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Induction of Inflammatory Cell Infiltration and Necrosis in Normal Mouse Skin by the Combined Treatment of Tumor Necrosis Factor and Lithium Chloride

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Previously we reported that lithium chloride (LiCl) potentiates tumor necrosis factor (TNF)-mediated cytotoxicity* in vitro *and* in vivo. *Here, using a murine normal skin model, it is shown that a subcutaneous injection of TNF plus LiCl induces acute dermal and subcutaneous inflammation and necrosis. Histology showed a marked initial dermal and subcutaneous neutrophil infiltrate by approximately 2 hours, followed by a predominantly mononuclear infiltrate by 24 hours, which remained present for several days. Tumor necrosis factor or LiCl alone induced negligible inflammation, disappearing after 6 hours; furthermore there was never necrosis or ulceration of the overlying skin in case of single-agent application.* In vitro *studies showed that the combination of TNF and LiCl, but not either agent alone, was directly cytotoxic to fibroblastic cells of murine skin. No inflammatory infiltration was visible in tumors treated intratumorally or perilesionally with TNF plus LiCl, although the latter treatment resulted in a perilesional leukocyte infiltration. Furthermore the combination of TNF and LiCl had no effect on macrophage cytotoxicity to L929 tumors. (Am J Pathol 1991, 138:727–739)

Tumor necrosis factor (TNF) initially was defined by its ability to cause tumor necrosis *in vitro* and *in vivo*.[1] Additional biologic activities of TNF, such as activation[2,3] and chemotaxis[4] of macrophages and neutrophils, suggest that TNF also is an important mediator of inflammation.

Receptors for TNF are abundant in the skin,[5] and approximately 30% of an intravenously administered dose of TNF may be recovered from skin.[6] Furthermore TNF-induced regression of tumors *in vivo* is particularly evident on subcutaneous implantation of the tumors.[7] It has been reported also that TNF can recruit neutrophils locally into rabbit skin.[8]

Recently we showed that lithium chloride (LiCl) potentiates the *in vitro* cytotoxic effect of TNF against several transformed cell lines.[9] Furthermore when mice carrying subcutaneous tumors were treated perilesionally with TNF and LiCl, the antitumor effect of TNF was considerably enhanced.[9] Our preliminary results showed that this potentiating effect of LiCl was less pronounced on intraperitoneal administration of TNF and LiCl. This observation, together with the finding that the combined local injection of TNF and LiCl in our earlier experiments induced a macroscopically visible skin reaction, led us to assume that, besides the direct cytotoxic action of TNF plus LiCl on tumor cells, also indirect mechanisms might be operative in the observed tumor-necrotizing reactions. In this paper we report that the combination of TNF and LiCl induces inflammatory cell infiltration and necrosis in normal murine skin. We also present evidence that these phenomena do not play an important role in the *in vivo* antitumor action of TNF plus LiCl.

Materials and Methods

Cytokine Preparations

Recombinant human TNF (hTNF) and murine TNF (mTNF) were produced in *E. coli* and purified to at least 99% homogeneity.[10,11] The preparations used had a specific activity of 1×10^8 and 1.9×10^8 international

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units (IU) per milligram of protein, as determined in a TNF cytolysis assay,[9] (units as defined in Ostrove and Gifford[12]; reference hTNF [code 87/650] and mTNF [code 88/532] were from the National Institute for Biological Standards and Control, Potters Bar, UK) and contained less than 13 ng and 4 ng endotoxin per milligram protein, respectively. The preparations used were stored in phosphate-buffered saline (PBS, free of Ca^{2+} and Mg^{2+}) at −70°C.

Purified recombinant human lymphotoxin (hLT), expressed in *Escherichia coli*,[13] was provided by Dr. A. Porter, National University of Singapore, Singapore. This hLT had a specific activity of 2.2×10^8 IU/mg.

Recombinant murine interferon-γ (mIFN-γ) was derived from the culture medium of a transformed Chinese hamster ovary cell line, as described for human IFN-γ.[14] The purified product had a specific activity of about 10^7 IU/mg as determined in a cytopathic reduction assay,[15] and was stored in PBS with 0.1% gelatin at −70°C (reference mIFN-γ [code Gg02-901-533] was from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD).

Recombinant human interleukin-1β (hIL-1β; 5×10^8 U/mg as assessed by proliferation of RPMI1788 cells[16]) and recombinant murine granulocyte monocyte colony-stimulating factor (mGM-CSF; 2.5×10^8 U/mg according to proliferation of FDCp1 cells[17]) were gifts from Dr. A. Shaw and Dr. J. Delamarter, respectively, Glaxo Institute for Molecular Biology, Geneva, Switzerland.

Recombinant human interleukin-6 (hIL-6; 1×10^8 IU/mg as assessed by 7TD1 proliferation[18]) was cloned, expressed in yeast and purified in our laboratory (Y. Guisez, J. Demolder, and R. Contreras, unpublished data).

Rabbit polyclonal antiserum against recombinant hTNF was provided by J. Van der Heyden (Roche Research Gent, Gent, Belgium).

Chemicals

BW755C (3-amino-1-[3-trifluoromethylphenyl]-2-pyrazoline hydrochloride; Wellcome Research Laboratories, Beckenham, UK), WEB2086 (3-{4(2-chlorophenyl)9-methyl-6*H*-thieno-[3,2f]-[1,2,4-triazolo]-[4,3a]-[1,4]-diacetine-2-yl}-1-(4-morpholinyl)-1-propanone; Boehringer-Ingelheim, Ingelheim, FRG), hydrocortisone hemisuccinate (Diosynth, Oss, The Netherlands), diethylcarbamizine (DEC; Sigma Chemical Co., St. Louis, MO), and cycloheximide (Sigma Chemical Co.) were dissolved in PBS. Nordihydroguaiaretic acid (NDGA; Sigma Chemical Co.), ketoconazole (Janssen Pharmaceutica, Beerse, Belgium), ebselen (2-phenyl-1,2-benzisoselenazol-3(2*H*)-one; Nattermann & Cie, Köln, FRG), SKF86002 (5-[4-pyridyl]-6-[4-fluorophenyl)-2,3-dihydroimidazo-[2,1b]-thiazole; Smith Kline & French Laboratories, Philadelphia, PA), AA861 (2,3,5-trimethyl-6-[12-hydroxy-5, 10-dodecadiynyl]-1,4-benzoquinone; Takeda Chemical Industries, Osaka, Japan), and VZ65 (4-(11-hydroxy-1, 9-undecadiin)-brenzcatechin; Grnüenthal, Stolberg, FRG) were dissolved in ethanol and further diluted in PBS to a final concentration of 1%. Indomethacin (Sigma Chemical Co.) was dissolved in 1.4% $NaHCO_3$ and further diluted in PBS. Dexamethasone sodium phosphate solution was from Merck Sharp & Dohme (Brussels, Belgium).

Lipopolysaccharide (LPS) from *E. coli* 0111:B4 was purchased from Difco Laboratories (Detroit, MI).

Mice

Female nude mice (Swiss nu/nu; Iffa-Credo, Saint Germain-sur-l'Arbresle, France), 7 to 9 weeks old, were used for most experiments. C3H/HeJ mice (Iffa-Credo) were used to investigate the role of residual endotoxin. BALB/c and DBA2/n mice were from Charles River Breeding Laboratories (Wiga, FRG).

Preparation of Skin Fibroblastic Cells

Normal skin was dissected from the back of nude mice that had been killed. Specimens were immersed immediately in RPMI1640 medium (Gibco Bio-Cult, Paisley, UK) to which 10% fetal calf serum, 100 U penicillin/ml, and 0.1 mg streptomycin/ml had been added. While suspended, skin was cut into 3-mm pieces and placed in 10-cm^2 wells, epidermal surface up. Sufficient medium to cover 80% of the explant surface area was added and the explants were incubated at 37°C under 5% CO_2-humidified air. When substantial outgrowth was observed, the center of the explant was removed with a pincet. The remaining attached cells were allowed to grow out until the whole surface of the well was covered. Most of the cells obtained in this way had a fibroblastic morphology.

In Vitro TNF Cytolysis and Cytostasis Assays

Tumor necrosis factor cytotoxicity was assayed as described previously.[9]

Assessment of the Inflammatory Skin Response

One hundred microliters (or 50 μl in case of multiple-site injections) of PBS containing the test substances were

injected subcutaneously into the backs of the mice. In experiments with tumor-bearing nude mice, tumors were generated by subcutaneous injection of 1×10^6 L929 murine fibrosarcoma cells (Rega Institute, Leuven, Belgium). Tumors became palpable 3 to 5 days after tumor inoculation and the mice were used when tumors reached a diameter of approximately 0.5 cm. In this case, test substances were injected perilesionally at 1 cm distance from the tumor. At different time intervals, skin lesions and a piece of the outer site of the tumor were removed and fixed in 4% formalin. Routine hematoxylin and eosin sections were prepared. All sections were evaluated by a pathologist who was not aware of the treatment. The presence of macrophages was confirmed by nonspecific esterase staining according to the method of Li et al.[19]

Macrophage-mediated Cytotoxicity

Macrophages were isolated by peritoneal lavage of nude mice and seeded in microtiter plates at 1×10^5/well in RPMI1640 medium, supplemented with 10% fetal calf serum and 50 micromolar β-mercaptoethanol. Four hours later, the following substances were added, alone or in combinations: hTNF, mIFN-γ, LPS, and LiCl. Eighteen hours later, medium was removed from the effector cells and replaced by fresh culture medium without stimulating additives, but containing neutralizing anti-hTNF antibodies. After two hours of further incubation, 5000 L929 cell targets were added to wells containing effector cells or, as controls, to wells without effector cells. After 72-hour incubation at 37°C, cell viability was determined *via* viable cell staining with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co.).[20] The cytotoxicity percentage was determined using the equation

$$\left[1 - \frac{(A_{tc+ec}) - (A_{ec})}{A_{tc}}\right] \times 100$$

where A = absorbance, tc = target cells, and ec = effector cells.

Results

The Combination of TNF and LiCl Induces a Skin Reaction in Mice

A single subcutaneous injection of hTNF plus LiCl induced a macroscopically visible skin reaction (Figure 1). This reaction became visible after 2 hours and was most prominent at 24 hours. Strong reactions could reach up to 1 cm in diameter. The reaction appeared as a local erythema and swelling, while at high TNF doses necrosis became visible after 1 or 2 days (Table 1). Neither hTNF nor LiCl, administered alone, led to a visible skin reaction. Also intraperitoneal injection of LiCl (1 mg), in combination with subcutaneous injection of TNF (10 μg), was ineffective in inducing a skin response. Furthermore injection of hTNF and LiCl at the same site, but with a time interval of 6 hours, did not lead to an inflammatory response, independent of which agent was injected first (data not shown). A similar skin reaction could be obtained by the injection of TNF plus other lithium salts (LiBr or Li_2SO_4), while the combined injection of hTNF plus NaCl or RbCl did not induce a skin reaction (data not shown). Skin reactions could be inhibited by 0.5 hour pretreatment of the hTNF plus LiCl injection mixture with polyclonal neutralizing anti-hTNF serum (data not shown). The following controls also argue against the involvement of LPS in the observed responses. First heating the LiCl for 3 hours at 180°C could not impair the observed effects. Second the skin reactions described could be obtained after hTNF plus LiCl injection in C3H/HeJ mice known to be unresponsive to LPS.[21] Last coinjection of LPS (0.5 μg), either with hTNF (10 μg) or LiCl (1 mg), was ineffective in inducing the skin response.

Daily subcutaneous injections of hTNF (10 μg) plus

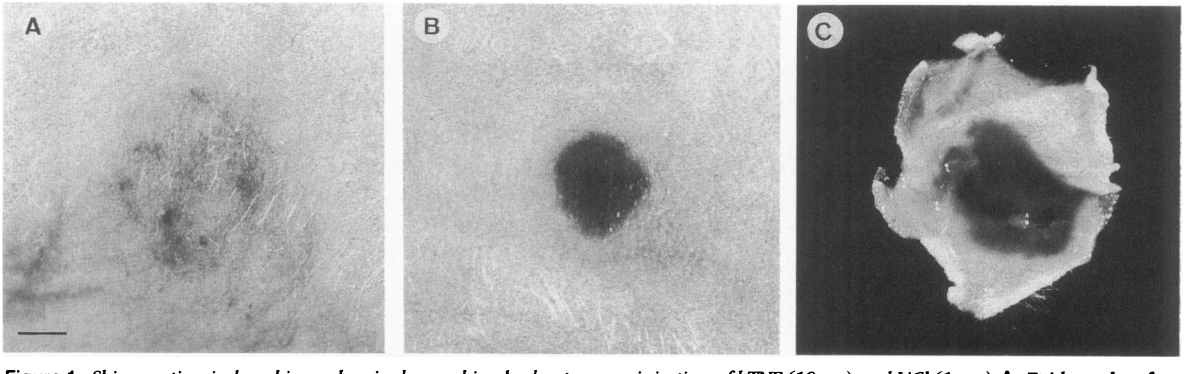

Figure 1. *Skin reaction induced in nude mice by combined subcutaneous injection of hTNF (10 μg) and LiCl (1 mg).* **A:** *Epidermal surface after 6 hours, showing erythema, reddening, and swelling.* **B:** *Epidermal surface after 24 hours, showing beginning necrosis and ulceration.* **C:** *Dermal surface after 6 hours, showing erythema. Scale bar: 2 mm (identical for all panels).*

Table 1. *Dose Dependency of the Action of Various Cytokines as Assessed* via In Vitro *Cytotoxicity and Induction of a Skin Reaction* In Vivo

	*In Vitro**	*In Vivo†*	
Cytokine	50% killing (pg/ml)	Erythema/ edema (ng/site)	Necrosis (ng/site)
hTNF	1.80 (14)	10 (50)	780 (6.6)
mTNF	0.25 (100)	5 (100)	50 (100)
hLT	1.80 (14)	40 (13)	>3000 (<1.6)
hIL-1β	$>10^4$ (<0.003)	$>10^4$ (<0.003)	$>10^4$ (<0.5)

* Dose resulting in 50% killing of WEHI164cl13 cells in an 18-hour assay in the presence of 1 μg actinomycin D/ml; the number of surviving cells incubated in medium with actinomycin D alone was considered 100%. Values between brackets show the relative potency of the cytokines, the amount of mTNF needed to induce the indicated effect being considered 100%.

† Minimal dose required to induce the indicated responses. Erythema/oedema and necrosis were checked macroscopically, 6 hours and 2 days, respectively, after subcutaneous coinjection in the back of a nude mice of a serial dilution of cytokine with LiCl (1 mg). Three mice were used per group. Values between parentheses are defined above.

LiCl (1 mg) at sites close to each other led to desensitization: while a weaker skin response was already observed after the second injection, a third injection was no longer effective. However, when the same mouse was injected 5 days later with hTNF plus LiCl, a clear inflammatory reaction could be induced again. No desensitization to a subcutaneous injection of hTNF plus LiCl could be obtained with a daily intraperitoneal injection of both substances at equal concentrations, as mentioned above (data not shown).

Dose Dependency and Specificity of the Skin Reaction

To study whether the effect of hTNF plus LiCl on murine skin was dependent on the hTNF dose, nude mice were injected subcutaneously at eight sites on the back with a serial dilution of hTNF combined with a constant 1-mg LiCl dose. Skin reactions could be induced with as little as 10 ng hTNF, while skin necrosis was observed at 780 ng hTNF (Table 1). Similar experiments with a serial dilution of LiCl, combined with a fixed hTNF dose of 5 μg, showed that the minimal dose of LiCl required for a skin reaction was 250 μg. hTNF alone did not cause a visible skin reaction, even with injection of a 100-μg dose. Injection of 4 mg LiCl alone produced a skin reaction, which became visible after 5 minutes. However this presumably was due to the high salt concentration because 4 mg NaCl induced a similar reaction (data not shown).

We compared the ability of hTNF and mTNF to induce skin inflammation *in vivo* with their potency to induce cytolysis of TNF-sensitive tumor cells *in vitro*. As expected,[22] some species preference for mTNF was evident in our murine model (Table 1). Also some other cytokines in combination with either LiCl (1 mg) or hTNF (5 μg) were tested for their ability to induce a murine skin reaction. Human lymphotoxin was as effective as hTNF when tested in a cytolysis assay, but the minimal required dose to induce a skin reaction in the presence of LiCl was more than four times higher. Furthermore the hLT-induced skin reaction always was less intense than observed with hTNF and necrosis was never seen. All other cytokines (5 μg hIL-6, 10 μg hIL-1β, 5 μg mIFN-γ, 1.8 μg mGM-CSF) tested in combination with either LiCl or hTNF were ineffective (partially shown in Table 1).

Histologic Effects of TNF Plus LiCl

Histologic examination of murine skin treated for 2 hours with hTNF (10 μg) plus LiCl (1 mg) demonstrated vasodilatation of the dermal blood vessels, intravascular polymorphonuclear leukocytes, and beginning extravasation of neutrophils and erythrocytes (Figure 2A and B). This was followed by further infiltration of neutrophils in the surrounding fibrous tissue of the dermis, but also in the underlying muscle and fat (Figure 2C). This reaction increased until 12 hours after hTNF plus LiCl injection. By 24 hours, most neutrophils had disappeared and the number of mononuclear cells in the dermis had increased (Figure 2D). Most of the latter infiltrating cells were identified as macrophages by staining for nonspecific esterase (Figure 2D, inset). After 24 to 48 hours, the fibrous tissue showed necrosis with fragmentation of the collagenous fibers and ulceration of the overlaying epidermis (Figure 3). After 48 hours, some granulomas also were found. They consisted of a necrotic center surrounded by epitheloid macrophages and some giant cells. Four to five days after injection, epithelialization of the epidermis occurred again. At this time, the dermis consisted of a dense collagenous fibrous tissue, resulting in scar formation (data not shown).

In skin sites that were injected with LiCl or hTNF alone, only vasodilatation of the dermal blood vessels with a slight intravascular increase in the number of neutrophils was apparent 2 hours after treatment (Figure 4). After 6 hours, there was some extravasation of neutrophils into the dermis (data not shown). However the inflammatory reaction of the skin was much less intense after single-agent injections than after the combined treatment. In the former case, necrosis, ulceration, or increase in the number of macrophages were never observed and the inflammatory reaction completely disappeared after 6 to 12 hours, without residual lesions. As expected from our macroscopic observations, neither hIL-1β nor hIL-1β plus LiCl induced infiltration of neutrophils or macrophages in the murine skin (data not shown).

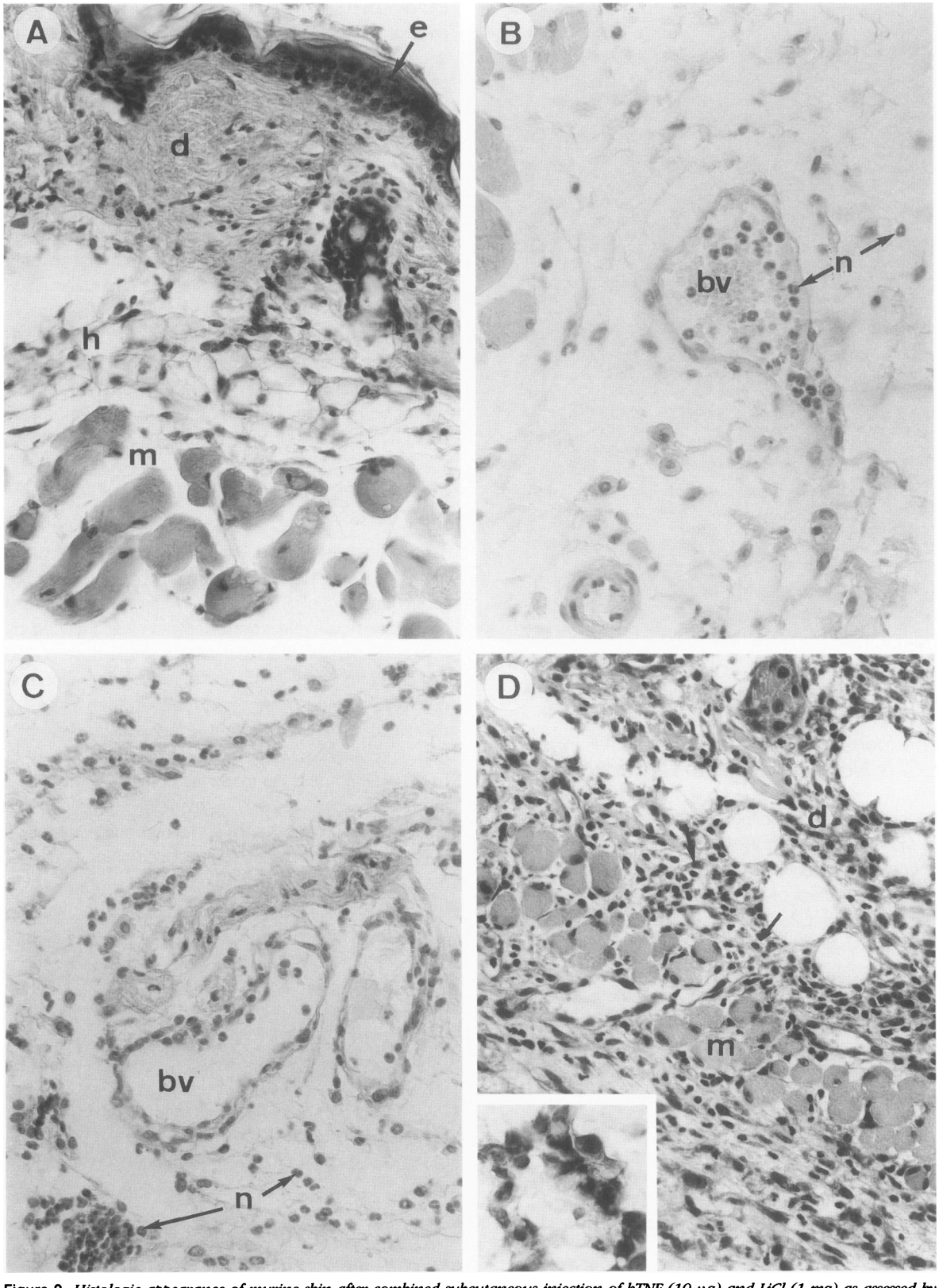

Figure 2. *Histologic appearance of murine skin after combined subcutaneous injection of hTNF (10 μg) and LiCl (1 mg) as assessed by hematoxylin and eosin staining.* **A:** *Untreated; normal skin without inflammatory cells. e, epidermis; d, dermis; h, hypodermis, m, muscle [×260].* **B:** *Two hours after injection: dilatated blood vessels (bv) and beginning extravasation of neutrophils (n) [×300].* **C:** *Six hours after injection: vasodilatation and extravasated neutrophils in the surrounding tissue [×300].* **D:** *Twenty-four hours after injection: strong macrophage infiltration (arrows) in dermis and muscle [×260]. The insert shows a nonspecific esterase staining for macrophages of a section of the same skin piece [×360].*

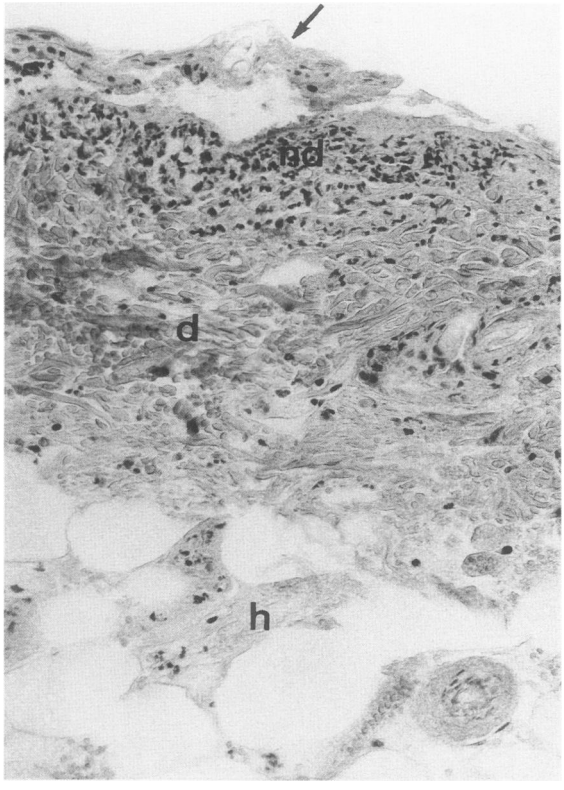

Figure 3. *Ulceration (arrow) and loosening of epidermis with formation of nuclear dust (nd) 48 hours after combined subcutaneous injection of hTNF (10 μg) and LiCl (1 mg). d, dermis; h, hypodermis. Hematoxylin and eosin staining [×120].*

The Skin Reaction Induced by TNF Plus LiCl Is Not a Shwartzman Reaction

Because some of the effects described here were similar to those observed in the Shwartzman-like reaction induced by sequential injections of LPS and TNF,[23] we studied the effect of hTNF plus LiCl in DBA2/n mice. These mice are C3a and C5a deficient, so they show no Shwartzman-like reaction.[24] BALB/c mice were used as a positive control. Animals were treated subcutaneously with a combination of hTNF (10 μg) and LiCl (1 mg). Effects on skin were evaluated 2 days later. In both strains of mice, a similar skin reaction could be observed macroscopically and histologically (data not shown). These results show that the effects seen after TNF plus LiCl injection are not related to a Shwartzman reaction.

Evidence Against an Involvement of the Phospholipase A2 Pathway in TNF Plus LiCl-induced Skin Reaction

Evidence for an involvement of products of the phospholipase A_2 pathway in inflammation has been reported.[25] To investigate the role of these metabolites in TNF plus LiCl-induced skin reaction, mice were injected subcutaneously with hTNF (10 μg) plus LiCl (1 mg) in the presence of drugs known to inhibit different steps in the phospholipase A_2 pathway. BW755C (1 mg), which inhibits cyclooxygenase and lipoxygenase, and the more specific lipoxygenase inhibitor NDGA (50 μg) both completely inhibited the hTNF plus LiCl-induced erythema and necrosis when checked macroscopically (Table 2). However, when the same skin samples were examined microscopically, neither drug could inhibit the TNF plus LiCl-induced neutrophil and macrophage infiltration (data not shown). In addition, coadministration of other inhibitors, which also are supposed to act on the phospholipase A_2 pathway, did not prevent TNF plus LiCl-induced skin reaction as evaluated both microscopically and macroscopically (Table 2). These results argue against an involvement of the phospholipase A_2 pathway in TNF plus LiCl-induced leukocyte infiltration.

Direct Effects of TNF Plus LiCl on Murine Skin Cells

To investigate whether TNF plus LiCl-induced skin necrosis *in vivo* is due to a direct effect on murine skin cells, we tested the effect of hTNF plus LiCl in animals depleted of white blood cells. Neutrophil depletion was induced by total-body gamma irradiation with a single dose of 750 rads. This treatment was found to reduce the number of neutrophils in the peripheral circulation from values of 4500/μl to values below 150/μl 7 days after irradiation. At that time, animals were injected subcutaneously with hTNF plus LiCl and the response of the skin was monitored macroscopically and histologically. Despite a very weak induction of neutrophil and macrophage infiltration by TNF plus LiCl in the skin of irradiated animals, erythema and necrosis were still as strong as in the controls (data not shown). These results suggest that necrosis does not depend on the presence of neutrophils and macrophages, which confirms our inhibitor studies.

More evidence for a direct effect of TNF plus LiCl on murine skin cells came from *in vitro* cytotoxicity studies on murine skin fibroblasts. Neither hTNF nor LiCl alone were toxic to these cells within 3 days of treatment, but their combination resulted in considerable cell death, which began after 1 or 2 days of treatment (coinciding with the time required to observe necrosis *in vivo*) and which was dependent on both the hTNF and LiCl concentration (Figure 5).

Evidence that Tumor Regression and Skin Inflammation Are Two Independently TNF Plus LiCl-induced Effects

We previously reported that LiCl potentiates the antitumor effect of TNF when injected perilesionally for several

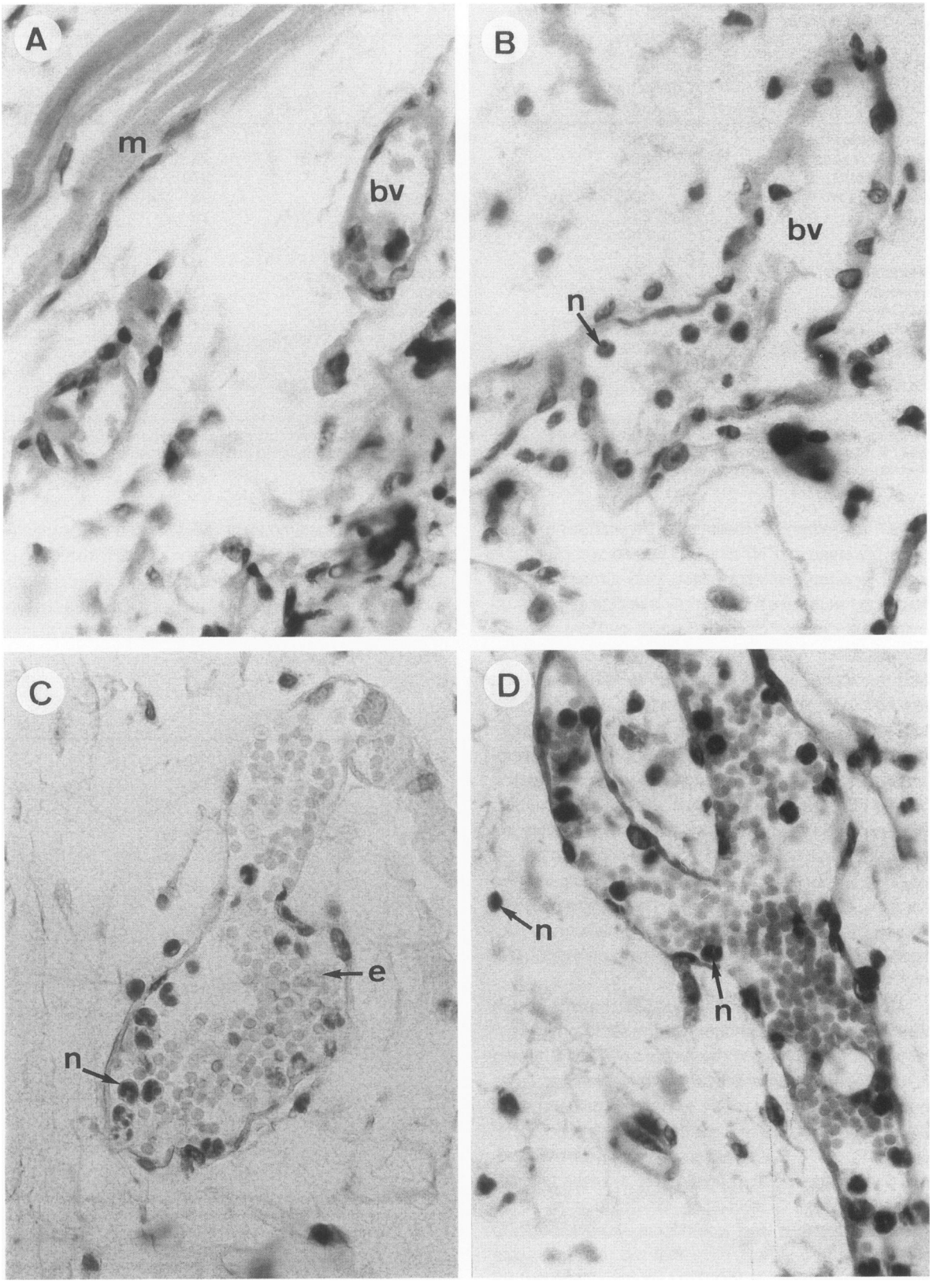

Figure 4. *Histologic changes assessed 2 hours after subcutaneous injection of solvent (PBS), LiCl, hTNF, or their combination.* **A:** *PBS; normal blood vessels (bv) without neutrophil infiltration. m, muscle.* **B:** *LiCl (1 mg); vasodilatation and a limited number of intravascular neutrophils (n).* **C:** *hTNF (10 μg); dilatated blood vessel (bv) filled with erythrocytes (e) and a limited number of intravascular neutrophils (n).* **D:** *hTNF (10 μg) + LiCl (1 mg); strong vasodilatation with a large number of intravascular neutrophils and some extravasated neutrophils. Hematoxylin and eosin staining [×575].*

Table 2. *Concentrations and Putative Targets of Drugs Tested for Their Effect on TNF + LiCl-induced Erythema and Necrosis of the Skin*

Drug	Concentration (μg)	Putative target	Ref.	Erythema and necrosis inhibition
Dexamethasone	666	PLA_2 inhibitor	26	–
Hydrocortisone	200	PLA_2 inhibitor	26	–
WEB2086BS	20	PAF-antagonist	27	–
Indomethacin	100	cyclooxygenase	28	–
VZ65	250	5-lipoxygenase	29	–
AA861	250	5-lipoxygenase 12-lipoxygenase	30	–
Ketoconazole	10	5-lipoxygenase	31	–
Ebselen	10	lipoxygenase	32	–
NDGA	50	lipoxygenase	33	+
DEC	1000	lipoxygenase	34	–
BW755C	1000	lipoxygenase cyclooxygenase	35	+
SKF86002	1000	lipoxygenase cyclooxygenase	36	–

Drugs were injected subcutaneously together with hTNF (10 μg) + LiCl (1 mg). The final ethanol concentration for drugs dissolved in ethanol was 1%. Such concentration was shown not to affect the skin reaction. Effects on TNF + LiCl-induced erythema and necrosis were checked macroscopically after 6 hours and 24 hours (– means no inhibition; + means total inhibition).

days.[9] To determine whether the skin-infiltrating leukocytes observed in hTNF plus LiCl-treated skin play a role in the reported antitumor effect, L929 tumors and surrounding tissue were histologically investigated after subcutaneous injection of TNF plus LiCl at 1 cm distance from the tumor. Also in this case an infiltrate of neutrophils and macrophages was visible at the injection site and reached the border of the tumor after a single TNF plus LiCl injection (Figure 6A and B). However neither an intratumoral increase in inflammatory cells nor tumor necrosis were observed after a single TNF plus LiCl injection (Figure 6C). The latter correlates with our earlier finding that daily perilesional TNF plus LiCl injections are needed to induce tumor regression.[9] However this treatment led again to desensitization to leukocyte infiltration and skin necrosis, even in peritumoral sites. These results indicate that an involvement of intratumoral and peritumoral neutrophils or macrophages in the antitumor effect of TNF plus LiCl is unlikely.

More evidence for the latter suggestion came from *in vitro* studies on macrophage cytokine secretion and cytotoxicity. Table 3 shows the amount of TNF, IL-6, and IL-1 secreted on activation of peritoneal macrophages either with LiCl and/or hTNF, or with the potent macrophage activators LPS and/or IFN-γ.[37] While a combination of LPS and IFN-γ considerably increased TNF, IL-6, and IL-1 levels, only IL-6 levels were increased slightly after hTNF plus LiCl stimulation. When the same stimulated macrophages were tested for *in vitro* cytotoxicity to L929 tumor cells, it was clear that macrophages were activated synergistically for L929 tumor-cell killing by LPS and IFN-γ, whereas hTNF and LiCl were ineffective in this respect (Table 3). The range of cytotoxicity correlated very well with the amount of TNF secreted by the macrophages, suggesting a participation of TNF in the killing of L929 cells by the LPS- and IFN-γ–activated macrophages. These results further support the idea that inflammatory cells do not play a direct role in the antitumor effect of TNF plus LiCl.

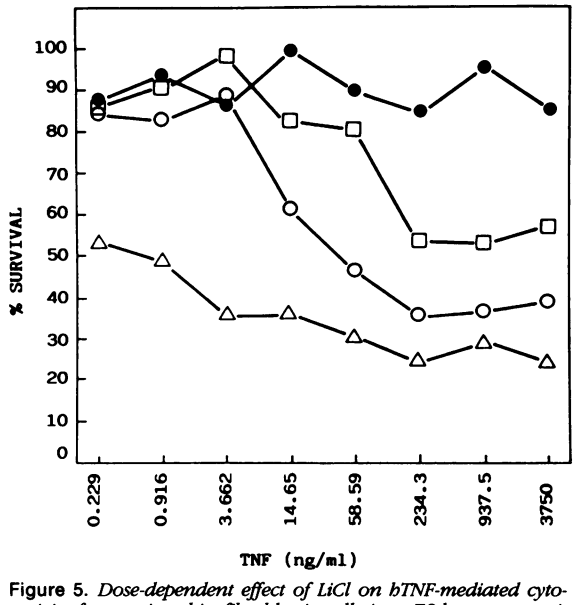

Figure 5. *Dose-dependent effect of LiCl on hTNF-mediated cytotoxicity for murine skin fibroblastic cells in a 72-hour cytostasis assay. The survival percentage is plotted against hTNF concentration for LiCl concentrations of 0 mmol/l (●), 5 mmol/l (□), 10 mmol/l (○), and 20 mmol/l (△). The survival percentage is the cell-staining value obtained after treatment with hTNF + LiCl, expressed as a percentage of the cell-staining value obtained in an untreated culture. LiCl alone, at the concentrations used, did not affect cell viability. Data are from a representative experiment.*

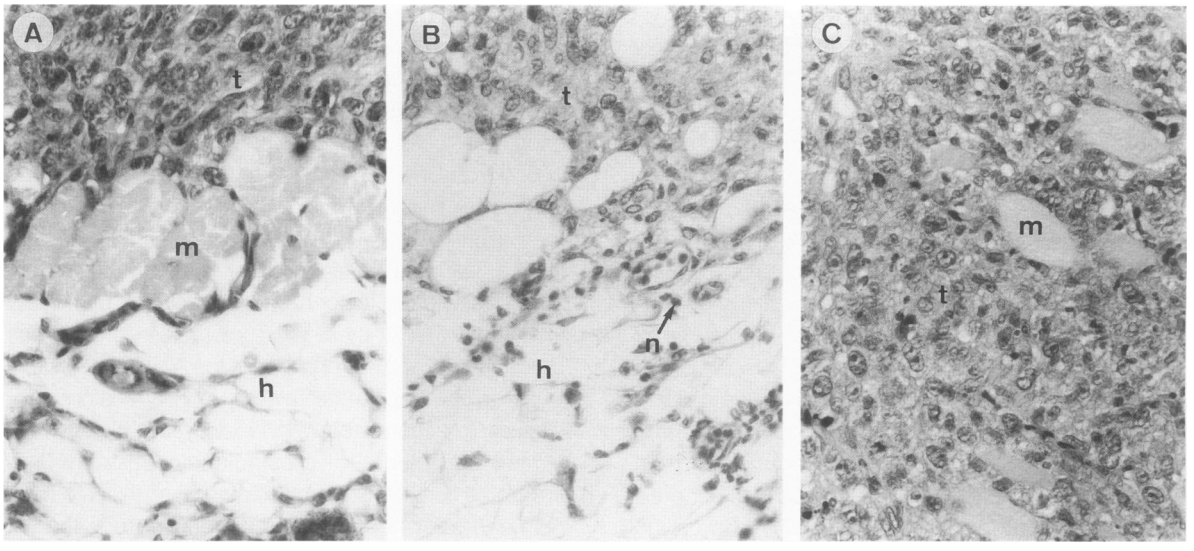

Figure 6. *Histologic effect of a single perilesional injection of hTNF (10 μg) + LiCl (1 mg) on an L929 tumor.* **A**: *Six hours after solvent (PBS) injection; absence of inflammatory cells in the tumor (t) or surrounding muscle (m) and hypodermis (h).* **B**: *Six hours after TNF + LiCl injection; neutrophils (n) in the hypodermis (h) at the border of the tumor.* **C**: *Twenty-four hours after TNF + LiCl injection; absence of inflammatory cells or necrosis in the tumor. Hematoxylin and eosin staining [×420].*

Discussion

The data presented in this report demonstrate that a combination of TNF and LiCl, but not either agent alone, induces a strong macroscopic skin reaction in mice. This response was histologically shown to consist of an initial vigorous vasodilatation and neutrophil infiltration, followed by infiltration of macrophages and finally by necrosis. A certain degree of TNF species specificity for induction of this effect could be observed, which correlates with the earlier reported species preference of TNF.[22] A combination of hLT and LiCl resulted in a response similar to that obtained with TNF plus LiCl, but less intense; necrosis was never observed at the doses tested (up to 3 μg). This correlates with the results of Broudy et al,[38] who demonstrated that LT has lower proinflammatory properties *in vitro* than TNF. Rampart et al[8] reported that TNF, when coinjected with prostaglandin E2, induced neutrophil infiltration in rabbits. In contrast, Sharpe et al[39] reported a neutrophil infiltration in the footpad of mice on injection of TNF alone. However none of these authors observed a clear macroscopic skin reaction and necrosis after a single TNF injection. Recently Piguet et al[40] described massive tissue necrosis in mice, but this was observed after subcutaneous perfusion of high TNF doses for several days.

Our results suggest that macroscopic erythema and necrosis are independently induced from the histologically observed neutrophil and macrophage infiltration. This suggestion is based on the following observations: 1) erythema and necrosis are not reduced in animals with a white blood cell content 30 times lower than that of

Table 3. *Cytokine Production and Tumor Cell Killing by Murine Macrophages*

Macrophage pretreatment*	Amount of cytokine secreted†			
	TNF (pg)	IL-6 (pg)	IL-1 (pg)	Cytotoxicity %‡
None	<0.3	70 ± 20	<3	9 ± 5
LiCl	<0.3	110 ± 10	<3	8 ± 4
TNF	<0.3	200 ± 20	<3	7 ± 4
LiCl + TNF	<0.3	370 ± 80	<3	5 ± 3
LPS	0.8 ± 0.1	73,400 ± 410	28 ± 7	18 ± 4
IFN-γ	1.8 ± 0.4	1,950 ± 60	<3	31 ± 3
LPS + IFN-γ	8.1 ± 0.2	181,000 ± 220	22 ± 5	83 ± 2

* Macrophages were pretreated for 18 hours with the indicated agents at concentrations of 10 mM LiCl, 5000 IU/ml hTNF, 1 μg/ml LPS, and 250 IU/ml IFN-γ, respectively.

† Amount of cytokine secreted in the supernatant by 1 × 10^5 peritoneal macrophages in a volume of 200 μl during an activation period of 18 hours. The supernatant was preincubated for 1 hour with neutralizing anti-hTNF antibodies before cytokine secretion was assayed. TNF was determined by a cytolysis assay on WEHI164cl13 cells.[9] IL-6 and IL-1 were assayed *via* proliferation of 7TD1 cells[18] or RPMI1788 cells,[16] respectively.

‡ The cytotoxicity percentage to L929 cells was determined as described.[9] The target/effector ratio was 20/1.

control animals; 2) inhibition of erythema and necrosis with NDGA or BW755C was not associated with an inhibition of neutrophil and macrophage infiltration; 3) high TNF plus LiCl concentrations (which can be reached locally at the injection site) are directly cytotoxic to murine skin fibroblastic cells *in vitro*, and the time required for such observation is comparable with that needed to observe skin necrosis *in vivo*. Hence we believe that TNF plus LiCl-induced necrosis is due to a direct cytotoxic effect, the mechanism of which is still unclear. Recently TNF was shown to be cytostatic for normal keratinocytes.[41] A possible cytotoxic effect of TNF plus LiCl on keratinocytes in our model needs further investigation.

The observation that LiCl renders normal skin cells sensitive to TNF cytotoxicity is the first example of a sensitizing effect of LiCl on TNF-resistant cells. In our previous studies,[9] we demonstrated that LiCl could only increase TNF sensitivity of cells sensitive to TNF as such. However, when compared with the TNF-sensitive L929 fibrosarcoma cells,[9] 50% skin fibroblastic cell killing could only be obtained at a 1×10^5-fold higher hTNF concentration when tested in the presence of the minimal required LiCl dose (5 mmol [millimolar]). Remarkably normal human foreskin fibroblasts (FS4) could not be killed by cotreatment with hTNF and LiCl (data not shown). Certain drugs, such as actinomycin D, cycloheximide, or mitomycin C, are known to induce sensitivity to TNF in FS4 cells and some other normal cells.[42] Also we already reported that a combination of IFN-γ and TNF is cytotoxic to normal mouse and rat embryo fibroblastlike cells.[43] In correlation with these results, the murine skin fibroblastic cells also were sensitized to TNF cytotoxicity *in vitro* by cotreatment with actinomycin D or with mIFN-γ (data not shown).

So far, the primary target cell for and the mediator(s) of TNF plus LiCl-induced neutrophil and macrophage infiltration are unknown. Lithium was shown to correct polymorphonuclear cell defects and to induce leukocytosis *in vivo*.[44,45] Furthermore LiCl induces degranulation of neutrophils from normal and psoriatic patients[46,47] and activates monocytes.[48] Recombinant TNF, on the other hand, has been reported to be chemotactic for neutrophils *in vivo*[49] and to activate a number of functions in neutrophils *in vitro*.[2–4] It is tempting to speculate that some of these TNF-mediated and/or LiCl-mediated effects on neutrophils and macrophages are operative in the leukocyte infiltration system described here. Preliminary results excluded an effect of LiCl, at the concentrations used, on the number of leukocytes in circulation (data not shown). In addition to neutrophils and macrophages, the skin contains a variety of other cell types that may participate in the generation of a leukocyte infiltration. It has been reported that TNF stimulates expression of endothelial/leukocyte adhesion molecules in endothelial cells.[50] LiCl could, however, not increase the TNF-induced expression of these adhesion molecules in human endothelial cells (K. Schulze-Osthoff, oral communication, October 1990). Furthermore the effects of TNF on endothelial cells could be obtained equally with IL-1, while we observed no effect of IL-1 plus LiCl in the murine skin model. The latter observation also makes an involvement of the recently identified neutrophil-attracting factor, IL-8, in TNF plus LiCl-induced leukocyte infiltration unlikely because this factor has been shown to be equally induced by TNF or by IL-1.[51] Tumor necrosis factor has been found to activate phospholipase A_2 in fibroblasts,[52] which might lead to the release of inflammatory substances. In accordance with this, Dayer et al[53] demonstrated the release of prostaglandin E2 from TNF-stimulated fibroblasts. This mediator, when injected intradermally, was shown to be a powerful vasodilator that produces a long-lasting erythema.[54] Other investigators reported a TNF-mediated release of leukotriene B_4 (LTB_4) and platelet-activating factor (PAF) by human neutrophils.[55] Leukotriene B_4 can activate many functions of neutrophils and augment macrophage and monocyte cytotoxic activities.[56] However our observation of no inhibition of TNF plus LiCl-induced skin reaction by different drugs known to inhibit leukotriene, prostaglandin, or PAF production renders an involvement of these metabolites unlikely. The inhibitory effect of NDGA and BW755C on TNF plus LiCl-induced skin necrosis might be due to other effects than lipoxygenase blocking.[57,58] Because LiCl has been shown to induce TNF secretion by human monocytes,[48] we tested the possibility that a LiCl-induced increase in TNF production might be responsible for the strong neutrophil and macrophage infiltration. However such an increase does not seem to be responsible for the increased leukocyte infiltration: injection of up to 100 μg hTNF, in the absence of LiCl, did not result in a response similar to the one observed after 10 μg hTNF plus LiCl administration.

Recently we reported that the combination of TNF and LiCl was quite efficient in inhibiting the growth of murine and human tumors in nude mice.[9] Philip and Epstein[59] found that TNF can activate macrophages to kill TNF-sensitive tumor cells, although other investigators reported that TNF alone cannot activate macrophages for cytotoxicity.[60] In our system, an involvement of leukocytes in the TNF plus LiCl-induced antitumor response is unlikely for several reasons. First we could not demonstrate any effect of TNF, LiCl, or their combination on the induction of macrophage cytotoxicity against L929 tumor cells. Second L929 tumors treated with TNF plus LiCl *in vivo* were not infiltrated with leukocytes, although the perilesional 'normal' skin was infiltrated. Third, while multiple TNF plus LiCl injections were needed to obtain a clear antitumor effect, such a treatment resulted in desensiti-

zation to TNF plus LiCl-induced effects on normal skin. Finally we reported previously that tumors of HeLa H21 cells, which are insensitive to TNF plus LiCl *in vitro*, do not regress by TNF plus LiCl treatment *in vivo*.[9] These observations strongly suggest that mainly direct effects were involved in the previously observed tumor destruction by TNF plus LiCl, despite the obvious effect of TNF plus LiCl on the host skin.

The present study provides evidence that in addition to the previously reported antitumor effect, the combination of TNF and LiCl offers the unique property to induce at least two, possibly independent, skin reactions (leukocyte infiltration and necrosis). These effects strongly suggest a major role for TNF in the regulation of inflammatory skin diseases. Because the skin reaction has been observed at serum LiCl concentrations (1.5 mmol/l) that are at the limit of toxicity in humans,[61] our observation may have important clinical implications. One of the major side-effects in the treatment of manic-depressive illness with lithium is the development of several skin diseases, especially psoriasis.[62] Bloomfield and Young reported the enhanced release of inflammatory mediators from lithium-stimulated neutrophils in psoriasis.[47] Although the specific role played by TNF in these processes requires further study, the recent finding that TNF levels are increased in extracts of psoriatic stratum corneum[63] makes an involvement of endogenous TNF in LiCl-induced skin disease very likely.

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