## *Brief Definitive Report*

# INDUCTION AND DETECTION OF A HUMAN ENDOTHELIAL ACTIVATION ANTIGEN IN VIVO

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Considerable evidence now supports an active role for vascular endothelial cells in the initiation of inflammatory and immunological responses, including adhesion of circulating leukocytes, stimulation of intravascular coagulation, and presentation of antigen to T cells. In previous studies (1–4), we have shown that some of these endothelial effects can be induced in vitro by well-defined monokines, lymphokines, and other stimuli. In particular, IL-1, tumor necrosis factor (TNF), and bacterial LPS stimulate the synthesis and surface expression of a tissue factor–like procoagulant activity in cultured human endothelial cells (1, 2) and increase endothelial adhesivity for leukocytes (3, 4). Similar results have been reported by others (5, 6). These inducible functions are dependent on protein and RNA synthesis, are maximal 4–6 h after exposure to the monokines, and decline by 24 h (4). We recently reported (7) that both IL-1 and TNF also induce the expression of a new endothelial cell surface protein, identified by specific binding to an mAb, H4/18, which had been developed by immunization with IL-1-treated endothelial cells. The time sequence of induction of the H4/18 binding antigen and its susceptibility to metabolic inhibitors were similar to those observed for increased procoagulant activity and leukocyte adhesion, suggesting that H4/18 binding could be a useful marker for these inducible endothelial functions.

Using immunocytochemical techniques, we now report that the H4/18 binding antigen can be induced in microvascular endothelium of human skin in delayed hypersensitivity reactions (DHR), and that it can be detected in a variety of human inflammatory conditions with an immune background. These studies indicate that H4/18 is a useful marker for activated endothelium in situ, and lend credence to the relevance of inducible endothelial functions initially observed in vitro, to inflammation in vivo.

## Materials and Methods

*mAbs and Immunohistochemical Techniques.* mAb H4/18, developed by immunizing BALB/c mice with cultured human umbilical vein endothelial cells (HEC) pretreated for 4 h with 5 U/ml of affinity-purified human IL-1, has been described previously (7). H4/18

This work was supported by National Institutes of Health grants HL-22602 and HL-36003. J. S. Pober is an Established Investigator of the American Heart Association. Address correspondence to Dr. Ramzi S. Cotran, Department of Pathology, Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115

was detected by immunoperoxidase screening of binding to IL-1-treated vs. control HEC; the antibody is of the IgG1K isotype. For the present study, the antibody was used as the culture supernatant, diluted in 10% horse serum to a concentration of ~0.05 mg/ml of mouse Ig. In some experiments, $F(ab')_2$ fragments of antibody were prepared from ascites fluid (8) and reconstituted to ~3.4 mg/ml.

For immunohistochemical studies, tissues were frozen in isopentane liquid nitrogen; 4–6 μm frozen sections were fixed in acetone for 10 min, washed three times in PBS, and incubated for either 1 h at room temperature or occasionally overnight at 4°C with optimally diluted mAbs. Sections were then washed and incubated with peroxidase-conjugated rabbit anti-mouse IgG (Dako Corp., Santa Barbara, CA), diluted in PBS containing 20% normal human serum for 30–60 min. Peroxidase activity was detected using either diaminobenzidine (DAB, 6 mg/ml in 0.1 M Tris/saline buffer at pH 7.6 with 0.03% $H_2O_2$), or acetylethylcarbazol (AEC, 0.02% in 5% dimethyl formamide, 0.2% sodium acetate buffer, pH 5, with 0.03% $H_2O_2$) containing reaction mixtures. The sections were then washed in distilled water, lightly post-stained with hematoxylin, and mounted.

Control antibody was routinely tested on step sections of the same tissue specimens. The control used was an irrelevant IgG1K supernatant, K16/16. Six other control antibodies prepared against rat renal structures, and having the same isotype and Ig concentration, were tested on selected specimens. Step sections were also stained with anti-von Willebrand factor (anti-vWF) mAb (Cappel Laboratories, Cochranville, PA); these served as positive controls for normal endothelial staining.

*Delayed Hypersensitivity Reactions.* Two sets of DHR were elicited on forearm skin of a healthy male volunteer using streptococcal varidase (Lederle, The Netherlands, B.V.; containing 100,000 IU/ml of streptokinase and 25,000 IU/ml of streptodornase) as antigen. In the first study, the streptococcal varidase reaction site, an area of erythema and induration ~35 mm inches in diameter, was biopsied under local anaesthesia at 23 h. In the second study, a control punch biopsy was obtained, and immediately thereafter 0.1 ml of varidase was injected intracutaneously in two sites. At 16 h, both sites were strongly reactive, with erythema and induration measuring 35 mm in diameter. One of the two sites was biopsied at 16 h, and the second at 6 days, when the induration and erythema had largely disappeared.

*Tissues.* A variety of normal and pathologic tissues (see Results) were selected from fresh surgical specimens or from a bank of frozen tissue in the Department of Pathology at the University of Leiden School of Medicine, The Netherlands, or at the Brigham and Women's Hospital. In addition to the immunohistochemical studies, representative sections were stained with hematoxylin and eosin, and other special stains as required, and most of the lymph nodes were subjected to immunophenotypic analysis of lymphoid cells, using commercially available antisera (9).

## Results

*Normal Tissues.* There was no staining in H4/18 antibody in the microvascular endothelium (arterioles, capillaries, and venules) of the following normal tissues examined: skin (chest, arm, abdomen, neck); kidney; heart; skeletal muscle; liver; spleen; ovaries; lung; or subcutaneous fat. Four normal lymph nodes were also negative. One lymph node obtained from a lymph node dissection for prostatic carcinoma showed fine specks of positive staining along the vascular endothelium of postcapillary venules. In some skin sections, there was focal staining of squamous epithelium and eccrine duct epithelium. When the same tissues were stained with anti-vWF antibody, there was the expected strong staining of normal endothelial cells. Attempts to increase the sensitivity of the immunoperoxidase-labeling technique including the avidin-biotin procedure (10), did not uncover endothelial staining in the normal tissues.

*Delayed Hypersensitivity Reactions.* As expected from the initial screening of

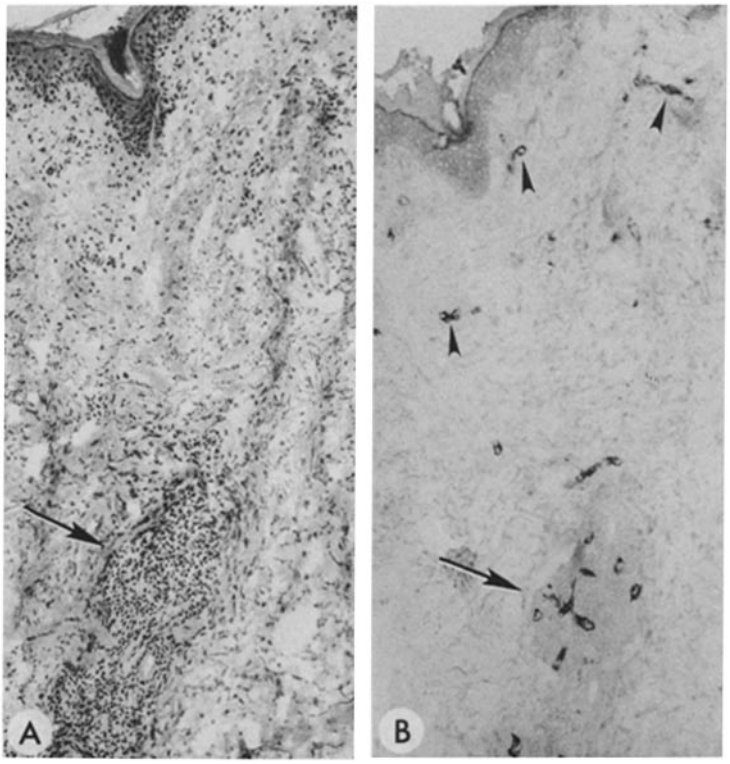


FIGURE 1. Photomicrographs of serial frozen sections from skin biopsy 23 h after eliciting a DHR. (*A*) Stained with hematoxylin and eosin, and (*B*) with immunoperoxidase technique using H4/18 antibody. The peroxidase-positive outlines in *B* correspond to small vessels in the deep mononuclear infiltrate in *A* (*long arrows*). *Arrowheads* in *B* point to staining in the more superficial dermal vessels. Original magnification, × 40.

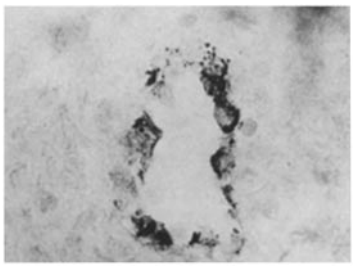

FIGURE 2. High magnification photomicrograph of positively staining vessel from same section as in Fig. 1*B*. Note that the staining is confined to the endothelium. Original magnification, × 400.

normal skin, the control punch biopsy showed no endothelial staining with the mAb H4/18. In contrast, the well-developed hypersensitivity reaction sites, at 16 and 23 h, exhibited strong reaction product in numerous small vessels in the hypodermis and dermis (Fig. 1). The staining outlined the vessel lumens, and on high magnification (Fig. 2) appeared to be exclusively in endothelium; it was most prominent in the deeper venules exhibiting a dense perivascular infiltrate,

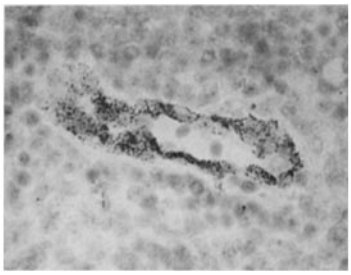

FIGURE 3. Hyperplastic vessel from a lymph node of a patient with angioimmunoblastic lymphadenopathy and developing T cell lymphoma, stained for H4/18 binding antigen. The staining is restricted to the lining endothelium. Original magnification, × 300.

but was also present in the more superficial smaller vessels. The reaction product was granular in both DAB- and in the AEC-reacted slides. Except for endogenous peroxidase in polymorphonuclear leukocytes (present also in sections stained with control antibodies), there was no staining of inflammatory cells either in vessel lumina or in the perivascular infiltrate. The skin biopsy taken at 6 d showed some foci of persistent perivascular inflammatory infiltrate, but there was no staining with H4/18 in endothelium. Control antibodies failed to reproduce the endothelial staining obtained with H4/18. Anti-vWF antibody stained microvascular endothelium in all sections at all time intervals.

*Pathological Tissues.* Our initial survey of pathological tissues with the H4/18 antibody has shown specific labeling of vascular endothelium in several clinicopathologic settings. There was extensive staining in a lymph node of a patient with angioimmunoblastic lymphadenopathy with developing T cell lymphoma (11). The reaction product was exclusively in the endothelium (Fig. 3), most prominently in the hyperplastic venules characteristic of this entity. There was no staining of the highly pleomorphic mononuclear infiltrate within the lymph node. No similar distribution of staining was seen with other mAbs used to immunophenotype this patient's lymph node. The endothelium was also reactive in lymph nodes of two cases of acute granulomatous lymphadenitis (consistent with cat scratch disease) and in lymphocyte-rich thyroiditis (one case), hyperplastic tonsillitis (three cases); and active inflammatory dermatitis (2 cases). Endothelial staining was also observed in lymph nodes or skin of eight patients with Hodgkin's or T cell lymphoma; the staining was strongest in the hyperplastic vessels which were often present in these conditions. In contrast, we have thus far found minimal or no staining in lymph nodes with B cell lymphoma (four cases), dermatopathic lymphadenitis (three cases), chronic sarcoid granuloma (three cases), and in renal allograft rejection (eight cases), foreign-body granuloma (one case), chronic pneumonitis (two cases), viral hepatitis (two cases), and around burn wounds (three cases). In two cases of appendicitis, endothelial staining was noted in vessels surrounded by inflammatory infiltrate.

## Discussion

In this study we have shown that the murine mAb H4/18, which binds an antigen induced in cultured endothelium by IL-1, also recognizes an endothelial

molecule induced in vivo by DHR in human skin. The antigen can be also detected by immunocytochemical techniques in pathological tissues mostly in clinical settings of immunological activation. To our knowledge, this is the only reported endothelial-specific mAb that fails to react with normal endothelium, but identifies activated endothelium in vivo.

The immunohistochemical results reported here are consistent with the kinetics of H4/18 binding in vitro. H4/18 uniformly fails to bind normal endothelial cells, or normal and activated leukocytes in situ, as is the case in vitro. The IL-1-induced binding in culture is transient; in the induced DHR reactions in the skin, H4/18 staining disappeared as the lesions faded. Our preliminary screening of pathological tissue also suggests the antigen is transiently inducible by mediators. The staining was focal, and was most prominent in hyperplastic endothelium in settings of inflammatory or neoplastic reactions associated with activated lymphocytes and macrophages.

Although evidence for the active role of the endothelial cell in inflammation and the importance of inducible endothelium functions has come from recent work, the notion of endothelial cell activation is a relatively old one. Early light and electron microscopic studies of DHR described plump, hypertrophied endothelium with increased numbers of intracellular organelles, and it was suggested that the endothelium was activated in these reactions (12). It now seems likely that these changes of activation reflect some of the alterations in structure, function, and growth induced in endothelium by specific lymphokines and monokines. The studies reported here support the concept of endothelial activation and provide a useful marker for its further investigation in vivo.

## Summary

We used a murine mAb, H4/18, raised by immunization with IL-1-treated human umbilical vein endothelial cell cultures, to localize an endothelial activation antigen in induced human delayed hypersensitivity reactions (DHR) and in pathological tissues. We used streptococcus varidase to elicit DHR in human skin and we examined sequential skin biopsies with the immunoperoxidase technique. There was no staining for H4/18 binding antigen in normal endothelium of skin and other tissues; strong positive staining, localized to vascular endothelium, was seen at 16 and 23 h but disappeared by 6 d, when the DHR had faded. H4/18 binding antigen, also confined to endothelium, was detected in lymph nodes, skin, and other tissues exhibiting immune/inflammatory reactions. The studies indicate that H4/18 is a useful marker for activated endothelium in vivo and they support the relevance of in vitro studies on inducible endothelial cell functions.

Most of the immunohistochemical studies reported here were performed in the Department of Pathology, University of Leiden, The Netherlands. We thank the staff for invaluable help and useful discussions, and particularly Dr. R. Willemze, Dr. P. Kluin, A. Dijkstra, E. Dreef, and J. C. van Rijn.

*Received for publication 28 April 1986.*

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