) and 7 hours (lane 7) was completely inhibited by pretreatment with DEX (lanes 5 and 6, DEX + EDB 4 hours; lanes 8 and 9, DEX + EDB 7 hours).

Effect of DEX on Liver Cell Proliferation Induced by LN

The next question we asked was whether the inhibition of TNF-α mRNA expression by DEX could result in an inhibition of hepatic DNA synthesis induced by LN or EDB. Because our previous studies have shown that LN induces a peak of S phase at approximately 28 to 40 hours after treatment,[30,31] the timing of administration of tritiated thymidine was selected to cover this time period. As shown in Figure 5, A, DEX administration completely abolished the incorporation of [^{3}H]thymidine into hepatic DNA induced by LN (8 cpm/μg DNA in DEX + LN group *versus* 39 cpm/μg DNA in LN group, $P < 0.005$).

The inhibition of thymidine incorporation into hepatic DNA was associated with a dramatic decrease in the mitotic activity of hepatocytes induced by LN (Figure 5, B). In fact, DEX pretreatment caused a fourfold decrease in mitotic index induced by LN at 48 hours (0.38 *versus* 1.75% of LN-treated rats). As a result of this inhibitory effect, total hepatic DNA content was found to be much lower in DEX + LN-treated

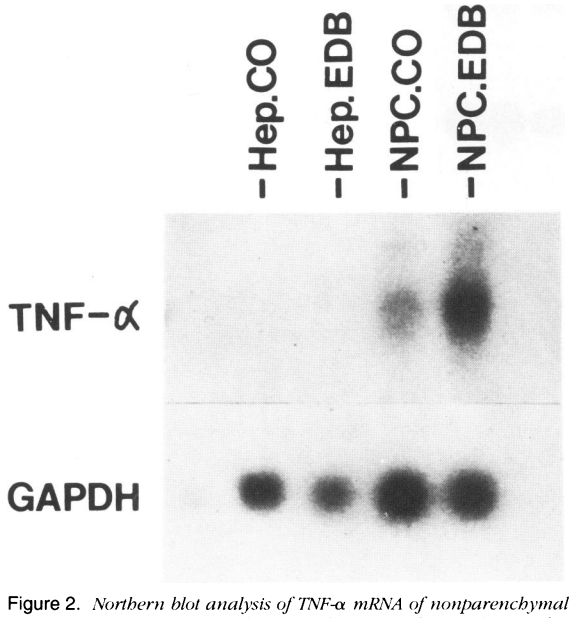

Figure 2. *Northern blot analysis of TNF-α mRNA of nonparenchymal and parenchymal liver cell fraction of rats sacrificed 4 hours after EDB or an equivalent volume of the vehicle (CO). Hepatocyte (HEP) and crude NPC fractions were separated after collagenase digestion of the liver and (5×) low centrifugation.*

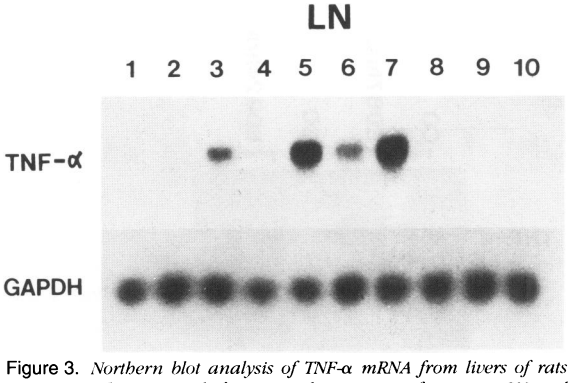

Figure 3. *Northern blot analysis of TNF-α mRNA from livers of rats given DEX 60 minutes before a single injection of water or LN and sacrificed 3 and 6 hours thereafter. Controls (lanes 1 and 2); LN, 3 hours (lane 3); DEX + LN, 3 hours (lane 4); LN, 6 hours (lanes 5 to 7); DEX + LN, 6 hours (lanes 8 to 10).*

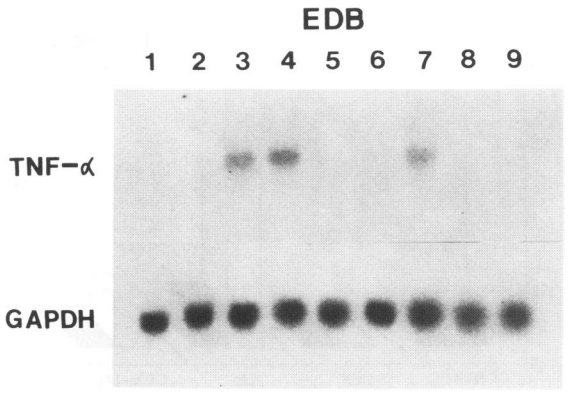

Figure 4. *Northern blot analysis of TNF-α mRNA from livers of rats given DEX 60 minutes before EDB administration. Oil (lanes 1 and 2); EDB, 4 hours (lanes 3 and 4); DEX + EDB, 4 hours (lanes 5 and 6); EDB, 7 hours (lane 7); DEX + EDB (lanes 8 and 9).*

rats when compared with animals receiving the mitogen alone (Table 1). Inhibition of LN-induced liver cell proliferation by DEX was not a transitory effect because a lower mitotic activity was still present in the liver of DEX-administered rats even at 3 days after treatment (Figure 5, B).

Effect of DEX on Liver Cell Proliferation Induced by EDB

Pretreatment with DEX also completely abolished cell proliferation induced by EDB. In fact, as shown in Figure 6, although EDB administration caused an approximately sevenfold increase in thymidine incorporation (20 cpm/μg DNA *versus* 2.8 cpm/μg DNA of controls), DEX pretreatment resulted in a complete inhibition of DNA synthesis (1.4 cpm/μg DNA). From these results it appears that abolishment of TNF-α expression by DEX might be responsible for the inhibition of LN- and EDB-induced liver cell proliferation, suggesting a critical role for TNF-α during cell proliferation induced by primary mitogen.

Discussion

It is known that liver cells may be induced to proliferate by several chemicals, primary mitogens, in the absence of any cell loss. This type of cell proliferation, defined as direct hyperplasia, differs from compensatory regeneration in many aspects including the pattern of hepatic expression of immediate early genes,[32] ploidy state,[33] and the effect on initiation of chemical hepatocarcinogenesis.[23] The cellular and molecular mechanisms of hepatocyte proliferation induced by primary mitogens are not known. Possible involvement of HGF and TNF-α, which are thought to be primary stimuli for compensatory liver regeneration, has been considered. Administration of primary mitogens either acutely or chronically, unlike compensatory regeneration, did not induce any significant change in hepatic expression of growth factor mRNA, such as HGF and TGF-α.[10,11] On the other hand, the primary mitogen LN was able to stimulate a rapid increase in the levels of TNF-α mRNA at a time preceding the onset of hepatocyte DNA synthesis, thus raising the possibility that TNF-α might be the critical factor in triggering this type of cell proliferation.

The results of this study lend further support to this notion. We have shown that: 1) a single treatment with two different liver mitogens, LN and EDB, induced similar patterns of enhanced TNF-α mRNA expression in rat liver; 2) DEX completely abolishes the increase in the expression of TNF-α mRNA induced by LN and EDB; and 3) an inhibition of TNF-α mRNA was associated with inhibition of liver cell proliferation induced by both mitogens. As expected, TNF-α mRNA was detectable only in nonparenchymal cell fraction of the liver and its levels were enhanced after EDB treatment. Even though no further fractionation of NPC was made, the source of TNF-α production in the liver is most probably Kupffer cells.

The increase-in hepatic levels of TNF-α mRNA was not accompanied by a detectable increase in TNF-α levels in the serum, monitored by the L929 cytotoxicity assay. However, when a low dose of lipopolysaccharide, which by itself does not produce any toxicity in L929, was given after LN, a dramatic increase in cytotoxicity was observed (data not shown). The increased toxicity was associated with liver necrosis and death of the animals.[11] Thus, it is possible that the amount of TNF-α needed to produce liver cell proliferation in the absence of severe toxicity may not be enough to be detected in the blood. In this respect, it is interesting to note that Akerman et al[20] were not able to find increased levels of TNF-α in the blood after pH, although inhibition of liver regeneration was achieved by treating hepatectomized animals with antibodies against TNF-α.

The participation of TNF-α in triggering cell proliferation has been proposed by several investigators.[14–20] They showed that administration of nonnecrogenic dosages of recombinant murine or human TNF-α to rats induced a mitogenic response in the liver.[16–19] However, although Feingold et al[16] reported that TNF-α induced proliferation only in nonparenchymal cells, other studies demonstrated a proliferative effect by TNF-α on hepatocytes, with an increase in mitotic index ranging from 12- to 15-fold.[17,18] The effect appears to be dependent on experimental conditions, because continuous infusion or repeated injections of TNF-α induced hepa-

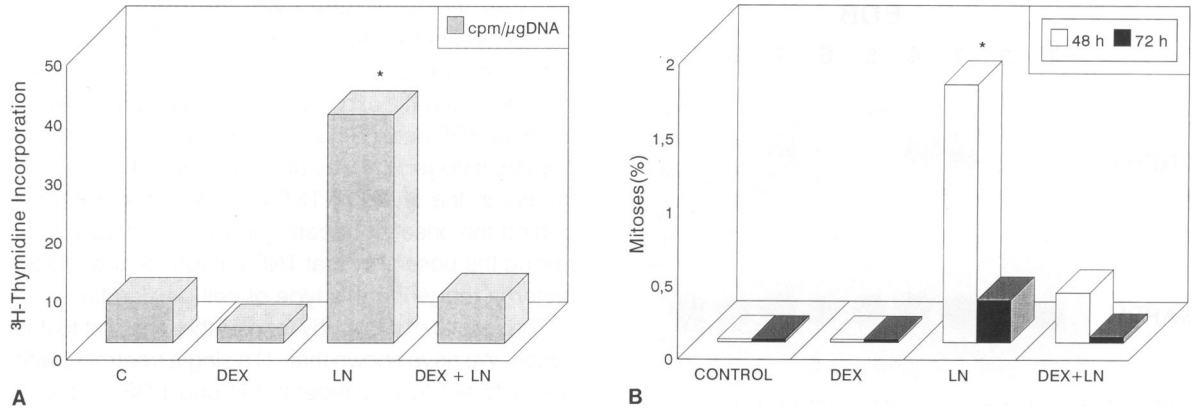

Figure 5. **A:** *Effect of DEX on [³H]thymidine incorporation induced by LN. Rats were given LN 90 minutes after treatment with DEX (2 mg/kg) or the vehicle. [³H]thymidine (10 μCi/100 g body weight) was given at 26, 30, and 36 hours after LN and rats were sacrificed at 48 hours. Radioactivity of extracted DNA was determined and expressed as mean ± SD counts per minute per microgram DNA. *Significantly different from DEX + LN; P < 0.005.* **B:** *Effect of DEX on LN-induced mitoses. Rats were given LN with or without DEX pretreatment and were sacrificed 2 and 3 days later. Mitotic index was calculated in hematoxylin and eosin-stained liver sections by scoring at least 2000 hepatocyte nuclei per rat. Mean of four rats per group. *Significantly different from DEX + LN-treated rats; P < 0.010.*

Table 1. *Effect of DEX on LN-Induced Liver Growth and Hepatic DNA Content*

Treatment	Relative Liver Weight (g/100 g body weight)	DNA Content (mg/100 g body weight)
Control	4.62 ± 0.61	11.04 ± 0.81
DEX	4.35 ± 0.17	12.13 ± 1.26
LN	6.51 ± 0.37*	16.41 ± 2.34†
DEX + LN	5.22 ± 0.41	10.82 ± 1.21

Rats were given DEX (2 mg/kg, intraperitoneal) 90 minutes before LN (100 μmol/kg body weight, intravenous) and were sacrificed 48 hours after treatment. Mean ± SD of four rats per group.
* Significantly different from DEX + LN; $P < 0.005$.
† Significantly different from DEX + LN; $P < 0.050$.

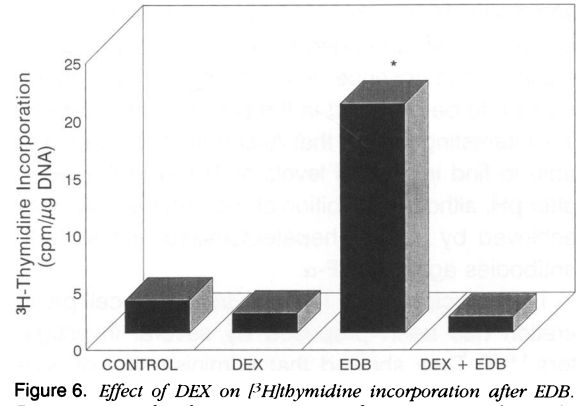

Figure 6. *Effect of DEX on [³H]thymidine incorporation after EDB. Rats were treated with EDB 90 minutes after treatment with DEX (2 mg/kg) or an equivalent amount of the vehicle. Rats were given tritiated thymidine (10 μCi/100 g body weight) at 20, 22, and 24 hours after EDB and were sacrificed 2 hours later. *Significantly different from DEX + EDB; P < 0.001.*

tocyte mitoses. It is not clear, however, whether TNF-α exerts a direct proliferative effect on hepatocytes or its action is mediated through secondary effects generated by this multifunctional cytokine. TNF-α is known to stimulate induction of other lymphokines such as interleukin-1 (IL-1), IL-6, and IL-8,[34,35] though very little information is currently available regarding the effects of these lymphokines on liver cell proliferation. Studies *in vitro* on the effect of TNF-α on hepatocytes have not established a definitive answer. Although Satoh et al[14] and Beyer and Theologides[15] showed stimulation of DNA synthesis by TNF-α of mouse and rat hepatocytes in primary culture, other investigators did not observe such stimulatory effect of TNF-α on cultured rat hepatocytes (T. Nakamura and G. Michalopoulos, personal communication).

Our findings that LN, a metal salt, and EDB, a halogenated hydrocarbon, induce a similar pattern of induction of liver TNF-α mRNA and that their mitogenic effects were completely abolished by inhibiting TNF-α induction by DEX suggest common pathways of triggering hepatocyte proliferation by primary mitogens. Whether or not a similar pathway is involved in other classes of primary mitogens, such as peroxisome proliferators and the antiandrogen cyproterone acetate, has to be answered in future studies. Although our studies focused on a possible role of TNF-α on liver cell proliferation induced by primary mitogens, there are several reports that suggest potential roles of TNF-α on cell proliferation during compensatory regeneration such as after partial hepatectomy. Satoh et al[19] showed that partial hepatectomy in mice is associated with an increased secretion of TNF-α and an inhibition of TNF-α secretion by glucocorticoid results in inhibition of liver regeneration. Akerman et al[20] showed that antibodies against TNF-α administered before partial hepatectomy inhibited rat liver regeneration and suggested that

TNF-α may prime hepatocytes to make them more sensitive to stimulatory effects of other known growth factors. Preliminary experiments performed in our laboratory indicate that a single treatment with antibodies against rat TNF-α (gift from Dr. G.J. Bagby, Louisiana State University) did indeed inhibit the liver cell proliferation elicited by LN (data not shown), suggesting that TNF-α produced in response to LN or EDB treatment may have a similar priming effect on hepatocytes. Further studies are necessary to unravel critical differences in the mechanisms responsible for triggering cell proliferation involved in direct hyperplasia and compensatory regeneration.

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