0022-1767/87/13912-1883$02.00/0
THE JOURNAL OF IMMUNOLOGY
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Vol. 139, 1883-1888, No. 12, December 15, 1987
Printed in U.S.A.

ENDOTHELIAL ACTIVATION DURING INTERLEUKIN 2 IMMUNOTHERAPY

A Possible Mechanism for the Vascular Leak Syndrome[1]

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A major sequela of immunotherapy with interleukin 2 (IL-2) is development of a vascular leak syndrome. The pathogenesis of this toxic effect is not known. We have examined pre- and post-treatment skin biopsies from 14 patients undergoing systemic administration of IL-2 for evidence of endothelial cell activation. Specifically, we have used the immunoperoxidase technique to detect the expression of three different activation antigens: endothelial-leukocyte adhesion molecule 1, detected with monoclonal antibody H4/18; intercellular adhesion molecule 1, detected with antibody RR1/1; and histocompatibility leukocyte antigen-DQ, detected with antibody Leu 10. Each of these antigens may be induced on cultured endothelial cells by various cytokines (although not by IL-2) and is expressed during endothelial cell activation in vivo at sites of delayed hypersensitivity and other immune responses. Pretreatment biopsies from each patient showed no endothelial expression of endothelial-leukocyte adhesion molecule 1 and only weak to moderate expression of intercellular adhesion molecule 1 and histocompatibility leukocyte antigen-DQ (except for one specimen unreactive with Leu 10). After 5 days of treatment, every patient showed marked endothelial expression of all three antigens (except for the same patient who remained unreactive with Leu 10). Endothelial-leukocyte adhesion molecule-1 expression was confined to postcapillary venular endothelium whereas intercellular adhesion molecule-1 and Leu 10 also were expressed on stromal cells and mononuclear cells. Thus, we conclude that i.v. administration of IL-2 leads to endothelial cell activation. Because IL-2 fails to induce the same antigens on cultured endothelial cells, we infer that IL-2 acts in vivo by inducing the production of other cytokines (e.g., interleukin 1, tumor necrosis factor, lymphotoxin, and interferon-γ). Finally, since endothelial cell activation at sites of cell-mediated immune responses is well known to result in vascular leakiness to macromolecules, we propose that the vascular leak syndrome accompanying IL-2 therapy may arise from widespread inappropriate endothelial cell activation.

Treatment with interleukin (IL)[3] 2 alone or in combination with lymphokine-activated killer (LAK) cells is currently being used in several clinical trials as a form of therapy for patients with metastatic cancer (1–5). Although partial or complete regression of tumor metastases has been noted in some treated patients, therapy is associated with a number of toxic reactions (3). The most important dose-limiting toxic effect is the "vascular leak syndrome," which usually begins within 24 hr of an infusion of IL-2 and results in accumulation of extracellular fluid including ascites, hydrothorax, and pulmonary edema. Although the precise pathogenesis of this syndrome is unknown, rapid clearance of radiolabeled albumin in these patients suggests increased vascular permeability as the cause of edema. Skin lesions consisting of erythematous eruptions often accompany the syndrome. Histologic changes in the skin are nonspecific, consisting of mild dermal papillary edema and scant to moderate perivascular mononuclear infiltrate (6).

We have recently shown that certain lymphokines and monokines can induce or increase expression of specific cell surface antigens on cultured human vascular endothelial cells (7–11). These antigenic changes correlate with specific cytokine-induced functional changes. For example, the induction of the antigen recognized by monoclonal antibodies H4/18 (9) or H18/7 (11) by IL-1, tumor necrosis factor (TNF), or lymphotoxin (LT) occurs concomitantly with the development of increased adhesiveness for leukocytes (11, 12). Monoclonal antibody H18/7 has recently been shown to block the endothelial-dependent adhesion of polymorphonuclear leukocytes and the antigen has accordingly been named endothelial-leukocyte adhesion molecule-1 (E-LAM 1) (11). Subsequent detection of E-LAM 1 expression in vivo at sites of

Received for publication May 15, 1987.
Accepted for publication September 21, 1987. Delayed in publication from December 15, 1987, due to scheduling errors by the editorial office and the printer.
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[1] This work was supported by National Institutes of Health Grants HL-36028 and HL-36003. J. S. P. is an Established Investigator of the American Heart Association.
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[3] Abbreviations used in this paper: IL, interleukin; E-LAM 1, endothelial-leukocyte adhesion molecule 1; ICAM 1, intercellular adhesion molecule 1; IFN-γ, interferon-γ; LAK, lymphokine-activated killer; LT, lymphotoxin; TNF, tumor necrosis factor.

acute immunologic inflammation (13) has been used to infer that functional activation of the endothelium may be occurring in these settings. Other markers of endothelial activation may also be used for in vivo correlative evidence of endothelial cell functional alterations. For example, expression of intercellular adhesion molecule-1 (ICAM 1), which may confer adhesivity for lymphocytes (14) is increased by IL-1, LT, TNF, and interferon-γ (IFN-γ) in vitro (10, 11). Class II major histocompatibility complex antigens (including both HLA-DR and DQ), which may confer antigen-presenting function, are induced by IFN-γ in vitro (7, 8).

Herein, we have examined skin biopsies from patients receiving IL-2 therapy for the presence of E-LAM 1, ICAM 1, and HLA-DQ as markers for endothelial cell activation. We show that despite the nonspecific histologic changes in these biopsies, there is consistent expression of these endothelial activation antigens after IL-2 infusion. The studies suggest that endothelial activation, induced by cytokines, is an important component of the skin lesions and may play a role in the vascular leak syndrome seen in these patients.

MATERIALS AND METHODS

Patient skin biopsies. All 14 patients participating in this study had metastatic renal cell carcinoma, colonic carcinoma, osteogenic sarcoma, or malignant melanoma and were being treated on a protocol designed to evaluate therapy with IL-2 and transferred LAK cells. This protocol had previously been approved by the Investigational Review Board, National Cancer Institute, and the Food and Drug Administration. All patients signed informed consents and agreed to repetitive cutaneous biopsies in this instance. All biopsies were done at times during IL-2 administration and were not performed during the period of reinfusion of LAK cells. Recombinant IL-2, kindly provided by the Cetus Corp. (Emeryville, CA) was administered i.v. as bolus infusions at 30,000 to 100,000 U/kg every 8 hr; 4-mm punch biopsies were taken from the skin under local anesthesia before treatment and at intervals during therapy. These biopsies were flash-frozen in OCT compound (Miles Laboratories, Naperville, IL) at −70°C or alone as tissue fragments. Tissues were prepared as detailed below for staining.

In the first set of 10 patients biopsies were taken before treatment and 5 days after IL-2 therapy from the presternal area, because this was a common area of involvement by erythema during immunotherapy (6). Subsequently in four patients biopsies were taken at 0, and at 24, 48, 72, or 96 hr after IL-2 therapy from the forearm, an area that is not particularly prone to develop erythema.

Immunohistochemical studies. For immunohistochemical studies, 6-μM cryostat sections were cut, fixed in acetone for 10 min, washed three times in phosphate-buffered saline, and incubated for 1 hr at room temperature with the monoclonal antibodies. Sections were then washed and incubated with peroxidase-conjugated rabbit anti-mouse IgG (Dako Corp., Santa Barbara, CA), diluted in phosphate-buffered saline containing 20% normal human serum for 60 min. Peroxidase activity was detected using acetylethylcarbazol (0.002% in 5% dimethylformamide, 0.2% sodium acetate buffer pH 5, with 0.03% H_2O_2). Sections were then washed in distilled water, lightly poststained with hematoxylin, and mounted. The monoclonal antibodies used were H4/18 (anti-E-LAM 1) (9, 11); RR1/1 (anti-ICAM-1) (10, 14); and Leu 10 (polymorphic anti-HLA-DQ, Becton Dickinson, Mountain View, CA). Control antibodies were routinely tested on step sections of the same tissue specimens. The negative staining control used was an irrelevant IgG K supernatant K16/16. In some experiments, step sections were also stained with anti-von Willebrand factor monoclonal antibody (Cappel, Cochranville, PA) serving to identify endothelium; with antibody TS2/9, reactive with LFA-3; or with antibody E1/1.2, an anti-mesenchymal cell reagent that recognizes a 100-kDa surface protein. None of these three control antibodies shows changes in staining of cultured endothelial cells as a result of cytokine treatments (J. S. Pober, unpublished observations). All grading of stained sections was done without knowledge of the identity of the patients or the time of biopsy.

In vitro studies. Recombinant IL-2 (Cetus), from the same lots as used to treat the patients, was tested for its ability to alter antigen expression on cultured human umbilical vein endothelial cells. Confluent monolayer cultures in replicate microtiter wells were refed with standard culture medium or with the same medium supplemented with recombinant IL-2 (1000 U/ml), recombinant TNF (50 U/ml) (15), or recombinant IFN-γ (200 U/ml) (16); both TNF and IFN-γ, used for comparison with IL-2, were kindly provided by Prof. Walter Fiers, State University of Ghent, Ghent, Belgium. Antigen expression was quantitated in triplicate at the end of the cytokine treatment period by a two-step radioimmunobinding assay as described elsewhere (7, 9). The antibodies tested were H4/18, RR1/1, and LB3.1, a monomorphic anti-HLA-DR reagent. Nonspecific radioactivity, present after binding of irrelevant monoclonal antibodies, was also determined in triplicate and subtracted to determine specific binding values for each antibody. Data are expressed as mean specific binding ± the SD.

RESULTS

Immunohistochemical Studies

Pre-IL-2 treatment biopsies. In accordance with our previous observations in normal skin (13), pre-IL-2 biopsies in all patients showed no staining of the microvascular endothelium in sections treated with H4/18 antibody. On the other hand, RR1/1 stained normal endothelium, as has been reported previously (14), with variable intensity. HLA-DR antigens are strongly expressed on normal human skin endothelium in situ (17). Because in vitro studies had indicated that endothelial cell expression of HLA-DQ is always coincident with HLA-DR, but that HLA-DQ expression and mRNA levels are only 10% of that of HLA-DR (8), we elected to examine HLA-DQ expression in the biopsies in order to increase our ability to detect induction. We found weak to moderate staining of endothelial cells in all but one of the pre-IL-2 treatment biopsies.

Post-IL-2 biopsies. In all 10 biopsies taken from the presternal area 5 days after IL-2 administration had begun, H4/18 stained the cutaneous small blood vessels, predominantly the venules of the dermis (Fig. 1 *A* to *C*). The intensity of the staining and the number of positively reacting vessels varied among different patients. The staining was limited exclusively to the endothelium and did not involve the lymphoid cells in the perivascular infiltrate. Although some of the lesions exhibited a mild to moderate perivascular infiltrate, staining could be seen in many vessels that showed scant or absent infiltrate. As previously reported for reactivity in vessels from delayed hypersensitivity reactions, much of the staining with H4/18 was distinctly granular, and some appeared to be intracytoplasmic. However, our previously published data (9) and unpublished electron microscopic studies unequivocally establish that the antigen is also expressed on the cell surface.

In sections stained with RR1/1, there was an obvious increase in the number of microvascular profiles that stained positively and in the strength of staining reaction, as compared with pre-IL-2 biopsies (Figs. 1 *D* and *E* and Fig. 2). In contrast to the H4/18-stained sections in which only endothelium was reactive, there was significant staining in many of the cells within the perivascular lymphoid infiltrates and in fibroblast-like cells in the perivascular connective tissue. There was also focal staining in keratinocytes of the basal layer.

Post-IL-2 treatment biopsies stained with anti-HLA-DQ also showed increased staining of microvascular endothelium (Figs. 1 *F* and *G*), except in one patient in whom there was absent staining in the pre-IL-2 biopsy. We interpret the lack of staining with Leu 10 in this patient

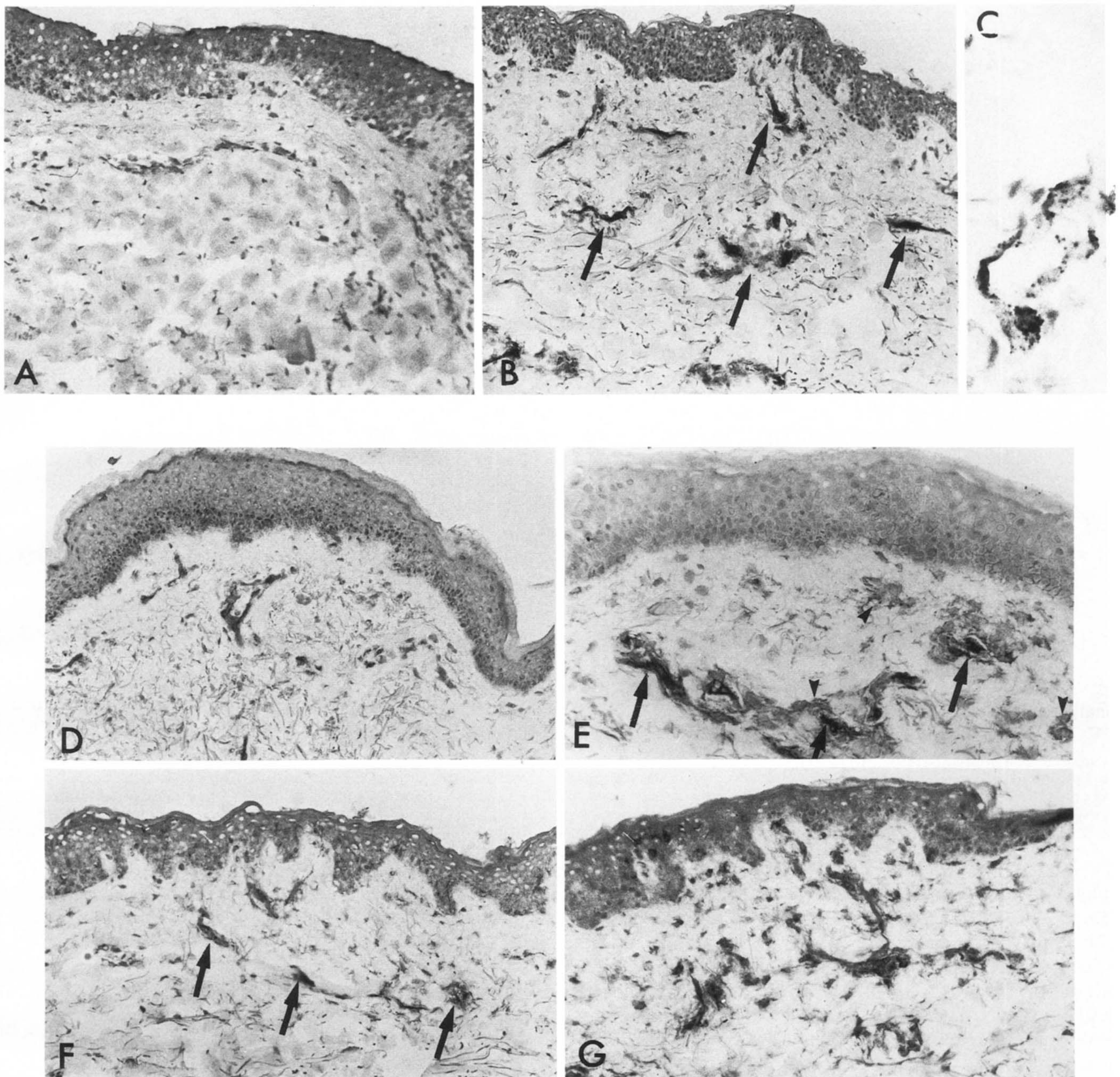

Figure 1. A, Pre-IL-2 skin biopsy stained with H4/18. Note absence of staining in microvessels of the dermis ×80. B, Post-IL-2 biopsy (5 days) stained with H4/18. Several vessels (*arrows*) show intense staining ×80. C, Higher magnification of vessel stained with H4/18 showing staining confined to endothelium ×400. D, Pre-IL-2 biopsy stained with RR1/1. The blood vessels in the dermis are stained ×75. E, Post-IL-2 biopsy stained with RR1/1. Increased numbers of blood vessel profiles (*arrows*) are stained. There is also staining of perivascular lymphoid and interstitial cells (*arrowheads*) ×100. F, Pre-IL-2 biopsy stained with anti-HLA-DQ. Dermal vessels are stained (*arrows*) as are occasional intraepidermal dendritic cells ×80. G, Post-IL 2 biopsy stained with anti-HLA-DQ. More profiles of dermal vessels are stained and there are positively staining lymphoid cells in the connective tissue ×80.

to suggest that his DQ antigens are not recognized by this polymorphic antibody (i.e., Leu 10 does not recognize DQ linked to DR3 or 7, according to the supplier, Becton Dickinson). In all of the other patients there was also strong expression of HLA-DQ in a proportion of the mononuclear infiltrate present around vessels, and focal significant increases in HLA-DQ-positive cells within the epidermis. Post-IL-2 biopsies examined showed no difference in endothelial staining with antibodies TS2/9 (anti-LFA 3) or E1/1.2, compared with pre-IL-2 biopsies.

We attempted to study the time of appearance of the activation antigens in four patients in whom sequential biopsies were performed at 0, 24, and 48 hr, or at 0, 48, and 96 hr from areas in the forearm. Unequivocal although weak staining for H4/18 was present in some vessels at 24 hr, increased appreciably by 48 hr, and remained unchanged at 96 hr. Increased staining with RR1/1 in the endothelium became appreciable at 48 hr and at 96 hr was associated with staining of lymphoid cells and fibroblasts. Similarly, intensified staining for HLA-DQ was obvious at 48 hr, at which time there were also distinct increased numbers of intraepidermal reactive cells.

In Vitro Studies

Previous experiments from our laboratory had found that recombinant IL-2, kindly provided by Biogen (Cam-

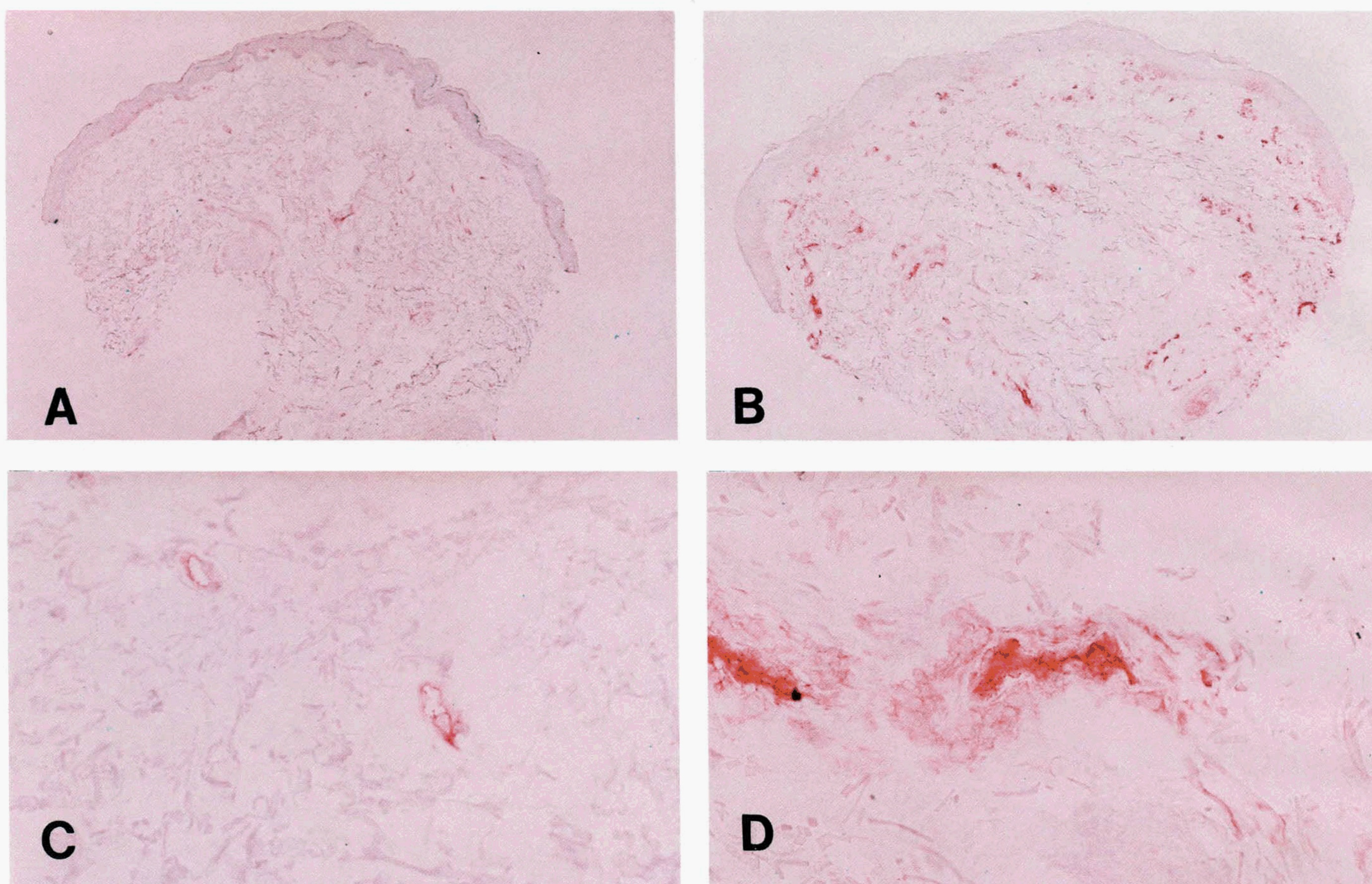

Figure 2. Pre- (*A* and *C*) and post- (*B* and *D*) IL-2 skin biopsies taken from the same patient and stained with antibody RR1/1 for ICAM 1. Sections from both biopsies were cut at the same thickness and processed for immunocytochemistry simultaneously, with identical reagents. *A* and *B* are low power magnification micrographs (×40) and show increased numbers of positively staining vascular profiles. There is also a more intense staining in *B* (post-IL-2) than in *A* (pre-IL-2). *C* and *D* are higher power micrographs (×200). In *C* (pre-IL-2) the endothelium is clearly stained. In *D* (post-IL-2), note the increased intensity of the staining in the central vascular profile and the membrane staining of lymphoid cells in the perivascular infiltrate.

bridge, MA), did not affect endothelial cell expression of E-LAM 1, ICAM 1, HLA-DR, or HLA-DQ, at a wide range of concentrations up to 100 U/ml. In light of the immunohistochemical studies, we wished to confirm these results using recombinant IL-2 provided by Cetus (the reagent used to treat the patients) and to extend our examination up to concentrations of 1000 U/ml, which markedly exceeds the serum concentrations observed during IL-2 infusion. As shown in Figure 3, this concentration of IL-2 neither induces E-LAM 1 nor HLA-DR (a more sensitive marker than HLA-DQ), nor augments the basal expression of ICAM-1 at 6 or 72 hr of treatment. Positive control experiments show that these cultures could respond because, in accord with our previous reports, TNF rapidly and transiently induced E-LAM 1 expression, both TNF and IFN-γ enhance ICAM-1 expression, and IFN-γ caused induction of HLA-DR expression (7–11). Binding of antibody E1/1.2 varied by less than 10% among the treatment groups (data not shown).

DISCUSSION

In this study we have demonstrated the induction of endothelial activation antigens in the skin of patients undergoing IL-2 therapy for metastatic cancer. We used three antibodies to document this phenomenon. Antibody H4/18, which recognizes E-LAM 1, gives the most unequivocal results, because normal control pre-IL-2 biopsy endothelium is devoid of the antigen and the antigen was seen in all biopsies examined 5 days after therapy. The studies with ICAM 1 and HLA-DQ serve to confirm these findings. In addition, these latter antigens that can also be stimulated in activated lymphoid cells and fibroblasts in vitro (8, 14), were demonstrated on the perivascular infiltrates and connective tissue fibroblasts, and in the case of HLA-DQ on epidermal dendritic cells after IL-2 therapy. Endothelial staining was most prominent in biopsies from erythematous areas, but was also present from biopsies of the forearm, an area not prone to develop skin lesions. The explanation for this regional variation is unclear, but could relate to the normal pretreatment distribution of IL-2 responsive cell types (e.g., lymphocytes, monocytes, etc.).

Although the precise sequence of events leading to induction of these antigens in endothelium after IL-2 therapy is unclear, our in vitro studies suggest possible mechanisms. As shown in Figure 3, none of these three antigens can be induced in umbilical vein endothelial cultures with IL-2, including the specific preparation of IL-2 administered to these patients. These data do not preclude a direct effect of IL-2 on microvascular endothelium in vivo, but other explanations appear more likely. Previous studies have shown that E-LAM 1 is induced by IL-1, TNF, and LT; ICAM 1 by IL-1, TNF, LT, and IFN-γ; and HLA-DQ by only IFN-γ (7–11). IL-2 has been shown to boost IFN-γ biosynthesis in vitro (18, 19) and IL-2 infusions result in detectable serum levels of IFN-γ (20),

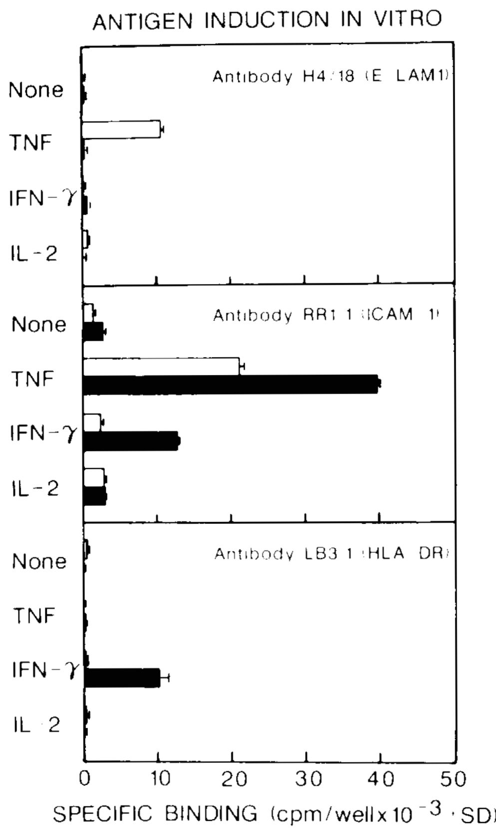

Figure 3. Expression of activation antigens on cultured human umbilical vein endothelial cells. Replicate cultures were treated as confluent monolayers in microtiter wells by supplementing the culture medium with no mediator, 50 U/ml recombinant TNF, 200 U/ml recombinant IFN-γ, or 1000 U/ml recombinant IL-2 for 6 hr (*open bars*) or 72 hr (*black bars*), before determination of monoclonal antibody binding. Specific binding of each antibody is reported as the mean of triplicate determinations ± SD.

so that the increased expression of both ICAM 1 and DQ levels in endothelium and lymphoid cells could thus be attributed, at least in part, to IFN-γ effects. In addition, it has been recently reported that IL-2 stimulates both TNF and LT synthesis in vitro (21) offering a potential explanation for the induction of E-LAM 1, localized to the endothelium in the post-IL-2 skin biopsies. We therefore suggest that IL-2 therapy may stimulate endogenous production of other lymphokines and monokines, possibly including IL-1, TNF, LT, and IFN-γ, and these in turn serve as a stimulus for the activation of endothelial cells. This model of indirect IL-2 action is consistent with a recent demonstration that the vascular leak accompanying IL-2 infusion in mice depends on the presence of T lymphocytes (22). These putative second cytokines may be locally produced in far greater quantities than are detectable in serum. Indeed, preliminary attempts to examine the effects of serum from these patients on endothelial cells in vitro have revealed only weak and equivocal results.

Our kinetic experiments indicate that E-LAM 1 expression progressively increased and was then sustained during the 5 days of IL-2 infusion. Our in vitro experience has been that individual mediators induce only transient expression of this antigen, after which the cells become resistant to restimulation (9–11). A possible explanation for this difference is that endothelial cells stimulated by one mediator (e.g., IL-1) can be restimulated by a second mediator (e.g., TNF or LT), potentially allowing more sustained expression (9, 11). Alternatively, the disappearance of E-LAM 1 and/or the development of tachyphylaxis may be abrogated by a signal present in vivo but lacking in the culture experiments.

What is the relationship of the expression of endothelial cell activation antigens reported herein to the cutaneous manifestations and the vascular leak syndrome caused by IL-2? It is well established that delayed hypersensitivity reactions elicit a localized vascular leak in the postcapillary venules (23). Such leaky endothelial cells concomitantly display the activation antigens examined in the present study (13) and acquire "high venular" or "activated" morphology (23). In vitro, the same cytokine signals that induce E-LAM 1, ICAM 1, and HLA-DQ expression also cause morphologic rearrangements of endothelial cell culture (11, 25), opening gaps between cells. Collectively, these observations suggest the following hypothesis: Endogenous synthesis of cytokines (e.g., IL-1, TNF, LT, and IFN-γ) induced by administration of exogenous IL-2 causes endothelial cells (as well as fibroblasts, lymphoid cells, and dendritic cells) to become "activated," both displaying new antigens and undergoing morphologic changes. The latter processes may underlie the leak. Thus, the vascular leak syndrome may be an example of a normally beneficial effect leading to toxicity when it is converted from a local event (i.e., at a site of a response to antigen) to a systemic process. If correct, our model has two important implications. First, agents that limit the synthesis of endogenous cytokines may limit toxicity. The clinical value of this approach will depend on to what extent endogenous cytokine synthesis mediates the anti-tumor effects of IL-2 and LAK therapy. Second, if immune effector cells such as T cells and macrophages have different receptors for cytokines than do vascular endothelial cells, then selective endothelial cell receptor blockade may offer another means of limiting toxicity. In conclusion, our studies suggest that extensive endothelial cell activation occurs in the course of therapy with IL-2 and that such endothelial cell activation, dependent on IL-2-mediated induction of other cytokines, may account, at least in part, for the vascular leak syndrome that accompanies IL-2 administration.

Acknowledgments. We thank Drs. Donna L. Mendrick and Michael P. Bevilacqua for collaborations in the use of antibody H4/18, Mr. George Stavrakis and Ms. Lynne Lapierre for expert technical assistance, and Ms. Julie Smith for preparation of the manuscript.

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