0022-1767/87/13810-3319802.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 138, 3319-3324, No. 10, May 15, 1987
Printed in U.S.A.

ACTIVATION OF CULTURED HUMAN ENDOTHELIAL CELLS BY RECOMBINANT LYMPHOTOXIN: COMPARISON WITH TUMOR NECROSIS FACTOR AND INTERLEUKIN 1 SPECIES[1]

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Recombinant human lymphotoxin (LT) was compared with recombinant human tumor necrosis factor (TNF) for direct actions on cultured human endothelial cells (HEC). At equivalent half-maximal concentrations (based on L929 cytotoxicity units) LT and TNF each caused rapid and transient induction (peak 4 to 6 hr) of an antigen associated with leukocyte adhesion (detected by monoclonal antibody H4/18), a rapid but sustained increased expression (plateau 24 hr) of a lymphocyte adhesion structure (ICAM-1), a gradual (plateau 4 to 6 days) increase in expression of HLA-A,B antigens, and gradual (4 to 6 days) conversion of HEC culture morphology from epithelioid to fibroblastoid, an effect enhanced by immune interferon (IFN-γ). Induction of H4/18 binding by maximal concentrations of LT or TNF could not be augmented by addition of the other cytokine, and 24 hr pretreatment with LT or TNF produced hyporesponsiveness to both mediators for reinduction. H4/18 binding can be transiently induced by tumor-promoting phorbol esters. Pretreatment with either LT or TNF also fully inhibited induction of H4/18 binding by phorbol ester, whereas phorbol ester pretreatment only variably and partially inhibited reinduction by LT or TNF. These actions of LT on endothelium shared with TNF may serve in vivo to promote lymphocyte and inflammatory leukocyte adhesion and transendothelial migration. Recombinant human interleukin 1 species (IL 1α and IL 1β) shared many of the actions of LT and TNF and were indistinguishable from each other. However, IL 1 species could be distinguished from LT/TNF by their relative inability to enhance HLA-A,B expression, by their ability to augment H4/18 binding caused by maximally effective concentrations of LT or TNF, and by their inability to inhibit reinduction of H4/18 binding by LT or TNF. In contrast to the actions of LT or TNF, pretreatment with IL 1α or IL 1β only partially inhibited induction of H4/18 binding by phorbol ester, and phorbol ester pretreatment consistently, albeit partially, inhibited induction by IL 1 species. These studies suggest that activated T cells through the secretion of LT can in turn activate the local endothelial lining so as to promote homing and extravasation of inflammatory cells. Furthermore, these LT actions can be augmented or complemented by other locally produced mediators such as IFN-γ or IL 1.

Lymphotoxin (LT)[3] was initially defined as a protein factor generated in response to T cell activation that could mediate cytolysis of tumor cells and other targets (1). Its spectrum of tumoricidal activity is strikingly similar to that of the activated macrophage product tumor necrosis factor (TNF) (2). Subsequently, proteins with both LT and TNF activities have been purified to homogeneity, sequenced, and cloned (3–6). Detailed structural analysis has revealed regions of homology between LT and TNF. Furthermore, the purified proteins bind competitively to receptors on certain tumor cells (7). Interestingly the genes for LT and TNF appear to be arranged in tandem within the major histocompatibility complex (MHC) (8), although the significance of this finding is unknown.

The recent availability of recombinant TNF has revealed that this cytokine is a potent pleiotropic inflammatory mediator (9). Remarkably, many of the actions of TNF are similar or identical to those observed with natural or recombinant interleukin 1 (IL 1) species. We have been studying the actions of TNF and IL 1 upon vascular endothelial cells. For example, by using cultured human endothelial cells (HEC), we and others have found that TNF and IL 1 cause transient cell surface expression of tissue factor procoagulant activity (10–12) and transiently render endothelial cells markedly more adhesive for inflammatory leukocytes and related cell lines (13–16). IL 1 has been reported to render HEC more adhesive for lymphocytes, although this effect appears to persist longer than the adhesiveness for other leukocytes (17). Changes in HEC adhesiveness can be correlated with changes in surface antigen expression. Specifically, both TNF and IL 1 induce de novo synthesis and transient

Received for publication December 19, 1986.
Accepted for publication February 6, 1987.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] This work was supported by NIH grants HL-36003 and 22602. Jordan S. Pober is an Established Investigator of the American Heart Association. Research on TNF is supported by Biogen.
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[3] Abbreviations used: LT, lymphotoxin; HEC, human endothelial cells; TNF, tumor necrosis factor.

expression (peak 4 to 6 hr) of an endothelial surface antigen associated with monokine-stimulated leukocyte adhesion (16) recognized by murine monoclonal antibody H4/18 (18), and cause a rapid but sustained increase in expression (above basal levels) of a lymphocyte adhesion structure (called intercellular adhesion molecule or ICAM-1), recognized by murine monoclonal antibody RR1/1 (19). TNF has been found to induce a gradual (4 to 6 day) increase in expression of class I MHC antigens (20), the recognition structures for cytolytic T lymphocytes. Coincident with the enhanced expression of class I antigens, TNF causes a rearrangement of cultured HEC monolayers. Treated cultures appear fibroblastoid, exhibit numerous intercellular gaps, and lose basement membrane components (21). (IL 1 effects on HEC morphology have also been reported (22), but these have not been studied with recombinant IL 1 species free of potential TNF contamination. Furthermore, it cannot be assessed from these reports whether IL 1-induced morphological changes are similar to those induced by TNF). Collectively, these and other studies have suggested that vascular endothelium is an important target cell for the proinflammatory actions of TNF, as well as for those of IL 1. It is not presently known whether LT will share with TNF its proinflammatory, as well as tumoricidal activities. In the present study we have compared the actions of recombinant human LT on cultured HEC with those of recombinant TNF and of recombinant IL 1 species. We find that in most assays of antigenic and morphologic modulation, LT appears both qualitatively and quantitatively similar to TNF in its spectrum of activities. Furthermore, in some of the assays used, LT and TNF, although indistinguishable from each other, may be clearly differentiated from IL 1α and IL 1β.

MATERIALS AND METHODS

HEC were isolated from umbilical veins (23) and were serially subcultured (24) using conditions and reagents described elsewhere (18). Cytokines and other mediators were added to the culture medium for the times and at the concentrations indicated in each experiment. The cytokines used in these studies were recombinant LT (expressed in CHO cells and partially purified to final concentration of 3.2×10^5 U/ml in phosphate-buffered saline, specific activity 3×10^6 U/mg protein), recombinant TNF (expressed in *E. coli* and purified to homogeneity, specific activity 2.5×10^7 U/mg) (25), recombinant IL 1α (Genzyme, Boston, MA) (26), recombinant IL 1β (Cistron, Pinebrook, NJ) (27), and recombinant immune interferon (IFN-γ, expressed in CHO cells with final concentration of 2.5×10^5 U/ml) (28). In some experiments, affinity-purified natural IL 1 preparations (from Genzyme or Cistron) were used instead of recombinant IL 1 molecules. TNF and LT units were determined in L929 cytotoxicity assays (performed at Biogent courtesy of Drs. Jan Tavernier and Jose Van der Heyden); IL 1 units are based on information provided by the suppliers for thymocyte co-stimulating activity; and IFN-γ activity was established in standard viral protection assays (28). Note that the two cytokines used in this study that are naturally glycosylated (i.e., LT and IFN-γ) were produced in mammalian cell expression systems and are thus glycosylated in recombinant form as well. Other mediators used in these studies include the calcium ionophores A23187 and ionomycin (Calbiochem, San Diego, CA), the tumor promoting phorbol esters 4-phorbol 12-myristate 13-acetate (PMA), and mezerein (Sigma Chemical Co., St. Louis, MO), as well as the less active 4-O-methyl derivative of PMA (4-O-methyl PMA, also from Sigma). Stock solutions of these compounds were made in dimethyl sulfoxide (Sigma), and equivalent carrier concentrations were added to control cultures in the relevant experiments. For short term (less than 24 hr) induction experiments, cytokines or mediators were added in 20% fetal bovine serum in medium 199 without additional growth factors; no differences were noted whether growth factors were included in the medium, whether serum was replaced with albumin, or whether stimulation was conducted in protein free medium. Finally, in some experiments, RNA (actinomycin D and daunorubicin) and protein synthesis (cycloheximide and emetine) inhibitors (all from Sigma) were added 15 to 30 min before the addition of cytokines or mediators at the concentrations indicated (found in other experiments to inhibit [^{35}S]methionine incorporation into trichloroacetic acid precipitable protein by 95% or greater without inducing cell detachment or loss of trypan blue exclusion, either in the presence or absence of mediators).

Morphology experiments were performed on early subcultured cells (first or second passage level) in microtiter wells. Cytokines were introduced at visual confluence. Cell monolayer organization was assessed by phase contrast microscopy after 2.5% glutaraldehyde fixation, methanol dehydration, and air drying. Morphology was rated semiquantitatively by two independent observers by using the following scale: 0 = cells are uniformly polygonal and nonoverlapping ("epithelioid"); 1 = most cells are polygonal; 2 = most cells are elongated; and 3 = cells are thin and highly elongated, whorl patterns are evident, "dendritic" forms may be present, and *no* clusters of polygonal cells are found ("fibroblastoid"). These changes were illustrated (21).

Surface antigen expression was quantitated on serially subcultured cells (passage levels 3 to 12) either by radioimmunobinding assay to replicate monolayers in microtiter wells, or by fluorescence flow cytometry on trypsin-versene suspended cell populations (18, 20). Monoclonal antibodies used in these studies were H4/18 (18), RR1/1 (anti-ICAM-1) (29), W6/32 (anti-HLA-A,B) (30), E1/1.2 (anti-mesenchymal cell) (19), and isotype matched non-binding controls. In some radioimmunobinding experiments, test antibody binding was normalized to E1/1.2 binding, but any experiment revealing significant variation in E1/1.2 binding, indicative of changes in cell number, was excluded.

RESULTS

Human recombinant LT, like TNF, induces expression of specific H4/18 binding, (Fig. 1), increases expression of ICAM-1 (measured by specific RR1/1 binding; Fig. 2) and increases expression of HLA-A,B antigens (measured by specific W6/32 binding, Fig. 3 and Table I). The maximal increases produced by LT occasionally varies from

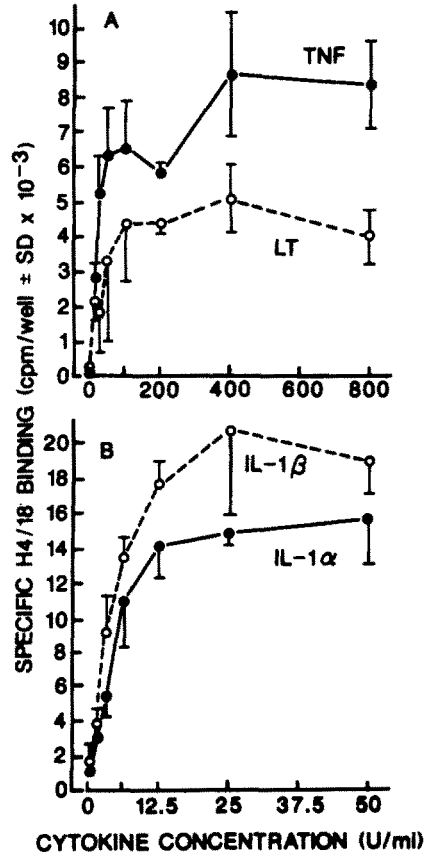

Figure 1. Induction of H4/18 binding by cytokines assessed by radioimmunoassay at 4 hr. *Panels A* and *B* are separate experiments.

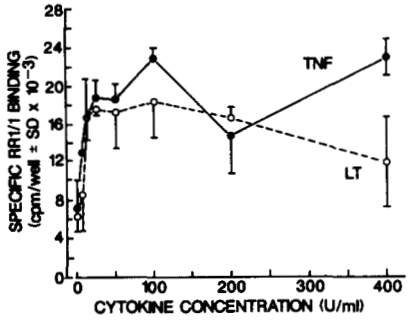

Figure 2. Enhancement of ICAM-1 expression by cytokines assessed by radioimmunoassay at 4 hr.

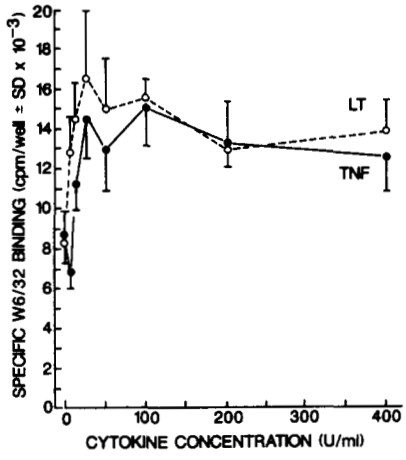

Figure 3. Enhancement of HLA-A,B antigen expression by cytokines assessed by radioimmunoassay at 4 hr.

TABLE I

HLA-A,B Antigen modulation

Cytokine (conc)	Specific W6/32 Binding
None	1.0[a]
IL 1α (5 U/ml)	1.1
IL 1β (5 U/ml)	1.6
TNF (20 U/ml)	3.1
LT (25 U/ml)	2.5

[a] Ratio at 72 hr of treated to control model specific W6/32 binding per cell determined by FACS analysis. In this experiment, some IL 1α treated cells appeared to show diminished HLA-A,B expression, although the model fluorescence did not decrease.

those obtained with TNF, but no consistent pattern was found. However, the concentrations of LT and TNF that induce half-maximal stimulation are consistently comparable (10 to 50 U/ml) when standardized by cytotoxic activity in L929 cells (Figs. 1 to 3). Like TNF, LT causes the organization of HEC monolayers in endothelial cultures to change from epithelioid to fibroblastoid; cells in treated cultures are elongated, overlapping, and arranged in whorls (Table II). Both TNF and LT synergize with IFN-γ in producing morphological rearrangement (Table II).

To investigate the mechanisms of LT and TNF actions on HEC, we used induction of specific H4/18 binding as an assay to test potential second messengers. We found no effect at 4 to 6 hr of incubation of ionophores that elevate intracellular calcium (A23187 or ionomycin; Table III). In contrast, we observed transient induction of H4/18 binding with the tumor promoting phorbol ester PMA (Table III and Fig. 4), but not with the less active derivative 4-O-methyl PMA (Table III). Other tumor promoting phorbol esters such as phorbol 12,13-dibutyrate

TABLE II

Morphological rearrangement in HEC cultures at 96 hr

Cytokine Concentrations (U/ml)	Morphological Appearance			
	IL 1α	IL 1β	TNF	LT
1[a]	1[b]	2	0	2
3	2	3	1	3
10	3	3	2	3
30	3	3	3	3

[a] Numbers represent thymocyte costimulating activity for IL 1 species or L929 cytotoxicity units × 10^{-1} for TNF and LT.

[b] Scale: 0, fully epithelioid; 1, mostly epithelioid; 2, mostly fibroblastoid; 3, fully fibroblastoid. Criteria are described in *Materials and Methods*. In replicate cultures in this experiment, treatment with IFN-γ at 200 U/ml scored 1; co-addition of IFN-γ (200 U/ml) to any other cytokine at any concentration scored 3, indicating potential synergy.

TABLE III

Induction of H4/18 binding: specificity of mediators

	Mediator (Conc)	Specific H4/18 Binding (mean cpm/well ± SD × 10^{-3})
Expt. 1.	None	0.8 ± 0.2
	IL 1 (5 U/ml)[a]	4.6 ± 0.4
	PMA (5 × 10^{-8} M)	6.7 ± 0.4
	4-0-methyl PMA (5 × 10^{-8} M)	0.8 ± 0.5
Expt. 2.	None	1.6 ± 0.6
	TNF (100 U/ml)	15.1 ± 1.2
	A23187 (5 × 10^{-6} M)	1.5 ± 1.3
	Ionomycin (1 × 10^{-6} M)	2.0 ± 1.2

[a] In this experiment, natural IL 1 was used. Binding was assessed at 4 hr in both experiments.

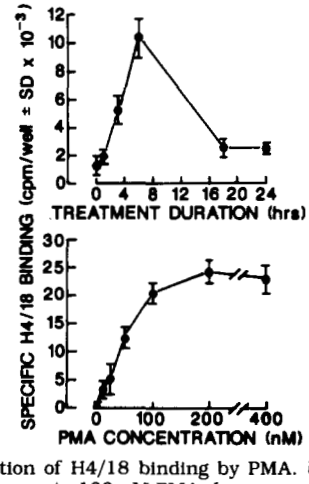

Figure 4. Induction of H4/18 binding by PMA. *Upper panel* shows time course in response to 100 nM PMA; *lower panel* shows expression at 4 hr as a function of PMA concentration. Each *panel* is derived from a separate experiment.

TABLE IV

Induction of H4/18 binding: phorbol ester tachyphylaxis

Pretreatment (24 hr)	Percent Inhibition of Restimulation (4 hr)[a]	
	PMA	Mezerein
PMA (100 nM)	90	94
Mezerein (300 nM)	100	91

[a] Percent Inhibition = $1 - \frac{\text{Induced specific H4/18 binding to mediator pretreated HEC}}{\text{Induced specific H4/18 binding to sham pretreated HEC}} \times 100$

In this experiment, H4/18 binding to mediator-pretreated cultures in the absence of restimulation (i.e., at 28 hr) was less than 20% of binding at 4 hr after stimulation of sham-pretreated cultures.

(not shown) or mezerein, (Table IV) were also effective. Similar to induction by IL 1 species (18) or TNF (shown for comparison in Table V), induction of H4/18 binding by PMA is inhibited by RNA or protein synthesis inhibitors. Interestingly, HEC treated with PMA or mezerein for 24 hr become refractory to restimulation with either tumor-promoting phorbol ester (Table IV).

To compare LT (and TNF) actions with those of IL 1, we next investigated the effects of recombinant IL 1α and IL 1β in the various endothelial cell assays. Recombinant IL 1α and IL 1β each induce expression of H4/18 binding (Fig. 1). IL 1α and IL 1β also mimic LT/TNF effects on endothelial cell morphology, and show similar synergy with IFN-γ (Table II). Half maximal effects with IL 1 species for both actions are seen between 1 and 5 thymocyte co-stimulatory U/ml. None of the assays we have used distinguish IL 1α from IL 1β. In contrast, IL 1 species can be differentiated from LT and TNF in several ways. First, IL 1 species are consistently less effective at increasing the expression of HLA-A,B antigens (Table I), often showing no effect at all. Despite the lack of effect on HLA-A,B expression, IL 1 species at the same concentrations are consistently active in other assays performed on replicate cells in the same experiment (e.g., ICAM-1 enhancement, shape change, etc.). Second, co-addition of IL 1 species to HEC treated with maximally-effective concentrations of LT or TNF leads to small but measurable augmentation of H4/18 binding, whereas the combination of LT with TNF is no better than either cytokine alone (Table VI). Similarly, the combination of maximally effective concentrations of IL 1α and IL 1β is no better that either cytokine alone, whereas each can be augmented by LT or TNF. Third, although all four cytokines produce tachyphylaxis after 24 hr of treatment for reinduction of H4/18 binding by the same cytokine, LT and TNF produce cross-tachyphylaxis to each other but not to IL 1α or IL 1β (Table VII). Similarly, IL 1α and 1β produce cross-tachyphylaxis to each other but not to LT or TNF. Finally, pretreatment with PMA can produce partial hyporesponsiveness for reinduction of H4/18 binding by IL 1 species, but does not significantly inhibit reinduction by TNF (Table VII). Interestingly, PMA pretreatment does in some experiments (Table VII) partially inhibit reinduction by LT, but experimental variability in this result cautions against interpreting this effect as a difference between LT and TNF. On the other hand, pretreatment with TNF or LT markedly and consistently blocks reinduction by PMA, whereas pretreatment with IL 1 species only partially inhibits reinduction by PMA (Table VII).

TABLE V

Induction of H4/18 binding: metabolic inhibition[a]

	TNF (100 U/ml)	PMA (50 nM)
Cycloheximide (20 μg/ml)	77%	83%
Emetine (5 μM)	97%	89%
Actinomycin D (5 μg/ml)	97%	90%
Daunorubicin (5 μg/ml)	97%	89%

[a] Percent Inhibition

$$= \left(1 - \frac{\text{Induction of specific H4/18 binding with inhibitor}}{\text{Induction of specific H4/18 binding without inhibitor}}\right) \times 100$$

assessed at 4 hours.

TABLE VI

Induction of H4/18 binding: combined addition of cytokines

First Cytokine	Percent Change[a] Second Cytokine			
	IL 1α	IL 1β	TNF	LT
IL 1α (25 U/ml)	—	(−22)	+33	+17
IL 1β (25 U/ml)	(−29)	—	+21	+17
TNF (500 U/ml)	+54	+54	—	+2
LT (500 U/ml)	+17	+18	(−12)	—

[a] Percent Change

$$= \left(\frac{\text{Specific H4/18 binding after two cytokines} - \text{binding after first cytokine}}{\text{Binding after first cytokine}}\right) \times 100$$

in which specific H4/18 binding is normalized to percent of specific E1/1.2 binding in each treatment group.

DISCUSSION

The observations presented in this report demonstrate that LT shares with TNF a number of direct endothelial cell actions. Specifically, LT causes enhanced expression of endothelial cell receptors for various leukocyte recognition structures (e.g., the antigen recognized by H4/18, ICAM-1, and HLA-A,B). The LT altered endothelial cell surface may serve to facilitate leukocyte and lymphocyte adhesion at a site of cell-mediated immunity. In addition, LT, like TNF, can cause morphological reorganization of HEC monolayers, a change that may serve to facilitate subsequent transendothelial trafficking of lymphocytes and other inflammatory leukocytes. We have hypothesized that these in vitro changes represent the same process that results in the in situ specialization of the high endothelial venule (21). Individual experiments show some variations of the relative magnitude of the maximal responses to recombinant LT and TNF, although no consistent pattern has been noted. However, the concentrations of LT and TNF that produce half maximal changes on HEC are consistently comparable when assessed by bioassay units determined from murine L929 cytotoxicity. Overall, we find no evidence that these recombinant mediators can be distinguished by their HEC actions. It is unlikely that these recombinant mediators are cross-contaminated or contain other HEC-active mediators (e.g., endotoxin), a conclusion reinforced by our ability to selectively neutralize both TNF and LT with separate polyclonal sera purchased from Endogen (Boston, MA) (data not shown). Furthermore, a natural LT preparation (kindly provided by Dr. Berish Rubin, New York Blood Center), produced similar effects on H4/18 binding and HLA-A,B antigen expression (J. S. Pober and J. L. Strominger, unpublished), confirming that recombinant LT expressed in CHO cells behaves like a human lymphocyte-derived product.

The second major point of this report is that although LT and TNF appear to be similar to IL 1α and IL 1β in some endothelial cell assays, these two pairs of mediators can be distinguished by other assays. We interpret these observations as consistent with the hypothesis that LT and TNF share one receptor (7) but that IL 1α and 1β share a second (31, 32). As we have argued previously (19), it is unlikely that tachyphylaxis for reinduction of H4/18 binding results simply from receptor disappearance, because sustained treatment with cytokine is necessary to induce and maintain fibroblastoid morphology and other gradual changes.

Our PMA experiments are of interest with regard to the mechanism of action of LT, TNF, and IL 1 species. The fact that PMA and other phorbol esters can induce

TABLE VII

Induction of H4/18 binding: tachyphylaxis to cytokines[a]

Pretreatment (24 hr)	Percent Inhibition of Restimulation (4 hr)[b]				
	IL 1α	IL 1β	TNF	LT	PMA
IL 1α (10 U/ml)	*68*	*71*	(−21)	24	22
IL 1β (10 U/ml)	*92*	*97*	14	(−5)	44
TNF (100 U/ml)	36	27	*82*	*78*	*93*
LT (100 U/ml)	29	24	*71*	88	*86*
PMA (100 nM)	*70*	*68*	(−6)	62	*90*

[a] Each horizontal row is representative of three to six separate internally controlled experiments; comparisons of absolute values between experiments may be misleading. Italicized values indicate significant (i.e., greater than 2/3) inhibition. The inhibition of reinduction by LT after PMA treatment has been variable among six separate experiments, and is therefore underlined.

[b] Defined as in Table IV. In each experiment, H4/18 binding to mediator-pretreated cultures without restimulation was less than 30% of binding at 4 hr after stimulation of sham-pretreated cultures.

expression of H4/18 binding suggests potential involvement of a protein kinase C-dependent process (33). The fact that LT and TNF are active in cells that have become completely unresponsive to PMA (i.e., after PMA pretreatment) suggests that a pathway independent of protein kinase C must exist. The mechanism by which LT or TNF pretreatment inhibits PMA induction of H4/18 binding is not clear. It is interesting that HEC that have been pretreated with TNF for 24 hr and become completely refractory to stimulation of H4/18 binding expression by PMA, still can be induced to secrete von Willebrand factor by PMA (B. M. Ewenstein and J. S. Pober, unpublished observations); i.e., not all PMA responses are lost after TNF pretreatment. Perhaps LT or TNF pretreatment uncouples protein kinase C from induction of the H4/18 binding protein, or perhaps LT or TNF inactivates a topologically or biochemically distinct subset of protein kinase C molecules; more than one protein kinase C species have recently been identified (34). Alternatively, PMA may activate expression of H4/18 binding protein (or von Willebrand factor release) through a mechanism independent of a protein kinase C.

The key implication of our studies is that T lymphocytes, activated by antigen, can secrete a factor (LT) that has many of the same endothelial directed actions previously described for monocyte-derived inflammatory mediators (IL 1 and TNF). We propose that antigen presentation by endothelium to circulating T helper cells (35) would trigger LT release, leading to subsequent local activation of the vascular lining. The LT-induced endothelial changes would then serve in turn to amplify a cell-mediated immune response by enhancing local adhesion of lymphocytes and other leukocytes to the endothelial cell surface and facilitating transendothelial migration. Our studies also suggest that specific LT effects could be augmented by coincident generation of other cytokines (e.g., IL 1 or IFN-γ). The reciprocal activation of T cells and lymphocytes may be critical in the development of cell mediated responses in vascularized tissues.

Acknowledgments. We thank Dr. Ramzi Cotran for his encouragement and support. We wish to thank Drs. Jan Tavernier, Jose Van der Heyden, and Rao Movva for preparation of recombinant LT. We thank Mr. Paul Cohill and Ms. Patricia Riendeau for excellent technical assistance and Ms. Donna Hickey for assistance in preparation of the manuscript.

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