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Expression of VCAM-1 and E-Selectin in an *in Vivo* Model of Endothelial Activation

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Vascular cell adhesion molecule 1 (VCAM-1) and E-selectin (or endothelial-leukocyte adhesion molecule 1) are inducible endothelial cell adhesion molecules that play a role in the recruitment of leukocytes into sites of inflammation. Information about the spatial and temporal pattern of induced expression of these leukocyte adhesion molecules in vivo is limited. This study reports the expression profile of VCAM-1 and E-selectin in various mouse tissues after lipopolysaccharide administration. Using rat complementary DNA probes for VCAM-1 and E-selectin, Northern blot analysis showed a marked increase in transcript levels for both adhesion molecules in lung, heart, and kidney. Maximal transcript levels for both VCAM-1 and E-selectin were observed at 3–6 hours and declined to low, constitutive levels of expression at 48 hours. Consistent with the Northern blot results, immunoperoxidase analysis revealed focal endothelial cell expression of VCAM-1 in control animals. Following lipopolysaccharide administration, VCAM-1 expression increased dramatically in all vascular beds examined, although the response was heterogeneous. Widespread induced expression of VCAM-1 on cells other than vascular endothelium was not seen. Neither basal nor induced expression correlated with leukocyte adhesion. Signals other than the expression of endothelial leukocyte adhesion molecules are required in vivo for leukocyte infiltration in this murine model of systemic endothelial activation. (Am J Pathol 1993, 143:725–737)

The inflammatory mediators interleukin-1β (IL-1β) and tumor necrosis factor α (TNF-α) induce new endothelial cell surface molecules during the initial phases of an inflammatory response. Several cytokine-inducible endothelial leukocyte adhesion molecules have been cloned and characterized: E-selectin (or endothelial-leukocyte adhesion molecule 1, ELAM-1); vascular cell adhesion molecule 1 (VCAM-1), and intercellular adhesion molecules 1 (ICAM-1). E-selectin is a member of the family of structurally related molecules, designated selectins (reviewed in Ref. 1), that participate in endothelial-leukocyte adhesion. It is a receptor for sialylated Lewis X and related oligosaccharides on leukocyte glycoproteins (reviewed in Ref. 2). E-selectin mediates the adhesion of polymorphonuclear cells,[3] monocytes,[4,5] and some memory T cells[6,7] to cytokine-activated endothelial cells. It is expressed by venular and capillary endothelial cells at sites of active inflammation.[8] VCAM-1, a member of the immunoglobulin gene superfamily, is a cell surface adhesion molecule that mediates the adhesion of leukocytes to human umbilical vein endothelial cells.[9] VCAM-1 interacts with cells expressing the integrin α4β1 (VLA4), such as monocytes, lymphocytes, basophils, and eosinophils, but not neutrophils.[10] The third leukocyte adhesion molecule, ICAM-1 (CD54), is a member of the immunoglobulin gene superfamily and is a receptor for LFA-1 (αLβ2) and Mac-1 (αMβ2) integrins, found on all leukocyte types (reviewed in Ref. 11).

The three inducible endothelial adhesion molecules have distinct time courses of cytokine induction. In cultured human endothelial cells, ELAM-1 first appears on the cell surface within 1–2 hours of cytokine treatment.[3] Maximal expression occurs at 4–6 hours and then rapidly declines in the continued presence of cytokine, reaching basal levels by 24 hours.

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VCAM-1 first appears at 4–6 hours, reaches maximal levels at 12–18 hours, and then gradually declines over several days.[9] In contrast, ICAM-1 reaches peak levels by 18–24 hours and persists at peak levels as long as cytokine is present.[12] The time course of cytokine-induced expression suggests that the three endothelial adhesion molecules have distinct functions (reviewed in Ref. 13). The early rise in ELAM-1 expression may mediate transient neutrophil adhesion (rolling),[14,15] whereas the delayed rise in VCAM-1 may correspond to the switch to preferential adhesion and infiltration of mononuclear leukocytes. ICAM-1 may support subsequent leukocyte spreading and transendothelial migration.

An important question generated by the *in vitro* observations is whether endothelium *in vivo* will display similar cytokine-regulated responses. In one approach to this issue, the response of baboon dermal microvessels to injected cytokines or lipopolysaccharide (LPS) was examined. Local intradermal injection of TNF caused sustained expression of ELAM-1 and VCAM-1 and a late (24–48 hours) T-cell infiltrate. Injection of LPS induced a neutrophil-rich infiltrate and the transient expression of both ELAM-1 and VCAM-1, without significant T-cell infiltration.[16,17] These results support the proposal that sustained cytokine-induced VCAM-1 expression contributes to T-cell extravasation at sites of chronic inflammation. This correlation was previously noted in the development of T-cell-rich infiltrates in cardiac and renal allograft rejection.[18] A second approach to the issue of cytokine-regulated endothelial responses *in vivo* employed a baboon model of septic shock.[19] Intravenous injection of LPS caused widespread expression of E-selectin throughout the vasculature. Expression of this leukocyte adhesion molecule was not associated with a neutrophilic infiltrate in the peripheral tissues. The current studies expand the latter approach and explore the pattern of induced VCAM-1 and E-selectin expression in an *in vivo* murine model of endothelial activation. In this report we describe the effects of endotoxin in terms of both VCAM-1 and E-selectin gene expression and endothelial phenotypic changes and relate these changes to leukocyte accumulation in the mouse.

Materials and Methods

Cloning of Rat E-Selectin cDNA using the Polymerase Chain Reaction

Male Sprague-Daley rats (250 g; Charles River, Boston) were injected (per kg body weight) with 250 μg *Escherichia coli* LPS (serotype 0.55:B5; Sigma Chemical Co., St. Louis, MO). Rat lungs were harvested at 4 hours, and total RNA was prepared by a guanidine isothiocyanate protocol.[20] To obtain a complete open reading frame, an RNA polymerase chain reaction (PCR) was employed using nested oligonucleotide primers. Primers were chosen from regions of the E-selectin molecule that contained the highest degree of conservation based on nucleotide homologies between the human, rabbit, and mouse sequences. Reverse transcriptase was used to synthesize first-strand complementary DNA (cDNA) during a 1-hour incubation (42 C; final reaction volume, 40 μl), using 1 μg of total RNA, 50 ng of an oligonucleotide primer complementary to exon 13 (cytoplasmic domain and 3′UT), ACAA(A/G)GACTAAGAAAAACTT(G/A)AATT, and 100 units of avian myeloblast virus reverse transcriptase (Molecular Genetic Resources, Tampa, FL). Two rounds of PCR were performed using nested primers, and 10 μl of first-strand cDNA or first-round PCR product as templates.[21] Three hundred ng of primers were used for the first round of PCR and 150 ng for the second round. A primer to the 5′ lectin domain of the mRNA, GCCAAGAACTGGGCTCCAGGTGAAC, and the same 3′ primer used in cDNA synthesis were used for first-round PCR. The nested set of primers consisted of GATGAGGACTGCGTGGAGATCTA (lectin domain) and a sequence complementary to AAAGCAAAGAAATTTGTTCCTGC (transmembrane and cytoplasmic I domain). Conditions of the PCR were the same as outlined previously.[21,22] The prominent 1446-bp reaction product from a parallel set of six PCR reaction tubes was pooled, purified, and subcloned.[20] From three independent subclones DNA sequence analysis was performed on both strands by standard dideoxynucleotide sequencing in order to detect possible PCR amplification artifacts. The same strategies were used to obtain the 5′ and 3′ ends of the open reading frame. The following oligonucleotides were used for the PCR at the 3′ end: as primer for the reverse transcriptase reaction the complement of ACAA(G/A)GACTAAGAAAACTT(A/G)AATT located in the 3′ untranslated region; for the first round 5′, CTGTGAGCACAGTTTCACA (exon 7), and for the second round 5′, GGGTACAGTGTTCAAGCC (exon 10), while for the 3′ end the same primer was used as for the reverse transcriptase reaction. At the 5′ end the following oligonucleotides were obtained: as primer for the reverse transcriptase reaction the complement of GTCCGAAGGTACCAGTCCCA(T/C)AACC located in exon 5; for the first round 3′, the same primer as for the

reverse transcriptase reaction; for the second round 3′, the complement of GGTTGACGTCCTTACA-CAACCATGTCC, while both rounds of PCR were carried out with the same 5′ primer, CTCG(A/G)-TGACT(A/T)CACA(G/T)CAAAAC, located in 5′ untranslated region. Products of the expected size from six reactions each were pooled, purified, and subcloned as outlined before. DNA sequences of the PCR products were determined by direct sequencing using the CircumVent thermal cycle dideoxy DNA sequencing technique (New England Biolabs, Inc., Beverly, MA) and α-^{35}S-dATP (New England Nuclear, Boston, MA) according to the manufacturer's instructions. After the authenticity of the amplification productwas verified, the PCR generated products were subcloned.

Nucleotide sequences of all subclones were determined by the dideoxynucleotide chain termination procedure with modified T7 DNA polymerase (United States Biochemical Corp., Cleveland, OH) and α-^{35}S-dATP. Oligonucleotide primers were synthesized using an oligonucleotide synthesizer (Applied Biosystems, Foster City, CA), deprotected, and desalted by passage through NAP-10 columns (Pharmacia, Piscataway, NJ).

RNA Blot Analysis

Pathogen-free, adult CD1 mice, weighing 23–25 g (Charles River, Wilmington, MA) were injected intraperitoneally with 0.5 ml of phosphate-buffered saline (PBS) containing 100 μg of LPS (serotype 0.55: B5) or PBS alone as control. Two animals per time point (1.5, 3, 6, 12, 24, 48, 72, 168 hours) were sacrificed by cervical dislocation after injection, and the brain, heart, lung, and kidney were harvested. Total RNA was extracted with guanidine isothiocyanate from each organ and purified by centrifugation through a cesium-chloride cushion.[20] Fifteen μg of RNA from each organ at each time point were separated on a 1% agarose gel, containing 0.4 mol/L 3-*N*-morpholinopropanesulfonic acid (pH 7.0), 0.1 mol/L sodium acetate, 0.01 mol/L EDTA, and 8% formaldehyde. The gel was washed three times in 10× standard saline citrate (SSC), and RNA transfer was performed on Hybond membrane (Amersham, Arlington Heights, IL) overnight in 20× SSC. After UV cross-linking, the membrane was incubated for 4 hours at 42 C in prehybridization solution containing 50% deionized formamide, 5× SSC, 50 mmol/L sodium phosphate (pH 6.5), 10× Denhardt's solution, 0.125% sodium dodecyl sulfate (SDS), and 0.5 ml preboiled salmon sperm DNA (5 mg/ml; Sigma) in a final volume of 40 ml. cDNA probes were prepared from plasmid constructs containing a 1.8-kb cDNA insert for the rat VCAM-1[22] or a 1.3-kb cDNA insert for the rat E-selectin (see the section above). Inserts were excised by restriction enzyme digestion, fragments were isolated and labeled by random priming (MegaPrime, Amersham) using [α-^{32}P]dCTP (3000 Ci/mmol; New England Nuclear), and the nonincorporated isotope was removed by column chromatography. Northern blot hybridizations were performed at 42 C overnight. Filters were rinsed and then washed at 65 C for 1.5 hours in buffer containing (per liter) 7× SSC, 25 mmol/L sodium phosphate, 10× Denhardt's solution, and 0.25% SDS, followed by a half-hour wash with 0.5× SSC and 0.25% SDS per liter. Filters were exposed to Kodak XAR-2 film at −70 C with two intensifying screens for 1 hour to 2 days.

Light Microscopy

Pathogen-free, adult CD1 mice (Charles River, Cambridge, MA) of both sexes were injected intraperitoneally with PBS containing either 10 or 100 μg LPS or PBS alone and sacrificed by cervical dislocation at 1.5, 3, 6, 12, 48, 72, or 168 hours (2–3 animals per time point). Selected organs, including heart, lung, kidney, brain, liver, and spleen, were collected from animals that received either the low (10 μg) or high (100 μg) dose of endotoxin. In one set of eight animals, these tissues were prepared for light microscopy by fixation in 10% buffered formaldehyde, and after routine processing, sections were stained with hematoxylin-eosin. In another set of eight animals the same organs were collected and snap frozen to be used for immunoperoxidase studies. In the set of animals receiving 100 μg of LPS, the additional following organs/tissues were excised and prepared for immunoperoxidase (for method see below): tongue, thymus, esophagus, stomach, pancreas, jejunum, colon, paraaortic lymph nodes, adrenal glands, aorta, bladder, ovary, uterus, testis, seminal vesicles, prostate gland, striated muscle (thigh), skin, ear, and tail.

Immunoperoxidase

VCAM-1 was localized with the use of monoclonal antibody M/K-2 (generously provided by Dr. P. W. Kinkade, Oklahoma City, OK).[23] An irrelevant, isotype-matched rat immunoglobulin (Sigma) in the

appropriate concentration was used as a negative control. Factor VIII-related antigen was detected using a rabbit anti-human antibody (Calbiochem, San Diego, CA) at 1:100. Six-µm-thick frozen sections were cut from rat tissues previously embedded in OCT, snap frozen in liquid nitrogen, and mounted on Vectabond (Vector Laboratories Inc., Burlingame, CA) coated glass slides. After air drying for 30 minutes, sections were fixed in 100% ice-cold acetone for 10 minutes, air dried for 10 minutes, and stored at −70 C protected by plastic wrap until further use. Subsequently, sections were air dried for 30–60 minutes, fixed in 100% acetone at room temperature for 5 minutes, and washed in PBS (pH 7.4) containing 1% normal goat serum (Gibco BRL, Grand Island, NY) for 5 minutes. All subsequent steps were carried out at room temperature. Sections were incubated with the primary antiserum diluted in PBS in a humid chamber for 1.5 hours. Dilutions of the primary antiserum were optimized in pilot experiments to be 1/40. After three PBS washes, the last containing 1% normal goat serum, the slides were incubated for 1 hour in a humid chamber with a goat-anti-rat IgG (heavy and light chain) peroxidase-conjugated antibody (mouse absorbed; Caltag Laboratories, South San Francisco, CA) diluted with PBS. After three washes in straight PBS, followed by one wash in 0.1 mol/L acetate buffer (pH 5.2) for 5 minutes, the antibody conjugate was detected by incubating the slides in 0.1 mol/L acetate buffer containing 15 ml freshly filtered 3-amino-9-ethylcarbazole (0.1 g; Aldrich, Milwaukee, WI) in 25 ml *N,N*-dimethylformamide and 150 µl 30% hydrogen peroxide as described previously.[24] After a subsequent 5-minute wash in 0.1 mol/L acetate buffer, slides were counterstained in methyl green for 2 minutes and coverslipped using gelatin/glycerol.

The construction, expression, and functional characterization of a soluble form of human E-selectin have been described in detail elsewhere.[25] A rabbit polyclonal antibody was generated to recombinant human soluble ELAM-1. The metabolic labeling and immunoprecipiation were performed as described earlier.[26] An irrelevant, isotype-matched rabbit immunoglobulin (Sigma) was used as a negative control. The immunperoxidase detection protocol for ELAM-1 detection was similar to that described for VCAM-1. After acetone fixation, the sections were subjected to methanol: hydrogen peroxide (100:0.3, v/v) treatment for 8 minutes at room temperature, blocked with 20% normal swine serum (Organon Technika Co., Durham, NC), washed with PBS containing 3% normal swine serum, and then incubated with the rabbit polyclonal antibody, diluted in PBS overnight at 4 C in a humid chamber. Dilutions of primary antibody were optimized in pilot experiments to be 1:50. After three washes containing a normal swine serum, the sections were incubated with swine-anti-rabbit IgG (heavy and light chain) peroxidase-conjugated antibody (absorbed; Dako Co., Carpinteria, CA) for 1 hour at room temperature. The detection of the peroxidase labeled antibody, counterstaining, and coverslipping were done as outlined before.

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1       TAACAGAAAGTTTCTCCGGTCTAGCATCTGGATGAAAGGAACTGCTGGGGTCATGAATGCCTCGTGCCTTCTCTCTGCTCTCACCTTTGTTCTCCTCATT
                                                                M  N  A  S  C  L  L  S  A  L  T  F  V  L  L  I
101     GGAGAGAGCATAGCTTGGTACTACAATGCCTCCAGTGAGCTCATGACATATGATGAAGCAAGTGCGTATTGTCAACGGGACTACACACATCTGGTGGCGA
        G  E  S  I  A  W  Y  Y  N  A  S  S  E  L  M  T  Y  D  E  A  S  A  Y  C  Q  R  D  Y  T  H  L  V  A  I
201     TTCAGAACAAGGAAGAGATCAATTACCTAAACTCCACTCTGAGGTATTCACCAAGTTATTACTGGATTGGAATCAGAAAAGTCAATAATGTATGGATCTG
          Q  N  K  E  E  I  N  Y  L  N  S  T  L  R  Y  S  P  S  Y  Y  W  I  G  I  R  K  V  N  N  V  W  I  W
301     GGTGGGGACCCAGAAGCCTCTGACGGAGGAAGCTAAGAACTGGGCGCCAGGTGAACCAAACAACAAACAAAGAAACGAGGACTGTGTAGAGATCTACATC
          V  G  T  Q  K  P  L  T  E  E  A  K  N  W  A  P  G  E  P  N  N  K  Q  R  N  E  D  C  V  E  I  Y  I
401     CAAAGACCCAAAGACTCCGGCATGTGGAATGACGAGAGATGTGACAAAAAGAAACTGGCTCTGTGTTACACAGCTTCCTGTACCAACACATCCTGCAGTG
        Q  R  P  K  D  S  G  M  W  N  D  E  R  C  D  K  K  K  L  A  L  C  Y  T  A  S  C  T  N  T  S  C  S  G
501     GTCACGGTGAATGCGTTGAGACCATCAATAGTTATACCTGCAAATGTCACCCCGGCTTCCTGGGACCCAAGTGTGACCAAGTTGTGACCTGCCAAGAACA
          H  G  E  C  V  E  T  I  N  S  Y  T  C  K  C  H  P  G  F  L  G  P  K  C  D  Q  V  V  T  C  Q  E  Q
601     GGAATACCCTGACCATGGAAGCCTGAACTGCACCCACCCATTCGGCCTCTTCAGCTATAATTCATCCTGCTCCTTCAGCTGTGAAAGGGGCTATGTGCCC
          E  Y  P  D  H  G  S  L  N  C  T  H  P  F  G  L  F  S  Y  N  S  S  C  S  F  S  C  E  R  G  Y  V  P
701     AGCAGCATGGAGACCACAGTGTGGTGTACATCCTCTGGAGAGTGGAGTGCACCTGCTCCTGCCTGCCATGTGGTTGAATGTAAAGCTTTGACCCAACCTG
        S  S  M  E  T  T  V  W  C  T  S  S  G  E  W  S  A  P  A  P  A  C  H  V  V  E  C  K  A  L  T  Q  P  A
801     CCCACGGAGTCAGGAAATGTTCCTCAAATCCTGGGAGCTACCCATGGAATACAACGTGCACGTTTGACTGTGAGGAAGGGTACAGGAGAGTTGGAGCTCA
          H  G  V  R  K  C  S  S  N  P  G  S  Y  P  W  N  T  T  C  T  F  D  C  E  E  G  Y  R  R  V  G  A  Q
901     GAATCTACAGTGTACCTCATCCGGTGTCTGGGACAACGAGAAGCCATCGTGCAAAGCTGTGACCTGTGATGCCATCCCTCGGCCTCAGAATGGCTCTGTG
          N  L  Q  C  T  S  S  G  V  W  D  N  E  K  P  S  C  K  A  V  T  C  D  A  I  P  R  P  Q  N  G  S  V
1001    AGCTGCAGCAACTCAACGGCTGGAGCGCTTGCCTTTAAGTCTTTCTGTAATTTTACCTGTGAGCACAGTTTCACATTGCAGGGCCCAGCCCAGGTTGAAT
        S  C  S  N  S  T  A  G  A  L  A  F  K  S  F  C  N  F  T  C  E  H  S  F  T  L  Q  G  P  A  Q  V  E  C
1101    GCAGTGCACAAGGGCAGTGGACACCACAAATCCCAGTGTGCAAAGCTTCCCAGTGTGAAGCCTTATCTGCACCACAGCGAGGCCACATGAAATGTCTTCC
          S  A  Q  G  Q  W  T  P  Q  I  P  V  C  K  A  S  Q  C  E  A  L  S  A  P  Q  R  G  H  M  K  C  L  P
1201    CAGTGCCTCTGCACCCTTCCAAAGTGGGTCCAGTTGTAAGTTCTCCTGTGACGAAGGATTTGAATTGAAGGGATCAAGAAGGCTTCAGTGTGGTCCAAGA
          S  A  S  A  P  F  Q  S  G  G  S  S  C  K  F  S  C  D  E  G  F  E  L  K  G  S  R  R  L  Q  C  G  P  R
1301    GGGGAGTGGGACAGCGAAAAGCCCACATGTGCAGGGGTACAGTGTTCAAGCCTTGACCTTCCAGGAAAGATGAACATGAGTTGCAGTGGGCCAGCAGTTT
        G  E  W  D  S  E  K  P  T  C  A  G  V  Q  C  S  S  L  D  L  P  G  K  M  N  M  S  C  S  G  P  A  V  F
1401    TTGGCACGGTATGTGAATTTACATGTCCTGAGGGTTGGACACTCAACGGATCTTCAATCCTGACATGTGGTGCCACAGGACGCTGGTCTGCGATGCTGCC
          G  T  V  C  E  F  T  C  P  E  G  W  T  L  N  G  S  S  I  L  T  C  G  A  T  G  R  W  S  A  M  L  P
1501    TACTTGTGAAGCCCCAGCCAACCCTCCCCGTCCCTTGGTAGTGGCACTTTCTGTGGCAGCAACCTCACTTCTGACATTGTCCTCATTGATCTACGTGTTG
          T  C  E  A  P  A  N  P  P  R  P  L  V  V  A  L  S  V  A  A  T  S  L  L  T  L  S  S  L  I  Y  V  L
1601    AAGAGATTCTTTTGGAAGAAAGCAAAGAAATTCGTTCCTGCTAGTAGCTGCCAAAGTCTTCAATCGTTTGAAAACTATCAAGGGCCTTCTTACATCATTT
        K  R  F  F  W  K  K  A  K  K  F  V  P  A  S  S  C  Q  S  L  Q  S  F  E  N  Y  Q  G  P  S  Y  I  I  -
1701    AAGTTCAA
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Figure 1. *Nucleotide sequence of the rat E-selectin cDNA and predicted amino acid sequence of rat E-selectin protein.*

Results

Isolation and Characterization of Rat E-Selectin Gene

The rat E-selectin gene was cloned by PCR. Three sets of degenerate primers were synthesized from conserved sequences found in the human,[3] rabbit,[27] and mouse[28] E-selectin cDNAs and used in the initial amplification. Three overlapping amplification products were obtained, and a composite sequence of the open reading frame was derived (Figure 1). DNA sequences from three independent clones for each amplification product were obtained to eliminate PCR-generated sequence errors. Translation of the open reading frame generated a protein with a calculated molecular weight of 60,132 and an estimated isoelectric point of 6.6.

The rat E-selectin had a predicted hydrophobic signal sequence, a C-type lectin domain, five complement regulatory (CR) repeats, a single transmembrane domain, and a short cytoplasmic region. The rat E-selectin lectin domain was 76.6% homologous to the other E-selectins and fits the consensus for a C-type recognition domain.[29] This domain is most conserved in the E-selectins and is consistent with a functional role for this domain in the recognition of similar carbohydrate ligands. Recently an E-selectin region critical for carbohydrate recognition and cell adhesion was determined.[30] The location of amino acid side chains, the mutations of which appeared to affect sialylated Lewis X recognition, were found in a small region on the surface of one side of the lectin domain. Although conservation of the lectin domain is generally high (Figure 2), the specific residues believed to be involved in carbohydrate recognition[31] are completely conserved in all species consistent with the structural model.

Comparison of the rat E-selectin epidermal growth factor-like domain to the other E-selectins (Figure 2) revealed that the arrangement of cysteines characteristic of this domain[32] is conserved. Most of the residue changes in this domain were neutral substitutions. The deduced rat E-selectin sequence encoded five CR repeats, a striking difference from the six similar repeats found in the human and mouse genes. The rat E-selectin does not contain the fifth CR domain. Interestingly, the rabbit E-selectin lacks the fourth CR domain.[27] This lack of a CR element in the rat E-selectin could reflect alternative mRNA splicing events. Alternatively, the corresponding exon or its flanking sequences in the rat E-selectin gene could have been altered or deleted. The diversity in the CR repeats expressed in the E-selectins suggests that some variability in the number of these structures is tolerated in the function of E-selectins. The transmembrane region of the selectins is not well conserved (21%). The rat ELAM-1 has four potential *N*-linked glycosylation sites that are conserved in all four species.

Regulation of ELAM-1 and VCAM-1 transcript expression in vivo

VCAM-1 and ELAM-1 transcripts were dramatically induced in all organs examined after intraperitoneal endotoxin administration. These transcripts were detected by 1.5 hours after LPS treatment; maximal transcript expression occurred at 1.5–3 hours and then gradually declined (Figure 3). For each gene, only one major transcript was detected, 3.2 kb for VCAM-1 and about 4 kb for ELAM-1; no new adhesion molecule transcript species were detected by Northern blot analysis. The general kinetic pattern of transcript accumulation for the two adhesion molecules was similar in all organs examined. Induced expression of ELAM-1 and VCAM-1 was greatest in the lung, where maximal transcript content was greatest and detectible expression persisted for longer than 3 days.

Immunohistochemical Studies

Specificity of the immunohistochemical staining was demonstrated by complete absence of staining product using either a nonspecific rat immunoglobulin or the secondary antibody alone. Induced VCAM-1 expression in the vasculature was qualitatively similar over a range of LPS dosages (5–100 μg) and differed only in the staining intensity, which was slightly stronger at the high endotoxin dose.

Macro- and Microvasculature Expression of VCAM-1

Considerable differences were detected in the basal and LPS-induced levels of VCAM-1 expression in the micro- and macrovasculature. Basal expression of VCAM-1 was seen in small and medium-sized arteries in the lung and kidney, as well as in the aorta. Expression consisted of a strong staining pattern with one or more individual endothelial cells surrounded by immunohistochemically negative flanking cells.

Most endothelia were capable of expressing VCAM-1 after LPS administration. VCAM-1 expression was detectable 1.5 hours following LPS injection and peaked at 6 hours. VCAM-1 expression

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SP        RT   MNASCLLSALT--F
          M    MNASRFLSALV--F
          RB   MVASWLLSTLTFAL
          H    MIASQFLSALT--L

LECTIN    RT   VLLIGESIAWYYNASSELMTYDEASAYCQRDYTHLVAIQNKEEINYLNSTLRYSPSYYWIGIRKVNNVWIWVGTQKPLTE
          M    VLLAGESTAWYYNASSELMTYDEASAYCQRDYTHLVAIQNKEEINYLNSNLKHSPSYYWIGIRKVNNYWIWVGTGKPLTE
          RB   VLLIKETSTWTYHFSAENMTYDEASAYCQQNYTHLVAIQNKEEIDYLNSILDYSPSYYWIGIRKVNNYWIWVGTHKPLTE
          H    VLLIKESGAWSYNTSTEAMTYDEASAYCQQRYTHLVAIQNKEEIEYLNSILSYSPSYYWIGIRKVNNVWVWVGTQKPLTE

          RT   EAKNWAPGEPNNKQRNEDCVEIYIQRPKDSGMWNDERCDKKKLALCYT
          M    EAQNWAPGEPNNKQRNEDCVEIYIQRTKDSGMWNDERCNKKKLALCYT
          RB   GAKNWAPGEPNNKQNNEDCVEIYIKRPKDTGMWNDERCSKKKLALCYT
          H    EAKNWAPGEPNNRQKDEDCVEIYIKREKDVGMWNDERCSKKKLALCYT

EGF       RT   ASCTNTSCSGHGECVETINSYTCKCHPGFLGPKCDQ
          M    ASCTNASCSGHGECIETINSYTCKCHPGFLGPNCEQ
          RB   AACTEASCSGHGECIETINNYSCKCYPGFSGLKCEQ
          H    AACTNTSCSGHGECVETINNYTCKCDPGFSGLKCEQ

CR1       RT   VVTCQEQEYPDHGSLNCTHPFGLFSYNSSCSFSCERGYVPSSMETTVWCTSSGEWSAPAPACH
          M    AVTCKPQEHPDYGSLNCSHPFGPFSYNSSCSFGCKRGYLPSSMETTVRCTSSGEWSAPAPACH
          RB   VVTCEAQVQPQHGSLNCTHPLGNFSYNSSCSVSCERGYLPSSTETT-WCTSSGEWSAPPATCK
          H    IVNCTALESPEHGSLVCSHPLGNFSYNSSCSISCDRGYLPSSMETM-QCMSSGEWSAPIPACN

CR2       RT   VVECKALTQPAHGVRKCSSNPGSYPWNTTCTFDCEEGYRRVGAQNLQCTSSGVWDNEKPSCK
          M    VVECEALTHPAHGIRKCSSNPGSYPWNTTCTFDCVEGYRRVGAQNLQCTSSGIWDNETPSCK
          RB   VVECDTMGKPANGDVKCSPSQGSAPWNTTCTFDCEEGFTLLGARSLQCTSSGSWDNEKPTCK
          H    VVECDAVTNPANGFVECFQNPGSFPWNTTCTFDCEEGFELMGAQSLQCTSSGNWDNEKPTCK

CR3       RT   AVTCDAIPRPQNGSVSCSNSTAGALAFKSFCNFTCEHSFTLQGPAQVECSAQGQWTPQIPVCK
          M    AVTCDAIPQPQNGFVSCSHSTAGELAFKSSCNFTCEQSFTLQGPAQVECSAQGQWTPQIPVCK
          RB   AVSCDTIHHPQNGSVSCSNSSEGKFTFRSSCNFTCEWNFLLRGPAQVECTAQGQWTQQAPVCE
          H    AVTCRAVRQPQNGSVRCSHSPAGEFTFKSSCNFTCEEGFMLQGPAQVECTTQGQWTQQIPVCE

CR4       RT   ASQCEALSAPQRGHMKCLPSASAPFQSGSSCKFSCDEGFELKGSRRLQCGPRGEWDSEKPTCA
          M    AVQCEALSAPQQGNMKCLPSASGPFQNGSSCEFSCEEGFELKGSRRLQCGPRGEWDSKKPTCS
          H    AFQCTALSNPERGYMNCLPSASGSFRYGSSCEFSCEQGFVLKGSKRLQCGPTGEWDNEKPTCE

CR5       M    AVKCDDVPRPQNGVMECAHATTGEFTYKSSCAFQCNEGFSLHGSAQLECTSQGKWTQEVPSCQ
          RB   AVKCDPVHTLEDGFVKCTHPHTGEFTYKSSCTFNCREGFELHGSAQLECTSQGQWAQELPSCQ
          H    AVRCDAVHQPPKGLVRCAHSPIGEFTYKSSCAFSCEEGFELYGSTQLECTSQGQWTEEVPSCQ

CR6       RT   GVQCSSLDLPGKMNMSCSGPAVFGTVCEFTCPEGWTLNGSSILTCGATGRWSAMLPTCE
          M    VVQCPSLDVPGKMNMSCSGTAVFGTVCEFTCPDDWTLNGSAVLTCGATGRWSGMPPTCE
          RB   VVQCPSLAVLGKTNVSCSGEPVFGTVCNFACPEGWTLNGSAALMCGAEGQWSGMLPTCE
          H    VVKCSSLAVPGKINMSCSGEPVFGTVCKFACPEGWTLNGSAARTCGATGHWSGLLPTCE

TM        RT   APANPPRPLVVALSVAATSLLALSSLIYYLKRFFWKK
          M    APVSPTRPLVVALSAAGTSLLTSSSLLYLLMRYFRKK
          RB   EPIASNVPLAVGLSVSGTSFLTLTSFLLWFLKYFRKK
          H    APTESNIPLVAGLSAAGLSLLTLAPFLLWLRKCLR-K

CYTO1     RT   AKKFVPAS
          M    AKKFVPAS
          RB   AKKFVPAC
          H    AKKFVPAS

CYTO2     RT   S-CQSLQSFENYQGPSYII
          M    S-CQSLQSFENYHVPSYNV
          RB   SRYVGLEAHGNCQVPSHLI
          H    S-CQSLESDGSYQKPSYIL
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Figure 2. *Structural homology of the rat (RT), mouse (M), rabbit (RB), and human (H) E-selectin amino acid sequence. Conserved amino acids in all four sequences are printed in bold and boxed. Sequences are displayed according to their respective domains by aligning them visually according to conserved amino acids. SP, signal peptide; LECTIN, lectin domain; EGF, epithelial growth factor-like domain; CR 1–6, consensus complement-like repeats; TM, transmembrane domain; CYTO, cytoplasmic domain.*

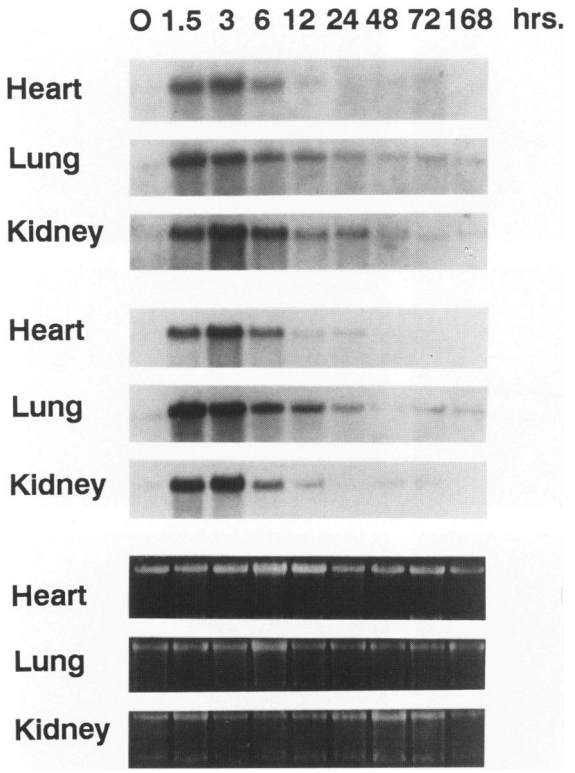

Figure 3. *Northern blot analysis of transcript levels for VCAM-1 (top) and E-selectin (middle) in three different organs before and at various times after LPS injection. (Bottom) comparable amounts of intact total RNA were loaded for each organ and time point as assessed by staining with ethidium bromide.*

was stronger in the arterial than in the venous system in brain, heart, lung, liver, kidney, and central vessels. The expression continued to be strong in arterial vessels for up to 72 hours, while at that time the venous system displays a much less prominent pattern of expression. The most remarkable difference in expression was observed in the microvasculature. Capillary endothelial cells in general showed a uniform but weak VCAM-1 expression of short duration at high LPS doses in most organs, including the lungs and the kidneys. Serial sections of brain, lung, and kidney stained for factor VIII-related antigen revealed that all capillary endothelial cells weakly expressed VCAM-1 (data not shown). This expression was highly restricted to the time points of maximal expression in the arterial system, while thereafter it dropped to undetectable levels. This finding contrasted to the marked capillary endothelial cell expression for VCAM-1 in the brain, which was sustained after LPS induction over more than 24 hours before returning to background levels. These results are consistent with proposals that capillary endothelium may be phenotypically different from the endothelial cells lining large vessels (eg, Ref. 33).

Expression of VCAM-1 in Specific Organ Systems

Aortic endothelium expressed VCAM-1 constitutively but was patchy and weak (Figures 4 and 5). VCAM-1 expression on the aortic endothelium peaked at 6 hours after endotoxin but positivity diminished to background levels by 1 week. Lower levels of inducible VCAM-1 expression were seen in aortic smooth muscle cells (Figure 5). In the lung, constitutive, patchy staining occurred in the endothelium of arteries and venules (Figure 4). It appeared that single endothelial cells were positive adjacent to numerous negative endothelial cells. At 6 hours, when maximal staining in the lung was observed, patchy, faint VCAM-1 expression developed in alveolar septa, suggesting that capillary endothelium was focally positive. Weak constitutive staining of endothelium in arteries, arterioles, and veins was also present in the kidneys as well as very faint capillary staining adjacent to the tubuli (Figure 4). By 6 hours after intraperitoneal administration of LPS, maximal signals for VCAM-1 were noted in the endothelium of all vessels, including capillaries. Glomeruli were predominantly negative, although a focal, faint mesangial expression occurred at 6 hours, which was undetectable at all other time points. In the heart, control animals demonstrated patchy, weak endocardial staining (Figure 6). Following LPS administration, the extent and intensity of endocardial VCAM-1 expression increased. Capillaries in the myocardium developed faint positivity, but arteries in both the myocardium and the epicardium were strongly positive. VCAM-1 expression diminished to background levels by 1 week, but there was residual staining on both endocardium and in a few of the arteries in the epicardium. As compared to other organs, capillary staining in the brain was more prominent, peaking at 6 hours, but as in other organs, it quickly decreased over time. Although there was faint VCAM-1 positivity in arteries of the portal triads of the liver, sinusoids and central veins remained negative. In addition to arterial and venular endothelial VCAM-1 expression in the spleen that followed kinetics similar to that of other organs, there was a moderate positivity of the reticulum of the red pulp. This increased in intensity by 6 hours after endotoxin inoculation and decreased but remained positive at 1 week. A similar, LPS-inducible pattern of VCAM-1 expression was observed in lymphoid reticulum outside the germinal centers. The adrenal gland displayed only a very faint VCAM-1

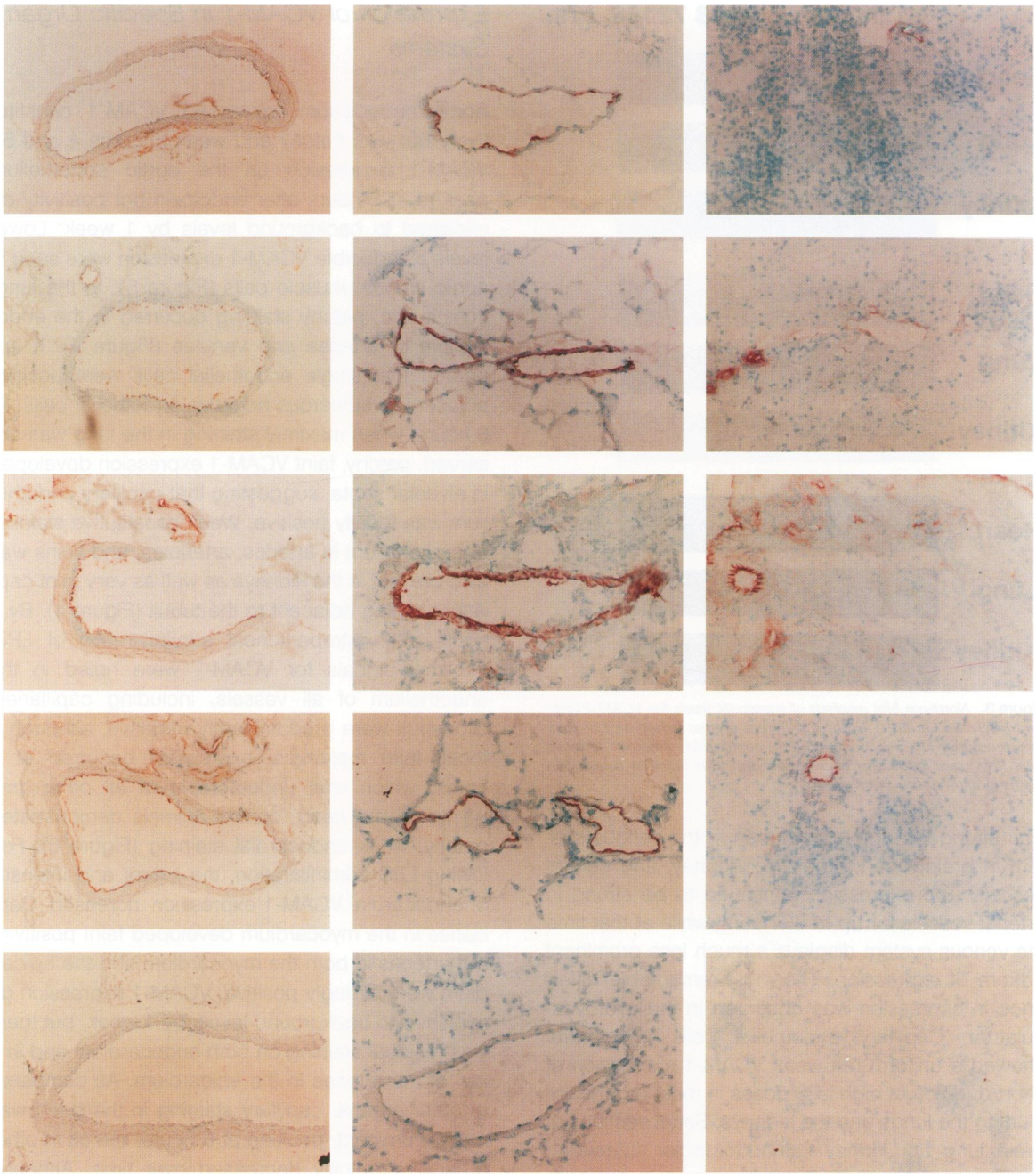

Figure 4. *Comparison of the pattern of VCAM-1 expression in the aorta, lung, and kidney following LPS induction. Sections of aorta (left column), lung (center column), and kidney (right column), following 0 (top row), 3 (second row), 6 (third row), and 72 hours (fourth row) after LPS administration. (Bottom row) sections stained with normal rat IgG, 6 hours after LPS administration.*

signal in its medullary capillaries 6 hours after induction. Other organs (ie, esophagus, stomach, jejunum, colon, bladder, muscle, skin, and male and female genital organs) demonstrated similar patterns and kinetics of vascular expression, particularly in the arterial circulation.

Histological sections in control and inoculated animals at 1.5, 3, and 6 hours after inoculation were studied to determine whether there was margination of leukocytes in the vessels at the time of maximal E-selectin and VCAM-1 expression. Heart, lung, kidney, brain, and liver were examined (data not shown). Endothelium in control organs did not contain adherent neutrophils, although scattered mononuclear cells were present. After systemic endotoxin administration, significant margination of leukocytes

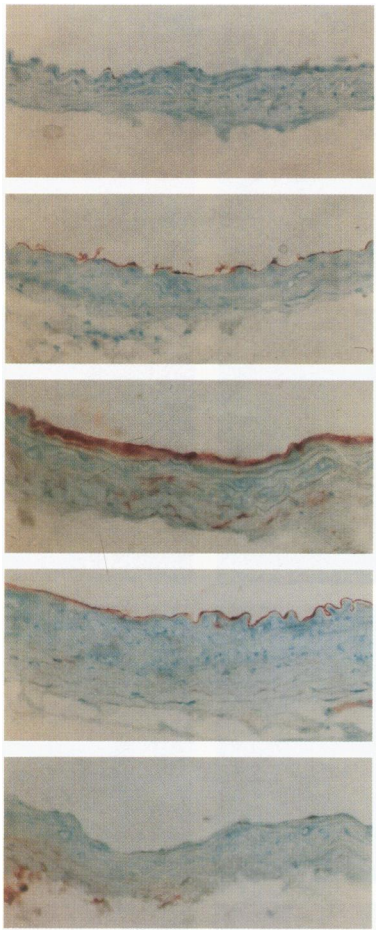

Figure 5. *Expression of VCAM-1 in the aorta. Sections of aorta following 0 (top row), 3 (second row), 6 (third row), and 72 (fourth row) hours after LPS administration. (Bottom row) negative control.*

was not detected in any organ. Notably, these foci occurred only where there was a very small amount of perivascular mononuclear inflammation.

Extravascular VCAM-1 Expression

VCAM-1 expression was not confined to vascular endothelial cells in the endotoxin-treated animals (Figure 6). Immunohistochemically detectable constitutive epithelial VCAM-1 expression was observed in the esophagus and the bladder, which was inducible by LPS and peaked at 6 hours. Stromal staining was observed in the testis and around the follicles in the ovary. The testicular staining was constitutive and did not increase. The VCAM-1 expression in the ovarian stroma was not present in uninduced animals but was seen maximally at 3 hours after LPS administration. Inducible extravascular staining was observed in the choroid plexus with a maximum intensity at 6 hours. In general, the intensity of extravascular staining was less than the degree of endothelial staining. VCAM-1 may have function(s) in these extravascular tissues independent of its role in leukocyte adhesion to endothelial cells. Alternatively, the extravascular staining by the VCAM-1 antibody is due to the recognition of an epitope on a distinct protein(s) in these tissues.

Expression pattern of ELAM-1

Specificity of the anti-ELAM-1 antibody was assessed by both immunoprecipitation and immunohistochemical criteria. Immuoprecipitation with the anti-soluble ELAM-1 antibody of extracts from metabolically labeled and detergent-lysed control and IL-1β-treated cultured human umbilical vein endothelial cells revealed that the antibody did not recognize anything from control cells (Figure 7). In contrast, the antibody recognized a protein of the expected size (110,000 d) in extracts of cytokine-treated cells. Specificity of the immunohistochemical staining was demonstrated by minimal endothelial staining using either a purified absorbed rabbit immunoglobulin or the secondary antibody alone. Some staining of tracheal epithelium and interstitial tissue was seen with this antibody (data not shown).

No immunohistochemically detectable constitutive expression of ELAM-1 could be found in any of the vascular beds investigated. After LPS treatment, ELAM-1 expression was observed in the endothelial cells in medium and small veins in the lung (Figure 8), the heart, and the kidney (data not shown). In contrast to the VCAM-1 pattern of expression, no ELAM-1 expression was detectable in the aortic endothelium with this antibody.

Discussion

The present study describes the *in vivo* pattern of gene expression of endothelial leukocyte adhesion molecules elicited by intraperitoneal administration of endotoxin. We find that endotoxin can stimulate VCAM-1 and ELAM-1 gene expression; however, the widespread modulation of the endothelial cell surface phenotype did not correlate with leukocyte infiltration. Analysis of VCAM-1 transcript content and endothelial surface phenotype revealed some adhesion molecule expression in the endothelium of control animals. Focal (or patchy) VCAM-1 staining was seen in multiple organs. For example, VCAM-1 expression was detected in individual endothelial cells in small and medium-sized vessels in the lung.

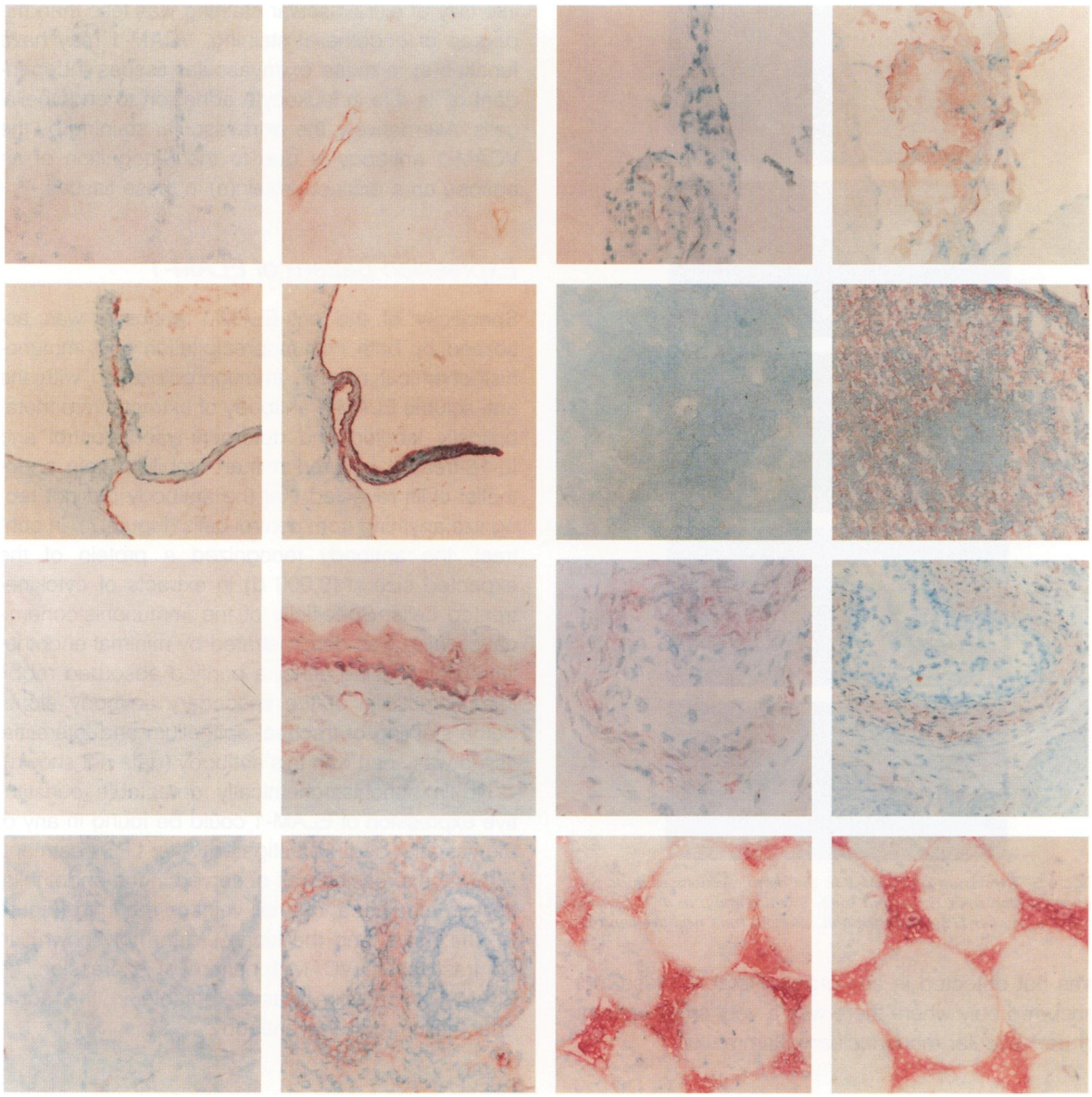

Figure 6. *Basal and induced VCAM-1 expression in selected organs. Basal expression (first and third columns from the left) and maximally induced (6 hours after LPS administration) expression (second and fourth columns from the left) in mid-brain (top row, first and second columns); choroid plexus (top row, third and fourth columns); heart valve (second row, first and second columns); lymph node (second row, third and fourth columns); esophagus (third row, first and second columns); bladder (third row, third and fourth columns); ovary (bottom row, first and second columns); testis (bottom row, third and fourth columns).*

Expression was confirmed by the presence of low levels of VCAM-1 transcript in RNA samples from control lungs. No evidence for constitutive expression of ELAM-1 on the large vessels was observed in this mouse model, in contrast to previous results with human biopsy samples.[33]

Endotoxin administration resulted in dramatically increased VCAM-1 and ELAM-1 gene expression and modulation of the endothelial phenotype. Endothelial activation was widespread, involving most organs examined, but was particular strong in the lung, heart, and aorta. Endothelial activation following LPS administration is due either to the direct effects of LPS on endothelium or to the indirect effects mediated by activated neutrophils, mononuclear cells, or products released from these cells. In general the endothelial induction of leukocyte adhesion molecules was much more dramatic than the induced expression of VCAM-1 in extravascular tissues. The high circulating levels of cytokines found in systemic sepsis (eg, reviewed in Ref. 34) may generate the extraordinary levels of leukocyte adhe-

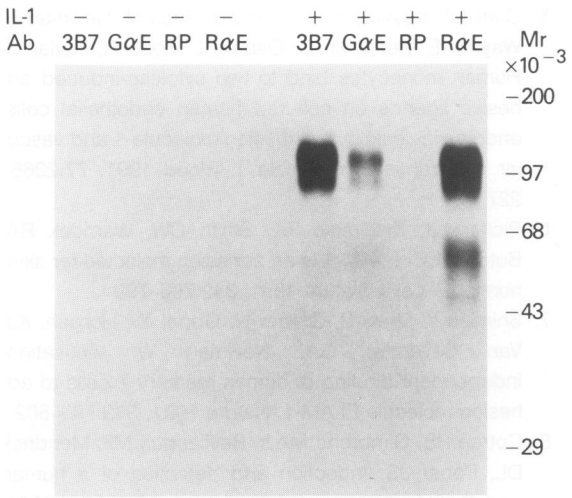

Figure 7. *Immunoprecipitation of ELAM-1 from extracts of IL-1β-treated human endothelial cells with a rabbit anti-soluble form of ELAM-1. Human umbilical vein endothelial cells were cultured for 4 hours in the absence (−) or presence (+) of 1 ng/ml of IL-1β. Detergent lysates of each were immunoprecipitated with monoclonal antibody 3B7 or the Ig fractions of goat anti-ELAM-1–420 (GαE), rabbit prebleed, or rabbit anti ELAM-1–420 (RαE).*

sion molecule expression in the vasculature, while having less of an effect on VCAM-1 expression in nonvascular tissues.

The kinetics of ELAM-1 and VCAM-1 transcript accumulation in several organs following intraperitoneal endotoxin administration were surprisingly similar. In cytokine-activated cultured human umbilical vein endothelial cells, both the *ELAM-1* and *VCAM-1* genes are transcriptionally activated by cytokines.[35,36] The large increases in endotoxin-induced VCAM-1 and ELAM-1 transcript content seen in these studies are consistent with the previous *in vitro* findings demonstrating that the induced expression of the *VCAM-1* and *ELAM-1* genes is the result of new gene transcription. In cultured human umbilical vein endothelial cells, cytokine-induced ELAM-1 transcript accumulation is maximal within 1–2 hours, followed by a return to a near-basal level by 24 hours. Cytokine-induced VCAM-1 transcripts accumulated more slowly, reaching maximal levels at 24 hours, and persisted for several days. The differences between the pattern of leukocyte adhesion molecule transcript accumulation seen in cultured endothelial cells and the pattern in this murine model raises the possibility that additional cytokines generated by resident cells *in vivo* changes the pattern of expression. This possibility is consistent with recent observations in which it was shown that IL-4 can augment the capacity of TNF-α to selectively promote VCAM-1 expression and the development of T-cell-rich infiltrates.[17]

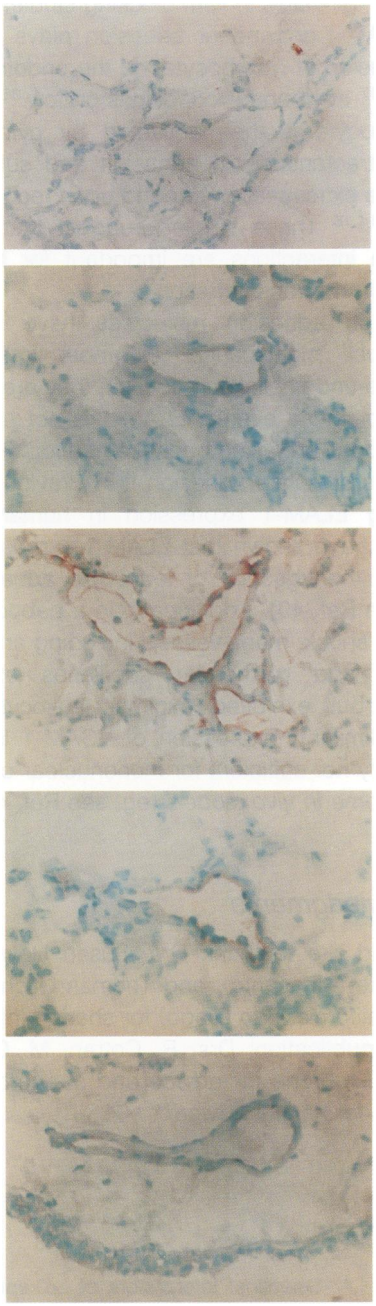

Figure 8. *Pattern of ELAM-1 expression in lung. Sections of lung following: 0 (top), 1.5 (second from top), 3 (third from top) and 6 (fourth from top) hours after LPS administration. (Bottom) negative control.*

Our data suggest that transiently increased expression of VCAM-1 and ELAM-1 is not sufficient in this *in vivo* model for the development of leukocyte adhesion. It was suggested that expression of selectins, such as E- and L-selectin, is important in the initial, low-affinity adhesive events (rolling) between leukocytes and activated endothelial

cells.[14,15] Similarly, *in vitro* blocking studies suggest that VCAM-1-dependent adhesion plays a role in the adhesion of lymphocytes to the endothelial cell surface,[37] even under conditions of flow.[38] The rolling leukocyte is then activated by cytokines or chemoattractants. Firm adhesion and subsequent leukocyte extravasation depend upon recognition of ICAM-1.[14,15] These studies suggest that multiple adhesive interactions are important for leukocyte adhesion and transmigration.

Although adhesion molecules have assumed conceptual significance in most models of leukocyte-endothelial interactions, they are not necessary for these events. ELAM-1 is not necessary for neutrophil adhesion because neutrophil-directed mediators stimulate adhesion.[16,39] Previous studies localizing ELAM-1 expression in human biopsy samples have shown that ELAM-1 expression can occur in the absence of leukocyte adhesion (reviewed in Ref. 40). Additionally, in a baboon model of septic shock, many vessels including arteries, sinusoids, and glomerular capillaries expressed ELAM-1 but exhibited minimal leukocyte adhesion.[19] Similarly, expression of VCAM-1 is neither necessary nor sufficient for mononuclear cell adhesion in some *in vivo* models (eg, see Ref. 41).

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