Disturbed Homeostasis of Lung Inter cellular Adhesion Molecule-1 and Vascular Cell Adhesion Molecule-1 During Sepsis

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Cecal ligation and puncture (CLP)-induced sepsis in mice was associated with perturbations in vascular adhesion molecules. In CLP mice, lung vascular binding of ¹²⁵I-monoclonal antibodies to intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 revealed sharp increases in binding of anti-ICAM-1 and significantly reduced binding of anti-VCAM-1. In whole lung homogenates, intense ICAM-1 up-regulation was found (both in mRNA and in protein levels) during sepsis, whereas very little increase in VCAM-1 could be measured although some increased mRNA was found. During CLP soluble VCAM-1 (sVCAM-1) and soluble ICAM-1 (sICAM-1) appeared in the serum. When mouse dermal microvascular endothelial cells (MDMECs) were incubated with serum from CLP mice, constitutive endothelial VCAM-1 fell in association with the appearance of sVCAM-1 in the supernatant fluids. Under the same conditions, ICAM-1 cell content increased in MDMECs. When MDMECs were evaluated for leukocyte adhesion, exposure to CLP serum caused increased adhesion of neutrophils and decreased adhesion of macrophages and T cells. The progressive build-up in lung myeloperoxidase after CLP was ICAM-1-dependent and independent of VLA-4 and VCAM-1. These data suggest that sepsis disturbs endothelial homeostasis, greatly favoring neutrophil adhesion in the lung microvasculature, thereby putting the lung at increased risk of injury. (*Am J Pathol* 2004, 164:1435-1445)

Multiple organ dysfunction syndrome is a frequent complication of sepsis.¹⁻³ Despite substantial advances, septic shock when accompanied by acute respiratory distress syndrome is still the leading cause of death in clinical intensive care units with a mortality that has remained ~50%.⁴⁻⁶ It has been known for some time that pulmonary accumulation of inflammatory cells such as neutrophils and macrophages is implicated in mediating

tissue damage by releasing toxic oxygen metabolites and proteases. During the complex array of cell-cell interactions in the development of inflammatory processes, expression of cell vascular adhesion molecules plays a fundamental role leading to adhesion of leukocytes to the activated endothelium and subsequent transmigration of leukocytes into the extravascular areas.⁷⁻¹² Intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1, both members of the immunoglobulin superfamily, have been described to be up-regulated in endothelial cells by inflammatory mediators such as cytokines and bacterial lipopolysaccharide.¹³⁻¹⁵ Clinical studies have further shown that increased plasma concentrations of ICAM-1 and VCAM-1 predict development of multiple organ dysfunction syndrome and death in neonatal and adult sepsis.^{16,17} Furthermore, the observation that blockade or genetic deletion of adhesion molecules reduces the severity and sequelae of experimentally induced sepsis has underscored the importance of VCAM-1 and ICAM-1 in endothelial-leukocyte interactions during the pathogenesis of multiple organ dysfunction syndrome.¹⁸⁻²⁰ VCAM-1, in contrast to ICAM-1, is not considered to be an important mediator of neutrophil (PMN) adhesion to the endothelium, because PMNs express little very late antigen-4 (VLA-4), the ligand for VCAM-1. Recent reports have described the expression of VLA-4 on PMNs during sepsis and arthritis.^{21,22} In addition, blood neutrophils from septic but not from control patients have shown expression of $\alpha_4\beta_1$ integrin, which caused increased adhesiveness to immobilized VCAM-1.²³ A novel function for the VLA-4/VCAM-1 pathway in PMN migration during human septic conditions was suggested. The precise role of VCAM-1 in the development of organ failure during sepsis is not well defined.

To elucidate the mechanism(s) of VCAM-1 and ICAM-1 expression and their possible involvement in leukocyte recruitment into lung during sepsis, we assessed the vascular binding of ¹²⁵I-antibodies to VCAM-1 and

Supported by the National Institutes of Health (grant GM-61656) and the American Lung Association (grant RG-057-N).

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Accepted for publication December 17, 2003.

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ICAM-1 in mouse lung after cecal ligation and puncture (CLP) and changes in leukocyte adhesion to MDMECs that had been exposed to CLP serum. The data suggest that sepsis induced by CLP causes a fundamental shift in the leukocyte-endothelial adhesion system to favor the binding of PMNs to the lung vascular endothelium.

Materials and Methods

Antibodies and Other Reagents

Hamster anti-mouse ICAM-1 (CD54) monoclonal antibody (clone 3E2) and rat anti-mouse CD49d (integrin α_4 chain) monoclonal antibody were purchased from BD Biosciences Pharmingen, Inc., San Diego, CA. Rat monoclonal anti-mouse VCAM-1 (clone M/K-2) antibody was purchased from Biosource, Inc. (Camarillo, CA), rat monoclonal anti-mouse ICAM-1 (clone KAT-1) and goat anti-mouse VCAM-1 antibodies were purchased from R&D Systems, Inc. (Minneapolis, MN). Hamster and rat IgG were obtained from (Jackson Labs, West Grove, PA). Among these antibodies, hamster anti-mouse ICAM-1 was used for *in vivo* binding study and *in vitro* adhesion assay, whereas rat anti-mouse ICAM-1 was used for flow cytometric assay. Rat anti-mouse VCAM-1 was used for *in vivo* binding study, *in vivo* blocking experiments, *in vitro* adhesion assay, and flow cytometric assay, whereas goat anti-mouse VCAM-1 was used for Western blot analysis.

RPMI 1640 containing heat-inactivated fetal bovine serum, penicillin-streptomycin, Fungizone, and L-glutamine were purchased from (Life Technologies, Inc., Grand Island, NY). Endothelial cell growth supplement was obtained from (Collaborative Biomedical, Inc., Bedford, MA) and Dispace II neutral protease from (Boehringer Mannheim, Inc., Indianapolis, IN). Collagenase IV and all other reagents were obtained from (Sigma Chemical Co., St. Louis, MO).

Mouse Model of CLP

Male BALB/c-specific pathogen-free mice 24 to 28 g (Harlan, Inc., Indianapolis, IN) were used in all studies. Anesthesia was induced by intraperitoneal injection of a ketamine/xylazine/DPBS (Dulbecco's phosphate-buffered saline) solution (11 μ l/g body weight), 1 ml of ketamine containing 9% xylazine was diluted with 7 ml of DPBS. After shaving the abdomen and using a 1-cm abdominal midline incision, the cecum was identified and was ligated involving approximately two-thirds of the entire cecum. The ligated part of the cecum was then subjected to a single through and through perforation with a 21-gauge needle and then gently squeezed to ensure patency of the perforation sites. After repositioning of the bowel, the abdomen was closed in layers using a 4.0 surgical suture (Ethicon Inc., Somerville, NJ) and metallic clips. Sham animals underwent the same procedure without ligation and puncture of the cecum. For animal sacrifice, the inferior vena cava was incised and ~500 μ l of blood withdrawn. The chest was then opened and the pulmonary artery was slowly perfused with 20 ml of cold

DPBS, with perfusion liquid leaking out of the open caval vein after perfusion of the arterial system. The organ color changed to white as the blood was completely removed. Thereafter, organs were removed and either snap-frozen or used immediately for radioactivity measurement or for myeloperoxidase (MPO) activity evaluations.

In Vivo Binding Studies

Rat anti-mouse VCAM-1 IgG or normal rat IgG was labeled with 125 I using the chloramine T-based method as previously described.²⁴ This protocol involves gentle oxidation. For the binding studies, sepsis was induced by CLP and mice were sacrificed 6, 12, 18, and 24 hours thereafter. Following the protocol as described elsewhere,²⁵ organ radioactivity was compared to that found in CLP animals sacrificed at 0 hour. One μ g of 125 I-labeled anti-mouse VCAM-1 IgG-specific activity of 125 I-IgG, anti-mouse ICAM-1 IgG, or whole IgG isotype control together with 4 μ g of nonlabeled antibody (cold antibody) as a carrier in a total volume of 200 μ l of DPBS was administered intravenously 15 minutes before sacrificing the animals. At the time point indicated, blood was withdrawn from the abdominal caval vein to determine the amount of radioactivity in blood. Thereafter, lungs were thoroughly flushed with DPBS, as described above, and then weighed. Radioactivity was measured in a gamma counter (1261 Multi; Wallac Co., Gaithersburg, MD). Data were expressed as counts per minute (cpm) per g tissue, divided by the cpm in 100- μ l blood sample for each individual animal.²⁵ In these experiments, unlabeled antibody was used to mask the influence of systemic soluble ICAM-1 or VCAM-1 in the *in vivo* binding assay. The uptake of 125 I-labeled anti-ICAM-1 or anti-VCAM-1 by organs was minimal if unlabeled antibodies were not included (data not shown), suggesting that 125 I-labeled anti-ICAM-1 and anti-VCAM-1 bind to soluble ICAM-1 or VCAM-1 in circulation, thus interfering with organ binding.

Collection and Preparation of Serum Samples

Approximately 500 to 800 μ l of blood were drawn from healthy control animals or from sham or CLP animals at various time points (as indicated) after laparotomy. The blood samples were placed on ice and allowed to clot before centrifugation at 3000 $\times g$ for 10 minutes. Supernatants were collected and immediately frozen at -80°C until used for enzyme-linked immunosorbent assay (ELISA) analysis or for *in vitro* stimulation of MDMECs. Before incubation with endothelial cell monolayers, serum was diluted to 10% with sterile phosphate-buffered saline (PBS) and filtered (0.2 μ m).

MDMEC Culture

Mouse dermal microvascular endothelial cells (MDMECs) were isolated from the ear dermis of 5- to 8-week-old mice as previously described.^{26,27} These endothelial cells were used because lung microvascular endothelial cells were consistently contaminated by ~50% fibrino-

blasts (data not shown). Ears were removed, split, and incubated in Dispase II (5 mg/ml in PBS) for 45 minutes, after which the epidermis was removed and discarded. Endothelial cells were expressed from the dermal sheets and placed into growth media (RPMI media supplemented with ECGS (endothelial cell growth supplement), 20% heat-inactivated fetal calf serum, L-glutamine, streptomycin-penicillin, and Fungizone) in gelatin-coated tissue culture dishes by applying lateral pressure with the blunt end of a scalpel. Cells grown to confluence in 4 to 6 days were trypsinized and used at 90 to 95% confluence for all experimental studies (passage 1). Cultured cells were characterized by a cobblestone appearance and specific staining for von Willebrand factor (vWF) as well as flow cytometric determination of uptake of Dil-Ac-LDL (1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate) according to previously described methods.²⁸ Results described were obtained using several separate isolates of endothelial cells, as indicated for each experiment.

RNA Isolation and Detection of VCAM-1 and ICAM-1 mRNA by Semi-Quantitative Reverse Transcriptase-Polymerase Chain Reaction

Lungs (and other organs as indicated) from mice were obtained 0, 3, 6, 12, 18, and 24 hours after induction of CLP and prepared as described above. Total RNA was isolated with the Trizol method (Life Technologies Inc., Rockville, MD) according to the manufacturer's instructions. RNA integrity was determined by the use of denaturing formaldehyde agarose gel electrophoresis. Digestion of any contaminating DNA was achieved by treatment of samples with RQ1 Rnase-free Dnase (Promega, Inc., Madison, WI). Complementary DNA was prepared using 1 μ g of total RNA isolated from whole lung tissues (MDMEC monolayers). VCAM-1 and ICAM-1 primers were used to amplify specific gene products and GAPDH was used as an internal control. Reverse transcriptase-polymerase chain reaction conditions for VCAM-1, ICAM-1, and GAPDH were 94°C for 5 minutes, 26 cycles of 95°C for 1 minute, 60°C for 1 minute (ICAM-1 62°C), 72°C for 1 minute, and a final elongation step of 72°C for 10 minutes. Amplification products (10 μ l for lung products and 15 μ l for MDMEC products) were separated by 1.2% agarose gel electrophoresis and visualized by UV light.

Detection of VCAM-1 and ICAM-1 on MDMECs by Flow Cytometric Analysis

Adhesion molecules of MDMECs could be determined by flow cytometric analysis as described elsewhere.²⁹ Briefly, confluent cell monolayers (passage 1) in 12-well plates (Corning, Elmira, NY) were incubated for 6 hours at 37°C with 10% mouse serum from healthy normal mice (control) or from mice at various time points after CLP. Ultimately, cells were placed on ice, washed, and incubated with anti-VCAM-1, anti-ICAM-1, or matching IgG

isotype antibody (10 μ g/ml) in 400 μ l of staining buffer (PBS with 0.1% sodium azide and 1% fetal bovine serum) on ice for 30 minutes. Cell monolayers were then washed and incubated with a secondary fluorescein isothiocyanate-conjugated IgG antibody (10 μ g/ml) on ice for 30 minutes, protected from light. After this incubation period, exposure to trypsin/ethylenediaminetetraacetic acid occurred. Cell suspensions were centrifuged at 700 $\times$ g for 5 minutes at 4°C, washed once, and resuspended in 1% paraformaldehyde/PBS solution with 0.1% sodium azide. Cells were analyzed using a flow cytometer (Coulter, Inc., Miami, FL).

Detection of VCAM-1 Protein in MDMECs by Western Blot Analysis

MDMEC monolayers (passage 1) were treated as described, washed, and homogenized in lysis buffer (1% Triton X-100, 10 mmol/L Tris, 50 mmol/L NaCl, and complete proteinase inhibitor cocktail). Thirty μ g of protein from endothelial cell whole lysates were electrophoresed in a NuPAGE 10% Bis-Tris gel (Invitrogen, Carlsbad, CA) and then transferred to a polyvinylidene difluoride membrane. Nonspecific binding sites were blocked with TBST (pH 7.6, 300 mmol/L NaCl and 0.1% Tween 20) containing 5% nonfat dry milk overnight. Membranes were incubated with goat anti-mouse VCAM-1 antibody in a 1:1000 dilution for 2 hours at room temperature. After five washes in TBST, membranes were incubated with horseradish peroxidase-conjugated donkey anti-goat IgG secondary antibody at 1:7000 for 2 hours. The signals were visualized with the enhanced chemiluminescence technique according to the manufacturer's protocol (Amersham Pharmacia Biotech Inc., Piscataway, NJ).

Quantification of VCAM-1 and ICAM-1 by ELISA

Lung, liver, kidney, and heart were thoroughly flushed intra-arterially with sterile saline and harvested at various time points after CLP. The organs were homogenized in RIPA lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.25% deoxycholic acid, 1% Nonidet P-40, 1 mmol/L ethylenediaminetetraacetic acid). To remove membrane-bound protein, the tissue homogenates were incubated in RIPA buffer for 30 minutes before centrifugation. Measurement of VCAM-1 and ICAM-1 was performed in whole tissue homogenates, in serum or in supernatant fluids from some cultured MDMECs using ELISA kits (Biosource Intl. Inc., Camarillo, CA) according to the manufacturer's instructions.

MPO Activity

Lungs were weighed and homogenized in a homogenate buffer, 0.5% hexadecyltrimethylammonium bromide and 5 mmol/L ethylenediaminetetraacetic acid in 50 mmol/L potassium phosphate buffer, pH 6.0. The samples were sonicated for 1 minute, and then centrifuged at 20,000 $\times$ g for 15 minutes. Ten μ l of each sample was added to a

96-well plate, followed by addition of 250 μ l of assay buffer, 0.005% H_2O_2 , and 0.5 mmol/L *o*-dianisidine dihydrochloride in 100 mmol/L of potassium phosphate, pH 6.0. The change in optical density at 460 nm was measured throughout a period of 6 minutes at 15-second intervals, using a kinetics mode in a spectrophotometer (Molecular Devices, Inc., Sunnyvale, CA). The slope of the change in optical density was calculated to reflect the rate of change in units per g of lung per minute. All samples were diluted one to five to guarantee a linear response.

Isolation and Preparation of Mouse Peritoneal Neutrophils and Macrophages and Murine EL4 T-Lymphocyte Cell Line for Cell Adhesion Assays

For isolation of peritoneal neutrophils and macrophages, BALB/c mice were injected intraperitoneally with 3 ml of 2.4% thioglycollate solution (Difco Laboratories, Detroit, MI) for either 5 hours (neutrophils) or 4 days (macrophages). After mice were sacrificed, a 1- to 2-cm patch of skin was dissected from the abdominal muscles. Ice-cold DPBS (3 $\times$ 10 ml) was injected intraperitoneally; the peritoneal lavage fluid was then collected and kept at 4°C. Fluids were spun down and washed. This isolation procedure yielded greater than 95% macrophages and neutrophils as demonstrated by cyto-spin and differential stain analysis. After hypotonic lysis of residual red blood cells, cells were resuspended in Hanks' balanced salt solution and fluorescein-labeled with BCECF (2',7'-bis(2-carboxyethyl)-5-(and 6)-carboxy-fluorescein acetoxymethyl ester) (Molecular Probes, Inc., Eugene, OR) for 30 minutes at 37°C. The murine T-lymphocyte cell line EL-4 (C57BL/6N) was purchased from the American Type Culture Collection (Manassas, VA) and cultured according to the manufacturer's instructions. T cells were also labeled with BCECF as described above.

Leukocyte Adhesion Assays

MDMECs were grown to confluence (passage 1) in 48-well plates (Corning, Elmira, NY). Monolayers were exposed for 6 hours to 10% serum from 6-hour CLP mice. Cells were then washed and preincubated with 20 μ g/ml of anti-ICAM-1 and anti-VCAM-1 IgG or isotype control antibody at 37°C for 15 minutes. Thereafter, supernatant fluids were removed and the monolayers incubated together with fluorescent labeled 1×10^6 PMN/well, 1×10^6 macrophages/well, or 4×10^6 T cells/well for 20 minutes. Nonadherent cells were washed away and remaining cells were counted. The number of adherent cells was determined by cytofluorometry (Molecular Devices, Inc.). For each measurement, triplicate samples were used. The profiling of adhesion molecules on peritoneal neutrophils and macrophages elicited by thioglycollate may be different from those on blood neutrophils and macrophages. Thus, the adhesion experiments were always controlled with nontreated cells.

Statistical Analyses

All values are expressed as mean $\pm$ SEM. Data sets in groups with equal variances were analyzed using one-way analysis of variance. Individual group means were then compared with the Student-Newman-Keuls multiple comparison test. In groups containing unequal variances, Kruskal-Wallis analysis of variance was performed followed by Dunnett's method for multiple comparisons. Significance was assigned where $P < 0.05$.

Results

In Vivo Lung Binding of 125 I-Anti-VCAM-1 and 125 I-Anti-ICAM-1 after CLP

To evaluate changes in VCAM-1 lung expression during sepsis, 1 μ g of 125 I-anti-VCAM-1 together with 4 μ g of unlabeled antibody were injected intravenously into mice 0, 6, 12, 18, and 24 hours after CLP, with a dwell time of 15 minutes before sacrificing the animals. The unlabeled antibody was used to mask the blocking of the antibody by the presence in serum of soluble ICAM-1 or VCAM-1. For control animals, 1 μ g of 125 I-normal IgG (125 I-IgG) together with 4 μ g of unlabeled IgG were administered at the same time points after CLP as described above. After sacrifice the lungs were extensively perfused with 30 ml of sterile saline to remove unbound 125 I-IgG. As shown in Figure 1A, 125 I-anti-VCAM-1 binding to lung was significantly decreased (by $\sim 50\%$) in the early phase of sepsis, reaching a nadir at 6 hours ($P = 0.028$), when compared to the values from animals obtained at time 0. By 12 and 18 hours was a gradual return to normal values. In contrast, no significant changes in 125 I-normal IgG binding in lung could be observed 6 hours after CLP when compared with binding at 0 hour. The binding of 125 I-normal IgG was $\sim 1\%$ of that found for 125 I-anti-VCAM-1 binding (data not shown). Similar technology was used to assess lung binding of 125 I-anti-ICAM-1. In contrast to decreased VCAM-1 binding, Figure 1B demonstrates that 125 I-anti-ICAM-1 binding to lung was significantly increased (by $\sim 50\%$) 6 hours after CLP, suggesting differential pulmonary vascular content of VCAM-1 and ICAM-1 during the early phase of sepsis. Binding of the radiolabeled isotype control for ICAM-1 was very low being $< 1\%$ of the binding of antibody to ICAM-1 (data not shown).

The decrease in binding of 125 I-anti-VCAM-1 and the increase in binding of 125 I-ICAM-1 to lung 6 hours after CLP correlated with the increased serum levels of soluble mouse VCAM-1 from 6 to 24 hours after CLP (Figure 1C) and a less dramatic increase in soluble ICAM-1 (sICAM-1 in serum) at 18 and 24 hours (Figure 1D). In addition, the VCAM-1 mRNA was increased in lung 3 and 6 hours after CLP and then appeared to return to the control levels, whereas the up-regulation of ICAM-1 mRNA appeared 6 hours after CLP and was maintained at 24 hours (Figure 1E). Although serum levels of sICAM-1 increased at 18 hours after CLP, lung levels of ICAM-1 did not fall at this time point (Table 1), suggesting that ICAM-1 shedding co-exists with ICAM-1 generation in lung during sepsis.

Figure 1. *In vivo* binding of ^{125}I -rat monoclonal antibody anti-mouse VCAM-1 (A) and ^{125}I -hamster monoclonal antibody anti-mouse ICAM-1 (B) to mouse lung tissue at times indicated after CLP-induced sepsis. Binding is expressed as the ratio of cpm per g of flushed organ to cpm in 100 μl of blood obtained 15 minutes after intravenous injection of ^{125}I -anti-VCAM-1 or ^{125}I -anti-ICAM-1. C and D: ELISA measurements for sVCAM-1 (C) and sICAM-1 in serum after CLP (D). In each case 1 μg of ^{125}I -antibody IgG and 4 μg of unlabeled antibody were injected. Binding of ^{125}I -irrelevant IgG was <1% the binding of anti-ICAM-1 or anti-VCAM-1 (see text). The data are representative of five to eight animals per group. *, $P < 0.05$; **, $P < 0.001$, represented as statistically significant changes between control and CLP-treated groups. E: VCAM-1 and ICAM-1 mRNA expression in lung, as a function of time after CLP, by reverse transcriptase-polymerase chain reaction. The data are representative of three separate experiments.

VCAM-1 and ICAM-1 Expression in Organs During Sepsis

To determine changes in VCAM-1 and ICAM-1 protein in organs (lungs, kidneys, liver, heart) during sepsis, VCAM-1 and ICAM-1 concentrations in organ homogenates were analyzed by ELISA. As shown in Table 1, basal levels of ICAM-1 were 10-fold to 40-fold greater than those of VCAM-1. VCAM-1 levels were not signifi-

cantly changed in lung after CLP, whereas they were increased significantly in kidney, liver, and heart. In contrast, ICAM-1 expression was up-regulated in all four organs after CLP.

Changes in VCAM-1 and ICAM-1 Expression on MDMECs Exposed to CLP Serum

VCAM-1 and ICAM-1 presence was evaluated quantitatively by flow cytometric analysis on MDMECs after incubation at 37°C for 6 hours with 10% serum obtained 0, 3, 6, 12, or 18 hours from CLP mice (Figure 2). MDMECs demonstrated constitutive levels of VCAM-1 and ICAM-1. VCAM-1 content of MDMECs exposed to serum from 3-, 6-, and 12-hour CLP mice declined significantly, with much less change after exposure to 18-hour CLP serum (Figure 2, A and B). In contrast, ICAM-1 content of MDMECs exposed to serum from CLP mice at the various time points increased early (3 hours after CLP) and remained constantly elevated when monolayers were exposed to serum from 6-, 12-, and 18-hour CLP mice (Figure 2C). Typical flow cytometry histograms for VCAM-1 and ICAM-1 content of MDMECs are shown in Figure 2, B and D. The isotype control IgG showed little or no staining of MDMECs. After exposure to 6 hours of CLP serum, VCAM-1 content of MDMECs declined to an average mean channel fluorescence of 4.4 ± 0.16 , from 7.6 ± 0.68 (Figure 2B). In contrast, in MDMECs exposed to 6 hours of CLP serum, the ICAM-1 content increased from a mean channel fluorescence value of 5.9 ± 0.51 to 13.8 ± 0.45 (Figure 2D). These data indicate that VCAM-1 expression on MDMECs is decreased after exposure to CLP serum whereas the opposite occurs with respect to ICAM-1 content, suggesting the possibility of altered expression of these adhesion molecules on microvascular endothelium during CLP induced sepsis.

Shedding of VCAM-1 (but Not ICAM-1) from MDMECs Exposed to Septic Serum

To investigate further the loss of VCAM-1 expression on MDMECs after exposure to serum from CLP mice (Figure 2), sVCAM-1 content was measured in supernatant fluids of MDMEC monolayers after a 6-hour exposure to 10% serum obtained 0 to 24 hours after CLP (Figure 3).

Table 1. Adhesion Molecule Expression (ng/ml) by ELISA During Sepsis

	Lung	Kidney	Liver	Heart
VCAM-1				
Ctrl	114.9 $\pm$ 9.1	47.1 $\pm$ 4.7	11.4 $\pm$ 1.9	10.4 $\pm$ 1.5
6 h	130.8 $\pm$ 10.6	67.6 $\pm$ 13.2*	21.3 $\pm$ 7.5	16.2 $\pm$ 2.7*
12 h	113.8 $\pm$ 11.9	66.1 $\pm$ 7.1*	21.7 $\pm$ 3.8	14.9 $\pm$ 1.4*
18 h	118.3 $\pm$ 10.3	62.6 $\pm$ 11.4*	25.7 $\pm$ 5.3*	15.2 $\pm$ 1.9*
ICAM-1				
Ctrl	4052 $\pm$ 250	640 $\pm$ 75	284 $\pm$ 53	127 $\pm$ 94
6 h	4767 $\pm$ 326*	783 $\pm$ 122	712 $\pm$ 131†	117 $\pm$ 33
12 h	4946 $\pm$ 298*	955 $\pm$ 147	710 $\pm$ 109†	139 $\pm$ 28
18 h	5057 $\pm$ 54*	992 $\pm$ 304*	683 $\pm$ 119†	180 $\pm$ 33†

* $P < 0.05$;
† $P < 0.001$.

Figure 2. Changes in ICAM-1 and VCAM-1 content on MDMECs after exposure to serum obtained from mice at the times indicated after CLP-induced sepsis. Confluent MDMEC monolayers (passage 1) were exposed for 6 hours at 37°C to 10% serum harvested from healthy normal mice (0 hour) or from mice after induction of CLP at various time points as indicated. ICAM-1 and VCAM-1 content on MDMECs was quantitatively determined by flow cytometric analysis using mean channel fluorescence (MDF). **A:** Time course of VCAM-1 content on MDMECs stimulated with CLP serum harvested at 6, 12, 18, and 24 hours after onset of sepsis. All values are the mean $\pm$ SEM and were run in duplicate ($n = 3$ for each data point). *, $P < 0.05$. Statistical comparisons are to the control group (0 hour) at each interval of time. **B:** Typical histogram for VCAM-1 staining of MDMECs with isotype control IgG, normal serum, and CLP serum (6-hour CLP) incubated 6 hours with MDMECs. **C:** Time course of ICAM-1 content on MDMECs similarly incubated and treated. All values are the mean $\pm$ SEM ($n = 3$ for each data point). Statistical comparisons were to the control group (0-hour serum sample) for each interval of time. **D:** Typical histogram for ICAM-1 staining of MDMECs exposed for 6 hours to normal serum or serum.

sVCAM-1 levels obtained in supernatant fluids from MDMECs after exposure to normal mouse serum were subtracted from the data in frame A. Basal sVCAM-1 levels from MDMECs exposed to 10% normal serum were low (8.0 ± 1.4 ng/ml). MDMECs exposed to 6-hour and 12-hour CLP serum showed significantly increased sVCAM-1 levels in supernatant fluids. In cells exposed to 18-hour and 24-hour CLP serum, sVCAM-1 levels in the supernatant fluids were in the same range found when MDMECs were cultured with normal mouse serum (time 0). sICAM-1 levels obtained in supernatant fluids from MDMECs after exposure to serum from septic mice were not higher than that in medium controls (containing 10% serum), suggesting there is little measurable shedding of ICAM-1 into supernatant fluids of MDMECs (data not shown).

To evaluate changes in VCAM-1 content in MDMECs, VCAM-1 protein from whole endothelial cell lysates was assessed by Western blot analysis (Figure 3B). Before the lysis step, MDMEC monolayers were exposed to serum from normal mice (0 hour) or to serum from mice 6 and 12 hours after CLP. There was a sharp reduction in VCAM-1 protein in MDMECs exposed to 10% mouse serum obtained 6 hours after CLP, but robust evidence of VCAM-1 was found in MDMECs exposed to 0- or 12-hour CLP serum.

A. ELISA of sVCAM-1

B. Western Blots from MDMEC Stimulated with 10% Mouse Serum after CLP [h]

Figure 3. Appearance of sVCAM-1 in supernatant fluids of MDMECs exposed to 6 hours of CLP serum. Cell monolayers were incubated for 6 hours at 37°C with 10% serum obtained from mice at various time points after induction of CLP-induced. **A:** ELISA measurements for sVCAM-1 in supernatant fluids of MDMECs. *, $P < 0.05$, when compared to results using 0-hour serum. **B:** Effects of exposure of MDMECs to normal serum (0 hour) or serum from 6-hour and 12-hour CLP mice on MDMEC VCAM-1 protein in whole cell lysates, as determined by Western blot measurements. Data are representative of two independent and separate experiments.

Effects of Anti-ICAM-1 and Anti-VCAM-1 on Lung MPO after CLP

To extend the data described above in the context of neutrophil sequestration in lung during CLP-induced sepsis, lung MPO levels were assessed at various time points after the onset of CLP (Figure 4). As would be expected, basal MPO activity in control lungs (0 hour) was low, with an optical density (460 nm) value of 8.0 ± 0.9 . After CLP there was a rapid increase in MPO by 3, 6, and 12 hours, with peak levels of approximately ninefold greater than control values. In contrast, MPO activity in lungs from sham-operated animals harvested 6 hours after laparotomy did not change significantly when compared to baseline values (data not shown). MPO levels remained significantly altered 12 hours after CLP and then declined progressively thereafter.

To investigate the possible roles of VLA-4, VCAM-1, or ICAM-1 in MPO buildup in lung after CLP, we performed MPO measurements on lungs 6 hours after CLP in mice were treated (at time 0) with the indicated antibodies to each of the three proteins. IgG antibodies (each at 50 μ g)

Figure 4. Lung MPO content after CLP-induced sepsis in mice. MPO activity was measured in lungs harvested from normal healthy animals (0 hour) or from animals at various time points after induction of CLP (6, 12, 18, and 24 hours). The data are representative of four to six animals per group. *, $P < 0.05$, statistically significant changes between 0-hour and CLP groups.

were injected intravenously immediately after CLP and animals were sacrificed 6 hours later. Intravenous injection of anti-VCAM-1 or antibody to its ligand, VLA-4, did not block the increase in MPO activity when compared to CLP animals that received isotype control antibody (Figure 5, A and B). However, as expected, anti-ICAM-1 reduced significantly the amount of MPO activity at this time point, indicating an ICAM-1 dependence on neutrophil sequestration in the lung 6 hours after CLP (Figure 5C).

Effects of Anti-VCAM-1 and Anti-ICAM-1 on Neutrophil, Macrophage, or T-Cell Adhesion to MDMECs Exposed to CLP Serum

As summarized in Figure 6, we examined adhesion of mouse peritoneal PMNs (Figure 6A), peritoneal macrophages (Figure 6B), or EL-4 T cells (Figure 6C) to MDMEC monolayers exposed for 6 hours to either 10% normal mouse serum or 10% serum obtained 6 hours after CLP, in the absence or presence of antibodies to VCAM-1 and ICAM-1. Neutrophil adhesion to MDMECs exposed to normal (ctrl) serum could be reduced by the presence of anti-ICAM-1 (10 μ g/ml) but not by similar concentrations of anti-VCAM-1 (Figure 6A). MDMECs exposed to 6-hour CLP serum showed significantly increased neutrophil adhesion when compared to control levels (Figure 6A). In the presence of anti-ICAM-1 (but not anti-VCAM-1), there was significantly reduced PMN cell adhesion, suggesting that the increased neutrophil adhesion to MDMECs after exposure to CLP serum is ICAM-1-dependent and VCAM-1-independent (Figure 6A).

Macrophage adhesion to MDMEC monolayers incubated with normal serum was reduced by addition of anti-VCAM-1, but not significantly after additions of anti-ICAM-1 (Figure 6B). In contrast to neutrophil adhesion, MDMECs exposed to 6-hour CLP serum demonstrated substantially decreased macrophage adhesion (60%, $P < 0.05$) when compared to MDMECs exposed to normal serum, and addition of antibody (anti-ICAM-1 or anti-VCAM-1) did not further reduce macrophage adhesion, consistent with the data presented above indicating that

A. Blockade of VLA-4

B. Blockade of VCAM-1

C. Blockade of ICAM-1

Figure 5. Effect of anti-VCAM-1, anti-VLA-4 or anti-ICAM-1 on MPO activity in lungs harvested from mice at 6 hours after the induction of CLP. As indicated, CLP animals received 50 μ g of isotype control antibody [ctrl IgG (A–C)], anti-VCAM-1 (A), anti-VLA-4 (B), or anti-ICAM-1 (C), which were given intravenously immediately after CLP. MPO content in normal healthy lungs was arbitrarily set at a value of 1.0. For each vertical bar, $n = 5$ to 8 animals for each time point.

exposure to MDMECs to 6-hour CLP serum causes loss of VCAM-1. The basis for the residual macrophage adhesion, which was both ICAM-1- and VCAM-1-independent, is not known.

Figure 6. Effect of anti-VCAM-1 and anti-ICAM-1 on neutrophil (A), macrophage (B), and T-cell (EL-4 cells) (C) adhesion to MDMECs exposed to serum from normal mice or mice 6 hours after CLP. MDMEC monolayers (passage 1) were exposed for 6 hours at 37°C to either 10% serum from normal healthy mice (ctrl) or 10% serum harvested from animals 6 hours after CLP. After stimulation, monolayers were incubated with 1×10^6 /ml neutrophils (A), 1×10^6 /ml macrophages (B), or 4×10^6 /ml EL-4 T cells (C) in the absence or presence of antibodies (20 μ g/ml) to ICAM-1 or VCAM-1 for 20 minutes at 37°C. Thereafter, the number of adhering cells was determined by cytofluorometry. Each condition was done in triplicate. Cell adhesion to MDMECs stimulated with control serum was arbitrarily set at a value of 100%. For each vertical bar, data represent results from three independent and separate experiments.

To investigate changes in lymphocyte adhesion to MDMECs after stimulation with CLP serum, the murine EL-4 T-cell line was used, because these cells are interactive with CAMs.^{30,31} When these cells were incubated with MDMECs that had been exposed to normal serum, there was substantial cell adhesion that was markedly reduced by addition of antibody to either VCAM-1 or ICAM-1 (Figure 6C). In contrast to assays with neutrophils or macrophages, T-cell adhesion to endothelial monolayers were greatly decreased after MDMECs had been exposed to 6-hour CLP serum. Under these conditions, subsequent treatment with anti-ICAM-1, but not with anti-VCAM-1, further reduced T-cell adhesion. Collectively, these data suggest that T-cell adhesion to MDMECs is both ICAM-1- and VCAM-1-dependent, the latter being sensitive to MDMEC contact with CLP serum.

Discussion

The principle objective of this study was to evaluate the lung vascular adhesion molecules, VCAM-1 and ICAM-1, with respect to their distribution and function during CLP-induced sepsis. Binding of 125 I-anti-VCAM-1 antibody to lung was significantly depressed 6 hours after CLP, correlating with increased sVCAM-1 levels in serum (Figure 1). The elevated blood levels of sVCAM-1 could interfere with the *in vivo* binding of 125 I-labeled anti-VCAM-1 to lung vasculature. A high dose of 1 μ g of radiolabeled antibody and 4 μ g of unlabeled antibody were used to reduce the interference by sVCAM-1 of binding of 125 I-anti-VCAM-1 to the vasculature. As shown in Figure 1, decreased binding of 125 I-anti-VCAM-1 to lung was found 6 hours after CLP, suggesting a decreased expression of VCAM-1 on the lung vasculature. The reduced binding of 125 I-anti-VCAM-1 gradually returned to normal levels 12 and 24 hours after CLP. In contrast, binding of 125 I-anti-ICAM-1 antibody to lung was increased 6 hours after CLP. Evidence for increased ICAM-1 expression in lung after CLP was confirmed by ELISA using lung homogenates (Table 1). Decreased VCAM-1 protein was detected in whole cell lysates of MDMECs exposed to CLP serum, correlating with shedding of VCAM-1 into cell culture fluids (Figures 2 and 3). Both ICAM-1 and VCAM-1 were transcriptionally up-regulated in lung during sepsis (Figure 1). However, only ICAM-1 protein in lung was shown to be elevated (Figure 1, Table 1). Exposure of endothelial cells to septic serum caused VCAM-1, but not ICAM-1, shedding. These data suggest that endothelial ICAM-1 and VCAM-1 are in a dynamic state during sepsis.

As expected, blockade of VCAM-1 and VLA-4 did not reduce lung neutrophil accumulation as measured by MPO activity after CLP, but MPO levels declined when anti-ICAM-1 was administered to CLP mice (Figure 5). Blockade of CD18 has been shown to reduce MPO build-up in lung in the same model of sepsis,³² underscoring the importance of ICAM-1 pathway in neutrophil migration during sepsis. Adhesion assays with MDMECs exposed to CLP serum in the absence or presence of anti-VCAM-1 and anti-ICAM-1 revealed that neutrophil

adhesion to MDMECs was ICAM-1- but not VCAM-1-dependent; macrophage adhesion was dependent on VCAM-1 but not ICAM-1; and T-cell adhesion was both VCAM-1- and ICAM-1-dependent (Figure 6). Taken together, these observations suggest that CLP-induced sepsis results in decreased lung vascular VCAM-1 and increased ICAM-1 expression, favoring PMN adhesion. PMN accumulation in lung could be blocked by anti-ICAM-1 (but not by anti-VCAM-1), and PMN adhesion to MDMECs exposed to CLP serum was ICAM-1- but not VCAM-1-dependent. It seems clear that neutrophil trafficking into CLP lungs is dependent on ICAM-1 and not VCAM-1. Decreased lung expression of VCAM-1 early after CLP may lead to delayed emigration of other inflammatory cells such as lymphocytes and monocytes/macrophages to the site of inflammation in the lung. It is possible that this could compromise both innate and adaptive immune responses facilitated by monocytes and lymphocytes.

The recruitment of circulating leukocytes to the sites of activated endothelium and subsequent pulmonary sequestration of leukocytes may be important in the pathogenesis of acute lung injury, including acute respiratory distress syndrome. Besides integrins and selectins,^{7,32} the cell adhesion molecules, ICAM-1 and VCAM-1, have been linked to leukocyte trafficking in various animal models of inflammation. In rats, up-regulation of hepatic ICAM-1 and VCAM-1 was associated with accumulation of neutrophils (after 3 hours) and monocytes (after 30 hours) after infusion of endotoxin.¹⁹ Up-regulation of ICAM-1 has been noted in distant tissues after hepatic ischemia/reperfusion.¹⁸ Monoclonal antibodies to ICAM-1 have been effective in decreasing pulmonary PMN accumulation in models of lung injury after hyperoxia, deposition of IgG immune complexes, and subsequent to hind limb ischemia/reperfusion.^{33–35} Mice genetically deficient in ICAM-1 are resistant to septic shock induced by endotoxin.²⁰ Enhanced pulmonary expression of VLA-4 and ICAM-1 and increased circulating soluble forms of VCAM-1 and ICAM-1 have been found in septic patients, correlating with multiorgan failure and death.^{17,36} Recently, increased expression of VLA-4 has been reported on PMNs from septic patients suggesting that a VCAM-1/VLA-4 pathway for PMN trafficking may be engaged in human sepsis.²³

Therapeutic strategies that target VCAM-1 have resulted in controversial outcomes in animal models of systemic inflammation.^{37,38} In mice with LPS-induced shock, anti-VCAM-1 therapy attenuated by 60% PMN accumulation in liver and reduced hepatic necrosis.³⁹ Anti-VCAM-1 therapy also reversed hypotension and the mortality rate and reduced intestinal PMN accumulation in a murine model of splanchnic artery ischemia/reperfusion.^{40,41} However, widespread induction of vascular VCAM-1 also occurs in the absence of tissue accumulation of PMNs in experimental sepsis in mice.⁴² VCAM-1 expression also occurs in endotoxemic myocardial dysfunction and appears to be independent of PMN accumulation,⁴³ suggesting that VCAM-1 expression is often dissociated from PMN accumulation. The present study indicates that PMN buildup in lung after CLP is ICAM-1-

dependent. ICAM-1-dependent and VCAM-1-independent adhesion of PMN and T cells to MDMECs exposed to CLP serum was observed. Anti-ICAM-1 reduced PMN accumulation in CLP lung, but antibodies to VCAM-1 or to VLA-4 did not. Our studies suggest that increased ICAM-1 expression is important in PMN accumulation in lung after CLP. ¹²⁵I-monoclonal antibodies have proven to be valuable tools for quantifying the *in vivo* expression of other cell adhesion molecules such as ICAM-1⁴⁴ and P-selectin⁴⁵ in different models of acute inflammation. This highly sensitive method allows precision not previously attained using immunostaining methods for VCAM-1 in CLP-inflamed lungs, especially in the presence of constitutive expression of VCAM-1 and ICAM-1 in lung.⁴⁶ A recent study describes the lack of immunostaining for VCAM-1 in two models of acute pulmonary inflammation.⁴⁷ Normal murine lungs showed VCAM-1 expression in arteries, arterioles, venules, and veins, but not in capillaries. In our study, VCAM-1 and ICAM-1 expression could be detected on primary cultures of MDMECs. Moreover, we were able to demonstrate differences in expression of VCAM-1 and ICAM-1 content after MDMEC exposure to CLP serum, correlating with reduced *in vivo* binding of ¹²⁵I-anti-VCAM-1 to lung. Investigating the role of the microvascular endothelium in VCAM-1- and ICAM-1-dependent lymphocyte-endothelial interactions was of interest, because, in contrast to postcapillary venules in the systemic circulation, pulmonary capillaries are known to be, the major site of PMN emigration.⁴⁸ Significant changes in lung binding of anti-VCAM-1 and anti-ICAM-1 occurred during sepsis as described in this report.

We postulate that decreased VCAM-1 expression during experimental sepsis is because of shedding of VCAM-1 from the microvascular endothelium because we demonstrated decreased VCAM-1 in the vasculature after CLP and on MDMECs after exposure to CLP serum, although the nature of the factor present in CLP serum is entirely unknown. In parallel to observations in human with sepsis,¹⁷ we detected elevated serum levels of VCAM-1 in CLP mice. Similar results have been described after inflammatory lung injury caused by abdominal sepsis, using the ascending colon in the stent peritonitis model (CASP).⁴⁹ In the CASP model, induction of ICAM-1 and VCAM-1 mRNA was weak or not detectable in lung after induction of peritonitis. In contrast, when gene expression of VCAM-1 was evaluated in different organs during CLP-induced sepsis, up-regulation of VCAM-1 expression occurred in pulmonary microvascular endothelial cells.⁴⁷

Down-regulation of VCAM-1 expression has been described in other conditions. It has recently been reported that endothelial expression of VCAM-1 and ICAM-1 on human umbilical vein endothelial cell monolayers is significantly decreased by exposure to plasma obtained from patients after coronary bypass procedures.⁵⁰ These studies are consistent with the decrease in VCAM-1 expression being related to shedding, which may restrict migration of lymphocytes and monocytes to the focus of inflammation. The marked accumulation in lung of PMNs may put the lung at risk of injury.

In summary, the current study provides evidence for decreased lung VCAM-1 expression during CLP-induced sepsis and increased expression of ICAM-1. The resulting accumulation of PMN in lung is dependent on ICAM-1 but not on VCAM-1 and VLA-4. Accordingly, sepsis alters vascular adhesion molecules in the lung in a manner that favors PMN accumulation. The changes noted would also likely reduce the influx of monocytes and lymphocytes, as has been suggested elsewhere⁵⁰ and put the lung at added risk of injury because of the reduced ability to recruit lymphocytes and monocytes, which may be critical for optimal adaptive immune responses.

Acknowledgments

We thank Beverly Schumann and Peggy Otto for secretarial assistance.

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