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# Characterization of a relationship between the T-lymphocyte derived differentiation inducing factor (DIF) and lymphotoxin: A common receptor system for DIF, lymphotoxin and tumor necrosis factor downregulated by phorbol diesters

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Here we describe results which show that recombinant lymphotoxin (rLT), like the T-lymphocyte derived differentiation inducing factor (DIF), inhibited the clonogenic growth of some myeloid leukemia cell lines by concentrations of 1 to 30 pmol/l. Wild type HL-60 cells were resistant at these concentrations but responded with differentiation into monocyte-like cells at higher concentrations. An antigenic relationship between DIF and LT was indicated because a neutralizing monoclonal anti-LT antibody bound to and neutralized both differentiation and growth inhibitory effects of DIF. An activity, which cochromatographed with DIF during all purification steps, competed with binding of both rLT and recombinant tumor necrosis factor (rTNF) to HL-60 cells. By use of radioiodinated ligand, 2100 binding sites for rLT were detected on HL-60 cells with a $K_d$ of 330 pmol/l. At 37°C bound ligand was transferred to lysosomes, followed by degradation. rTNF and rLT were shown to compete for binding sites on HL-60 cells. Receptors for both rLT and rTNF were downregulated by activators of protein kinase C such as phorbol diester or diacylglycerol; the number of cell surface receptors decreased while the $K_d$ remained unchanged. Our observations demonstrate a functional and antigenic relationship between DIF and LT and indicate that TNF, LT and DIF share binding sites on myeloid leukemia cells that are downregulated by activation of protein kinase-C.

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We (1, 2) and others (3, 4) have demonstrated that T-lymphocytes produce a polypeptide called differentiation inducing factor, DIF, which can induce maturation of the promyelocytic HL-60 cell line and inhibit the growth of other hematopoietic leukemia cell lines (5). Lymphotoxin (LT) is produced by activated lymphocytes (6–8) and has cytostatic and cytolytic effects toward

transformed cells in vitro (9, 10) and in vivo (11–14). Results from functional and biochemical studies suggested that LT is represented by a family of diverse molecular weight cytotoxins (15–17). Lymphotoxin purified from the lymphoblastoid cell line RPMI 1788 was sequenced and cloned by recombinant DNA methods (18, 19) and revealed 30% homology in the amino-acid sequence with tumor necrosis factor (TNF) (20). Like LT, TNF has also been associated with in vitro and in vivo killing of tumor cells (21–25). Activated macrophages may constitute the major cellular origin of TNF (21). Recent observations have indicated that TNF and LT share common receptors, which may be up-regulated by γ-interferon (26).

Our previous work (5, 27) has demonstrated that DIF and recombinant TNF (rTNF) have, in part, similar effects on hematopoietic cells but that they are biochemically different. In the present work we have investigated whether recombinant lymphotoxin (rLT) has properties in common with DIF and if these molecules may be related.

Although LT has been demonstrated to inhibit growth of malignant cells in vitro (9, 10), effects of LT on differentiation of leukemic hematopoietic cells have not been previously reported. Therefore we investigated growth inhibitory and differentiation effects of rLT on myeloid leukemia cell lines. A varying sensitivity of these cells to the antiproliferative effect of rLT was observed. Wild type HL-60 cells, which were resistant to clonal growth inhibition, were induced to differentiation by rLT. We showed that rTNF and rLT share common cell surface binding sites and that DIF competes with binding of rLT and rTNF to HL-60 cells, indicating that DIF, TNF and LT may share common binding sites.

## Material and methods

### Materials

Recombinant TNF (rTNF) and LT (rLT) (produced by Genentech, Inc) were supplied by Boehringer Ingelheim. The rTNF contained $38 \times 10^6$ U/mg (646 U/pmol) and the rLT contained $220 \times 10^6$ U/mg (3960 U/pmol). These activities were assayed as cytotoxic effects on L-929 fibroblast cells in the presence of 1 μg/ml actinomycin D (25).

DIF was isolated from conditioned medium of the T-lymphocyte line HUT-102 as described (5) using DEAE chromatography, gel filtration and high resolution ion exchange chromatography on MonoQ (Pharmacia, Uppsala, Sweden). The MonoQ chromatography represented a 37 000-fold purification and the preparation obtained was estimated to contain approximately 5% pure DIF (15 000 units/mg). 1 unit of DIF is defined as the amount necessary to increase by 10% the number of nitro blue tetrazolium (NBT)-positive (2) wild type HL-60 cells.

A rabbit antiserum to human rTNF was a gift from Dr. J. J. Mermod, Biogen, Geneva, Switzerland. A rabbit antiserum to purified human LT derived from the lymphoblastoid cell line RPMI 1788 was also used. A 1000-fold dilution of the LT-antiserum was able to completely neutralize the cytotoxic activity of 100 ng/ml LT. DIF, 1.5 μg obtianed by MonoQ chromatography, was incubated without or with 10 μl of rabbit antiserum at 37°C for 16 h in RPMI-10% FBS in a final volume of 300 μl. Serial dilutions of the incubate were assayed for growth inhibition on HL-60-10 cells and for differentiation on wild type HL-60.

A mouse neutralizing monoclonal antibody (LTB-lot A) to human lymphotoxin was also used. The neutralizing capacity was 550 U LT/μg. This antibody was coupled to cyanogen bromide activated Sepharose and 1 ml (5 mg antibody) was packed in a column and washed with 0.15 mol/l NaCl, 10 mmol/l Hepes pH 7.4 (column buffer). DIF, 9 μg, was added and elution was performed in 1 ml fractions with column buffer followed by 4 mol/l $SCN^-$. Bovine serum albumin, 5 mg, was added to each fraction, followed by extensive dialysis against column buffer. Of each fraction, 10 μl were used for assay of growth inhibition on HL-60-10 cells and 100 μl were used for assay of the differentiation effect on wild type HL-60.

Phorbol 12-myristate 13-acetate (PMA) and 1-oleoyl-2-acetyl-glycerol (OAG) were from Sigma, St. Louis, Mo. 4-α-phorbol 12-myristate 13-acetat (4-α PMA) was from LC Services Corporation, Woburn, Mass.

### Cell lines

Wild type HL-60 (28), a subclone of HL-60 (HL-60-10), U-937 (29), K-562 (30) and KG 1 (31) cell lines, was maintained in suspension culture in RPMI 1640 medium with 10% fetal bovine serum (FBS). The same medium was used for assays of differentiation and growth inhibition. The cells used in all experiments were in a logarithmic growth phase.

### Assays for growth inhibition and differentiation

The growth inhibitory effect of rLT on cell lines was determined by seeding 2000 cells in 1 ml of 0.3% agar on top of 1 ml 0.5% agar in growth medium with 15% FBS in 35 mm tissue culture dishes. Plates were scored after 7 d. For differentiation assays, 1-ml aliquots ($2 \times 10^5$/ml) wild type HL-60 (passage 20–45) were incubated with the cytokine preparation. After 4 d, the cell number, viability, the number of cells positive for unspecific esterase using α-naphthyl butyrate as substrate (32) and the number of cells capable of reducing NBT (2) were determined.

### Iodination of rLT and rTNF

The two-phase method of Tejedor & Ballasta (33) was employed. Borate buffer (50 µl 50 mmol/l pH 8.4), KI (10 µl 0.125 mmol/l in borate buffer) and 1 mCi of carrier-free $^{125}$I (Amersham, England) were mixed with 2 µg rLT or rTNF containing 0.05% Tween-20. Iodination was performed as described (33). The $^{125}$I-rLT was isolated on a Sephadex G-25 column. The specific activity was determined by self displacement analysis (34), assuming that the binding characteristics of labeled and unlabeled rLT and rTNF are the same.

The iodinated rLT was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (2). After electrophoresis, gels were dried on filter paper and exposed to X-ray film (Kodak X-Omat S) for 12 h. A single $M_r$ 18 000 component was seen on the fluorogram.

### Binding of $^{125}$I-rLT to cells

Cells were washed and incubated by rotation with radioiodinated rLT in binding buffer (RPMI, 2% FBS and 1% bovine serum albumin) in a total volume of 200 µl in 1.5 ml Eppendorf centrifuge tubes. Centrifugation was performed for 10 s at 8000 × *g*, and the pellet was resuspended and washed twice in ice-cold binding buffer to separate free and membrane-bound $^{125}$I-rLT. The radioactivity of the cell pellet was measured in a γ-counter. Specific binding was defined as the difference between total binding and the non-specific binding that occurred in the presence of a 20-fold excess of unlabeled rLT. The non-specific binding was 15–25% of the total binding. The assay was proportional to the number of cells added in the range $2 \times 10^6$ - $20 \times 10^6$ cells. As a routine $5 \times 10^6$ cells were utilized per incubation. The kinetics for binding of $^{125}$I-rLT were subjected to Scatchard analysis. The occurrence of degradation products of $^{125}$I-rLT in the incubation medium was determined by precipitation of nondegraded $^{125}$I-LT with 10% trichloroacetic acid (TCA) and the radioactivity of the TCA supernatant was taken as a measure of degraded $^{125}$I-rLT.

### Subcellular fractionation

Labelled cells mixed with $5 \times 10^7$ unlabelled carrier cells were homogenized at a concentration of $10^8$ cells/ml in 0.34 mol/l-sucrose/5 mmol/l Hepes (4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid) (pH 7.3)/0.5 mmol/l-EDTA (homogenization medium) with 40 strokes of a Dounce glass homogenizer. After dilution with the same solution, unbroken cells and nuclei were pelleted by centrifugation at 700 *g* for 10 min. For density gradient separations we used 6 ml of 12% Perkoll (density 1.069 g/ml) in Hepes/sucrose adjusted to give a final concentration of 15 mmol/l Hepes pH 7.4 and 0.25 mol/l sucrose layered on top of a 1-ml cushion of saturated sucrose. A 2.0-ml aliquot of the 700 *g* supernatant of the cell homogenate was layered on top of the Percoll followed by centrifugation at 32 000 *g* for 60 min at 4°C in a Sorvall RC-5B centrifuge using the SE-12 rotor. The gradient was collected in 19 fractions by use of a peristaltic pump and the cytosol was collected in fraction no. 20. Galactosyl transferase (marker for Golgi elements) and β-hexosaminidase (marker for lysosomes) were determined as described (35, 36). The distribution of plasma membranes was determined by $^{125}$I-lectin affinity as described (37).

## Results

### A functional and antigenic relationship between DIF and LT

We showed previously that DIF and rTNF induced differentiation of 50% of wild-type HL-60 cells at concentrations of 50 pmol/l and 400 pmol/l, respectively (5, 27). In the present work we investigated whether rLT also has the ability to induce differentiation of wild-type HL-60 cells. We found that approximately 500 pmol/l rLT induced 50% HL-60 cells into nonspecific esterase-positive and NBT-positive monocyte-like cells (data not shown). In addition, rLT inhibited clonal growth of the leukemia myeloid cell lines U-937, KG-1 and HL-60-10, but not of wild-type HL-60 and K-562 (Figure 1). All these effects are similar to those reported for DIF (5). Thus rLT has functional effects similar to those of DIF. It is emphasized that wild-type HL-60 was resistant

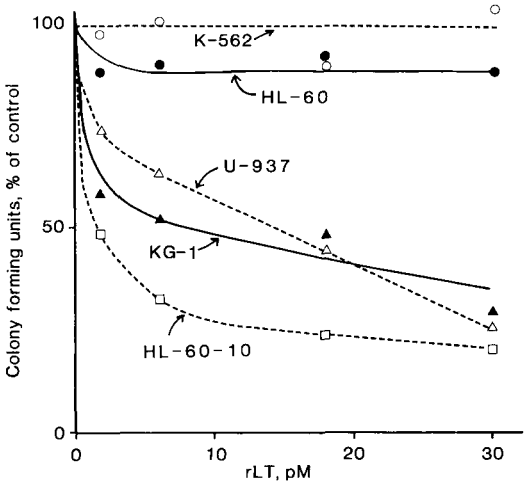


Figure 1. Proliferation inhibitory effects by rLT on the clonal growth in agar of the myeloid cell lines HL-60 (●), K-562 (○), KG-1 (▲), HL-60-10 (□) and U-937 (△). The experiments were performed two times on each cell line. Colony forming units are given as % of control after 7 d of culture. The plating efficiency was 30% for wild-type HL-60, 40% for K-562, 15% for KG-1, 30–50% for HL-60-10 and 40–50% for U-937.

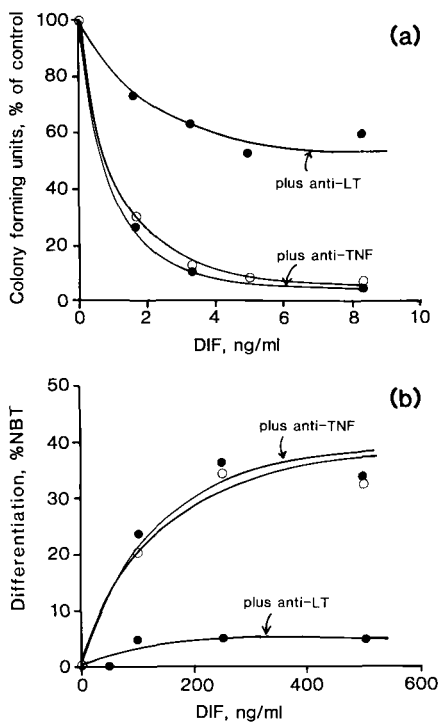


Figure 2. Effect of a neutralizing polyclonal anti-LT and an anti-rTNF antibody on the growth inhibitory effect on colony formation in agar of HL-60-10 cells (a) and the differentiation effect on wild-type HL-60 measured by NBT-reduction (b). Results are given from control incubations with DIF (○) and incubations with DIF and antiserum (●). For details see Material and methods.

to clonal growth inhibition by both rLT and DIF, but responded with differentiation to higher concentrations of these agents.

A neutralizing polyclonal antiserum to LT inhibited both growth inhibitory and differentiation effects of DIF (Figure 2). In contrast, neutralizing polyclonal antibodies to rTNF did not inhibit the growth inhibitory or the differentiation effect of DIF (Figure 2). The affinity of DIF for a monoclonal anti-rLT antibody, coupled to Sepharose, was also investigated (Figure 3). DIF bound to the antibody and could be eluted with 4 mol/l $SCN^-$. These data indicate an antigenic relationship between DIF and LT but not between DIF and TNF.

## Binding, internalization and degradation of $^{125}$I-rLT

In order to study the effect of DIF on the binding of rLT, the binding of $^{125}$I-rLT to HL-60-10 cells was first characterized. Binding sites for $^{125}$I-rTNF on such cells have been previously described (27). Radio-iodinated rLT migrated as a single Mr 18 000 component when analyzed with SDS-PAGE under reducing conditions, followed by fluorography (data not shown). On the basis of results from self-displacement analysis, the specific activity of $^{125}$I-rLT was found to vary between 0.12 and 0.56 × $10^6$ cpm/pmol in separate labelling experiments (rTNF radio-iodinated by use of the same method was found to have a specific activity of 0.56 × $10^6$ cpm/pmole).

Binding of $^{125}$I-rLT to all the myeloid cell lines in Figure 1 was competitively inhibited in presence of unlabelled rLT (data not shown).

The binding of $^{125}$I-rLT to HL-60-10 cells was measured at 4°C and 37°C. Maximum binding was observed after 120 min at 4°C and after 90

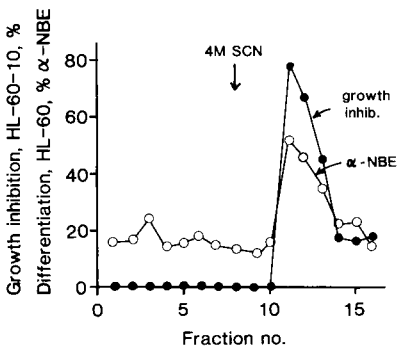


Figure 3. Binding studies of DIF to a neutralizing monoclonal anti-rLT antibody produced against recombinant LT. The antibodies were coupled to activated Sepharose. Bound material was eluted with 4mol/l $SCN^-$. For details see Material and methods. The dialyzed fractions were assayed for growth inhibitory effect on HL-60-10 and differentiation effect (α-NBE) on wild-type HL-60.

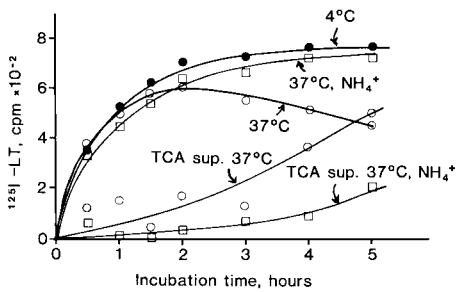


Figure 4. $^{125}$I-rLT binding to HL-60-10 cells at 4°C (●) and at 37°C (○) as a function of time of incubation. Incubations were also performed at 37°C in the presence of 10 mmol/l $NH_4^+$ (□). All values are corrected for nonspecific binding obtained at a 20-fold excess of unlabeled rLT. The occurrence of degradation products of $^{125}$I-rLT in the incubation medium was determined by precipitation with 10% trichloroacetic acid (TCA) in ice and by measuring the radioactivity of the supernatant. Data are given for the TCA supernatants obtained from binding at 37°C in the absence (○) and presence (□) of 10 mmol/l $NH_4^+$. These data are corrected for the radioactivity of TCA supernatants obtained from binding studies at 4°C. The latter showed no increase in radioactivity of TCA supernatants with time, indicating that no degradation occurred at 4°C.

min at 37°C (Figure 4). Extended incubation of $^{125}$I-rLT labelled cells at 37°C resulted in a decrease of the amount of cell-bound radioac-

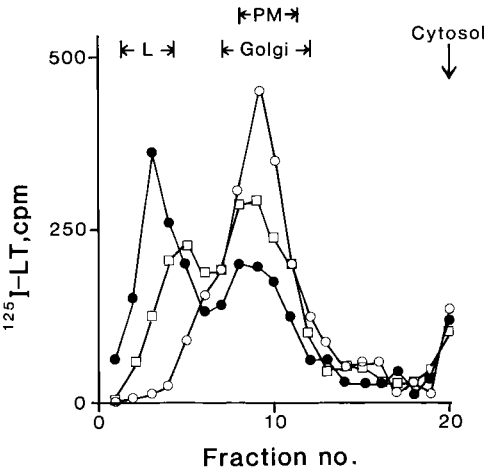


Figure 5. Demonstration of internalization of bound $^{125}$I-rLT in HL-60-10 cells by use of centrifugation of cell homogenates in 12% Perkoll. The localization in the gradient of markers for plasma membrane (PM) Golgi elements and lysosomes (L) is indicated. Fraction no. 20 represents the cytosol. The distribution in the gradient is shown for homogenates prepared after loading with $^{125}$I-LT at 4°C for 2 h and washed followed by incubation at 37°C for 0 (○), 10 (□) and 30 (●) min.

tivity. The decrease was accompanied by the presence of a corresponding amount of acid-soluble material released into the medium after 1–2 h of incubation at 37°C. This indicated a degradation of cell-bound $^{125}$I-rLT. No degradation was observed at 4°C. Binding experiments at 37°C in the presence of 10 mmol/l NH4+ showed no decrease of cell-associated $^{125}$I-rLT as a function of time, and presence of acid soluble material in the medium was low, indicating that NH4+ protected against degradation of rLT.

Temperature-dependent degradation of cell-bound $^{125}$I-rLT suggested that rLT was degraded after receptor-mediated endocytosis and transfer to lysosomes. Indeed, endocytosis was confirmed by use of a Perkoll density gradient system (Figure 5). The gradient system allowed separation of lysosomes from Golgi and plasma membrane fractions (see 'Material and methods'). $^{125}$I-rLT was allowed to bind to cells at 4°C for 2 h. The cells were thereafter washed and aliquots were incubated at 37°C for 0, 10 and 30 min followed by homogenization. Cell homogenates were frac-

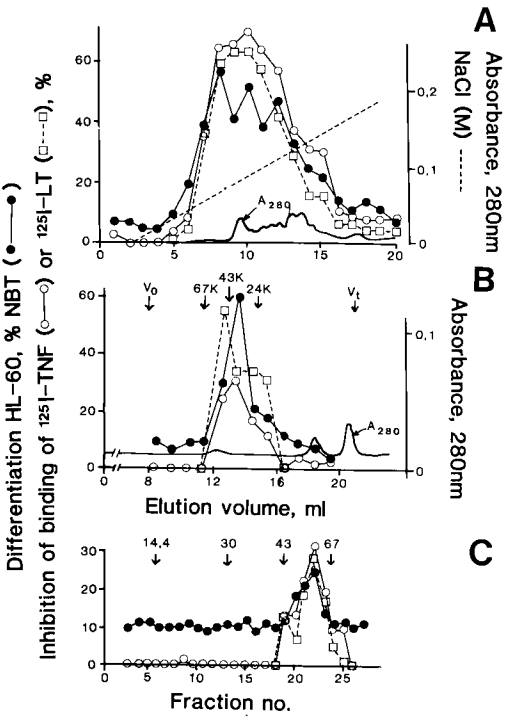


Figure 6. Competition by DIF for binding of $^{125}$I-rLT. Fractions obtained from purification of DIF (5) by Mono Q ion exchange chromatography (A), gel filtration on a Superose column (B) and SDS-PAGE (C) were included in the standard binding assay for $^{125}$I-rLT to HL-60-10 cells at 4°C in order to determine if DIF-containing fractions competed for the binding of $^{125}$I-LT or $^{125}$I-TNF. Data are given for differentiation induction of wild-type HL-60 as % NBT-positive cells (●), inhibition of $^{125}$I-LT binding (□), inhibition of $^{125}$I-TNF (○), as well as for absorbance at 280 nm in A and B (——) and for the NaCl gradient of A (-----). The positions of molecular weight markers are indicated in B and C. For details see Material and methods.

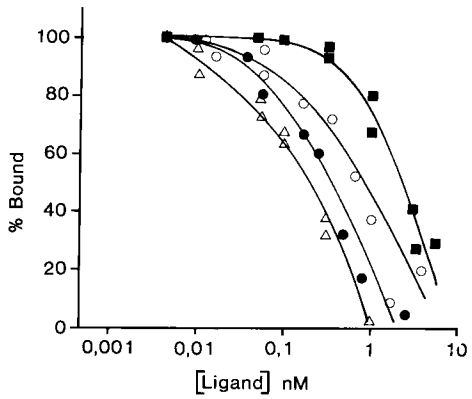


Figure 7. Evidence for a common receptor for rTNF and rLT on HL-60-10 cells. The results are plotted as specific binding relative to an assay with no added competitor. Shown is competition of $^{125}$I-rLT-binding with rLT (○) and with rTNF (△), competition of $^{125}$I-rTNF binding with rTNF (●) and with rLT (■).

tioned on Perkoll gradients as described above and fractions collected from the gradient were measured for presence of labelled material. Following binding at 4°C for 2 h, the majority of radioactivity was detected in fractions containing plasma membranes. When the washed, labelled intact cells were incubated at 37°C for 10 min prior to homogenization, $^{125}$I-rLT was detected in fractions with an intermediate density between plasma membrane and lysosomes, probably representing receptosomes. Incubation of the labelled cells at 37°C for 30 min resulted in the occurrence of radioactive material in the most dense fractions containing the lysosomes. Further extended incubation periods at 37°C resulted in an increasing accumulation of radioactivity in the lysosomal fractions with a concomitant decrease in the plasma membrane fractions. These data demonstrate that $^{125}$I-rLT is internalized and, at least partially, degraded in lysosomes.

## DIF competes for binding sites with LT and TNF

Because of the functional and antigenic relationship described above between DIF and LT, we also investigated whether DIF was competitively inhibiting binding of rLT and rTNF to the cell surface. For this, fractions obtained during different purification steps of DIF (5) were assayed for competition in binding assays for $^{125}$I-rLT and $^{125}$I-rTNF using HL-60-10 cells. Thus, fractions obtained by MonoQ chromatography, gel filtration on a Superose column and SDS-PAGE (5) were analyzed. As is shown in Figure 6, an activity cochromatographing with DIF in all

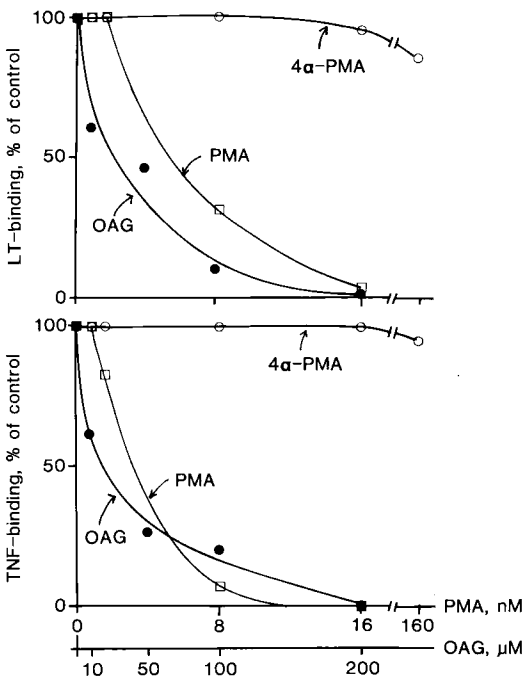


Figure 8. Inhibition by PMA or OAG of binding of rLT and rTNF to a cell surface receptor on HL-60-10 cells. Cells were incubated with various concentrations of PMA (□), 4 α-PMA (○) or OAG (●) for 30 min and washed, after which the binding of $^{125}$I-rLT and $^{125}$I-TNF at 4°C was determined.

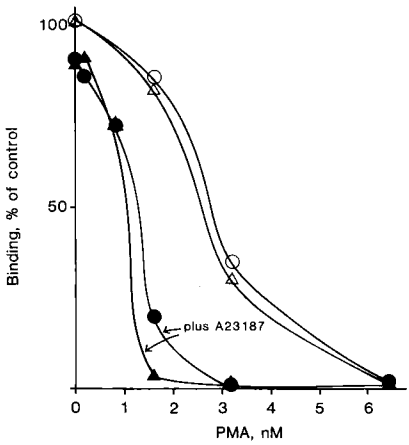


Figure 9. Synergistic inhibitory effect between PMA and the $Ca^{2+}$ionophore A23187 on the binding of rLT and rTNF to HL-60-10 cells. Cells were incubated with different concentrations of PMA with or without 0.5 μg/ml A23187 for 30 min and washed, after which the binding of $^{125}$I-rLT (△, ▲) and $^{125}$I-TNF (○, ●) at 4°C was determined.

three purification steps was competing for binding sites on the target cells with both rLT and rTNF.

As DIF competed for binding of both rLT and rTNF, evidence was sought for a common receptor for LT and TNF.

Figure 7 shows that binding of $^{125}$I-rLT and $^{125}$I-rTNF were competitively inhibited in the presence of unlabeled rTNF and rLT, respectively. These data indicate that both rLT and rTNF compete for binding to the same binding site(s). Therefore LT, TNF and DIF may all share common binding sites. However, a higher molar concentration of unlabelled rLT than of rTNF was needed to compete for binding of $^{125}$I-TNF. Similarly, a lower concentration of unlabelled TNF than of rLT was needed to compete for binding of $^{125}$I-rLT. Thus, it appears that rLT and rTNF share a common receptor and that rTNF may have a higher affinity for this receptor than LT. However, the data below (Figure 10) demonstrate an identical $K_d$ of both rLT and rTNF for binding to HL-60-10.

## Downregulation of receptors for LT and TNF by phorbol diester

HL-60-10 cells were treated at 37°C for 30 min with various concentrations of the phorbol diester phorbol 12-myristate 13-acetate (PMA). PMA-treated cells were washed and subjected to binding of $^{125}$I-rLT and $^{125}$I-rTNF at 4°C for 2 h. We found that 8 nmol/l PMA dramatically prevented binding of both rLT and rTNF (Figure 8). This effect was most probably mediated through activation of protein kinase C because 4 α-PMA, which does not activate protein kinase C (38), did not inhibit the binding (Figure 8).

The physiological activation of protein kinase C is mediated through diacyl-glycerol (39). Therefore we investigated whether not only PMA, but also 1-oleoyl-2-acetyl-glycerol (OAG) inhibited binding of rLT and rTNF. We found that the binding of $^{125}$I-rLT to HL-60-10 cells was

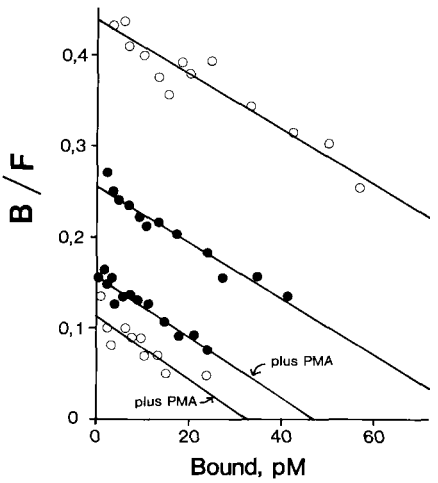


Figure 10. Scatchard analysis of data for the effects of PMA on binding of rLT and rTNF to HL-60-10 cells. The cells were preincubated with or without 3 nmol/l PMA, after which they were incubated at 4°C for 2 h with different amounts of $^{125}$I-rLT (●) or $^{125}$I-rTNF (○). Cell-associated and free $^{125}$I-ligand were separated by centrifugation and washing as described. Specific binding was defined as the difference between the total and the nonspecific binding occurring in the presence of 20-fold excess of unlabeled ligand. The binding of $^{125}$I-rLT showed a $K_d$ of 330 pmol/l with 2100 binding sites per cell; after treatment with PMA the $K_d$ was 310 pmol/l with 1100 binding sites per cell. The binding of $^{125}$I-TNF showed a $K_d$ of 330 pmol/l with 3500 binding sites per cell; after treatment with PMA the $K_d$ was 290 pmol/l with 800 binding sites per cell. Binding of rLT and of rTNF was performed in separate experiments, the difference in number of binding sites for rLT and rTNF is within the variation for different experiments.

inhibited by approximately 50% at 50 μmol/l OAG (Figure 8). This finding further indicates that prevention of binding was due to activation of protein kinase C. Treatment with the $Ca^{2+}$-ionophore A 23187 (2.0 μg/ml) resulted in a small reduction of $^{125}$I-LT or $^{125}$I-TNF binding. However, it synergistically amplified the inhibitory effect of PMA (Figure 9).

We investigated by Scatchard analysis whether PMA inhibited binding by decreasing the affinity of the ligand for the receptor or by decreasing the number of receptors (Figure 10). The dissociation constants and the maximum number of binding sites were calculated from the best fit equations. The binding of $^{125}$I-rLT showed a $K_d$ of 330 pmol/l with 2100 binding sites per cell. After treatment with 3 nmol/l PMA, the $K_d$ remained essentially unaltered (310 pmol/l) but the number of binding sites per cell was decreased (1100). Similarly, the number of binding sites for $^{125}$I-rTNF was decreased by PMA without a change of $K_d$ (Figure 10). These data indicate that activation of protein kinase C down-regulated the receptors for rLT and rTNF.

## Discussion

A major question addressed was whether there is a relationship between the T-lymphocyte-derived differentiation inducing factor (DIF) (1–4) and lymphotoxin. We have compared DIF with LT/TNF on the basis of functional, antigenic and biochemical similarities. In this study we show that rLT exhibits a growth inhibitory effect on some myeloid leukemia cell lines with 50% inhibition of clonal growth at approximately 15 pmol/l) concentration. However, there was a remarkable difference in susceptibility among various cell lines. Wild-type HL-60 was resistant to clonal growth inhibition at low rLT concentrations, while a higher concentration (500 pmol/l) induced differentiation of wild-type HL-60 into monocyte-like cells. The effects observed are consistent with those reported earlier for DIF and TNF (5, 27). Thus, DIF, LT and TNF all have similar growth inhibitory effects on myeloid leukemia cell lines. All three factors induced a fraction of wild-type HL-60 cells to mature into monocyte-like cells, although DIF may be the most potent inducer in this respect (5) and LT the least potent. In line with this, it was recently demonstrated that a differentiation inducing factor purified from medium conditioned with PHA-stimulated leukocytes was most likely identical with TNF (40).

Tumor necrosis factor and lymphotoxin are antigenically distinct molecules (41), which have been associated with in vitro and in vivo killing of tumor cells (9–14, 21–25). TNF and LT exhibit 30% homology in their amino-acid sequences (20). The conserved regions may be

crucial to the shared cytotoxic activities, most likely through binding to a common receptor expressed on the cell surface (25, 26). TNF produced by human monocytes and myeloid cell lines resolves as a 30 kD polypeptide by gel filtration, and as a 17 kD species on SDS-PAGE (21–26), which is clearly distinct from the behavior of DIF, the molecular weight of which corresponds to approximately 45 000 as judged by assay of biological activity of fractions obtained after SDS-PAGE (5). The elution pattern of DIF when subjected to high resolution ion exchange chromatography revealed some heterogeneity (5), which was also suggested by data from chromatofocusing revealing isoelectric points at pH 5.4–5.8. The isoelectric point of TNF is 6.0 (24). Thus DIF and TNF are biochemically distinct polypeptides, a conclusion supported by the present study which demonstrated a lack of antigenic similarities between DIF and TNF. Some biochemical characteristics also differ between DIF and LT. LT produced by a lymphoblastoid cell line resolved as a 20 kD or 25 kD species on SDS-PAGE with an isoelectric point of 5.8 (18). The heterogeneity in molecular size and charge reported for LT (16, 17) was suggested as being dependent on $NH_2$-terminal extension and aggregation behavior on gel permeation chromatography (18). Results from hybridization studies suggested that human LT is encoded by a single gene (19).

Our data indicated an antigenic relationship between DIF and LT since a neutralizing antiserum to LT neutralized both the growth inhibitory and differentiation inducing effects of DIF. However, the reactivity of one monoclonal antibody with both rLT and DIF does not constitute definitive evidence for the identification of the DIF as LT. Detailed biochemical studies with comparison of sequence data for DIF and LT will be required to establish the exact relationship.

Our finding of an activity, which cochromatographed exactly with DIF through all purification steps and which competed with binding of both rLT and rTNF, supports the notion that DIF shares common binding sites with LT and TNF on the surface of target cells. Characterization of the receptor system demonstrated high affinity ($K_d$ = 330 pmol/l) specific binding of radioiodinated rLT. Surface-bound ligand was internalized at 37°C followed by degradation in lysosomes. These results are consistent with our results reported earlier on binding and internalization of rTNF to leukemic cell lines (27). Competitive inhibition of binding of $^{125}$I-rLT by unlabeled rTNF as well as of $^{125}$I-rTNF by unlabeled rLT indicated that LT and TNF recognize the same receptor in agreement with previously published results (26). This observation may explain the similar effects that rTNF and rLT have on leukemic hematopoietic cells. Competition experiments (Figure 7) indicated that TNF binds to HL-60-10 cells with a higher affinity than LT. However, Scatchard analysis revealed an identical $K_d$ for both TNF and LT, 330 pmol/l. Native LT is known to have a strong tendency to polymerize (18) and if this is also true for rLT it makes the experiment difficult to interpret.

Our data showed that the phorbol diester PMA inhibited cell surface binding of rLT and rTNF. Phorbol diester tumor promoters are believed to exert their effects through activation of their cellular enzyme receptor, protein kinase C (42, 43). The specificity of this effect was apparent since the inactive 4α-PMA failed to alter the cell surface binding of rLT and rTNF. In addition, the physiological mediator of protein kinase C, diacylglycerol (39), also inhibited binding of rLT and rTNF. This further supports the notion that activation of protein kinase C leads to inhibition of binding of rLT and rTNF. The PMA-mediated inhibition of binding was apparently caused by a downregulation of the receptors, because Scatchard analysis demonstrated that the number of binding sites decreased, while the $K_d$ was unchanged. Membrane-bound substrates for protein kinase C include receptors for growth factors, such as EGF (44, 45, 46), whose phosphorylation leads to a reduction of EGF ligand binding to its high affinity class of receptors (46). Also the receptor for transferrin is a substrate for protein kinase C

(47) and treatment of HL-60 cells with phorbol diester induced a rapid down-regulation of surface transferrin receptor as a result of receptor internalization (48). Phosphoregulation of the transferrin receptor might serve as a transmembrane signal for rapidly dividing cells to cease proliferation and/or initiate differentiation (47). It is possible that protein kinase C could also phosphorylate a receptor for LT/TNF/DIF or, alternatively, phosphorylate an undefined substrate involved in receptor internalization. Such phosphorylation, which remains to be verified, could explain the receptor down-regulation which we observed upon incubation with PMA or OAG. A synergism has been demonstrated between $Ca^{2+}$ and phorbol diesters that leads to transferrin receptor phosphorylation and down-regulation in HL-60 cells (49). In analogy, we found that a $Ca^{2+}$-ionophore, A 23187, synergistically amplified the PMA-induced down-regulation of LT and TNF receptors.

In conclusion, we have demonstrated an antigenic relationship between DIF and LT and that DIF, TNF and LT share binding sites on myeloid leukemia cells that are down-regulated by activation of protein kinase C.

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