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Association of Murine CD31 with Transmigrating Lymphocytes Following Antigenic Stimulation

Steven A. Bogen,* H. Scott Baldwin,‡ Simon C. Watkins,† Steven M. Albelda,‖ and Abul K. Abbas*

From the Department of Pathology, Brigham and Women's Hospital, Boston, and the Harvard Medical School, Boston; the Dana Farber Cancer Institute and the Department of Physiology and Biophysics,† Harvard Medical School, Boston, Massachusetts; the Children's Hospital of Philadelphia,‡ Philadelphia; and the Hospital of the University of Pennsylvania,‖ Philadelphia, Pennsylvania*

Human CD31 is a recently characterized molecule present on leukocytes, platelets, and endothelium. Its function is not known. Because it is a member of the immunoglobulin superfamily and structurally homologous to carcinoembryonic antigen, a putative intercellular adhesion molecule, it is believed that CD31 may function also as an adhesion molecule. In this report, we characterize the cellular reactivity of a monoclonal antibody to a murine protein that is homologous to CD31. To delineate the cellular reactivity of the murine CD31 homologue recognized by our monoclonal antibody, we used immunoperoxidase and immunoelectron microscopic techniques. The most striking finding was that the putative murine homolog of CD31 is expressed in particularly high amounts on endothelium-adherent lymphocytes transmigrating across sinusoidal or venular vascular boundaries. Such a distribution was apparent in draining murine lymph nodes during the peak of an immune response after immunization with a protein antigen in adjuvant, a situation in which there are many transmigrating lymphocytes. Immunoelectron microscopic analysis also shows that CD31 is predominantly distributed on portions of transmigrating lymphocytes that are in contact with or adjacent to areas of contact with endothelial cells. These findings suggest a previously undescribed role for CD31 in lymphocyte recruitment and transmigration. (Am J Pathol 1992, 141:843–854)

Lymph nodes undergo striking morphologic changes after an antigenic stimulus, one of which is a tremendous influx of lymphocytes through postcapillary venules and afferent lymphatics. Naive T cells are believed to enter lymph nodes through postcapillary venules, a process controlled in part by gp90-MEL,[1] whereas memory T cells migrate preferentially to peripheral sites of antigen exposure, using a number of adhesion molecules including lymphocyte function-associated antigen-1 (LFA-1) and very late activation (VLA) molecules to bind to endothelium in peripheral vessels.[2] Previously described lymphocyte homing receptors, however, do not fully account for the induction of lymphocyte transmigration into lymphoid organs or sites of inflammation. Specific induction events presumably must occur to initiate the transmigration process itself, such as: 1) the polarization of the lymphocyte cytoskeleton to cause undirectional locomotion, and 2) the possible release of enzymes allowing digestion of the vascular basement membrane.

Although the mechanisms of lymphocyte activation and function are understood in great detail, largely because of *in vitro* analyses, relatively little is known about the patterns and control of lymphocyte localization and activation *in vivo*. Previous studies using an immunocytochemical stain for interleukin-2 (IL-2) as a marker for recent T cell activation have shown that after immunization with conventional protein antigens in adjuvant, T cells are first activated in perivascular locations of draining lymph nodes.[3] This observation raises the possibility that lymphocyte entry into lymphoid organs is associated with lymphocyte activation. To characterize early lymphocyte activation events *in vivo*, we have sought experimental approaches to analyzing changes in lymphocyte phenotype and function occurring in the perivascular area of lymphoid tissues.

In this report, we describe a new monoclonal antibody designated 2H8, which recognizes a murine cell surface protein that is homologous to CD31. CD31 has previously been described as a marker of endothelial cells and all

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Address reprint requests to Dr. S. A. Bogen, Department of Pathology, Boston University School of Medicine, 80 E. Concord St., Boston, MA 02118.

hematopoietic cell lines except the erythroid lineage.[4] Although the function of CD31 is unknown, it is a member of the immunoglobulin superfamily and is therefore believed to play a role in intercellular adhesion. Our immunohistochemical and immunoelectron microscopic studies suggest that the murine homolog of CD31 may function in lymphocyte recruitment and vascular transmigration in reactive lymph nodes after inflammation or antigenic stimulation.

Materials and Methods

Animals

Female Armenian hamsters were purchased from Toxicon, Woburn, Massachusetts. Female BALB/c mice, aged 6 to 10 weeks, were purchased from the Jackson Laboratories, Bar Harbor, Maine, and maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (DHEW publication No. [NIH] FS-23).

Generation of the 2H8 Hybridoma

An Armenian hamster was injected with 10^7 D10.G4.1 T cells emulsified in complete Freund's adjuvant (CFA) subcutaneously. The D10.G4.1 cell line was obtained from Dr. C. Janeway, Yale University, New Haven, Connecticut, and is an IL-4–producing $CD4^+$ T cell line specific for conalbumin + $I\text{-}A^k$.[5] Repeat subcutaneous injections of D10.G4.1 cells were done in incomplete Freund's adjuvant (day 17) and in phosphate-buffered saline (PBS) on days 32, 48, and 60. The hamster was killed on day 64 and the splenic lymphocytes were fused following standard protocols with the SP2/0 myeloma.[6] Supernatants of fusion products were screened for binding to D10.G4.1 by fluorescence-activated cell sorting (FACS) analysis. The 2H8 monoclonal antibody is an IgG isotype, as determined by a heavy chain molecular weight of approximately 52,000 on sodium dodecyl sulfate polyacrylamide eletrophoresis (SDS-PAGE) under reducing conditions.

Immunization

Reactive lymph nodes were generated by immunization with keyhole limpet hemocyanin (KLH, Calbiochem, La Jolla, CA) emulsified in CFA and injected into the hind foot pads of 6- to 10-week-old BALB/c mice.

Antibodies

The 2H8 hybridoma was grown in two different ways for purification of the monoclonal antibody. An ascites was generated by growing the 2H8 hybridoma in nude Swiss mice after Pristane priming. The ascites was passed over a CM Affigel Blue column (Biorad, Richmond, CA) to remove albumin. The effluent was analyzed by SDS-PAGE and found to be predominantly IgG. Monoclonal antibody 2H8 also was purified by adapting the 2H8 hybridoma to serum-free medium using Nutridoma SP (Boehringer-Mannheim, Indianapolis, IN). One liter supernatant was concentrated by ammonium sulfate precipitation to 5 ml.

Both antibody preparations were biotin-conjugated with N-hydroxysuccinimide biotin (Sigma Chemical Co., St. Louis, MO) as per the manufacturer's instructions. The 2H8 antibody did not lose binding activity after biotin conjugation, as judged by immunofluorescent flow cytometry.

Rat anti-mouse transferrin receptor hybridoma R17 217.1.3 (ATCC #TIB 219) was obtained from the American Type Culture Collection and used as a positive control for immunoprecipitation.[7] The hamster anti-mouse gamma interferon antibody H22 was a gift from Dr. R. Schreiber, Washington University, St. Louis, Missouri, and was used as a negative control. Other antibodies used were the rat anti-mouse CD3 monoclonal 29B, a gift of Dr. K. Bottomly, Yale University, New Haven, Connecticut, and rat anti-mouse B220 (6B2). Biotin-conjugated goat anti-mouse IgM was purchased from Southern Biotechnology Associates, Birmingham, Alabama.

Immunocytochemistry

Popliteal lymph nodes were removed 2 to 11 days after immunization and serial frozen sections were cut, dipped into cold acetone for 1 to 2 seconds, and then stored frozen at −20°C. Immediately before use, sections were fixed in cold acetone for 2 minutes and rehydrated in PBS with 1% fetal calf serum (FCS). Because only rare endogenous peroxidase-positive cells were present, lymph node sections were not quenched. Sections then were incubated with the hybridoma supernatant for 30 to 60 minutes at room temperature. Slides were rinsed in PBS/1% FCS, and incubated with a biotinylated rabbit anti-rat immunoglobulin, mouse Ig absorbed (Vector Laboratories, Burlingame, CA), 5 mg/mL in PBS with 2% normal mouse serum. Stains were developed with an avidin-biotin-horseradish peroxidase complex (Vectastain *Elite* ABC, Burlingame, CA), and enzyme activity detected with diaminobenzidine. Sections were counterstained with methyl green.

Flow Cytometric Analysis

FACS analysis was performed on spleen or lymph node lymphocytes, peripheral blood cells, or platelets by indirect immunofluorescence. Cells were incubated with the indicated primary antibodies for 45 minutes at 4°C. The cells then were washed with excess cold PBS/1% FCS/ 0.02% sodium azide and incubated for 30 minutes at 4°C with FITC-conjugated goat anti-rat or goat anti-hamster IgG, mouse immunoglobulin adsorbed (Southern Biotechnology Associates, Birmingham, AL). The cells then were washed using PBS without serum, fixed in 1% paraformaldehyde in PBS, and analyzed in a Becton-Dickinson FACSCAN.

Immunoelectron Microscopy

The methods used are essentially those of Tokoyasu and Singer.[8] Mice were anesthetized in chloroform, and the lymph nodes were fixed *in situ* by aortic perfusion with 0.01% glutaraldehyde and 2% formaldehyde in 0.15 mol/l (molar) NaCl, 0.05 mol/l phosphate buffer (pH 7.4). After perfusion for 15 minutes, the lymph nodes were dissected out and fixed for a further 45 minutes in fixative at 4°C, washed in PBS, cut into small cubes of 1 mm^3, and immersed in 2.3 mol/l sucrose overnight. The blocks then were mounted on cutting stubs and shock frozen in liquid nitrogen before sectioning. Thin sections were cut with a Reichert Ultracut Ultramicrotome fitted with an FC4D cryochamber at a temperature range of -110 to -130°C. The sections were mounted on Formvar/ carbon-coated 200 mesh $inch^{-1}$ grids and immunolabeled by a 30-minute incubation with or without biotinylated 2H8 monoclonal antibody, washed, and followed by streptavidin-coated 5-nm gold particles (Amersham Corp., Arlington Heights, IL). After counterstaining with urnayl acetate and embedding in 1.25% methylcellulose, sections were examined in a Phillips EM 300 electron microscope.

Immunoprecipitation of the 2H8 Molecule

3×10^7 CTLL (T cell receptor-negative, IL2-dependent line) or SP2/0 cells were harvested during log growth phase and labeled with 0.5 mCi ^{125}I (Amersham, Arlington Heights, IL) using lactoperoxidase and hydrogen peroxide (Sigma Chemical Co., St. Louis, MO) as per standard protocol.[9] The membrane proteins were extracted in TRIS-buffered saline/1% Triton X-100/ 10 mmol/l phenylmethylsulfonyl fluoride (PMSF)/75 mmol/l iodoacetamide (IAA), pH 7.6, for 1 hour at 4°C. Detergent isoluble material was removed by centrifugation at 10,000*g* in an Eppendorf microfuge. Fractions of the lysate then were added to Eppendorf tubes containing 0.03 to 0.05 ml packed volume antibody-coated agarose beads. The beads were either covalently coupled with antibody using cyanogen bromide–activated Sepharose 4B (Pharmacia, Piscataway, NJ, as per the manufacturer's instructions) or by indirect linkage on avidin-coated agarose beads (Vector Laboratories, Burlingame, CA). For the latter coupling, biotin-conjugated antibodies were preincubated with avidin-agarose beads. To precipitate transferrin receptor, the avidin-agarose beads were first coated with biotin-conjugated goat anti-rat IgG (Vector Laboratories), washed, and then incubated with the rat anti-mouse transferrin receptor antibody. After at least 1 hour at 4°C, the beads were washed and bound proteins were released by incubation in sample buffer, with or without 5% (vol/vol) 2-mercaptoethanol. A Protean II electrophoresis apparatus (Biorad, Richmond, CA) was used for SDS-PAGE. N-glycosidase (Genzyme, Boston, MA) treatment of immunoprecipitated 2H8 was performed overnight at 37°C.

N-terminal Peptide Sequencing

CTLL cells (4×10^9) were solubilized in lysis buffer as described above at a concentration of 2×10^7 cells/ml, and detergent insoluble material was removed by centrifugation. The lysate then was incubated with normal hamster IgG-conjugated Sepharose beads (1 ml packed volume) for 24 hours at 4°C on a rocking platform. The beads were packed into a column and the effluent transferred to 1 ml packed volume of washed 2H8-conjugated Sepharose beads. After a similar 24-hour incubation, the beads were packed into a column and washed with 20 ml cold 50 mmol/l PBS/0.1% Triton X-100, pH 7.6. Bound proteins were eluted with 50 mmol/l Glycine buffer, 140 mmol/l NaCl, and 0.1% Triton X-100, pH 2.3. Ten fractions of 1.5 ml each were collected. The pH was quickly neutralized with $NaHCO_3$. Fractions were individually dialyzed against PBS/0.1% Triton X-100 and then concentrated 10-fold by packing in Aquacide II (Calbiochem, La Jolla, CA) at 4°C. Fractions were run on an 8% SDS gel under reducing conditions, as described above. The proteins in the gel were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) in a Trans Blot Cell (Biorad) in CAPS buffer (3-[cyclohexylamino]-1-propanesulfonic acid, 10% methanol, pH 11.0) for 1 hour at 45v, 0.4A followed by 20 hours at 20v, 0.2A.[10] The PVDF membrane then was washed and stained in Coomassie Blue. Pre-stained high-molecular-weight markers (Biorad) were also run to assess the degree of transfer and the molecular weight of the Sepharose-2H8 eluted bands. N-terminal sequence analysis

was performed directly on the excised Coomassie blue-stained band at 120 to 125 kd, using an Applied Biosystems 477A Protein Sequencer (Dana Farber Molecular Biology Core Facility). Much fainter bands of molecular weights down to approximately 80 kd were also noted, presumably representing only partially glycosylated 2H8 molecule.

Cloning of the Murine CD31 Homolog

A mouse heart cDNA library (Stratagene #936306, La Jolla, CA) was screened with the full-length human CD31 cDNA,[11] and a 3.2-kb fragment cloned into the *Eco* RI restriction site of the Bluescript SK plasmid was rescued from the Lambda ZapII vector according to the manufacturer's directions. The initial 5′ and 3′ sequence of this cDNA was determined by the dideoxy-chain termination method using Sequenase DNA polymerase (United States Biochemical Co., Cleveland, OH). Comparison of these sequences with the human CD31 demonstrated a 72% similarity of amino acid composition and suggested that the full coding sequence of the mouse CD31 was contained within this cDNA clone (manuscript in preparation).

Transfection of COS-7 Cells and Immunofluorescent Staining

The 3.2-kb fragment was subcloned into the *Eco* RV site of the pcDNA/Neo SV40 expression vector (Invitrogen, San Diego, CA) and designated Mouse PECAM-1/pcDNA-neo. A 2.56-kb cDNA fragment containing the entire coding sequence for human PECAM-1 (CD31) was subcloned into the SV40 expression vector pESP-SVTEXP and designated PECAM/TEX as previously described.[12] COS cell transfections were performed by calcium phosphate–DNA coprecipitation.[13] Briefly, confluent T75 flasks of COS 7 cells were split 1:15 and plated on gelatin-coated coverslips in six-well tissue culture plates containing Dulbecco's minimum essential medium with 10% fetal bovine serum. After 24 hours, the cells were transfected with 6 μg mouse PECAM-1/pcDNA neo or 6 μg of PECAM/TEX cDNA in combination with 17 μg calf thymus DNA (Boehringer Mannheim, Indianapolis, IN). After incubation at 37°C for 5 hours, the cells were washed with PBS, and fresh Dulbecco's minimum essential medium with 10% fetal bovine serum was added.

The plates were maintained for an additional 36 hours and then fixed in 3% paraformaldehyde in HEPES-Hanks solution, washed in 0.1 mol/l glycine in PBS, and briefly permeabilized with ice-cold 0.5% NP-40 for 1 minute. After extensive washing, coverslips were incubated with undiluted 2H8 tissue culture supernatant (hamster anti-mouse CD31 mAb) or undiluted 4G6 tissue culture supernatant (a mouse anti-human CD31 monoclonal antibody [MAb]) for 1 hour. After rinsing, the coverslips were stained with 1:200 dilution of florescein isothiocyanate–labeled goat anti-hamster (Vector, Burlingame, CA) or goat anti-mouse (Organon-Teknika, West Chester, PA) IgG, respectively, for 1 hour. Cells were viewed on a Zeiss phase-epifluorescent microscope using a 40× planapochromatic lens with a 0.7 numerical aperture and photographed with Kodak TMAX p3200 film at an ASA of 3200.

Results

Tissue Distribution of the 2H8 Monoclonal Antibody Epitope

Immunoperoxidase analysis of the 2H8 monoclonal antibody on reactive lymph nodes (after immunization with KLH in CFA, as described in Materials and Methods) showed that the expression of the 2H8 epitope was largely restricted to lymphocytes adherent both to the sinusoidal endothelium and to postcapillary venular endothelium (Figures 1A, B). All of the marginating lymphocytes in Figures 1A and B are intensely stained, whereas cells in the central portion of the lumen and not marginating are unstained. The distinction of lymphocytes from high endothelial cells is not always straightforward on routine light microscopic stains such as these. The cells designated by arrows in Figure 1A, however, can be unequivocally identified as not of endothelial origin because of their presence within the lumen of the vessel rather than at its inner border. Figure 1B also demonstrates membrane staining of marginating lymphocytes that are still within the vascular lumen. The vessel in Figure 1B is a sinusoid, as determined by its cortical location and near continuity with the cortical sinus (not apparent in figure). Studies of the tissue distribution of the 2H8 molecule showed that it is present only weakly and irregularly on lymphatics and sinusoids. Therefore, the strongly stained cells in Figure 1B must be predominantly, if not exclusively, lymphocytes rather than endothelium. Control stains with normal rat immunoglobulin (NRIg) were negative (Figure 1C).

Because we are not aware of any molecule with such a unique tissue distribution (on marginating/transmigrating lymphocytes), we sought to further characterize and identify it. We next examined the expression of the 2H8 molecule on a panel of cultured lymphocyte cell lines, and freshly isolated lymphocytes, neutrophils, and platelets. Cell lines of B and T lineages expressed variable levels of the 2H8 epitope (Table 1). Note that *both* T- and B-cell–derived lines expressed the 2H8 mol-

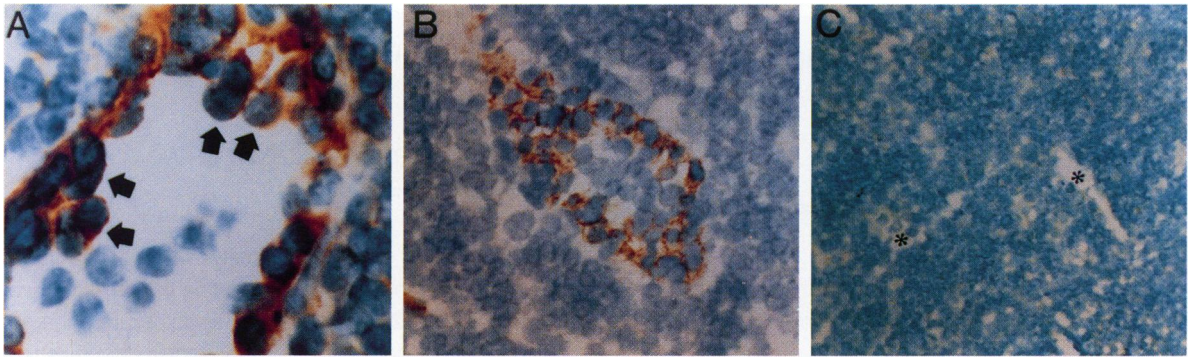

Figure 1. *Immunocytochemical detection of the 2H8 molecule in reactive lymph nodes. Note that marginating lymphocytes adjacent to the sinusoidal endothelium are strongly positive* **(A, B)**. *At least some of the marginating lymphocytes can be distinguished at this magnification from endothelial cells because of their position with respect to the vessel wall (arrows). The vessel in* **(B)** *is believed to be a sinusoid because of its proximity to the cortical surface and close relationship to the cortical sinus (not evident in the photo);* **(C)** *shows staining with normal hamster immunoglobulin (asterisks indicate two vessels). Magnification ×2000* **(A)**, *×800* **(B)**, *and ×330* **(C)**.

ecule. The CTLL cell line, a T cell receptor negative, IL-2–dependent line, had the highest level of surface expression and was subsequently used for biochemical analysis. The SP2/0 myeloma was the only completely negative cell line and was therefore used as a negative control in biochemical studies.

Freshly isolated lymphocytes from reactive lymph nodes and naive spleen were also analyzed by flow cytometry (Figure 2a, c). Reactive lymph node lymphocytes expressed little or no 2H8 (Figure 2a). This FACS graph emphasizes the differences in the abilities of the immunoperoxidase and flow cytometry techniques to detect small populations of cells constituting less than a few percentage points of the total. A FACS profile of 2H8-positive CTLL cells is shown for comparison (Figure 2b). The FACS profiles for naive spleen cells consistently demonstrated a slight shift to the right, indicating weak expression on most cells (Figure 2c). Immunocytochemistry of naive spleen showed few brightly positive splenocytes adherent to endothelial walls and a low level of background staining of parenchymal splenocytes (not shown), analogous to the pattern seen in lymph nodes (Figures 1a, b).

Freshly isolated peripheral blood leukocytes and platelets were also examined for expression of the 2H8 molecule by flow cytometry. Peripheral blood neutrophils were distinguished from lymphocytes by their different right-angle light scatter profiles. Neutrophils and platelets both expressed the 2H8 molecule (Figure 2f, d). Although there is a background level of fluorescence for a subpopulation of neutrophils in the negative control, the right shift in the 2H8 antibody FACS profile is quite dramatic. Peripheral blood lymphocytes expressed low levels of fluorescence on staining for the 2H8 molecule, comparable in intensity to naive splenocytes (Figure 2e).

We further characterized the tissue distribution of the 2H8 molecule by immunocytochemistry on frozen sections of murine tissues (Table 1). The 2H8 epitope was

Table 1. *Expression of 2H8 Epitope on Lymphocyte Cell Lines*

T-cell lines		Solid organs	
CTLL (TCR −)	+++*	Venules and arterioles (all tissues)	+++
HT2 (TCR −)	++	Brain, small population of neurons	+++
D10.G4 (TH2)	++	Kidney	−
2B4 (T hybridoma)	++	Pancreas	−
CDC25 (TH2)	+	Liver	−
CDC35 (TH2)	+	Uterus	−
D1.1 (TH1)	+	Heart	−
AR100.9 (TH1)	+	Skeletal muscle	−
DO11 (T hybridoma)	+	Thymus	−
EL4 (Thymoma)	+		
B-cell lines		Bone marrow	
11B11 (B hybridoma)	+	Freshly isolated blast cells	++
A20 (B lymphoma)	+		
WEHI 279 (B lymphoma)	+		
SP2/0 (Myeloma)	−		

* The degree of expression is graded semiquantitatively as moderate (+++), mild (++), faint (+), and absent (−).
Cell suspensions were stained with 2H8 tissue culture supernatant followed by FITC-anti-hamster Ig and analyzed by flow cytometry. Solid organs were analyzed by immunocytochemistry.

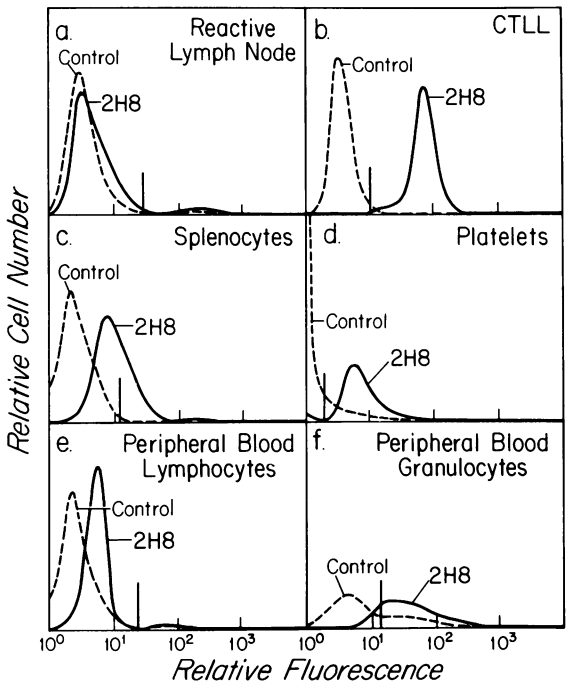

Figure 2. *FACS analysis of 2H8 expression on lymphocytes. The following cells were stained with a control hamster antibody (H22) or 2H8, followed by FITC-anti-hamster Ig:* (a) *lymph node cells from a BALB/c mouse immunized 7 days previously with KLH in CFA;* (b) *CTLL cell line;* (c) *BALB/c splenocytes cultured in medium at 37°C for 2 hours; and* (d) *BALB/c splenocytes cultured for 24 hours. N = 3 for the experiment in* (d).

found on venular and arteriolar walls throughout the body. Sinusoids of lymph nodes or tissue lymphatics were weakly and irregularly immunoreactive. The 2H8 epitope was also noted on an as-yet-unidentified population of neurons in the brain. Other tissues did not express the 2H8 molecule.

Appearance of 2H8-positive Cells After Immunization

The striking anatomic localization of the 2H8 epitope suggests that it is involved in the process of lymphocyte recruitment and vascular transmigration. Therefore, it was of interest to determine if the expression of the 2H8 molecule paralleled lymphocyte influx after an antigenic stimulus. Lymph nodes are known to dramatically increase in size after stimulation, eg, with antigens in CFA or adjuvants alone injected subcutaneously in the hind legs. The expansion is associated with a significant lymphocyte influx. As shown in Figure 3, the number of $2H8^+$ cells along vascular endothelium in the draining popliteal lymph nodes increased approximately 10-fold by days 7 to 9 after immunization with KLH in CFA. This increase was associated with a corresponding increase in the size and surface area of vascular channels. The number of $2H8^+$ cells sharply declined by day 11.

Ultrastructural Localization of the 2H8 Molecule

Immunoelectron microscopic analysis of reactive lymph nodes was performed to better define the ultrastructural location of the 2H8 molecule in endothelium-adherent lymphocytes. Additionally, it is important in distinguishing the expression of the 2H8 molecule on endothelium-adherent lymphocytes versus endothelial cells themselves (note that venular but not sinusoidal walls were immunoreactive in many tissues, Table 1). In agreement with the immunoperoxidase analysis, the 2H8 molecule was present on endothelium-adherent lymphocytes and at low levels on endothelial cells. Figure 4 shows three lymphocytes in various stages of transmigration through a lymph node sinusoid (so identified because of its size and the absence of collagen). In this low-magnification electron micrograph, chosen to demonstrate the physical relationships between lymphocytes and endothelium, the immunogold particles are not visible. Therefore, clusters of two or more immunogold particles, which were identified under a magnifying lens, are designated with arrowheads.

The cell labeled A has extended an attachment process to the endothelial cell. The 2H8 antibody, as detected by immunogold particles, binds to the cytoplasm

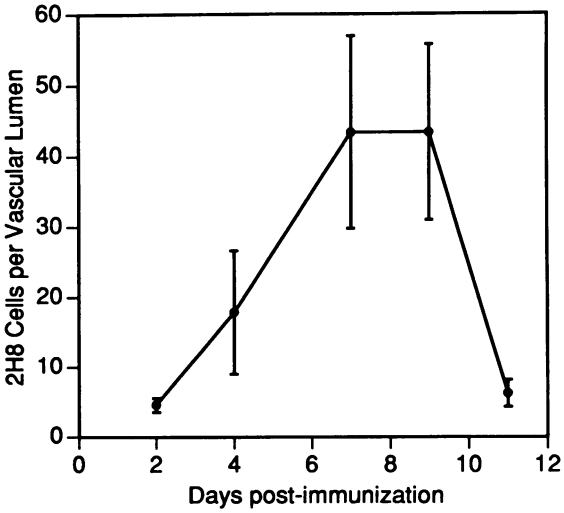

Figure 3. *Kinetics of 2H8 expression on endothelium-adherent lymphocytes in lymph nodes from immunized mice. Lymph nodes were removed from BALB/c mice 2,4,7,9, and 11 days after immunization with KLH in CFA, and frozen sections were stained with the 2H8 antibody. The numbers of 2H8+ lymphocytes adherent to vascular endothelium in two sections of lymph nodes from different mice were counted under light microscopy. Results are expressed as means ± SE.*

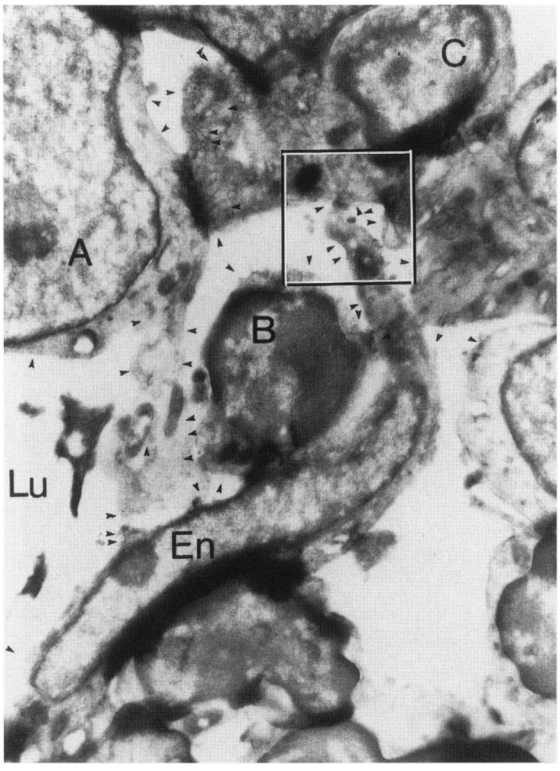

Figure 4. *Ultrastructural pattern of 2H8 expression on lymphocytes adherent to the endothelium of a sinusoid. Immunogold particles are not readily visible at this low magnification (×5800). Arrowheads denote clusters of two or more immunogold particles, which were located by a further fivefold magnification of the original print. Sections of a lymph node from a BALB/c mouse 7 days after immunization with KLH in CFA were stained with 2H8 and processed for electron microscopy as described in Materials and Methods. Three lymphocytes (A,B,C) in various stages of transmigration through a sinusoid are shown. Lu, lumen; En, endothelial cell. Note that lymphocytes not adherent to endothelium (lower right) are largely 2H8-negative.*

and membrane of the attachment process. The lymphocyte designated B is more closely adherent to the same endothelial cell. It has less immunogold label located mainly at the lateral edges of the endothelial cell–lymphocyte interface. The lymphocyte labeled C has nearly completely traversed the sinusoidal wall. The portion of the cell external to the sinusoid is largely devoid of immunogold label. Instead, the 2H8 epitope is located near the lymphocyte–endothelial cell junction (shown in high magnification in Figure 5) and intracytoplasmically in the trailing cell edge. Other lymphocytes external to the sinusoid are essentially devoid of immunogold label. Note that this latter finding is in agreement with the immunocytochemical data and provides a useful internal control. Additionally, immunoelectron microscopic analysis of a serial tissue section processed in the same way but without the 2H8 primary antibody showed no immunoreactivity. Thus, the distribution of the 2H8 epitope in each cell varies with the location of the cell with respect to the vascular wall. Figure 5 shows in high magnification the lymphocyte designated "C" in Figure 4, and its junction with endothelium. The immunogold particles, denoted by arrowheads, are evident at this magnification and are present at high density in the junctional area.

Biochemical Analysis of the 2H8 Molecule

To determine the nature of the 2H8 molecule, we performed immunoprecipitation and N-terminal amino acid sequencing. Immunoprecipitation of CTLL and SP2/0 iodinated cell lysates showed a specific band at approximately 120 to 130 kd under both reducing and nonreducing conditions (Figure 6). A nonspecifically precipitating band at approximately 95 kd (reducing) and 200 kd (nonreducing) is probably the transferrin receptor, which may have bound to transferrin present as a contaminant in the 2H8 and control (normal hamster immunoglobulin) preparations.

To exclude this nonspecific reactivity, the 2H8 hybridoma was adapted to culture in serum-free medium. IgG was concentrated by ammonium sulfate precipitation and biotin conjugated. Immunoprecipitation was repeated with I^{125}-labeled CTLL and SP2/0 cell lines using the biotinylated 2H8 antibody and avidin-agarose beads (Figure 7). In CTLL cells, the 2H8 molecule migrated at approximately 120 to 130 kd under both reducing (lane A) and nonreducing conditions, and had an apparent molecular weight of 95 to 100 kd after removal of N-linked sugars (lane B). Lanes C through H serve as specificity controls. No bands were seen with CTLL cells and a control hamster IgG monoclonal antibody (lane C), or with the 2H8 antibody on the SP2/0 cell line (lane G). Lanes E and H are positive controls for transferrin receptor using antibody R17 217.1.3. The molecular weight for the transferrin receptor appears to closely match the nonspecific band in Figure 6.

N-terminal sequence analysis was performed using the procedure of Matsudaira.[10] Coomassie blue staining of the PVDF membrane showed a split band at approximately 120 to 125 kd. The split bands were separately excised and sequenced. The consensus sequence is shown in Figure 8 and compared with the published sequences for CD31. The split bands were the same except for a slight difference in position 8. Both bands yielded signals for asparagine *and* valine at position 8, although the relative dominance of the signals at position 8 were opposite. The upper band yielded a dominant signal for valine and a weaker one for asparagine, whereas the lower band had the opposite pattern of dominance at position 8. The signal for position 2 was unclear, presumably because of glycosylation of this amino acid or noise in the system. Retrospective analysis of the data

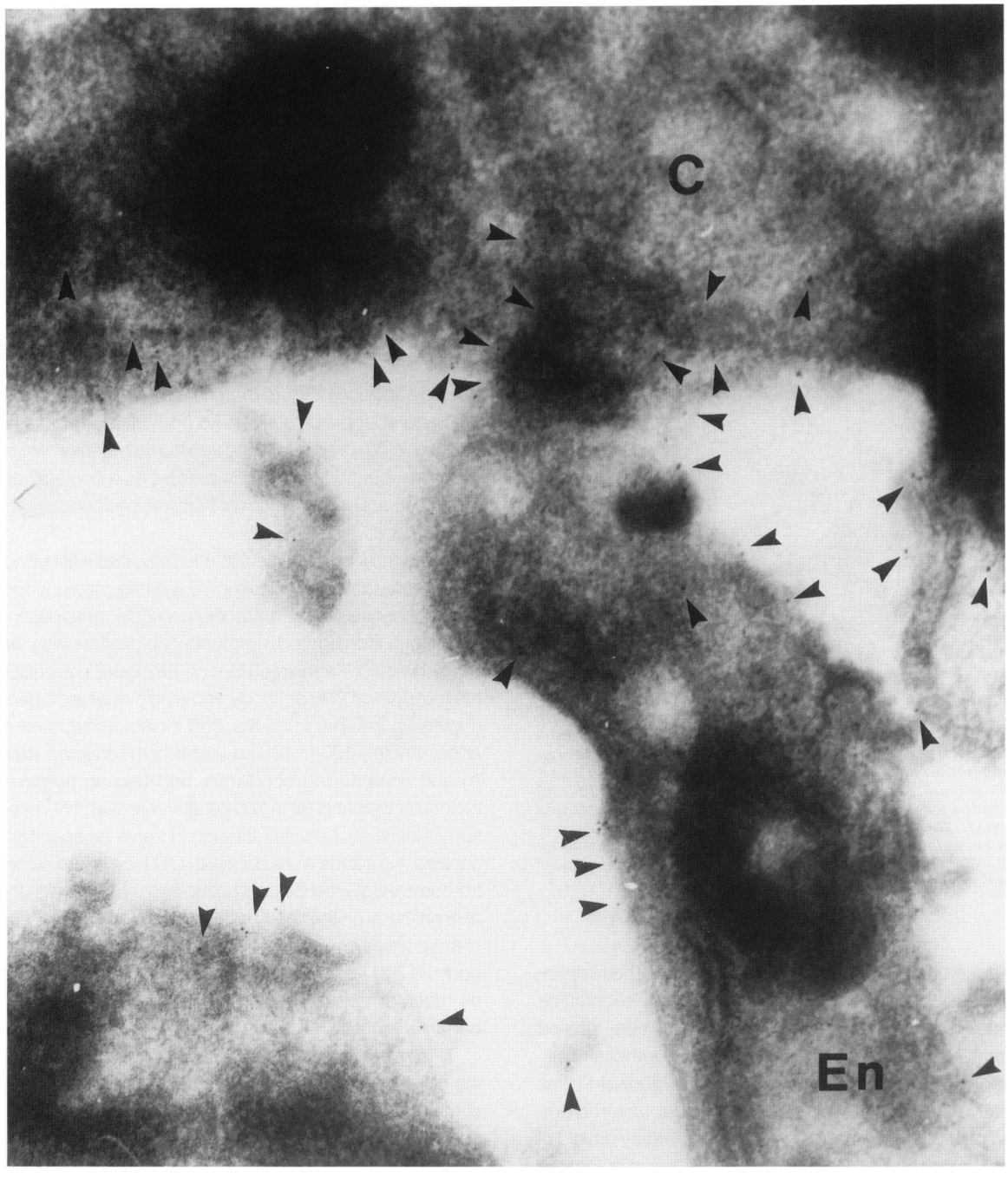

Figure 5. *2H8 expression at site of lymphocyte-endothelial contact. The inset is shown at high magnification, to illustrate the distribution of immunogold particles. Magnification* ×*63,800.*

on position 2 for a possible glutamic acid (E) showed that the signal peaked during that cycle and significantly decreased on the following cycle. The glutamic acid signal, however, was almost as high on cycle 1. Thus, the data are consistent with a glutamic acid peak, but not sufficiently definitive to call outright. A signal for isoleucine for position 4 was noted in the lower band (in addition to the serine peak seen in both bands) and is shown is parentheses.

Comparison of the N-terminus of the 2H8 molecule with two recently published cDNA sequences of human CD31[11,14] shows an exact match at seven or eight of nine residues (Figure 8). Human CD31 also has a molecular weight of approximately 120 to 145 kd (glycosy-

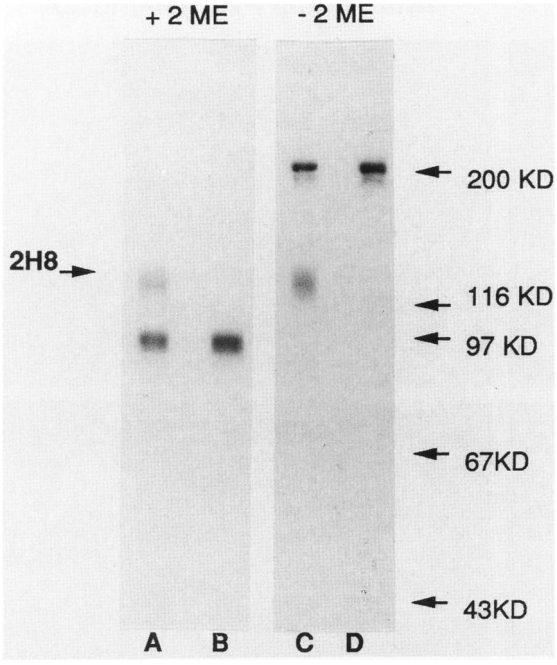

Figure 6. *SDS-PAGE analysis of 2H8. ^{125}I-labeled CTLL cell lysates were immunoprecipitated with 2H8-Sepharose* **(A,C)** *or control hamster Ig* **(B,D)** *and analyzed by SDS-PAGE under reducing* **(A,B)** *and non-reducing* **(C,D)** *conditions in an 8% gel. Bands at 95KD (reducing) and 200KD (nonreducing) are non-specific. The 2H8-specific band appears at 120–130 KD.*

lated) under both reducing and nonreducing conditions.[15–17] Moreover, it is expressed on comparable tissue/cell types—all hematopoietic cell lineages except mature erythrocytes and on endothelium.

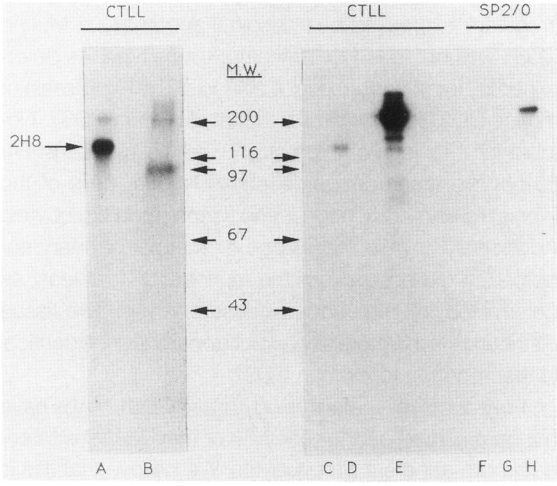

Figure 7. *SDS-PAGE analysis of 2H8 on CTLL and SP2/0 cells. ^{125}I-labeled CTLL and SP2/0 cell lysates were immunoprecipitated with various antibodies and electrophoresed under reducing* **(A,B)** *or nonreducing* **(C–H)** *conditions in a 7.5%–15% gradient gel. The lanes are the following:* **(A)** *2H8 immunoprecipitate;* **(B)** *2H8 immunoprecipitate treated with N-glycanase;* **(C,F)** *normal hamster Ig;* **(D,G)** *2H8;* **(E,H)** *anti-transferrin receptor antibody.*

NH2- Terminus

PECAM-1/CD31	**Q E N S F T I N S V D M K S . . .**
CD31	**Q E N S F T I N S V D M K S . . .**
Mouse PECAM-1	**E E N S F T I N S . . .**
2H8	**T X N S F T I N S . . .**
	E (I) V

Figure 8. *N-terminal sequence of the mouse 2H8 molecule, comparing both the direct N-terminal sequence data with the amino-acid translation of the murine (CD31 cDNA clone. A comparison to amino acid translation of published sequences of PECAM-1/CD31 (11) and CD31 (14) is also shown.*

Immunoreactivity of the 2H8 Monoclonal Antibody with a Cloned Murine CD31 Gene Product

To definitively confirm the identity of the glycoprotein recognized by monoclonal antibody (MAb) 2H8, the mouse homolog of CD31 was cloned by screening a murine heart cDNA library at high stringency using human CD31 cDNA as a probe. A 3.2-kb fragment was identified and partially sequenced. This cDNA showed a 72% homology at the amino acid level with the human CD31 over the first 300 base pairs sequenced and, except for the N-terminus amino acid, the amino acid translation was identical to that obtained from peptide sequencing of protein purified using the 2H8 antibody, as well as that obtained from translation of the sequence previously obtained for the human CD31 (Figure 8). When this murine CD31 cDNA was transfected into COS cells (Figure 9a), a subpopulation of cells clearly expressed an epitope recognized by the 2H8 antibody that was not recognized by the monoclonal antibody to human CD31 (Figure 9b). Likewise, COS cells transfected with the human CD31 cDNA expressed an epitope recognized by the anti-human CD31 MAb 4G6 (Figure 9d), but not by the anti-mouse CD31 MAb 2H8 (Figure 9c). This data not only verifies that 2H8 MAb recognizes mouse CD31, but also demonstrates a significant degree of species specificity for the monoclonal antibodies used in these studies.

Discussion

The goal of this study was to define the expression pattern and biochemistry of a surface molecule that is present on endothelium-adherent lymphocytes in lymph nodes draining sites of immunization. This molecule is the murine homolog of human CD31, based on biochemical data, pattern of tissue and cell expression, and immunoreactivity with a recently cloned murine CD31 homolog. Human CD31, also known as PECAM-1[11] or endoCAM[18] is a recently described glycoprotein produced by endot-

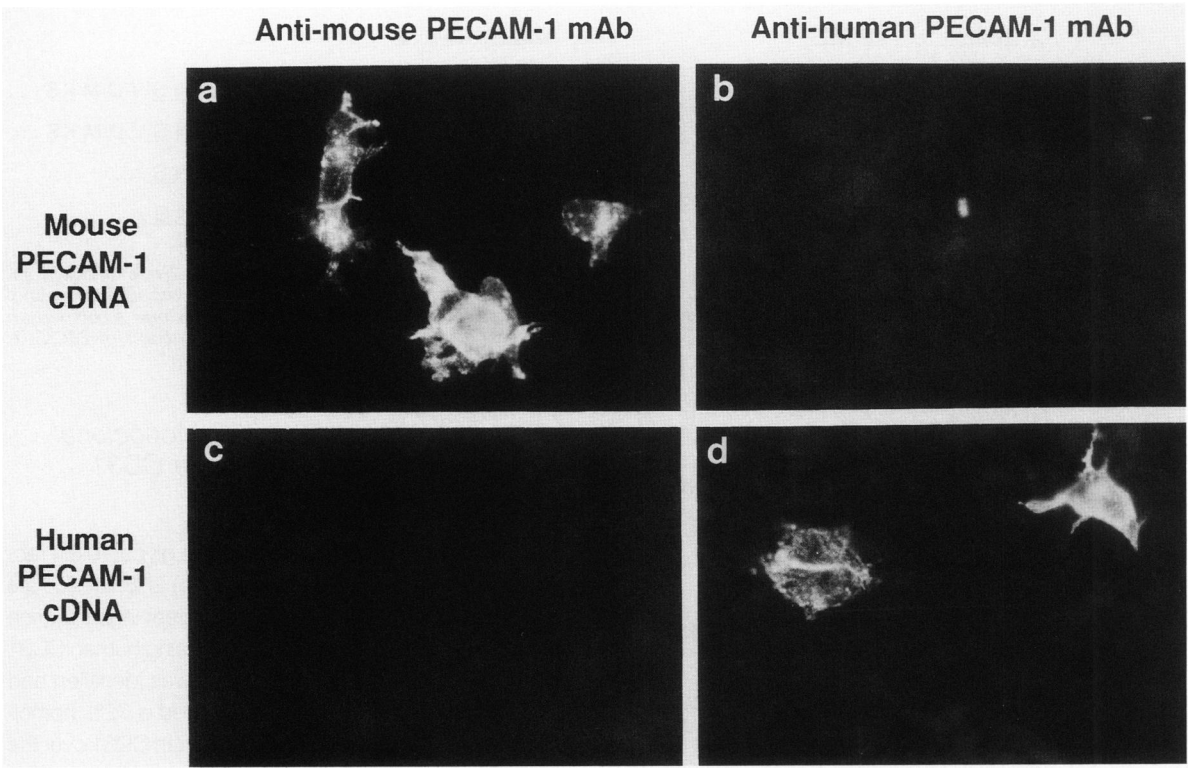

Figure 9. *Indirect immunofluorescent photomicrographs of COS-7 cells transfected with mouse CD31 cDNA* (a,b) *or human CD31 cDNA* (c,d) *and then stained with an anti-mouse CD31 MAb* (a,c) *or an anti-human CD31 MAb* (b,d). *The anti-mouse CD31 MAb recognizes an epitope on the subpopulation of COS cells transfected with the mouse CD31 cDNA* (a) *but did not stain untransfected COS cells in the same field or COS cells transfected with the human CD31 cDNA* (c). *Similarly, the anti-human CD31 MAb recognizes an epitope on the COS cells transfected with human CD31 cDNA* (d) *but did not stain untransfected cells or COS cells transfected with the mouse CD31 cDNA* (b). *A small amount of background staining of non-cellular material is seen in* (b).

helium and all hematopoietic cell lineages except mature erythrocytes.[4] It is a member of the immunoglobulin superfamily and is most closely related to carcinoembryonic antigen.[11,14] Endothelial cells express CD31 in areas of cell–cell contact.[16,18] Moreover, CD31 stably transfected into mouse L cells was shown to mediate calcium-dependent aggregation that was inhibitable by anti-CD31 MAbs.[12] For these reasons and because it is a member of the immunoglobulin superfamily, CD31 is postulated to play a role in intercellular adhesion.[11,14] Ohto et al[15] demonstrated that an antibody to human CD31 could inhibit endotoxin-induced chemotactic migration of neutrophils and monocytes through a micropore filter of a Boyden chamber. CD31 has also been proposed to be a marker for suppressor $CD4^+$ T cells.[19]

This is the first report that we are aware of describing a CD31 homolog in rodents. The molecule we refer to as "murine CD31" is expressed on some B and T lymphocyte cell lines and freshly isolated neutrophils, bone marrow blast cells (identified by low side scatter by flow cytometry), and platelets. Peripheral blood and splenic lymphocytes express murine CD31 weakly whereas lymph node lymphocytes appear to not express murine CD31 at all (by flow cytometry). Immunocytochemical stains, however, show a bimodal pattern of murine CD31 expression among lymphocytes, in that endothelium-adherent lymphocytes express murine CD31 intensely. Murine CD31 is also present on endothelial cells and a small population of neurons in the brain. It is weakly and irregularly expressed by sinusoidal endothelium. Murine CD31 was not found on any other tissue type, as determined by immunoperoxidase analysis. The expression of murine CD31 is quite similar to that of human CD31. Notable for their lack of expression were tissue-bound F4/80-positive macrophages such as Kupffer cells of the liver, splenic macrophages, and lymph node histiocytes. Additionally, thymic T cells and glomerular capillary endothelium did not express the murine CD31, at least as determined by immunoperoxidase analysis. The tissue distribution is therefore similar but apparently not identical to that reported for human CD31.

Previous investigators using human CD31 MAbs have not commented on the reactivity of their MAbs with endothelium-adherent lymphocytes. We believe that this is due to the paucity of transmigrating lymphocytes in a randomly obtained biopsy specimen. We found that in mice, the endothelium-adherent lymphocyte reactivity can be consistently observed only in tissue sections with many transmigrating lymphocytes. This situation was created experimentally by immunizing mice with protein an-

tigens such as KLH emulsified in CFA. In tissues lacking numerous transmigrating lymphocytes, the reactivity of the occasional endothelium-adherent lymphocyte cannot be distinguished from what would otherwise appear to be endothelial reactivity. We believe that such reactivity would be difficult to demonstrate in human tissues because of the rarity of such a synchronized influx of lymphocytes obtained from a surgical biopsy.

The presence of murine CD31 on lymphocytes attached to endothelium suggests that it is involved in lymphocyte recruitment and transendothelial migration. As would be expected for a molecule associated with lymphocyte recruitment, the number of cells expressing murine CD31 after an antigenic or inflammatory stimulus shows a characteristic kinetic profile. The number of murine $CD31^+$ cells attached to vascular walls increases 10-fold within 7 to 9 days after immunization with KLH in CFA. By day 11, as the immune response wanes, so too does the number of $CD31^+$ cells. This kinetic profile closely parallels the kinetics of a primary immune response as judged by lymph node size and lymphocyte influx, germinal center formation, and the appearance of perivascular IL-2^+ cells, as detected *in situ* by immunocytochemistry.[3] The kinetic profile is therefore compatible with and supports the hypothesis that murine CD31 is associated with lymphocyte transmigration.

The fact that CD31 is also present on endothelial cells raises the possibility that the apparent lymphoid expression is actually passively acquired from adjacent endothelium. We believe that the high level of murine CD31 on endothelium-adherent lymphocytes is of lymphocyte origin because: 1) lymphocyte cell lines produce CD31 *in vitro,* 2) CD31 can be visualized in lymphocyte attachment processes by immunoelectron microscopy as both membranous and intracytoplasmic, and 3) whereas sinusoidal endothelium expresses only low levels of murine CD31, as judged by immunoelectron microscopy, the attached intrasinusoidal lymphocytes express high levels. For these reasons, we believe that the murine CD31 detected on transmigrating lymphocytes is synthesized by lymphocytes rather than being passively acquired.

The presence of CD31 on both lymphocytes and endothelium suggests that this molecule may interact homotypically. This possibility has previously been raised because CD31-transfected cells spontaneously aggregate in culture and localize CD31 to sites of contact with other CD31-expressing cells.[12] Alternatively, both lymphocytes and endothelium may express complementary receptors for CD31. The physiologic ligand of CD31 is unknown.

Perhaps the most interesting finding from these studies is the relationship between murine CD31 expression and the area of lymphocyte contact during transmigration through endothelium. On endothelium-adherent lymphocytes within the vascular lumen, murine CD31 is concentrated at or near lymphocyte attachment processes to endothelium. On cells with a broad interface with endothelium, CD31 is found along the lateral edges of the interface. On lymphocytes that have already penetrated the endothelium and basement membrane, CD31 is present only in the trailing intravascular cytoplasmic tail. The apparent redistribution or shedding of murine CD31 during transmigration can explain the almost exclusive localization of the molecule to endothelium-adherent lymphocytes as seen by immunocytochemistry. These data suggest a specific pattern of redistribution for murine CD31 molecules on lymphocytes as they transmigrate past the endothelial barrier.

Thus, murine CD31 may function to bind lymphocytes to endothelium or to promote the migration of the cells through vessel walls. The localization of murine CD31 on lymphocytes noted by immunoelectron microscopy suggests that CD31 may be associated with the intracellular cytoskeleton, in a manner analogous to the f-met-leu-phe receptor on neutrophils.[20,21] Because the molecule is also present on neutrophils, it may serve a similar role in the trans-endothelial migration of such cells as well. The availability of an antibody to murine CD31 will be useful in future studies of lymphocyte traffic and in defining early lymphocyte activation events occurring in lymphoid organs *in vivo.*

References

1. Gallatin WM, IL Weissman, Butcher EC: A cell surface molecule involved in organ-specific homing of lymphocytes. Nature 1983, 304:30–34
2. Hamann A, Jablonzki-Westrich D, Duijvestijn A, Butcher EC, Baisch H, Harder R, Thiele HG: Evidence for an accessory role of LFA-1 in lymphocyte-high endothelium interaction during homing. J Immunol 1988, 140:693–699
3. Bogen SA, Weinberg DS, Abbas AK: Histologic analysis of T lymphocyte activation in reactive lymph nodes. J Immunol 1991, 147:1537–1541
4. McMichael AJ, Beverly PCL, Cobbold S, Crumpton MJ, Gills W, Gotch FM, Hogg N, Horton M, Ling N, MacLennan ICM, Mason DY, Milstein C, Spiegelhalter D, Waldmann SH: Leukocyte typing III, White Cell Differentiation Antigens. Oxford, Oxford University Press, 1987, pp 589–590
5. Kaye J, Gillis S, Mizel SB, Shevach EM, Malek TR, Dinarello CA, Lachman LB, Janeway CA: Growth of a cloned T helper cell line induced by a monoclonal antibody specific for the antigen receptor. Interleukin 1 is required for the expression of receptors for interleukin 2. J Immunol 1984, 133:1339–1345
6. Oi VT, Herzenberg LA: Immunoglobulin-producing hybrid cell lines, Selected Methods In Cellular Immunology. Edited by BB Mishell, SM Shiigi. San Francisco, WH Freeman, 1980, pp 351–372

7. Lesley J, Hyman R, Schulte R, Trotter J: Expression of transferrin receptor on murine hematopoietic progenitors. Cell Immunol 1984, 83:14–25
8. Tokoyasu KT, Singer SJ: Improved procedures for immunoferritin labelling of ultra-thin frozen sections. J Cell Biol 1976, 71:894–906
9. Marchalonis JJ: An enzymic method for the trace iodination of immunoglobulin and other proteins. Biochem J 1969, 113: 299–305
10. Matsudaira P: Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J Biol Chem 1987, 262:10035–10038
11. Newman PJ, Berndt MC, Gorski J, White GC II, Lyman S, Paddock C, Muller WA: PECAM-1 (CD31) cloning and relation to adhesion molecules of the immunoglobulin gene superfamily. Science 1990, 247:1219–1222
12. Albelda SM, Muller WA, Buck CA, Newman PJ: Molecular and cellular properties of PECAM-1 (endoCAM/CD31): A novel vascular cell-cell adhesion molecule. J Cell Biol 1991, 114:1059–1068
13. Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning: A Laboratory Manual. 2nd edition. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory, 1989 p 545
14. Simmons DL, Walker C, Power C, Pigott R: Molecular cloning of CD31, a putative intercellular adhesion molecule closely related to carcinoembryonic antigen. J Exp Med 1990, 171:2147–2152
15. Ohto J, Maeda H, Shibata Y, Chen R-F, Ozaki Y, Higashihara M, Takeuchi A, Tohyama H: A novel leukocyte differentiation antigen: Two monoclonal antibodies TM2 & TM3 define a 120KD molecule present on neutrophils, monocytes, platelets, and activated lymphoblasts. Blood 1985, 66:873–881
16. Muller WA, Ratti CM, McDonnell SL, Cohn ZA: A human endothelial cell-restricted, externally disposed plasmalemmal protein enriched in intercellular junctions. J Exp Med 1989, 170:399–414
17. Van Mourik JA, Leeksma OC, Reinders JH, de Groot PG, Zandbergen-Spaargaren J: Vascular endothelial cells synthesize a plasma membrane protein indistinguishable from the platelet membrane glycoprotein IIa. J Biol Chem 1985, 260:11300–11306
18. Albelda SM, Oliver P, Romer L, Buck CA: EndoCAM: A novel endothelial cell-cell adhesion molecule. J Cell Biol 1990, 110:1227–1237
19. Torimoto Y, Rothstein DM, Dang NH, Schlossman SF, Morimoto C: CD31, a novel cell surface marker for CD4 cells of suppressor lineage, unaltered by state of activation. J Immunol 1992, 148:388–396
20. Schmitt M, Keller H: Video intensification microscopy of binding and processing of the fluoresceinated N-formyl chemotactic peptide TRITC-FNLPNTL by human neutrophils, Proceedings of the International Congress on the Biological And Clinical Aspects Of Phagocyte Function. New York, Pergamon Press, 1986, 285–294
21. Sullivan SJ, Zigmond SH: Asymmetric distribution of the chemotactic peptide receptor on polymorphonuclear leukocytes. J Cell Biol 1984, 99:1461–1467