THREE DISTINCT CLASSES OF REGULATORY CYTOKINES CONTROL ENDOTHELIAL CELL MHC ANTIGEN EXPRESSION

Interactions with Immune γ Interferon Differentiate the Effects of Tumor Necrosis Factor and Lymphotoxin from those of Leukocyte α and Fibroblast β Interferons

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Endothelial cells (EC)[1] can function as APCs (1–4) and, as the lining cells of the vasculature, are uniquely positioned to use antigen presentation as a means of selectively recruiting those circulating T cells specific for an eliciting antigen into a site of developing immune inflammation (5). Since T cells can only recognize antigen presented in association with MHC molecules (6), the quantitative level of MHC molecule expression on the surface of a presenting cell is a determinant of antigen-presenting capacity (7, 8). EC expression of MHC molecules, as for many other cell types, is regulated by cytokines. Under standard culture conditions, human umbilical vein–derived EC express class I but not class II MHC antigens (9). Previous studies from our laboratory (10–13) as well as from others (14, 15) have shown that leukocyte IFN-α and fibroblast IFN-β increase expression of class I antigens on EC without inducing class II antigens, whereas immune IFN-γ both increases class I and leads to *de novo* expression of class II molecules (HLA-DR > HLA-DP > HLA-DQ). In EC, MHC antigen expression is, at least in part, controlled by steady-state mRNA levels. IFN-α, IFN-β, and IFN-γ treatment led to increased levels of class I transcripts; only IFN-γ led to the *de novo* appearance of the heavy and light chain transcripts for the three class II molecules as well as for the non-MHC–encoded invariant chain (12). Subsequent studies from our laboratory (16, 17) revealed that TNF and, more recently, that lymphotoxin (LT) as well, act like IFN-α/β by increasing both steady-state mRNA levels and surface expression of class I molecules without inducing class II transcripts or surface expression. Although functionally indistinguishable in these assays, we found that TNF differed from IFN-α/β at the level of mechanism, in that the protein synthesis inhibitor cycloheximide led to enhancement of the IFN-α/β–mediated increase of class I mRNA levels (and

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[1] *Abbreviations used in this paper:* EC, endothelial cells; LT, lymphotoxin.

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revealed induction of class II mRNA), whereas this agent blocked the actions of TNF (16); cycloheximide appeared to have no effect on IFN-γ actions on EC. These data raised the possibility that IFN-α or IFN-β, induced by TNF or LT, could mediate the actions of TNF and LT on MHC antigen expression. Here we report that the actions of TNF and LT can be clearly distinguished from those of IFN-α/β by examining the effects of these cytokines in the presence of IFN-γ. Specifically, LT and TNF act synergistically with IFN-γ to enhance class I MHC expression and do not significantly affect IFN-γ-mediated class II induction, whereas IFN-α and IFN-β are, at most, additive with IFN-γ for class I enhancement and strikingly inhibit the actions of IFN-γ on class II expression. In addition, LT or TNF can further enhance the expression of MHC antigens induced by optimal levels of IFN-α or IFN-β. These new data make it seem highly unlikely that LT or TNF regulate MHC antigen expression in EC by inducing the synthesis of IFN-α or IFN-β. Finally, experiments with rIL-6 (previously called 26-kD protein, IFN-β_2 or B cell stimulating factor 2) do not support the alternative hypothesis (18, 19) that this cytokine mediates the actions of LT or TNF upon MHC expression in human EC since our IL-6 preparation has no detectable effect on the levels of MHC antigen expression. We conclude that there are at least three different classes of regulatory cytokines, LT/TNF, IFN-α/β, and IFN-γ, each of which has distinct effects upon EC expression of MHC antigens.

Materials and Methods

Human EC, isolated by collagenase treatment from umbilical vein segments, were pooled (two to six donors) and serially subcultured using conditions described elsewhere (20, 21). Selected experiments, where indicated, were performed with EC cultured from a single umbilical vein segment (i.e., derived from one donor). All cultures in these experiments were used between passage level 3 and 8. Cytokines, expressed as products of recombinant cDNA clones or genes, were added to the cultures in complete standard medium (Medium 199; Biofluids, Rockville, MD) supplemented with 20% FCS, glutamine, antibiotics (all from Gibco Laboratories, Grand Island, NY), endothelial cell growth supplement (50 μg/ml; Collaborative Research, Lexington, MA), and porcine intestinal heparin (100 μg/ml, Sigma Chemical Co., St. Louis, MO) at the final concentrations indicated. The specific recombinant cytokine preparations used in these studies included TNF (expressed in *Escherichia coli* and purified to 2.5×10^7 U/mg in L929 cytotoxicity assays, reference 22), LT (cDNA cloned from RPMI 1788 cells and expressed in Chinese hamster ovary cells, partially purified to a concentration of 1.5×10^5 U/ml in L929 cytotoxicity assays), IFN-β (expressed in *E. coli* and purified to 10^8 U/mg, antiviral activity, reference 23), IFN-γ (cDNA expressed in Chinese hamster ovary cells, partially purified to a concentration of 2×10^5 U/ml antiviral activity, reference 24), and IL-6 (cDNA expressed in yeast, partially purified to a concentration of 8.7×10^5 U/ml in B cell stimulating factor 2 assays, reference 25). In addition, we obtained IL-1α (2.5×10^7 U/mg in thymocyte costimulation assays) and IL-1β (5×10^7 U/mg in thymocyte costimulation assays) as gifts of Dr. Alan Shaw (Biogen, Geneva, Switzerland), and IFN-α (10^8 U/mg antiviral activity, consensus sequence), purchased from Amgen (Thousand Oaks, CA). It should be noted that our preparations of LT and IFN-γ were expressed in mammalian cells and the preparation of IL-6 was expressed in a yeast system, resulting in glycosylated molecules. All of the cytokines used in these studies (except IL-6, for which specific antiserum is not available) could be neutralized by appropriate specific antisera and were unaffected in their endothelial-directed actions by 50 μg/ml polymyxin B sulfate (Sigma Chemical Co.).

Surface expression of MHC antigens was quantitated by indirect immunofluorescence

using a FACS Analyzer (Becton Dickinson & Co., Mountain View, CA). Staining was performed as previously described (10). mAbs used were W6/32 (26) for HLA-A,B antigens and LB3.1 (27) for HLA-DR antigens; results with these antibodies were confirmed with BBM.1 (28) (reactive with β_2 microglobulin) and L243 (29) (purchased from Becton Dickinson & Co., reactive with HLA-DR), respectively. Backgrounds for the staining were determined with nonbinding isotype-matched controls. Second antibody was a FITC-conjugated rabbit anti–mouse Ig serum (ICN Biochemicals, Cleveland, OH).

Specific mRNA transcript levels for HLA-DR α chain were determined by Northern blotting on nylon membranes (GeneScreen; New England Nuclear, Boston, MA), using cytoplasmic RNA prepared by methods described elsewhere (12). The specific probe used was a 0.6-kb Pst I fragment of an HLA-DR α cDNA clone (containing the $\alpha 2$ conserved domain); the complete cDNA (DB10) was a gift of Dr. Jack Strominger, Harvard University, Cambridge, MA. The probe was labeled by random oligonucleotide priming and blots were washed to a stringency of 0.5× standard sodium citrate, 1.0% SDS at 65°C. The specificity of the hybridization was confirmed by the size of the relevant transcript (1.3 kb for DR-α), based on comparisons to end-labeled Hind III λ phage markers, electrophoresed, and blotted in parallel.

Results

The effects of cytokines upon EC expression of MHC antigens were examined by indirect immunofluorescence and FACS quantitation. As reported previously, EC express class I antigens but not class II antigens under standard culture conditions (9). The surface density of class I antigens is quite homogenous, forming a tight log normal distribution, despite the fact that our EC cultures are derived from pooled donors. As a consequence of this homogeneity, it is reasonable to represent the population by the modal fluorescence (determined by the position of the peak channel for data expressed in the histogram mode of the FACS analyzer). In Fig. 1, the difference between the modal fluorescence of

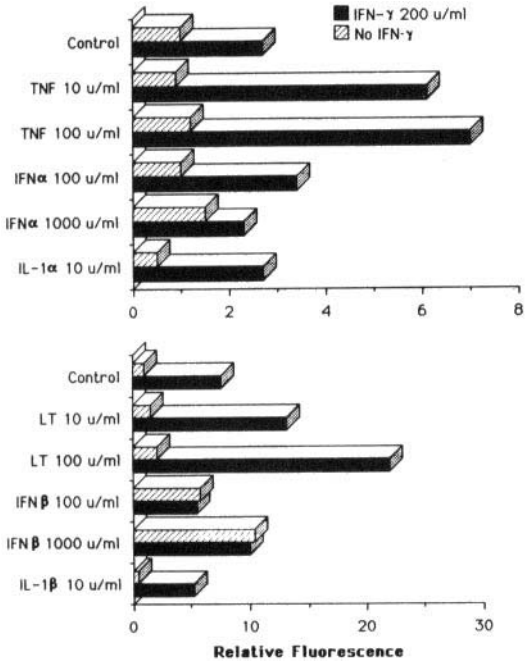

FIGURE 1. Modulation of class I MHC antigen expression by various cytokines with or without IFN-γ. Expression was quantitated after 24 h of treatment at the indicated concentrations. Scale represents relative modal fluorescence of W6/32 staining, defined as net modal fluorescence of cytokine-treated sample in arbitrary units divided by net modal fluorescence of control-treated sample. Net modal fluorescence is determined by converting the difference in modal channel numbers between W6/32 staining and staining with an irrelevant, isotype-matched control from a logarithmic to a linear fluorescence scale. One of five similar experiments.

TABLE I

Synergy of LT and IFN-γ on Surface Expression of Class I MHC Antigens (Relative W6/32 Modal Fluorescence)

Mediator	Without IFN-γ	IFN-γ (20 U/ml)	IFN-γ (200 U/ml)
None	1.0	4.4	8.9
LT (10 U/ml)	1.6	7.5	13.3
LT (100 U/ml)	2.2	10.0	18.9

One of nine similar experiments from pooled EC cultures; similar results were also obtained with two single donor EC cultures. Relative W6/32 modal fluorescence was as defined as in Fig. 1.

* 72 h of continuous treatment.

TABLE II

Effect of LT on IFN Modulation of Surface Expression of Class I Antigens (Relative W6/32 Modal Fluorescence)

Mediator	Without LT	LT (100 U/ml)
None	1.0	1.8
IFN-β (1,000 U/ml)	4.5	7.6
IFN-γ (200 U/ml)	4.2	7.0
IFN-β + IFN-γ	7.0	10.3

One of five similar experiments performed with pooled EC cultures; results of IFN-β and LT were also confirmed in two experiments with single donor EC cultures. Modal fluorescence was as defined as in Fig. 1.

* 24 h of continuous treatment.

W6/32 staining for class I antigens and that of isotype-matched irrelevant antibody is normalized to a relative fluorescence of 1.0. As can be seen in the top panel (*hatched bars*), IFN-α and, to a lesser extent, TNF cause a concentration-dependent elevation of class I MHC antigens at 24 h, whereas IL-1α does not; similarly, in the lower panel (*hatched bars*) IFN-β and, to a lesser extent, LT cause a concentration-dependent elevation of class I MHC antigens, whereas IL-1β does not. In both panels, an optimal concentration (200 U/ml) of IFN-γ by itself (*top line, black bars*) causes an elevation of class I expression. The interesting result (*remaining black bars*) is that TNF and LT appear to synergize with IFN-γ, whereas IFN-α and IFN-β show a combined effect with IFN-γ that is less than additive. IL-1 species did not affect the actions of IFN-γ in these experiments. The synergy of LT with IFN-γ is further illustrated in Table I, where at all concentrations tested, the enhancement caused by combined mediators is essentially the product of the actions of either mediator alone.

Interestingly, LT can also induce a further increase in class I MHC antigen expression beyond that produced by an optimal concentration of IFN-β (1,000 U/ml, Table II). This interaction is at least additive and may also be synergistic, as shown in Table III.

An additional difference between LT and IFN-β is illustrated in Fig. 2. In this experiment, the left hand FACS profiles show that neither LT nor IFN-β induce expression of class II MHC antigens by themselves. The right hand panels show that the maximally effective concentration of LT (100 U/ml) has little effect on

TABLE III

Interactions of LT and IFN-β on Surface Expression of Class I MHC Antigens (Relative W6/32 Modal Fluorescence)

Mediator*	Without IFN-β	IFN-β (10 U/ml)	IFN-β (100 U/ml)	IFN-β (1,000 U/ml)
None	1.0	2.1	3.8	4.9
LT (1 U/ml)	1.4	2.9	5.1	6.5
LT (10 U/ml)	2.2	4.1	6.6	10.7
LT (100 U/ml)	1.9	5.3	8.7	9.4

One of three similar experiments. Fluorescence was as defined as in Fig. 1.
* 24 h of continuous treatment.

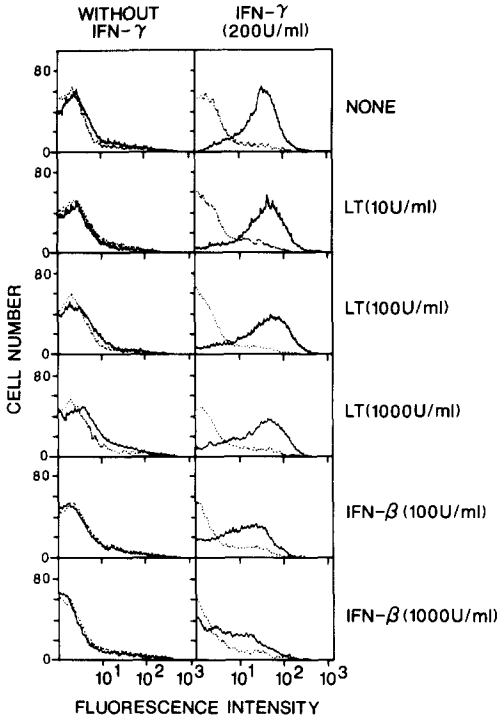

FIGURE 2. FACS analyses of class II MHC antigen expression on cytokine-treated EC after 48 h of treatment. The solid lines show staining of HLA-DR with antibody LB3.1; the dotted lines show staining with an irrelevant mouse myeloma protein. One of four similar experiments.

the expression of HLA-DR induced by IFN-γ, whereas IFN-β, at its maximally effective concentration (1,000 U/ml), is profoundly inhibitory. The positive actions of LT and IFN-β in the presence of IFN-γ on class I expression on the same samples (not shown) rule out the possibility that inhibition of class II expression by IFN-β is due to toxicity. Other experiments (not shown) indicate that TNF, like LT, has little effect on IFN-γ-mediated class II induction, whereas IFN-α, like IFN-β, is inhibitory. IL-1α and IL-1β have no effect on IFN-γ induction of class II (not shown). The distinction between LT and IFN-β on class II expression was confirmed by Northern blot analysis in the experiment shown in Fig. 3; 100 U/ml LT was only minimally inhibitory, whereas 1,000 U/ml IFN-β completely blocked the appearance of DRα mRNA caused by 200 U/ml IFN-γ in the control cultures.

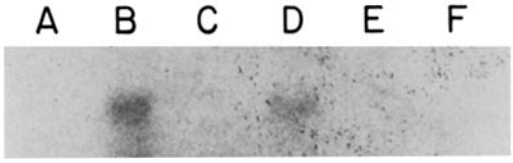

FIGURE 3. Northern blot, quantifying HLA-DR mRNA levels after 24 h continuous treatment with cytokines. Each lane contained 30 μg/ml cytoplasmic RNA, prepared from EC treated as follows: (*A*) no cytokine; (*B*) IFN-γ (200 U/ml); (*C*) LT (100 U/ml); (*D*) LT (100 U/ml), and IFN-γ (200 U/ml); (*E*) IFN-β (1,000 U/ml); and (*F*) IFN-β (1,000 U/ml) and IFN-γ (200 U/ml). FACS analysis of a sample of the cells used for RNA extraction confirmed that, in this experiment, LT and IFN-β each further enhanced IFN-γ-mediated increases in class I expression, whereas only IFN-β inhibited IFN-γ-mediated class II induction (not shown). One of two similar experiments.

TABLE IV

Effect of IL-6 on Surface Expression of Class I MHC Antigens (Relative W6/32 Modal Fluorescence)

Mediator*	Without IFN-γ	IFN-γ (200 U/ml)
None	1.0	4.7
LT (100 U/ml)	1.7	11.0
IL-6 (8.7×10^3 U/ml)	1.1	4.4

One of two similar experiments. Fluorescence as defined as in Fig. 1.
* 24 h continuous treatment.

Previous studies (16) had suggested that TNF (and by implication LT) elevates class I transcripts by induction of an intermediary protein. Since the functional effects of LT and TNF have now been shown to be distinct from those of IFN-α/β, it seems highly unlikely that the actions of TNF or LT on MHC expression in EC could be mediated by stimulating the secretion of IFN-α or IFN-β. It has been alternatively suggested that IL-6 (referred to by some investigators as IFN-β_2) might serve as the autocrine or paracrine second message for TNF modulation of MHC expression (18, 19). To test this possibility, a recombinant preparation of IL-6 was compared with the actions of LT. As shown in Table IV, even at concentrations of 2.5 μg/ml (equivalent to 8.7×10^3 U/ml of B cell stimulating factor 2 activity), our preparation of IL-6 does not mimic the actions of LT on EC; i.e., it neither directly enhances class I MHC expression nor synergizes with IFN-γ to produce a further increase of class I.

Discussion

The data presented in this report functionally distinguish the actions of LT and TNF from those of IFN-α and IFN-β in assays of MHC modulation on cultured EC. Specifically, although all four cytokines act to enhance EC expression of class I MHC antigens without inducing class II antigens, TNF and LT act in synergy with IFN-γ on class I, whereas IFN-α and IFN-β act at most with additivity. Furthermore, LT and TNF have little effect on class II expression induced by IFN-γ whereas IFN-α and IFN-β are profoundly inhibitory. Finally,

LT or TNF cause further increases in class I antigen expression on cells already optimally stimulated by IFN-α or IFN-β (i.e., with a concentration of cytokine greater than the maximally effective level). Collectively, these observations suggest that there are at least three separate classes of MHC modulating cytokines: TNF/LT; IFN-α/β; and IFN-γ. We propose that the net regulatory effect on EC expression of MHC antigens in vivo will depend upon the quantity and timing of cytokine generation in situ.

The responses of EC to cytokines should be considered in the context of what is known regarding the responses of other cell types. In EC, we have seen little effect of LT or TNF on EC expression of class II antigens, and certainly no positive effect. However, certain tumor cell lines appear to express class II molecules directly in response to these cytokines (30, 31). Furthermore, although IFN-γ is a sufficient signal to induce class II expression in EC as in most other cell types (32), recent studies have shown that TNF or LT serve as necessary cofactors with IFN-γ for class II induction on pancreatic islet cells (33). It remains to be seen if the EC pattern is the norm or one of several common variants.

The comparison of TNF or LT with the IFN family extends beyond the issue of MHC regulation. Like the IFNs, TNF and LT are growth inhibitory (34, 35) and produce an antiviral state (36, 37). Since the actions of TNF (and presumably LT) on the MHC appear to involve synthesis of a protein (i.e., the mRNA increase is blocked by cycloheximide). IFN-α or -β were considered as potential second signals. Our inability to find IFN-β mRNA induction in EC or dermal fibroblasts treated with TNF (16) was confirmed by others who found, instead, the induction of IL-6 (18), originally called IFN-β_2 (38) or 26-kD protein (25), or B cell stimulating factor 2 (39). It has been proposed that IL-6 mediates the actions of TNF (and presumably LT) on MHC antigen expression (19). Our initial experiments with a recombinant preparation of IL-6, biologically active as a B cell stimulating factor, are not consistent with this hypothesis in that IL-6 did not serve to regulate MHC expression in human EC. It remains to be determined whether other preparations or forms of IL-6 can modulate MHC expression, as there are reported discrepancies as to whether different preparations of this cytokine have antiviral activity (38, 40).

Our comparisons of LT and TNF with IFN-α and IFN-β have both biological and clinical implications. For example, we would predict that TNF or LT will augment the immune enhancing actions of IFN-γ. In the specific context of EC functions, TNF or LT would dramatically boost the IFN-γ effect on expression of class I MHC antigens, rendering EC more potent at interacting with cytolytic T lymphocytes (8). Furthermore, the coincident induction of membrane-associated IL-1 by TNF or LT (41) and of class II MHC antigens by IFN-γ may show "biological synergy" in promoting the activation of helper T lymphocytes. Since the same T cells that produce IFN-γ also appear to produce LT (42), the coincident exposure of EC to both cytokines appears quite likely. In contrast, we would predict that IFN-α and IFN-β, which have also been found to inhibit IFN-γ-induced class II antigen expression on murine mononuclear phagocytes (43–45), will be immunosuppressive. With specific regard to EC functions, the inhibition of class II MHC expression may, in turn, inhibit the recruitment of antigen-specific helper T lymphocytes and thus block the development of im-

mune inflammation. The signals required for IFN-α and IFN-β secretion (e.g., dsRNA, virus infection, etc.) are quite distinct from those that elicit IFN-γ and LT (e.g., antigen, T cell mitogens, etc.), and the inhibitory effects of IFN-α and IFN-β may not normally come into play in an immune response to antigen. However, the recent use of recombinant cytokines in various clinical settings (e.g., treatment of tumors or of viral infections) may have to be reevaluated in terms of whether an immunostimulatory agent (as in acquired immunodeficiency syndrome) or immunoinhibitory agent (as in a transplant recipient) would be preferable.

Summary

Recombinant preparations of TNF and lymphotoxin (LT) increase the expression of class I MHC antigens on cultured human endothelial cells (EC) without inducing expression of class II antigens. These actions are similar to those of rIFN-α or rIFN-β. However, TNF and LT differ from IFN-α/β in that the former synergize with IFN-γ for class I regulation whereas the latter do not. Furthermore, LT or TNF do not affect IFN-γ-mediated class II induction at optimal class I inducing concentrations (100 U/ml), whereas IFN-α and IFN-β (at their optimal concentrations of 1,000 U/ml) are strikingly inhibitory. LT and TNF also can further increase expression of class I antigens on cells already maximally stimulated by IFN-α or IFN-β. A recombinant preparation of IL-6 (formerly called 26-kD protein, IFN-β_2, or B cell stimulating factor 2) was without effect on class I expression in EC. These data make it seem unlikely that the actions of LT or TNF on EC expression of MHC antigens are mediated through autocrine or paracrine production of IFN-α, IFN-β or IL-6. More importantly, they suggest that LT or TNF are more likely to be immunostimulatory, whereas IFN-α or IFN-β are more likely to be immunoinhibitory in vivo, a consideration of potential relevance for cytokine administration to various patient populations.

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