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OVERLAPPING PATTERNS OF ACTIVATION OF HUMAN ENDOTHELIAL CELLS BY INTERLEUKIN 1, TUMOR NECROSIS FACTOR, AND IMMUNE INTERFERON[1]

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We have used the quantitative binding of murine monoclonal antibodies to the surface of cultured human umbilical vein endothelial (HUVE) cells to study the responses of HUVE cells to three different immune mediators: interleukin 1 (IL 1), tumor necrosis factor (TNF), and immune interferon (IFN-γ). Antibody H4/18, reactive with an endothelial cell-specific activation antigen, does not bind to unstimulated HUVE cells but shows rapidly and transiently inducible binding (peak 4 to 6 hr) to cells stimulated by IL 1 or TNF that declines to basal levels by 24 hr, even in the continued presence of mediator. Binding of H4/18 is unaffected by IFN-γ. Antibody RR1/1, reactive with intercellular adhesion molecule 1, binds to unstimulated HUVE cells, but binding is rapidly increased (plateau 24 hr) after stimulation by IL 1 or TNF and slowly increased (over several days) by IFN-γ. In contrast to H4/18 binding, the increase in RR1/1 binding is sustained in the continued presence of mediator. Antibody W6/32, reactive with HLA-A,B antigens, binds to unstimulated HUVE cells and shows gradually progressive increases (over several days) in binding upon treatment with IFN-γ or TNF. These observations demonstrate that HUVE cells show distinct but overlapping patterns of antigenic modulation in response to three different lymphokines, and suggest that the "activation" of endothelial cells observed in situ may represent a complex integration of several lymphokine-mediated signals.

Vascular endothelial cells in situ undergo morphological and functional alterations at sites of cell-mediated immune responses (1–6). These changes have been described as "endothelial activation" (1), but the nature of the activation process and the specific signals that induce it were not identified. More recently, it has been shown that similar localized vascular changes can be produced by intradermal injection of crude lymphokine preparations (7). Cultured human umbilical vein endothelial (HUVE)[3] cells also respond morphologically to crude lymphokine, providing an in vitro model for the in situ activation (8, 9). Furthermore, the recent availability of purified and recombinant lymphokines has made it possible to further dissect the HUVE cell response. Interestingly, distinct mediators appear to induce different but partially overlapping patterns of endothelial activation. For example, interleukin 1 (IL-1) and tumor necrosis factor (TNF), but not immune interferon (IFN-γ), cause the normally "anti-coagulant" HUVE cell surface to become procoagulant (10–12). Similarly, IL 1 and TNF cause HUVE cells to become markedly adhesive for polymorphonuclear leukocytes, blood monocytes, lymphocytes, and leukocyte cell lines (13–16). In contrast, both TNF and IFN-γ cause HUVE to become elongated and extensively overlap at confluence (17). IFN-γ uniquely confers upon HUVE cells the ability to stimulate T helper cells (18). All of these changes may be combined to some unspecified degree in the process of in situ activation.

We have used the specific binding of murine monoclonal antibodies to define more precisely the patterns of activation induced by individual mediators. We have found that IFN-γ uniquely causes HUVE cells to express class II major histocompatibility complex (MHC) antigens of the HLA-DR, -DP, and -DQ loci (19–21). IFN-γ, as well as leukocyte interferon (IFN-α), fibroblast interferon (IFN-β), and TNF, but not IL 1, increase HUVE cell expression of class I MHC antigens up to 10-fold (22). IL 1 and TNF, but none of the interferons, induce de novo expression of an endothelial cell activation antigen recognized by monoclonal antibody H4/18 (23). H4/18 partly blocks the augmented adhesion of the leukocyte cell line HL-60 to IL 1-stimulated endothelial cells (unpublished observations, M. P. Bevilacqua and M. A. Gimbrone Jr.). Recently, some of us raised monoclonal antibody RR1/1 against lymphocytes from a patient congenitally deficient in the LFA-1/Mac-1/gp150,95 leukocyte adhesion complex.[3] This antibody inhibits lymphocyte homotypic adhesion stimulated by phorbol esters and natural adhesion of lymphocytes to fibroblasts. The antigen recognized by RR1/1 is a widely distributed, approximately 90,000 dalton glycoprotein that has been designated intercellular adhesion molecule 1 (ICAM-1). The expression of ICAM-1 on cultured dermal fibroblasts is increased by IL 1 and IFN-γ (24). Here we describe the expression and regula-

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[3] Abbreviations used in this paper: HUVE, human umbilical vein endothelial; ICAM-1, intercellular adhesion molecule 1; TNF, tumor necrosis factor.

tion of ICAM-1 on cultured HUVE cells and compare these parameters with those of the antigen recognized by H4/18 and the class I MHC antigens.

MATERIALS AND METHODS

HUVE cells were isolated and serially cultured as described (23, 25); all experiments used cells at subculture levels 3 through 8. Mediators used in these experiments included natural, affinity-purified IL 1 (Genzyme, Boston, MA); recombinant TNF, expressed in *E. coli* and purified to homogeneity (26); recombinant human IFN-α (Amgen, Thousand Oaks, CA); recombinant human IFN-β, expressed in *E. coli* and purified to homogeneity (27); and recombinant human IFN-γ, expressed in CHO cells (28). These agents were added to the cultures at the concentrations and times indicated in the figure legends; units are defined in bioassays as described (26–28) or in the descriptions of commerical suppliers. In some experiments, recombinant human IL 1α and IL 1β species were used (Genzyme) with similar results to those obtained with natural IL 1 (not shown).

Murine monoclonal antibodies H4/18 and RR1/1 (anti-ICAM-1), both of the IgG1 isotype, have been described in detail (23, 24).[4] Binding to HUVE confluent monolayers in flat-bottomed microtiter wells was determined in triplicate by using ^{125}I-sheep anti-mouse Ig $(Fab')_2$ fragments (New England Nuclear, Boston, MA) as a second reagent (19). To obtain specific binding, the nonspecific binding determined with a nonbinding IgG1 control antibody was subtracted. E1/1.2 is an IgG2b murine monoclonal antibody raised against nonenzymatically harvested endothelial cells from umbilical veins; this antibody precipitates a 90,000 dalton mesenchymal cell surface antigen. E1/1.2 binding to individual HUVE cells is not modulated by any of the mediators used, as assessed by flow cytometry (not shown). Therefore, the specific binding of antibodies H4/18 and RR1/1 were normalized to the specific binding of antibody E1/1.2 so as to compensate for the decrease in cell number caused by cytostatic mediators such as TNF and IFN-γ (17). In some experiments, class I MHC antigen expression was quantitated by specific binding of murine antibody W6/32 (IgG2a) (29) and was similarly normalized to E1/1.2 binding. Radioactivity was measured in a Beckman 8000 gamma counter (Beckman Instruments, Irvine, CA).

RESULTS AND DISCUSSION

HUVE cells maintained under standard culture conditions do not specifically bind antibody H4/18. In contrast, antibody RR1/1 (anti-ICAM-1) does bind specifically to the same unstimulated cell population (Figs. 1 and 2). As shown previously (23), treatment of the HUVE cell cultures with either IL 1 or TNF rapidly induces specific binding of H4/18, which rises to peak expression at 4 to 6 hr and then declines to baseline by 24 to 48 hr. IL 1 and TNF also cause a rapid rise in ICAM-1 expression, which is about 50% maximal for TNF and 80% maximal for IL 1 at 4 to 6 hr, and plateaus by 24 hr for both mediators (Figs. 1 and 3). There is no decline in ICAM-1 expression from 24 to 72 hr in the continued presence of IL 1 or TNF (Fig. 3). HLA-A,B antigens, as detected by specific W6/32 binding, are expressed by unstimulated HUVE cells. Specific W6/32 binding in the TNF-treated cultures rose from 52% of specific E1/1.2 binding at 4 hr (indistinguishable from untreated replicate cultures) to 70% at 24 hr and to 89% at 72 hr; during this same time period, the HUVE became progressively elongated as judged by phase-contrast microscopy (not shown).

This gradual and progressive increase in HLA-A,B antigen expression (22) and the progressive endothelial cell shape change (17) both depend upon the continuous presence of TNF and are reversed by TNF withdrawal. Withdrawal of TNF for 24 hr causes a decline in ICAM-1 expression to basal levels (not shown) and is also necessary to permit reinduction of H4/18 binding by fresh TNF

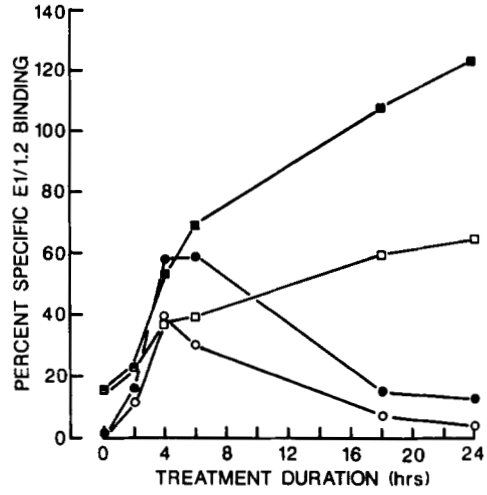

Figure 1. Specific binding of monoclonal antibodies H4/18 (○, ●) and RR1/1 (□, ■) to HUVE cells normalized to the specific binding of antibody E1/1.2. Replicate cultures were treated with 5 U/ml IL 1 (*open symbols*) or 100 U/ml TNF (*closed symbols*) for the indicated period of time before the binding assay. All samples were then assayed simultaneously.

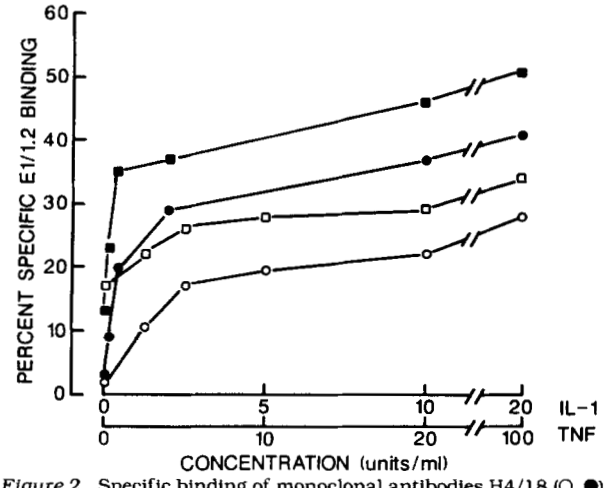

Figure 2. Specific binding of monoclonal antibodies H4/18 (○, ●) and RR1/1 (□, ■) to HUVE cells normalized to the specific binding of E1/1.2. Replicate cultures were treated for 4 hr with mediator-free medium (0 U/ml) or the IL 1 (*open symbols*) or TNF (*closed symbols*) concentration indicated on the *abscissa*. All samples were then assayed simultaneously.

in HUVE cells previously stimulated by TNF (23, 30). Collectively, these observations show that HUVE cells remain responsive to TNF for several days and that active TNF remains in the culture medium. Therefore, the decline in H4/18 binding after 4 hr and the plateau of ICAM-1 expression after 24 hr cannot be attributed either to loss of TNF activity or to global loss of TNF responsiveness by the HUVE cells. Instead, it appears that the three distinct patterns of antigenic changes detected by H4/18, RR1/1, and W6/32 respectively represent three different responses to the same active mediator by the same continuously responsive endothelial cell.

The concentration dependence of the modulation of H4/18 binding and ICAM-1 expression were similar both for IL 1 and for TNF (Fig. 2). IFN-γ, but not IFN-α and -β, also increased ICAM-1 expression (Figs. 2 and 4). These IFN-γ-induced changes were slower and quantitatively smaller than those observed with IL 1 and TNF. All of the interferon preparations were active on HUVE cells, because all produced increases in HLA-A,B antigen expression (Fig. 4). The effects of IFN-γ upon HLA-A,B antigen expression occurred at lower concentrations

[4] **Rothlein, R., M. L. Dustin, S. D. Marlin, and T. A. Springer.** 1986. An intercellular adhesion molecule (ICAM-1) distinct from LFA-1. *Submitted for publication.*

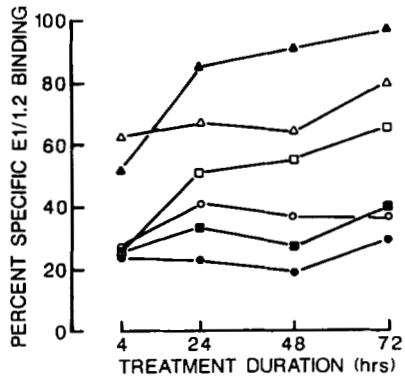

Figure 3. Specific binding of monoclonal antibody RR1/1 to HUVE cells normalized to the specific binding of E1/1.2. Replicate cultures were treated with mediator-free medium (●) or medium containing IFN-α (1000 U/ml, ○), IFN-β (1000 U/ml, ■), IFN-γ (200 U/ml, □), TNF (20 U/ml, ▲), or IL 1 (5 U/ml, △) for the indicated period of time before the binding assay. All samples were then assayed simultaneously.

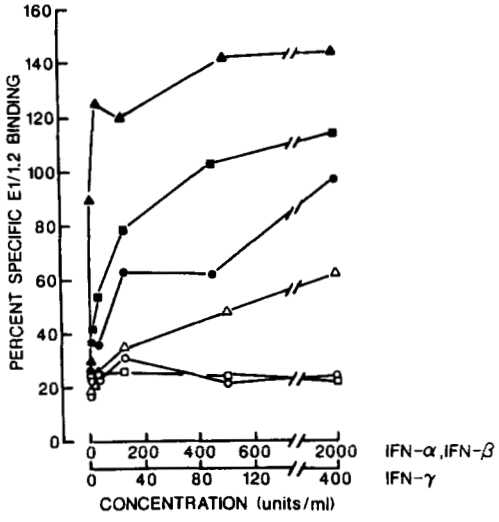

Figure 4. Specific binding of monoclonal antibodies RR1/1 (*open symbols*) or W6/32 (*closed symbols*) to HUVE cells normalized to specific binding of E1/1.2. Replicate cultures were treated for 72 hr with mediator-free medium (0 U/ml) or IFN-α (○, ●), IFN-β (□, ■), or IFN-γ (△, ▲) at the concentrations indicated on the *abscissa*. All samples were then assayed simultaneously.

than those effective in elevating ICAM-1 expression (Fig. 4). None of the interferons modulated H4/18 binding at any time or concentration examined.

The results reported in this study have two implications. First, the different patterns of response by the surface antigens recognized by antibodies W6/32, H4/18, and RR1/1 (anti-ICAM-1) point up potential functional differences among these three distinct molecules. W6/32 recognizes a nonpolymorphic portion of HLA-A,B antigens (29); the polymorphic determinants of these molecules form part of the target structure recognized by cytolytic T lymphocytes (31). IFN and TNF, but not IL 1, would be predicted to increase cytolytic T lymphocyte-mediated killing by increasing HLA-A,B antigen expression on the target cell. This effect has in fact been demonstrated for IFN (32). The molecules recognized by RR1/1 and H4/18 have both been implicated by antibody blocking experiments to be involved in the adhesion of leukocytes (24, 33; and unpublished observations, M. P. Bevilacqua and M. A. Gimbrone Jr.). The inducible expression of H4/18 binding parallels polymorphonuclear leukocyte and monocyte adhesion in that basal binding is low or absent, induction by IL 1 (or TNF) is rapid and transient, and there is no effect of IFN-γ (13, 14). In contrast, ICAM-1 expression more closely parallels T lymphocyte binding, showing high basal expression and sustained increases to both IL 1 and IFN-γ (15, 34). The inferences drawn from such correlations need to be tested by direct experimentation.

The second implication of our studies is to demonstrate that the patterns of endothelial cell activating lymphokines overlap. The parallel actions of IL 1 and TNF on endothelium, first noted for procoagulant activity (10–12), leukocyte adhesion (13, 14), and H4/18 binding (23), now extend to ICAM-1 expression. TNF and IFN-γ share the ability to increase class I MHC antigen expression (22). The increased expression of ICAM-1 by IL 1 and IFN-γ presented here is the first evidence that these two mediators have an action in common on endothelial cells. It is conceivable that the relatively slow induction of ICAM-I by IFN-γ depends upon IL 1 or TNF biosynthesis and secretion by HUVE cells, i.e., an autocrine/paracrine pathway. This seems unlikely inasmuch as IFN-γ treatment of HUVE cells does not lead to increases in detectable mRNA for either IL 1 or TNF (T. Collins and J. S. Pober, unpublished observations). Furthermore, IFN-γ treatment does not induce H4/18 binding as might be predicted if IFN-γ caused IL 1 or TNF secretion. In summary, the use of monoclonal antibodies reactive with different endothelial cell activation antigens provides a powerful approach to dissecting the details of endothelial cell activation processes.

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Note added in proof. In several recent experiments, treatment of HUVE cells with 5 units/ml of IL-1 for 3–4 days has decreased E1/1.2 binding. This may reflect a general decrease in protein synthesis and does not alter the results or conclusions of the present study.

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