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Human Mucosal Addressin Cell Adhesion Molecule-1 Is Preferentially Expressed in Intestinal Tract and Associated Lymphoid Tissue

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Lymphocyte homing to normal tissues and recruitment to inflammatory tissue sites are controlled, in part, by the selective expression of chemokines, pro-inflammatory cytokines and mediators, and various adhesion proteins and molecules. In the mouse, mucosal addressin cell adhesion molecule-1 (MAdCAM-1) is selectively expressed on endothelium of high endothelial venules in gut and gut-associated lymphoid tissue. By interaction with its integrin ligand, α4β7, lymphocytes presumed to be involved in mucosal immunity are selectively recruited to these intestinal sites. After generating monoclonal antibodies against a murine cell line expressing recombinant human MAdCAM-1, we qualitatively and semiquantitatively assessed MAdCAM-1 expression in human tissue sections from various normal and inflammatory disorders. We found that human MAdCAM-1, as in the mouse, is expressed in a tissue-selective manner. In normal tissues, MAdCAM-1 is constitutively expressed to endothelium of venules of intestinal lamina propria. Interestingly, using computer-assisted morphometric analysis, the proportion of venular endothelium within lamina propria that expresses MAdCAM-1 is increased, compared with normal tissues, at inflammatory foci associated with ulcerative colitis and Crohn's disease. Moreover, for the most part, MAdCAM-1 is not detected in the majority of normal or inflamed extra-intestinal tissues, including those with mucosal surfaces. These results are consistent with a role, as originally defined in the mouse, for human MAdCAM-1 in the localization of α4β7$^+$ lymphocytes in the gastrointestinal tract and associated lymphoid tissue. As such, the pathway defined by MAdCAM-1/α4β7 may be a relevant tissue-specific therapeutic target for the modulation of inflammatory bowel disease activity. (Am J Pathol 1997, 151:97–110)

Lymphocyte homing to both normal tissues and sites of inflammation is, in part, regulated by differential expression of cell surface homing receptors and their selective interactions with tissue-selective vascular addressins at sites of lymphocyte recruitment from the blood.[1–5] In the mouse, lymphocyte homing to mucosal lymphoid tissue such as the Peyer's patches and the intestinal lamina propria involves a single-chain 60-kd glycoprotein, the mucosal vascular addressin MAdCAM-1.[6,7] Murine MAdCAM-1 is expressed on endothelial cells in mesenteric lymph nodes, lamina propria of the small and large intestine, and the lactating mammary gland.[1,4,6] Murine endothelial cells can be induced to express high levels of MAdCAM-1 in response to pro-inflammatory cytokines.[8] Within inflammatory settings, expression of murine MAdCAM-1 has been observed on high

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endothelial venule (HEV)-like vessels in the pancreas of the non-obese diabetic mouse,[9,10] in venules of the central nervous system in chronic relapsing experimental allergic encephalomyelitis (EAE),[11] and on intestinal lamina propria venules from mice with experimentally-induced inflammatory bowel disease.[12,13] To our knowledge, MAdCAM-1 expression has not been observed at other nonmucosal sites of inflammation.

The lymphocyte integrin $\alpha4\beta7$ is the exclusive integrin receptor for MAdCAM-1.[14–16] Human lymphocyte cell lines and memory T cell subsets expressing high levels of $\alpha4\beta7$ have been shown to preferentially bind murine MAdCAM-1.[14–16] *In vitro* binding studies, using murine T cell lines and isolated human mucosal immunoblasts, have indicated that lymphocyte binding to appendix HEVs is largely mediated by $\beta7$ integrins, but not by utilization of alternative counter-receptors for $\alpha4\beta7$ such as VCAM-1 and fibronectin.[17] Albeit indirect, these data imply that human MAdCAM-1 is likely to be the major vascular counter-receptor for $\alpha4\beta7$ in these tissues.

We recently used functional expression cloning to identify the human homologue of murine MAdCAM-1, and analysis of RNA expression indicated that expression of human MAdCAM-1 is, as in the mouse, tissue selective and largely restricted to both large and small intestine and associated lymphoid tissue.[18] As an extension of these preliminary studies, we generated a panel of monoclonal antibodies (MAbs) specific for human MAdCAM-1 and used these antibodies for qualitative and semiquantitative assessment of MAdCAM-1 expression in human tissue sections from various normal and inflammatory disorders. We found that human MAdCAM-1 expression was predominantly limited to tissues associated with the gastrointestinal tract and associated lymphoid tissue. In normal tissues, MAdCAM-1 was constitutively expressed on endothelium of venules of intestinal lamina propria. Interestingly, using computer-assisted morphometric analysis, the proportion of venular endothelium within lamina propria that expressed MAdCAM-1 was increased, compared with normal tissues, at inflammatory foci associated with ulcerative colitis and Crohn's disease. Moreover, MAdCAM-1 was not detected, for the most part, in the majority of normal or inflamed extraintestinal tissues, including those with mucosal surfaces. Therefore, similar to findings in the mouse, human MAdCAM-1 represents an endothelial adhesion protein with a tissue-selective expression profile consistent with its role as a lymphocyte homing receptor for the gastrointestinal tract.

Materials and Methods

Generation of Cross-Reacting MAb LM20

The rear footpads of Fisher 411 rats were injected with 2 μg of immunoaffinity-purified recombinant murine MAdCAM-1 on days 1, 4, and 7. On day 10, both popliteal lymph nodes were removed and the lymphocytes fused with SP2/0 cells using standard fusion protocols.[19] Supernatants were screened for positive reactivity against murine MAdCAM-1 L1–2 transfectants,[15] murine Peyer's patches, human tonsil, and human appendix. Any MAbs that were reactive against the L1–2 transfectants and with HEVs of Peyer's patches and appendix, although negative on peripheral lymph nodes, were considered potential cross-reacting MAbs. LM20 recognizes an epitope that is neuraminidase sensitive and detects recombinant murine MAdCAM-1 by Western blot analysis (not shown).

L1–2 and CHO Cell Transfectants

The genes encoding either the macaque or human cDNAs for MAdCAM-1 were subcloned into the pcDNA3 vector (Invitrogen, San Diego, CA), linearized by digestion with the restriction enzyme *Sca*I (Gibco BRL, Gaithersburg, MD), and transfected into the murine pre-B-lymphoma cell line L1–2 by electroporation as previously described.[20] After electroporation, the cells were diluted to 25 ml in standard L1–2 growth medium (RPMI 1640, 10% Hyclone fetal bovine serum, penicillin/streptomycin (Gibco BRL) and L-glutamine (0.29 mg/ml; Gibco BRL)) and returned to the incubator. After 48 hours, the cells were pelleted by centrifugation and resuspended in 50 ml of L1–2 medium supplemented with G418 (Gibco BRL) at 0.8 mg/ml. Dilutions of the cell suspension were plated in 96-well microtiter plates, and single colonies were grown up and analyzed for expression of MAdCAM-1 by FACS analysis with the LM20 MAb, using fluorescein-isothiocyanate-conjugated anti-rat immunoglobulin (Jackson ImmunoResearch Laboratories, West Grove, PA). Surface expression of MAdCAM-1 in L1–2 cells was also confirmed by the ability of these cells to mediate aggregation with TK1 cells, a murine T cell lymphoma that expresses high levels of $\alpha4\beta7$.[21] Specificity of this interaction was further demonstrated by inhibition of aggregation by pretreatment of TK1 cells with anti-$\beta7$ MAb Fib 504, which had previously been shown to block $\alpha4\beta7$ binding to MAdCAM-1.[22]

CHO cells stably transfected with human MAdCAM-1 cDNA were prepared as described above with the following exceptions. Media for CHO cell growth was minimum essential medium-α with deoxyribonucleosides, 10% fetal calf serum (FCS), penicillin/streptomycin, and L-glutamine (0.29 mg/ml; Gibco BRL). Selection medium consisted of the same medium further supplemented with 0.55 mg/ml G418 (Gibco BRL). Single clones were grown up and analyzed for their ability to exhibit $\alpha 4\beta 7$-dependent binding to the B-cell lymphoma RPMI 8866, as previously demonstrated,[16,18] and by FACS staining with the LM20 MAb. Using these criteria, a cell line, CHO HuMAd-4, was established.

Monoclonal Antibodies

MAbs against human MAdCAM-1 were generated by immunizing C57BL/6 mice with L1–2 MAdCAM-1 transfectants. Mice were immunized intraperitoneally three times at 2-week intervals with 10 million cells resuspended in Hanks' balanced salt solution, and a final fourth immunization was injected intravenously.

Four days after the intravenous immunization, a fusion was performed by standard protocols[19] that generated approximately 5000 hybridomas. Ten days after the fusion, supernatants from the wells were screened for reactivity against CHO human MAdCAM-1 transfectants using a fluorescently labeled (fluorescein isothiocyanate) anti-mouse antibody (Jackson ImmunoResearch). A total of 45 antibodies were selected for strong reactivity against the CHO MAdCAM-1 transfectants. These supernatants were subsequently classified by their ability to block adhesion of RPMI 8866 cells to CHO human MAdCAM-1 transfectants. Inhibition experiments were performed by preincubation of CHO HuMAd-4 transfectants, which were plated in a 24-well format, with neat culture supernatants for 15 minutes at 4°C before the start of adhesion assays as previously described.[18] Selected blocking hybridomas were subcloned using limiting dilution. Supernatants from these hybridoma cultures were screened for their ability to specifically recognize high endothelial venular endothelium in human mesenteric lymph nodes using standard immunohistochemical techniques. Based upon these analyses, two hybridoma clones (10G3 and 10A6) were used for all subsequent immunohistochemical studies.

Immunoprecipitation and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis

L1–2 and L1–2 human MAdCAM-1 transfectant cells were generated as described above. These cells were surface labeled with biotin by incubating 10^7 cells/ml with 50 μg/ml sulfosuccinimydyl 6-(biotinamido) hexanoate (Pierce, Rockford, IL) in 10 mmol/L sodium borate, 150 mmol/L NaCl, pH 8.8, for 15 minutes at room temperature. Biotinylation was stopped by adding NH_4Cl to 10 mmol/L final concentration. Cells were then washed by centrifugation in 50 mmol/L Tris/HCl, pH 7.4, 25 mmol/L KCl, 5 mmol/L $HgCl_2$, 1 mmol/L EGTA. Cells were subsequently lysed with 0.5% Nonidet P-40, Tris-buffered saline, pH 8.0. Lysates were precleared with protein A/Sepharose beads and then immunoprecipitated with MAb 10G3 or an irrelevant isotype-matched MAb UPC 10 (Sigma Chemical Co., St. Louis, MO). Immunoprecipitates were then run on a 4 to 12% nonreducing SDS-PAGE gel (Novex, San Diego, CA) and transferred to nitrocellulose with a BioRad transblot apparatus per the manufacturer's instructions. The nitrocellulose was blocked with 5% nonfat milk in PBS, pH 7.2 (blocking buffer), at room temperature for 2 hours. Biotinylated, immunoprecipitated proteins on the nitrocellulose blot were then reacted with avidin-biotin-horseradish peroxidase complexes (Amersham, Arlington Heights, IL) diluted 1:100 in blocking buffer and detected by chemiluminescence with Renaissance^R-enhanced luminol reagent and oxidizing reagent (Dupont NEN, Boston, MA) according to the manufacturer's instructions.

Tissues, Immunohistochemistry, and Image Analysis

Normal and inflamed human tissue, colon from patients with ulcerative colitis, small and large intestine from patients with Crohn's disease, and multiple other normal mucosal and nonmucosal specimens were obtained from the Cooperative Human Tissue Network, a service organization funded by the National Cancer Institute (Table 1). Additional intestinal tissue from patients with and without ulcerative colitis or Crohn's disease was obtained retrospectively from pathology archival tissue at the University of Liverpool. Select tissue from patients with ulcerative colitis or Crohn's disease were obtained by endoscopic biopsy or by intestinal resection, and the diagnosis was con-

Table 1. *Tissues Used for Immunohistochemical Detection of MAdCAM-1*

Number of specimens	Tissue	Cells Immunoreactive
Intestinal tissue		
10	Normal colon (various sites)	Vascular endothelium-lamina propria/submucosa
3	Normal small intestine	Vascular endothelium-lamina propria/submucosa
2	Uninvolved ulcerative colitis, colon	Vascular endothelium-lamina propria/submucosa
3	Uninvolved Crohn's disease, small intestine	Vascular endothelium-lamina propria/submucosa
1	Uninvolved Crohn's disease, colon	Vascular endothelium-lamina propria/submucosa
1	Crohn's disease, colon	Vascular endothelium-lamina propria/submucosa
3	Crohn's disease, small intestine	Vascular endothelium-lamina propria/submucosa
3	Ulcerative colitis, colon/cecum	Vascular endothelium-lamina propria/submucosa
1	Rectal radiation injury	Vascular endothelium-lamina propria/submucosa
1	Appendicitis	High endothelial venular endothelium; dendritic cells of follicular centers
Lymphoid tissue		
1	Tonsil	Rare light venular endothelium
3	Axillary (2) and mediastinal lymph nodes	Light dendritic labeling to some follicular centers
1	Inguinal lymph node (rhesus monkey)	Dendritic labeling to follicular centers
2	Mesenteric lymph node (one from rhesus monkey)	High endothelial venular endothelium; dendritic cells of follicular centers
Normal tissue		
2	Lung	Negative
3	Liver	Negative
3	Kidney	Negative
1	Thymus	Rare, light venular endothelium in medulla
3	Pancreas	Venular endothelium
4	Mammary gland	Negative (3); occasional venular endothelium (1)
2	Heart (left ventricle)	Negative
4	Brain (cerebellum, cerebrum, midbrain)	Rare leptomeningeal epithelium from cerebral sample
2	Skin (one from rhesus monkey)	Negative
1	Spleen	Sinusoidal lining cells bordering PALS and rare venular endothelium of red pulp
1	Salivary gland	Negative
1	Uterus	Negative
1	Ovary	Negative
1	Skeletal muscle	Negative
Inflamed tissue		
1	Status asthmaticus lung	Negative
1	Rheumatoid arthritis synovium	Negative
2	Skin—rhesus monkey—LPS injection (4 and 24 hr post injection)	Negative
1	EAE brain—cynomolgus monkey	Negative

firmed by routine histopathological examination. Tissues from rhesus monkeys were derived from frozen tissue banks (Table 1), the experimental details of which have been previously described in part.[23] Immunohistochemical analysis for MAdCAM-1 and factor VIII was performed on both frozen and formalin-fixed, paraffin-embedded human tissue samples, using techniques previously described.[24–26] Human mesenteric lymph node or colonic mucosa were used as positive control tissue and were processed identically as the test tissue in all instances. Detection of the peripheral lymph node addressin was performed using frozen tissue samples only. Briefly, for detection of MAdCAM-1, factor VIII, and the peripheral lymph node addressin on frozen specimens, tissue was sectioned at a thickness of 6 μm, dessicated, and then fixed in 2% paraformaldehyde/0.5X PBS or acetone (factor VIII) for 10 minutes at 4°C. In some instances, duplicate formalin-fixed, paraffin-embedded tissues were used in immunohistochemical analyses for MAdCAM-1 and factor VIII expression so that greater morphological detail could be achieved. In these instances, sections were deparaffinized in xylenes and brought to PBS before proceeding. After PBS washing, both frozen and paraffin-embedded sections were incubated in 0.6% hydrogen peroxide in methanol or 1.0% hydrogen peroxide in PBS for 20 minutes at room temperature to remove nonspecific peroxidase activity. In some instances, examination of MAdCAM-1 immunoreactivity was re-examined in select tissues without this quenching step to increase sensitivity of the assay. Nonspecific antibody binding sites were then blocked with 10% normal goat serum/5% human AB serum/PBS for 30 minutes at room temperature. For MAdCAM-1 detection in frozen sections, the murine

antibody 10G3 was applied to tissue sections as tissue culture supernatant diluted 1:100 with PBS/1% FCS/0.1% sodium azide overnight at 4°C in a humidified chamber. For MAdCAM-1 detection in paraffin sections, the murine antibody 10A6, which recognizes a formalin-resistant epitope, was applied to sections overnight as tissue culture supernatant diluted 1:10 with 0.3% Triton X-100/0.2% Tween 20/1% FCS/5% human AB serum, and 0.1% sodium azide. For the detection of the peripheral lymph node addressin, MAb MECA 79[27] was used as undiluted tissue culture supernatant. For factor VIII localization in human colon, mouse MAb F8/86 (Dakopatts Corp., Carpinteria, CA) was used at 13 μg/ml diluted in PBS/1% FCS/0.1% sodium azide and incubated overnight. Isotype-matched, irrelevant MAbs were used as negative control reagents on step sections from each tissue examined. Subsequently, biotinylated goat anti-mouse IgG (for 10G3, 10A6, and F8/86) or biotinylated goat anti-mouse IgM (for MECA 79; Vector Laboratories, Burlingame, CA) and avidin-biotin-peroxidase complexes (Vector Laboratories) were added in the sequence. Diaminobenzidine was used as the chromogen and Mayer's hematoxylin as a counterstain for all analyses.

To examine the relative reactivity for MAdCAM-1 compared with factor VIII, quantitative, computer-assisted, morphometric analysis of human small intestine, mesenteric lymph node, rectum, and colon sections was performed using a Leica Quantimet 500 image analyzer. The relative percent area of mucosa stained for each antibody was determined on at least four random fields per section. Immunoreactive endothelial cells were selected based on the color wavelength generated from the brown diaminobenzidine reaction product, and color selection criteria were identical on all sections analyzed for each MAb marker. Data are expressed as the ratio of mean percent area stained for MAdCAM-1/mean percent area stained for factor VIII ($\pm$ 1 SEM).

Results

Monoclonal Antibodies 10G3 and 10A6 Recognize Human MAdCAM-1

A putative human cross-reacting anti-murine MAdCAM-1 MAb (LM20) was shown by FACS to stain the surface of CHO cells transiently expressing human MAdCAM-1 (not shown).[18] These same CHO transfectants were also shown to express functional human MAdCAM-1 as assessed by their ability to mediate adhesion to the human B-cell lymphoma, RPMI 8866, that was completely inhibited by the anti-$\alpha 4\beta 7$ MAb ACT-1 (not shown).[18] Subsequently, the cDNA encoding human MAdCAM-1 was stably introduced into CHO cells and the murine pre-B-cell line L1–2. Both CHO and L1–2 cells expressing high levels of human MAdCAM-1 were identified by positive staining with the LM20 MAb (Figure 1a on CHO transfectant cells) and by their ability to adhere to cell lines expressing $\alpha 4\beta 7$.

L1–2 cells expressing human MAdCAM-1 were used to immunize C57BL/6 mice. A total of 1000 wells from a single fusion were screened for selective reactivity against CHO HuMAd-4 transfectants (*versus* untransfected CHO cells), and 45 hybridomas were positive (Figure 1b for antibody 10G3). Moreover, immunoprecipitation using the MAb 10G3 and SDS-PAGE analysis from lysates of L1–2 human MAdCAM-1 transfectant cells, but not from untransfected L1–2 cells, produced a discrete band at 78 kd, corresponding to a glycosylated species of human MAdCAM-1 (Figure 1c). After expansion of the positive wells, supernatants from all 45 MAbs were screened for their ability to block adhesion of RPMI 8866 lymphoma cells to CHO-HuMAd transfectants, and 11 were judged to recognize functional epitopes as they completely inhibited binding of these cells. Preliminary imunohistological characterization of these 11 MAbs demonstrated specific staining of HEVs within human mesenteric lymph nodes whereas peripheral lymph nodes were negative. Two antibodies in particular, designated 10G3 (Figure 1) and 10A6, were chosen for additional immunohistochemical analyses.

Because murine MAdCAM-1 is selectively expressed in HEVs in mesenteric lymph nodes compared with lymphoid tissue in the periphery,[6] we next examined the reactivity of MAb 10G3 on these two classes of lymph nodes (Table 1). Immunohistochemical analysis of human mesenteric lymph node from a patient with Crohn's disease or a normal mesenteric lymph node from a rhesus monkey using 10G3 demonstrated intense immunoreactivity to endothelium of HEVs within interfollicular paracortical regions (Figure 2a). As expected, labeling of the peripheral node addressin recognized by MECA 79 in mesenteric lymph nodes was confined to HEVs of interfollicular paracortical regions. In contrast, MAdCAM-1 was not expressed on paracortical HEVs in axillary (human) or inguinal (rhesus monkey) lymph nodes (Table 1; Figure 2b), although these same HEVs were intensely immunoreactive for the peripheral node addressin defined by MECA 79 (not shown). Interestingly, the

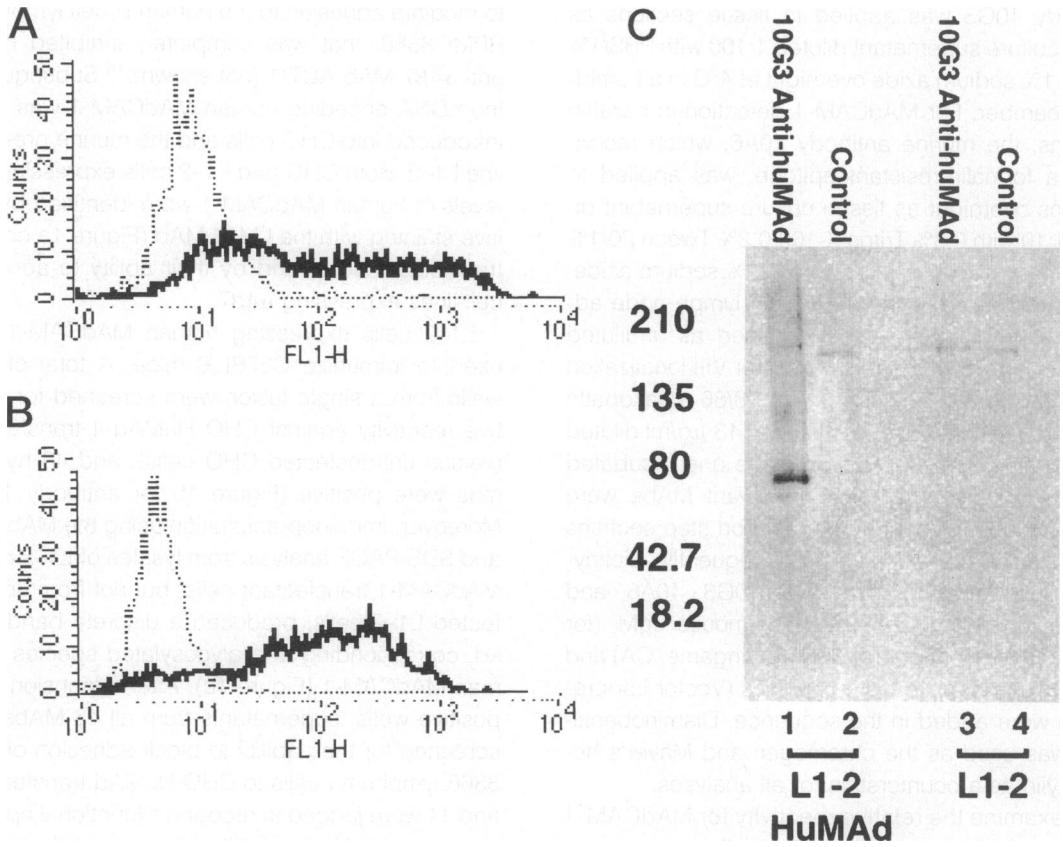

Figure 1. A: *Using flow cytometry, a MAb raised against murine MAdCAM-1, LM20, specifically recognizes CHO cells stably transfected with human MAdCAM-1 cDNA. The dark tracing in the histogram represents immunoreactivity against the CHO HuMAd-4 cell line, and the light tracing represents the background staining against parental CHO cells. An isotype-matched irrelevant antibody failed to stain this MAdCAM-1 transfected cell line (not shown).* **B:** *A similar profile using the anti-human MAdCAM-1 MAb 10G3 is obtained on the same cells as described in* **A**. **C:** *Immunoprecipitation and SDS-PAGE analysis using the 10G3 MAb* (**lanes 1** *and* **3**) *or an irrelevant isotype-matched MAb* (**lanes 2** *and* **4**) *on biotinylated lysates of L1–2 human MAdCAM-1 transfectants* (**lanes 1** *and* **2**) *or untransfected L1–2 cells* (**lanes 3** *and* **4**). *MAb 10G3 immunoprecipitates a protein of 78 kd, consistent with a glycosylated species of human MAdCAM-1.*

endothelium lining capsular small venules was occasionally immunoreactive for MAdCAM-1. In addition, within the follicular cortex of both mesenteric and peripheral lymph nodes, subtle delicate reticular staining to dendritic processes within some, but not all, germinal centers and mantle zones was observed (Figure 2c). In macaque tissue, this staining pattern was most pronounced in mantle zones and was consistent with our previous description of labeling patterns for follicular dendritic cells in nonhuman primates.[28] This immunoreactivity was not related to recognition of VCAM-1 as the antibodies used in the study did not bind to human VCAM-1 CHO transfectants (not shown). Interestingly, hyperplastic human tonsil (Table 1) was intermediate between mesenteric and peripheral lymph node in both the numbers and intensity of HEVs expressing MAdCAM-1, as only rare immunoreactive vessels within interfollicular T-cell regions were observed (Figure 2d). Collectively, therefore, these results demonstrated that MAb 10G3 recognized human MAdCAM-1, and when used in immunohistochemical analyses on lymphoid tissue, it identified a pattern of expression similar to that described previously for murine MAdCAM-1 (M. C. Szabo, E. C. Butcher, and L. M. McEvoy, manuscript submitted).[6]

MAdCAM-1 Is Expressed in Normal Colon and Is Increased in Intestinal Tract from Patients with Inflammatory Bowel Disease

Because human MAdCAM-1 was most consistently and preferentially localized to HEVs of mesenteric lymph node, we next examined the expression of MAdCAM-1 in normal human intestinal tract using the 10G3 MAb in frozen sections and the 10A6 antibody in paraffin-embedded tissue sections (Table 1). Both antibodies produced a similar

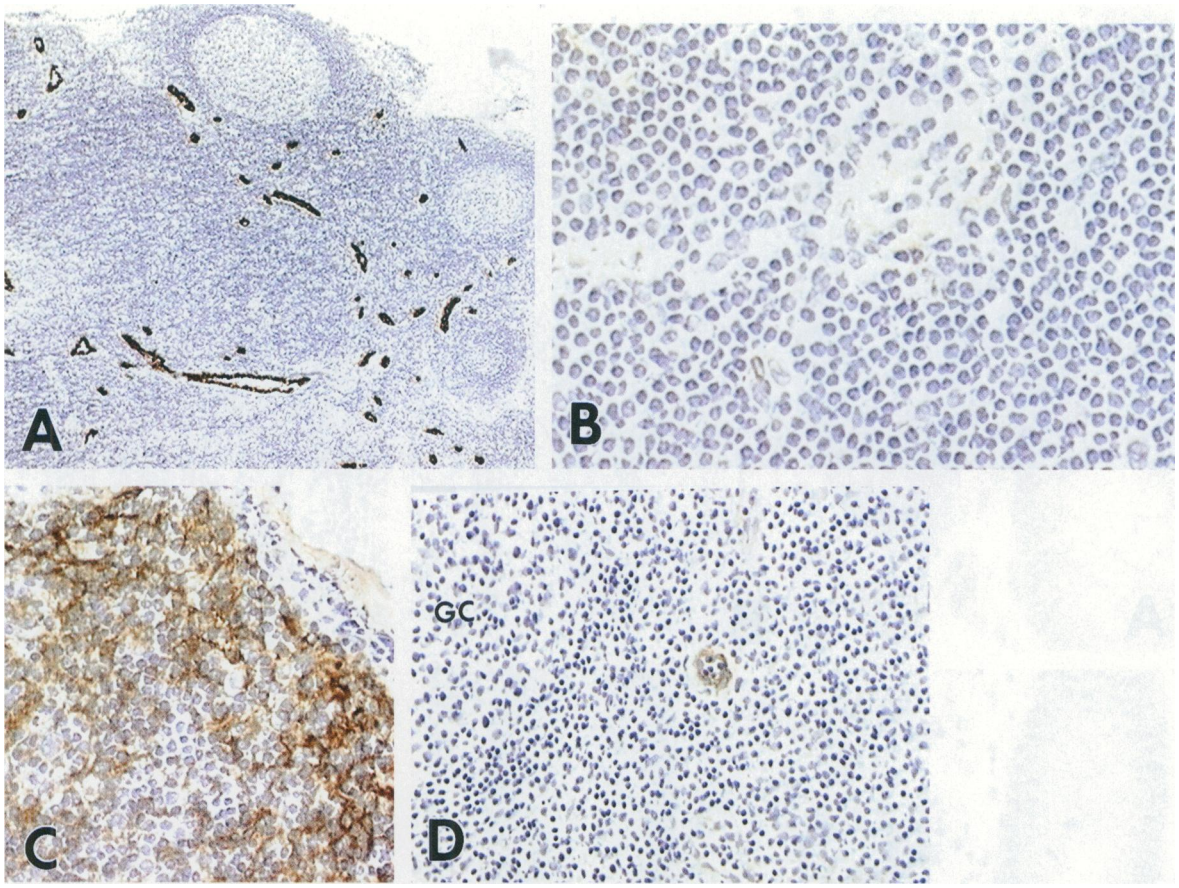

Figure 2. *Human MAdCAM-1 immunoreactivity in frozen lymphoid tissue.* **A:** *In mesenteric lymph node, MAdCAM-1 is consistently localized to endothelium associated with high endothelial venules (HEVs) within the paracortex. In contrast, paracortical HEVs in axillary or inguinal* (**B**) *lymph node are devoid of MAdCAM-1 immunoreactivity. In some, but not all, follicular centers in most lymphoid tissue samples, MAdCAM-1 is localized in a dendritic pattern consistent with reactivity to follicular dendritic cells* (**C**, *inguinal lymph node*). *In tonsil* (**D**), *MAdCAM-1 is localized to rare venular endothelium in interfollicular T cell regions adjacent to germinal centers (gc). Avidin-biotin-peroxidase immunohistological technique with Mayer's hematoxylin counterstain; magnification, ×100* (**A**), *×1000* (**B**), *×500* (**C**), *and ×250* (**D**).

pattern of cellular immunoreactivity with the exception that the staining of 10G3 in frozen sections appeared more intense. In normal colon and small intestine, MAdCAM-1 was constitutively expressed to venular endothelium of lamina propria and submucosa (Figure 3a). When found in sections, endothelium lining HEVs of Peyer's patch tissue was intensely immunoreactive, whereas staining to dendritic processes within germinal centers of Peyer's patch tissue was occasionally observed, particularly using the 10G3 MAb on frozen sections. Serosal venular endo-thelium was consistently devoid of staining forMAdCAM-1 in these normal tissues.

We next examined tissue from patients with various inflammatory disorders of the intestinal tract to assess whether changes in MAdCAM-1 expression were spatially associated with inflammatory activity (Table 1). In small intestine and colon from patients with Crohn's disease, the pattern of MAdCAM-1 expression paralleled that found in normal intestinal tract. Venular endothelium within lamina propria and submucosa was intensely immunoreactive (Figure 3b), and venules adjacent to mononuclear cell aggregates were especially immunoreactive (Figure 3c). Despite transmural inflammatory activity and peritonitis in some sections, venular endothelium of the tunica muscularis and serosa were consistently nonreactive. Colonic sections from patients with ulcerative colitis were indistinguishable in terms of MAdCAM-1 immunoreactivity (Figure 3d). Better morphological preservation inherent in formalin-fixed, paraffin-embedded tissue allowed more precise localization of MAdCAM-1 to endothelium in a pattern consistent with expression to both the luminal and abluminal aspects (Figure 3d). Interestingly, in some venules within lamina propria, MAdCAM-1 expression was multifocal in that endothelial immunoreactivity did not always encircle the venular lumen (Figure 3d). Lastly, one sample of rectal radiation

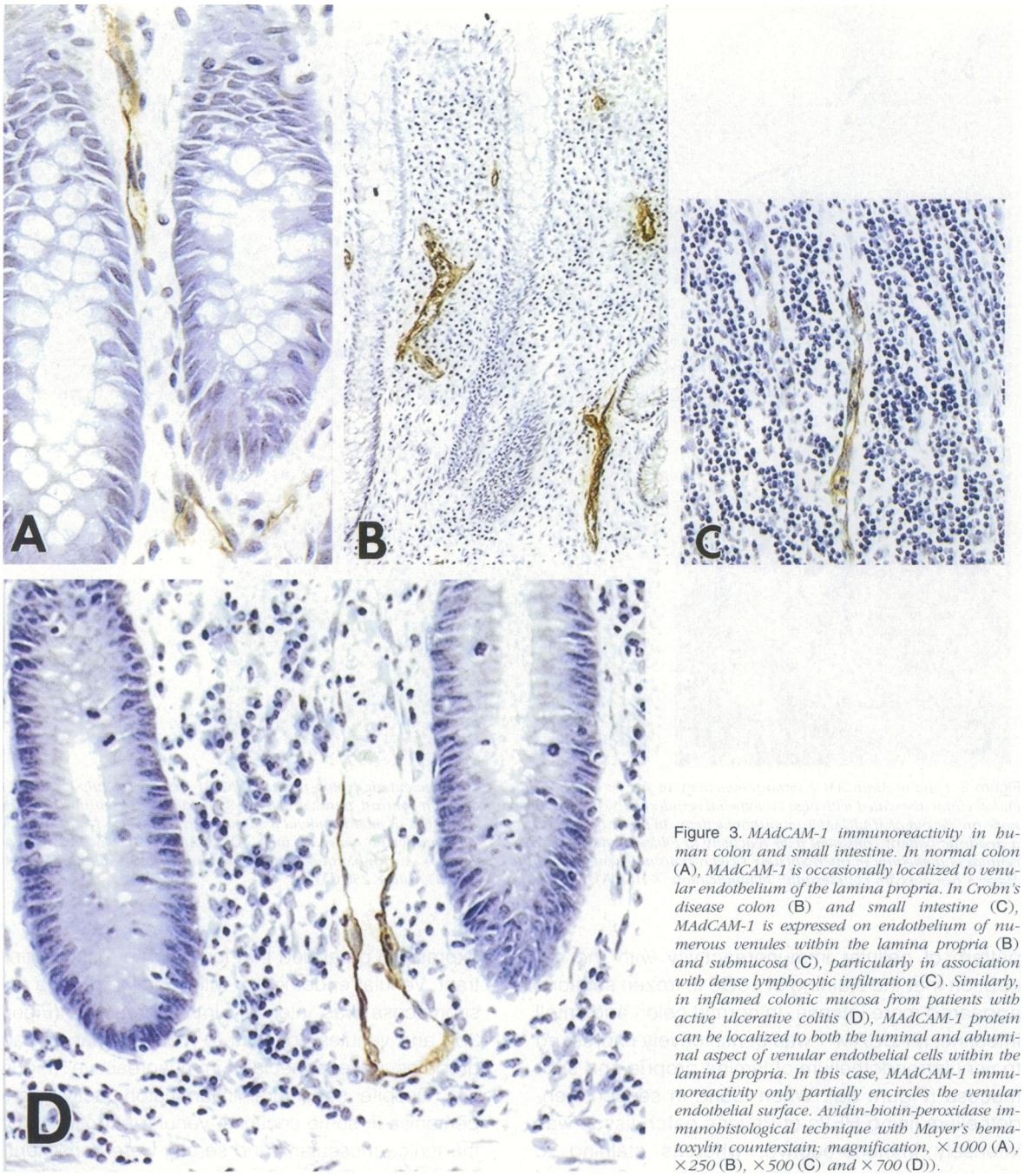

Figure 3. *MAdCAM-1 immunoreactivity in human colon and small intestine. In normal colon* (A), *MAdCAM-1 is occasionally localized to venular endothelium of the lamina propria. In Crohn's disease colon* (B) *and small intestine* (C), *MAdCAM-1 is expressed on endothelium of numerous venules within the lamina propria* (B) *and submucosa* (C), *particularly in association with dense lymphocytic infiltration* (C). *Similarly, in inflamed colonic mucosa from patients with active ulcerative colitis* (D), *MAdCAM-1 protein can be localized to both the luminal and abluminal aspect of venular endothelial cells within the lamina propria. In this case, MAdCAM-1 immunoreactivity only partially encircles the venular endothelial surface. Avidin-biotin-peroxidase immunohistological technique with Mayer's hematoxylin counterstain; magnification,* ×*1000* (A), ×*250* (B), ×*500* (C) *and* ×*700* (D)).

injury and one of appendicitis showed a pattern of MAdCAM-1 expression consistent with that from the other intestinal tissues, except that occasional serosal venules in the appendix were lightly labeled (not shown).

Despite significant constitutive expression of MAdCAM-1 in normal small intestine and colon, the relative area of endothelium expressing MAdCAM-1 appeared to increase in inflammatory settings. Increased expression of MAdCAM-1 was most obvious in sections containing multifocal inflammatory disease, as relatively normal regions contained only occasional venules that expressed MAdCAM-1 (Figure 4a; Crohn's disease), whereas areas typified by mononuclear and purulent inflammatory infiltrates contained numerous venules lined by intensely immunoreactive MAdCAM-1^+ endothelial cells (Figure 4b; Crohn's disease).

To quantitatively assess changes to MAdCAM-1 expression in the context of various inflammatory

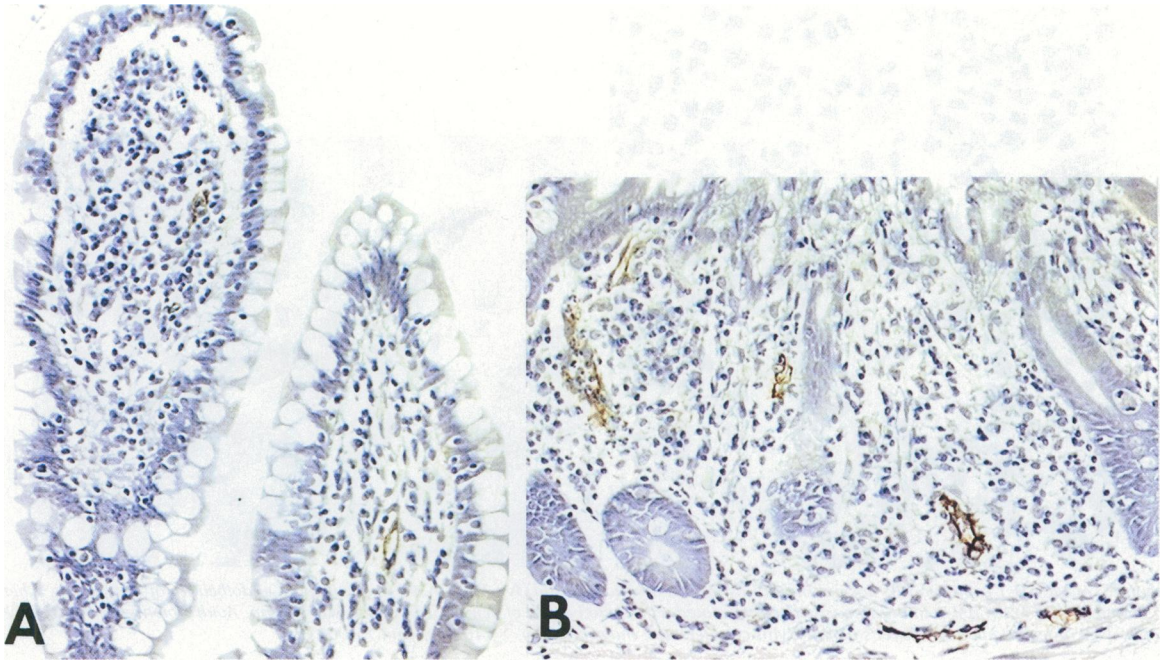

Figure 4. *MAdCAM-1 expression in uninvolved and involved small intestinal Crohn's disease.* **A**: *In uninvolved ileum, small intestinal villi are characterized by occasional, light immunoreactivity for MAdCAM-1 on venular endothelium of the lamina propria. In contrast, in the same section but at an inflammatory focus* (**B**), *mucosal villi are atrophic, there is loss of small intestinal glands, and there is a mixed inflammatory infiltrate. Numerous venules within the lamina propria are lined by endothelial cells that are brightly immunoreactive for MAdCAM-1. Avidin-biotin-peroxidase immunohistological technique with Mayer's hematoxylin counterstain; magnification, ×500.*

settings of the intestinal tract, computer-assisted morphometric analysis was used to examine the relative mucosal labeling for MAdCAM-1 as a function of endothelial labeling for factor VIII in both normal and inflamed intestinal tissue. Using MAb 10A6 on paraffin-embedded tissues, in normal human colon and small intestine, approximately 10% of the area within lamina propria stained for factor VIII was also stained for MAdCAM-1 (Table 2). In contrast, in noninvolved ileum from a patient with Crohn's disease, MAdCAM-1 immunoreactivity increased to 19.2 ± 5.6%, whereas involved small intestine from patients with Crohn's disease ranged from 17.0 ± 11.0 to 81.3 ± 36.7% (Table 2). Similarly, in involved colonic or cecal mucosa from patients with ulcerative colitis, MAdCAM-1 immunoreactivity ranged from 21.8 ± 11.8% to 33.0 ± 4.2%, whereas in a patient with colonic stricture and polymorphonuclear and mononuclear infiltration in colonic lamina propria, MAdCAM-1 immunoreactivity was 45.1 ± 8.2% (Table 2). Similar increases in MAdCAM-1 expression in inflamed mucosa were observed on frozen sections using the 10G3 MAb (data not shown). The only difference observed between the analyses using the two antibodies was that the relative comparative values adjusted to factor VIII obtained for the 10G3 MAb were generally higher, likely as a consequence of greater sensitivity for MAdCAM-1 in frozen tissue sections.

Table 2. *Ratio Percent of MAdCAM-1/Factor VIII Immunoreactivity**

Tissue	% (MAdCAM-1/ factor VIII)* ± 1 SEM
Normal colon	
case 96-437	13.5 ± 4.3
Normal small intestine	
case 96-439	9.6 ± 5.3
Noninvolved Crohn's disease, small intestine	
case 96-442	19.2 ± 5.6
Involved Crohn's disease, small intestine	
case 95-442A	17.0 ± 11.0
case 96-441	55.2 ± 21.7
case 96-440	81.3 ± 36.7
Ulcerative colitis, Colon/Cecum	
case 96-445; colon	21.8 ± 11.8
case 96-446; cecum	33.0 ± 4.2
Colonic stricture and mucosal inflammation	
case 96-438; colon	45.1 ± 8.2

Results are expressed as fractional positive area for MAdCAM/fractional positive area for factor VIII using the 10A6 MAb on formalin-fixed, paraffin embedded tissue.

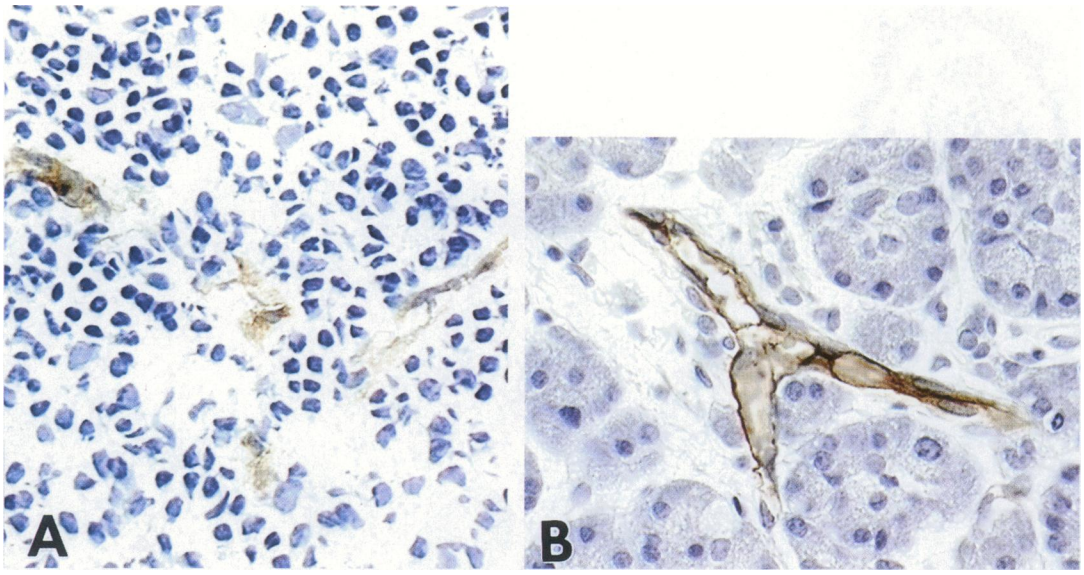

Figure 5. *MAdCAM-1 expression in normal human tissues. In thymic medulla* (**A**), *MAdCAM-1 is localized to endothelium of rare vessels, whereas in pancreas* (**B**), *MAdCAM-1 immunoreactivity is associated with endothelium of venules within the interstitium. Avidin-biotin-peroxidase immunohistological technique with Mayer's hematoxylin counterstain; magnification,* ×*1100* (**A**) *and* ×*1000* (**B**).

MAdCAM-1 Is Selectively Expressed in Intestinal Tract and Associated Lymphoid Tissues

To assess whether human MAdCAM-1, as in the mouse, is selectively expressed in intestinal tract and associated lymphoid tissue, we next examined various human tissues for MAdCAM-1 immunoreactivity. In normal human spleen, sinusoidal lining cells bordering periarterial lymphatic sheaths and rare venular endothelium of red pulp were observed to be immunoreactive. In addition, in one case of brain tissue from the cerebrum, leptomeningeal epithelium was multifocally immunoreactive. Excluding lymphoid and intestinal tissue, of the normal tissues examined, light staining of venular endothelium was only observed in thymic medulla (Figure 5a), pancreas (Figure 5b), and one of four samples of nonlactating mammary gland (Table 1). Venular endothelium of lung, liver, kidney, heart, salivary gland, uterus, ovary, skeletal muscle, skin, and brain parenchyma were consistently negative (Table 1).

MAdCAM-1 expression was also examined in a collection of inflamed tissues from sites other than the intestinal tract (Table 1). MAdCAM-1 immunoreactivity could not be demonstrated in human asthmatic lung or rheumatoid arthritis synovium. Moreover, MAdCAM-1 expression could not be induced in rhesus monkey skin by the intradermal injection of endotoxin (lipopolysaccharide), despite the fact that E-selectin and VCAM-1 were highly up-regulated in the same tissue samples.[23,29] Moreover, MAdCAM-1 was not detected in brain parenchyma from a macaque with fulminant EAE (Table 1).

Discussion

The accumulation of leukocytes within lymphoid tissues and extralymphoid sites of inflammation is a complex process, involving multiple adhesion receptors and activating events. A great deal of specificity lies, in part, on adhesion receptors expressed on specialized vascular endothelium, most notably (but not exclusively) in HEVs. The mucosal vascular addressin MAdCAM-1 is expressed on HEVs in gut-associated lymphoid tissues such as Peyer's patches and mesenteric lymph nodes[6] and is a principal ligand for circulating lymphocytes to these specialized lymphoid sites.[30–32] Expression of murine MAdCAM-1 is increased on endothelium of HEV-like vessels within islets in the pancreas of the NOD mouse, in the central nervous system in chronic relapsing EAE, and in response to inflammatory mediators in a murine endothelial cell line.[8–11] Most recently, murine models of inflammatory bowel disease have shown dramatic increases of MAdCAM-1 in the mucosal lamina propria, where basal expression in normal tissue is usually low.[12,13] The principal ligand for MAdCAM-1 is the lymphocyte integrin $\alpha 4\beta 7$, which defines a discrete subpopulation of memory T cells and probably B cells involved in mucosal immunity.[14–16,31,32,33] Antibodies to MAdCAM-1 or $\alpha 4\beta 7$ effectively inhibit migration of gut-derived

thoracic duct blasts and memory/effector T cells to the intestine.[30] In addition, *in situ* videomicroscopy has shown that antibodies to murine MAdCAM-1 inhibit both rolling and firm adhesion of lymphocytes within Peyer's patches.[31] These functional data, along with the demonstration of restricted expression of MAdCAM-1 in murine tissues, has firmly established the significance of this receptor in homing to mucosal tissues in the mouse.

The recent molecular cloning of human MAdCAM-1 has provided initial evidence that, at the RNA level, expression of this vascular addressin appears limited to specific anatomic sites, similar to that demonstrated in the mouse. However, the cellular distribution, degree of expression in various normal and inflamed tissues, and correlation with protein localization has not, to our knowledge, been performed. In the present study, we used anti-human MAdCAM-1 MAbs to show that, as observed in the mouse, human MAdCAM-1 is primarily expressed in endothelium in gut, related tissues (eg, pancreas), and lymphoid tissues. The expression of MAdCAM-1 appears to be regulated at sites associated with inflammatory bowel disease as greater intensity and numbers of MAdCAM$^+$ endothelial cells colocalize with inflammatory foci, yet MAdCAM-1 is not detected at various inflammatory extra-intestinal sites. Moreover, MAdCAM-1 protein appears to be localized to follicular dendritic cells in lymphoid tissue, regardless of anatomic association with the gastrointestinal tract. Additional work will be required to differentiate protein localization from cellular expression in many of the cells in these tissues.

Flow cytometry studies of peripheral blood lymphocytes has shown that, for both CD4$^+$ and CD8$^+$ T cells and B cell subsets, $\alpha4\beta7$ represents a unique mucosal subpopulation.[14,16,33] Using multicolor microflourimetry to compare lymphocyte subpopulations that bind to either MAdCAM-1 or VCAM-1, it was found that MAdCAM-1 selectively binds CD4 memory cells that are $\alpha4\beta7^{high}$ whereas VCAM-1, conversely, binds lymphocytes using $\alpha4\beta1$.[14] *In vitro* adhesion assays have shown, in accordance with these observations, that the anti-$\alpha4\beta7$ MAb, ACT-1, blocks binding to MAdCAM-1, whereas binding to the other vascular Ig-like adhesion receptor for $\alpha4\beta7$, VCAM-1, was unaffected.[16,18] The differences in lymphocyte subset binding to either MAdCAM-1 or VCAM-1 can be considered a major point of delineation between mucosal and nonmucosal trafficking compartments, allowing for separation of specialized immune responses. VCAM-1 expression is known to be associated with many inflammatory events such as delayed-type hypersensitivity, atherosclerosis, and rheumatoid arthritis. Expression of VCAM-1 within venules at mucosal sites, however, is very low, even in inflammatory bowel disease. In this study, we show that expression of human MAdCAM-1 was not detected at sites of inflammation where VCAM-1 expression is known to be induced, such as rheumatoid arthritis synovium and intradermal injection of endotoxin (lipopolysaccharide) in the rhesus monkey.

The selective expression of human MAdCAM-1 in specific anatomic vascular sites in human tissue adds supporting evidence to previous observations that, as in the mouse and sheep, lymphocyte populations use specific adhesion homing pathways both normally and in inflammatory disease. Intense MAdCAM-1 immunoreactivity was detected on HEVs within appendix and mesenteric lymph nodes in both human and nonhuman primate tissues, whereas expression in peripheral lymph nodes was negligible. The peripheral node addressin, as defined by the MECA-79 epitope and associated with L-selectin-mediated interactions, was abundantly expressed within these peripheral lymphoid tissues, demonstrating that tissue antigenicity was preserved in these lymphoid tissue samples. Interestingly, expression of human MAdCAM-1 at other mucosal sites, such as normal or inflamed lung, was not detected. Moreover, despite the observation that murine MAdCAM-1 could be induced on endothelial cells *in vitro* in response to pro-inflammatory cytokines, MAdCAM-1 could not be induced in rhesus monkey skin in response to lipopolysaccharide-induced injury and inflammation. In contrast, moderate expression of MAdCAM-1 was observed in hyperplastic human tonsil and thymic medulla. Therefore, taken collectively with *in vivo* homing data in the mouse, this pattern of MAdCAM-1 expression suggests that, in man, MAdCAM-1/$\alpha4\beta7$ interactions likely mediate lymphocyte trafficking to normal gastrointestinal mucosa and gut-associated tissues but not to peripheral lymph node. Moreover, because MAdCAM-1 expression is increased at sites of inflammatory bowel disease, this adhesion mechanism may also be intimately involved in lymphocyte recruitment and pathogenesis of ulcerative colitis, Crohn's disease, and other inflammatory diseases of the bowel mucosa. In this regard, we have recently demonstrated that administration of the anti-$\alpha4\beta7$ MAb ACT-1 to chronically colitic cotton-top tamarin monkeys inhibits recruitment of various leukocyte subclasses to inflamed colonic mucosa.[34] Furthermore, we have demonstrated a reduction of colonic inflammatory activity in *scid* mice reconstituted with CD45RB^{hi} CD4$^+$ T cells by the administration of antibodies to $\beta7$ and/or MAdCAM-1.[12] Therefore,

the MAdCAM-1/$\alpha 4\beta 7$ adhesion mechanism may represent a highly region-specific therapeutic target for the modulation of inflammatory bowel disease activity.

It is interesting that MAdCAM-1 was not expressed in endothelial cells associated with HEV-like vessels in tunica muscularis and serosa in tissue from patients with severe transmural Crohn's disease, fistulation, and localized peritonitis. Although dense lymphocyte infiltrates were observed in these areas, the mechanism by which these cells are recruited to these microcompartments is not known. Although MAdCAM-1 was not detected in these extramucosal sites, the relative contribution of mucosal inflammation, where MAdCAM-1 expression was associated with inflammatory disease, to transmural involvement needs to be defined. Furthermore, $\alpha 4\beta 7$-mediated adhesion events utilizing VCAM-1 and fibronectin as counterligands may be involved, along with numerous other adhesion pathways, in the recruitment and localization of lymphocytes to these sites. Additional work will be required to compare the immunophenotypic expression of $\alpha 4\beta 7$ in mucosal/submucosal *versus* nonmucosal lymphocytes, the degree of expression of other endothelial adhesion proteins, and *in vivo* blocking experiments in animals with transmural inflammatory involvement similar to Crohn's disease to address this issue.

In addition to vascular endothelium, MAdCAM-1 protein was putatively localized to follicular dendritic cells in peripheral and mesenteric lymph node and Peyer's patch lymphoid tissue. Similar results have been previously reported for other adhesion proteins of the immunoglobulin supergene family, such as ICAM-1 and VCAM-1,[35] and murine MAdCAM-1 has been reported to be expressed on splenic sinus-lining cells at the border of the white and red pulp and occasionally within germinal centers of the white pulp.[36] Although the function of MAdCAM-1 on these antigen-presenting cells cannot be determined from the results of the present study, it is reasonable to predict that, in addition to its role in the direction and regulation of cellular migration to tissues from the vascular compartment, MAdCAM-1 localization on lymphoid stromal cells may influence adhesion and retention of lymphocytes during the process of antigen presentation (M. C. Szabo, E. C. Butcher, L. M. McEvoy, manuscript submitted).

The functional significance of MAdCAM-1 localization to leptomeningeal epithelium in one of four samples of brain is not known. Previously, murine MAdCAM-1 was localized on choroid plexus epithelium[37] in mice with EAE and on brain parenchymal endothelium during the relapse phase of chronic murine EAE.[11] Although we did not appreciate an up-regulation of MAdCAM-1 expression in acute EAE in macaque monkeys, additional work will be required to examine MAdCAM-1 expression in various encephalitides of multiple etiologies to determine whether its expression on endothelium and epithelium is regulated in normal and inflammatory settings.

In summary, we have demonstrated, for the first time, that human MAdCAM-1 is expressed in a tissue-selective manner. Most MAdCAM-1 is localized to the intestinal mucosa and gut-associated lymphoid tissue, and expression appears to be increased in mucosal-associated inflammatory events. Moderate expression was demonstrated in thymic medulla, pancreas, spleen, and tonsil, whereas most other normal and inflamed tissues were without detectable expression. These results suggest that the adhesion pathway defined by MAdCAM-1/$\alpha 4\beta 7$ may represent, as demonstrated in the mouse, a tissue-selective homing mechanism for the gastrointestinal tract and associated tissue. As such, the pathway may be a relevant tissue-specific therapeutic target for the modulation of inflammatory bowel disease activity.

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