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Soluble E-Selectin is Found in Supernatants of Activated Endothelial Cells and Is Elevated in the Serum of Patients with Septic Shock[1]

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Abstract. A quantitative sandwich ELISA for E-selectin in the fluid phase (soluble E-selectin, sEs) has been developed that is sensitive to 100 pg/ml. The assay shows no reactivity with either L- or P-selectins. We have used this to determine the fate of E-selectin after cell-surface expression and to test whether levels measured in vivo may represent the state of endothelial activation. E-selectin was first detectable in supernatants of IL-1-stimulated endothelial cells at 24 h, and increased slowly up until 72 h. However, over this time period the total E-selectin detectable in the system (cells plus supernatants) declined dramatically. ^{125}I-surface-labeled endothelial cells cultured for 24 h show an E-selectin of reduced m.w. in the supernatant, indicating that the molecule is shed from the surface. The shed form also appears to be slightly smaller than the intact membrane form as determined from immunoprecipitation and molecular sieving studies. In addition, the cytoplasmic domain of the molecule found in supernatants of activated endothelial cells and in serum is not intact as determined by loss of reactivity with an antipeptide antibody specific for the cytoplasmic domain. We have examined the sera of 71 normal individuals. Without exception, sEs was found in serum in the range of 0.13 to 2.8 ng/ml, suggesting that even in the absence of overt inflammatory processes E-selectin is being synthesized and released into the bloodstream. In addition, bacteremic patients with hypotension, but not those without, showed markedly elevated sEs values. As determined by cell-binding studies, the blood-derived form of E-selectin is biologically active. *Journal of Immunology,* 1993, 150: 644.

The complexity of the inflammatory process is only beginning to be understood in molecular terms, but it seems clear that endothelial cells play a very active role in its amplification and maintenance. In part this is caused by the inducible nature of certain leukocyte adhesion proteins that appear on the luminal surface in response to a variety of stimuli including endotoxin, IL-1, IFN-γ, TNF-α, thrombin, and IL-4 (reviewed in References 1 and 2). Of particular interest to us is the ELAM-1/E-selectin molecule, a member of the selectin family with a lectin-like N-terminal domain (3) capable of

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recognizing the tetrasaccharide sialyl-Lewis^x or sialyl-Lewis^a of monocytes and granulocytes (4–7). E-selectin also interacts via an as yet unknown ligand with the memory CD4 subpopulation of T cells (8, 9). The other members of the selectin family include gp90^{mel} (L-selectin) and GMP-140/PADGEM (P-selectin, CD62) (reviewed in Reference 10). Our interest in E-selectin has extended to a consideration of its fate subsequent to placement in the plasma membrane. It was shown early on that endothelial cells induced to express E-selectin by stimulation with cytokines peaked in their expression at 4 to 6 h, and that by 24 h the molecule was no longer detectable on the cell surface (11). These findings raised the possibility that at least some of the E-selectin may be shed. A precedent for this is seen with the L- and P-selectins, which by distinct mechanisms have been shown to exist in soluble forms. If a soluble form of E-selectin could be demonstrated, it would allow us to evaluate the physiologic and clinical significance of detectable blood levels of E-selectin. One attractive feature of E-selectin is that since it is expressed only by activated endothelial cells and not other cell types, its presence in serum should reflect the state of endothelium in disease. We have begun to provide answers to these questions with a sandwich ELISA we have developed. These observations provide a basis for a more extensive analysis of the levels of E-selectin in patients with infectious and noninfectious inflammatory disorders. Preliminary studies in patients with bacteremia suggests that this assay may give us new insights into the state of endothelium during this disease process.

Materials and Methods

Reagents

mAb 3B7 and 7A9 to E-selectin have been previously described (12). These antibodies have been shown to react with E-selectin somewhere within the lectin-epidermal growth factor region (Walter Newman, unpublished observations). A synthetic peptide 32 mer of the cytoplasmic domain of the E-selectin molecule was synthesized and its sequence confirmed with published sequence data (3). It was conjugated to keyhole limpet hemocyanin by cross-linking lightly with glutaraldehyde, and used to immunize rabbits. The Ig fraction from multiply boosted animals was obtained after ammonium sulfate fractionation by anion exchange chromatography on a DEAE-HPLC column. This antibody was conjugated with biotin using NHS biotin as described below. In addition, a polyclonal antibody to the truncated ELAM-1–420 molecule was made in rabbits. ELAM-1–420 is described below. The Ig fraction from preimmune animals was purified and used as a control, and the Ig fraction from multiply boosted animals was also prepared as described above. This immune serum has no blocking activity in E-selectin-dependent adhesion assays. RIA grade BSA was purchased from Sigma, St. Louis, MO; OPD tablets were purchased from Zymed, South San Francisco, CA; NHS[3]-biotin was purchased from Pierce, Rockford, IL; and Affigel-10 was purchased from Bio-Rad, Richmond, CA. Seroclear was purchased from Calbiochem, La Jolla, CA. Nunc Maxisorp ELISA plates were purchased from PGC Scientific, Gaithersburg, MD. The Leu-8 mAb was purchased from Becton Dickinson (Mountain View, CA) and the TQ1 mAb was purchased from Coulter, Hialeah, FL. Superose 12 FPLC column (1 cm × 30 cm) and a gel filtration calibration kit were purchased from Pharmacia, Piscataway, NJ.

Cell culture

Endothelial cells were prepared from individual human umbilical cords and maintained as previously described except that in some cases cells were established and maintained in 10% heparinized human plasma of normal volunteers. Cells were not used after the fourth passage. Viability of cells at various time points was measured by the ethidium bromide/acridine orange technique of Lee et al. (13) rather than the less precise trypan blue technique. Confluent cultures of endothelial cells were cultured in gelatin-coated 35-mm tissue culture dishes in the absence or presence of 1 ng/ml of IL-1β for the indicated time periods. For measurement of E-selectin in soluble form, one dish was used at each time point as a source of supernatant and cells, such that E-selectin values were cumulative. Duplicate plates were set up and harvested at the indicated times by trypsinization to assess viability and cell number. Supernatants were harvested and passed through 0.2-μm filters, then centrifuged for 2 h at 100,000 × g. Monolayers were rinsed twice with D-PBS and lysed with Triton X-100 as previously described (12). Metabolic labeling of endothelial cells with [^{35}S]cysteine and immunoprecipitation was performed as previously described (12). Lactoperoxidase catalyzed iodination of endothelial cells was performed as follows. After 4 h of activation of confluent human umbilical vein endothelial cells with IL-1, cells were detached with EDTA, rinsed twice by centrifugation in PBS, and resuspended in 1 ml of D-PBS. To this was added 2.5 mg of lactoperoxidase, 1 mCi ^{125}I and H_2O_2 for 5 min. The reaction was terminated with 10 mM tyrosine HCl in D-PBS. The cells were washed twice, lysed with 0.5 ml 1% Triton X-100, centrifuged to remove debris, and the supernatant was used for immunoprecipitation. Nylon wool nonadherent T cells were prepared from the heparinized blood of normal donors as previously described (12). Platelets were obtained from blood of normal donors anticoagulated with citrate/

[3] Abbreviations used in this paper: NHS, normal human serum; sEs, soluble E-selectin; CHO, Chinese hamster ovary; D-PBS, Dulbecco's PBS; PCR, polymerase chain reaction.

glucose and centrifuged 200 × *g* for 20 min at room temperature to obtain platelet-rich plasma. The platelet preparation was fractionated by gel filtration at room temperature with Sepharose CL-2B (Sigma). Platelets were rinsed twice by centrifugation with PBS and lysed at 10^7/ml in 1% Triton X-100. HL60 cells were obtained from American Type Culture Collection (Bethesda, MD) and were shown to possess L-selectin by virtue of staining with the Leu-8 and TQ1 mAb in flow cytometry.

Blood donors

Preliminary studies to evaluate the best form of blood collection were performed on blood samples from normal laboratory personnel with informed consent. Blood was drawn into vacutainer tubes for serum collection or for anticoagulation by heparin, EDTA, or citrate buffers. Serum was collected at a 1-h clotting time and used immediately, plasma tubes were spun, and plasma was collected and used immediately. Both EDTA and citrate, which suppresses values, and heparin, which elevated values, appear to interfere in the E-selectin ELISA. Therefore, we have chosen to perform all our studies with serum. The sEs content of serum is unaffected by storage at 4°C for 7 days or by five cycles of freeze-thaw. Serum values for sEs are unaffected by prior centrifugation for 2 h at 100,000 × *g*. Serum samples from 71 normal blood donors aged 21 to 58 (median 39, mean 39) were obtained and stored at −20°C. There were 36 males and 35 females. These donors were selected randomly from donors to the Blood Bank at the Mayo Clinic (Rochester, MN). Informed consent was obtained. Blood was drawn into serum vacutainer tubes with a 19-gauge needle. Serum was collected immediately after centrifugation and frozen at −70°C. Twenty-one of these individuals were retested several months afterward. The medical records of each donor were reviewed by a single reviewer. A screening physical examination and laboratory studies included measurements of hemoglobin, white blood cell count, platelet count, erythrocyte sedimentation rate, and a chemistry profile including sodium, potassium, calcium, phosphorus, total protein, glucose, alkaline phosphatase, aspartate aminotransferase, bilirubin, uric acid, creatinine, and albumin. All donors had values within the normal range.

Sepsis patients were recruited from the primary medical-surgical intensive care units at either the University of California, Moffitt Hospital (San Francisco, CA) or San Francisco General Hospital (San Francisco, CA), as was done in our prior studies of critically ill patients (14, 15). Patients were identified as having suspected bacteremia on the basis of a temperature (>38.3 or <35.0°C) with a systolic blood pressure 20 mm Hg below their usual level. Patients were enrolled only if these temperature and blood pressure changes had developed within the prior 4 h. A total of 28 patients had blood drawn who met the above criteria. Of these 28 patients, 17 had a positive blood culture and were therefore included in the analysis for this study. These 17 blood culture-positive patients were then further subdivided into those patients with septic shock as indicated by a persistent systolic blood pressure below 100 mm Hg for more than 2 h in spite of volume resuscitation vs those patients in whom the systolic blood pressure stabilized above 100 mm Hg within 2 h with volume resuscitation alone. For additional control data, two normal individuals had serum drawn within 3 min of completing strenuous exercise (running five miles). The serum was then stored at −70°C until they were mailed in Dry Ice to Otsuka (Rockville, MD) for analysis. These studies were approved by the University of California at San Francisco Committee on Human Research.

Purification of plasma E-selectin

Heparinized plasma from five normal lab donors was pooled, adjusted to 2 mM PMSF, delipidated with Seroclear and immediately passed over a 7A9-Affigel 10 affinity column at 4°C. The column was washed with PBS and eluted with 5 M urea. Peak fractions by sEs ELISA were combined, concentrated and lyophilized. One milligram of protein was iodinated by the iodogen technique, free iodine removed by passage over a desalting column, and the labeled material immunoprecipitated as previously described (12).

Construction of a CHO cell line secreting sELAM-1-420

A truncated version of ELAM-1 containing 420 amino acids (ELAM-1-420) of the amino terminal end of the mature form, along with the signal sequence, was constructed by the PCR using the following oligonucleotide primers:

primer 1: 5′-GGGGGGCGGCC-
GCGAAGTCATGATTGCTTCACAG-3′

primer 2: 5′-GGGGGGGTCGAGT-
TACACCAAACCCTTCGGGGGCTG-3′

PCR and cloning into the expression vector was carried out as previously described (16).

ELAM-1-420 contains the lectin and epidermal growth factor domains and a portion of the C CR domain. The PCR amplified product was cloned into an expression vector containing the RSV LTR promoter driving ELAM-1-420, and SV40 "t" splice and poly(A) addition sequences from pMSG (Pharmacia) at the 3′ end of the ELAM-1-420 coding sequence. The expression plasmid also contains the human wild-type DHFR transcription unit with the SV40 early promoter and the SV40 mRNA

processing sequences at the 3′ end of the DHFR gene (17). This plasmid was transfected into the DHFR-CHO cell line, and methotrexate-resistant (600 mM) transfectants were isolated by sequential selection with increasing concentrations of methotrexate.

Purification of the Soluble Secreted Form of ELAM-1-420

CHO cells transfected with the ELAM-1-420 construct (CHO420 cells) were grown to confluence in (improved) MEM 20% FCS, nonessential amino acids and 600 nM MTX. At confluence, cultures were shifted to 5% FCS and left for 24 h to condition the medium. The harvested supernatant was passed through a 0.2-μm filter and adjusted to 1 M NaCl, 1 mM Mn^{++}, 0.1 M sodium acetate (pH 6) (Con A buffer) and passed through a 0.2-μm filter. It was then applied to a 200-ml packed gel column of Con A Sepharose. The column was washed with Con A buffer to remove nonspecifically adsorbed protein, and eluted with 250 ml of 1 M methylmannoside in D-PBS, pH 7.4. The eluted protein was concentrated with an Amicon device and a PM10 membrane. The buffer was exchanged to D-PBS and then passed several times over an anti-ELAM-1 affinity column. This column was prepared by conjugating 100 mg of purified 7A9 antibody to 50 ml of Affigel-10 according to the enclosed directions. Coupling efficiency was greater than 95%. Nonspecifically bound protein was washed from the column with D-PBS, and the ELAM-1-420 molecule was eluted with 5 M urea at 4°C in D-PBS. The peak of protein was dialyzed against D-PBS, and applied to a C-18 reversed phase column. Material was eluted with a gradient of acetonitrile in trifluoroacetic acid. Fractions were monitored for absorbance at 226 and for activity in an ELISA assay for sEs (see below). This material was judged to be authentic E-selectin by amino acid sequencing which, combined with amino acid analysis data, gave a working protein concentration and a purity of 90%. Nonreducing gels show that approximately 25% of this material exists as a disulfide-linked dimer. The material is biologically active, as shown previously (8).

Sandwich ELISA assay for E-selectin

We have previously shown that the 3B7 and 7A9 anti-E-selectin antibodies define non-overlapping epitopes of the molecule. The specificity of these antibodies has been shown previously by virtue of their adhesion to E-selectin transfected COS cells but not to mock-transfected COS cells (12). The sandwich ELISA assay was performed as follows. Biotinylated 7A9 was prepared by first dialyzing antibody against 0.1 M carbonate/bicarbonate buffer, pH 9.5, overnight at 4°C. Antibody concentration was adjusted to 2 mg/ml and to 1 ml was added 50 μl of NHS-biotin at 2 mg/ml in DMSO. After stirring for 1 h at room temperature, the reaction mixture was passed over a P-6DG desalting column to remove unreacted biotin. Purified antibody 3B7 was adjusted to 5 μg/ml in 0.1 M bicarbonate buffer, pH 9.5, and 100 μl were added to wells of Nunc ELISA plates. The plate was sealed and left overnight at 4°C. Wells were washed 10 times with PBS/Tween 20 at 0.05% and then blocked to prevent nonspecific adsorption by addition of 350 μl of D-PBS containing 1% BSA for 1 h at 37°C. After washing with PBS/Tween 20 10 times, test samples in twofold dilutions of D-PBS were added and allowed to incubate for 18 h at 4°C. Plates were washed 10 times with PBS/Tween and incubated with 100 μl of biotinylated 7A9 at 0.75 μg/ml in D-PBS for 3 h at 4°C. Wells were washed with PBS/Tween 10 times and incubated with 100 μl of a 1/333 dilution of streptavidin-horseradish peroxidase and left at 4°C for 30 min. Wells were washed 10 times with PBS/Tween and incubated with 100 μl of chromogen. This consisted of one table of *o*-phenylenediamine in 12 ml of 0.1 M citrate buffer, pH 5.0, plus 12 μl of 30% H_2O_2. After development with shaking for 15 min in the dark at room temperature, the reaction was stopped by addition of 25 μl of 12.5% H_2SO_4. Absorbance at 492 nM was read in a Titertek Multiscan plate reader. A standard titration of a known amount of ELAM-1-420 was included in each assay, and calculations of amounts of sEs in test samples were made by linear regression analysis.

This assay is linear in the range of 100 pg/ml to 3 ng/ml. Using a known amount of soluble ELAM-1-420, the ELISA assay was shown to be unaffected by the presence of up to 100% serum and 1% Triton X-100 (data not shown). This standard curve was run side-by-side in all subsequent experiments to allow a calculation of experimental values by regression analysis. The inter- and intra-assay coefficients of variation were less than 5%.

To exclude the possibility of any cross-reaction with the most closely related proteins, L-selectin and P-selectin, we have tested detergent lysates of HL60 cells and platelets as abundant sources of these respective proteins. The results shown in Table I indicate that only IL-1-stimulated endothelial cells, and to a much lesser extent, unstimulated endothelial cells show values for ELAM within the linear range of the assay. Hence there is no cross-reaction in this assay for P or L selectins.

Molecular sieving

One-milliliter samples of serum containing (0.5% Triton X-100 or 0.5% Triton X-100) lysates of 4-h IL-1 activated endothelial cells, 0.5% Triton X-100 lysates of resting endothelial cells, or supernatants from 24-h IL-1 activated endothelial cells adjusted to 0.5% Triton X-100 were loaded onto a Superose 12-column attached to a Waters model 820 HPLC. Samples were run at room temperature

Table I
Only IL-1-stimulated endothelial cells, and to a lesser extent, unstimulated endothelial cells show values for E-selectin within the linear range of the assay[a]

	E-Selectin/10^6 Cells (ng)
HUVEC	0.3
HUVEC + IL-1	22.2
Platelets	0.01
HL60	0.09
T cells	0.22 ± .003

[a] Endothelial cells (HUVEC) were cultured in medium alone or in medium plus 1 ng/ml IL-1 for 4 h at 37°C. Cells were rinsed twice with D-PBS and lysed with 1% Triton X-100, centrifuged at 12,000 × *g* for 30 min, and analyzed for E-selectin content. Platelets were harvested from heparinized plasma as described in *Materials and Methods* HL60 cells were washed twice to remove serum. Platelets and HL60 cells were washed twice with D-PBS, lysed with 1% Triton X-100, spun at 12,000 × *g* for 30 min, and supernatants were tested for E-selectin values. Neutrophils or T cells were purified as described in *Materials and Methods*, lysed with 1% Triton X-100, and centrifuged at 12,000 × *g* for 30 min. Detergent supernatants were analyzed for E-selectin content by ELISA. sEs values are means ±*SE*, *n* = 3.

at a flow rate of 0.5 ml/min with buffer containing 25 m Tris, 0.1 M NaCl, 0.5% Triton X-100, pH 7.4. 0.5 ml fractions were collected and 100 ul assayed the same day in the sEs ELISA.

Biologic activity of serum E-selectin

Biologic activity of serum E-selectin was measured in a capture-adhesion assay in which the nonblocking polyclonal rabbit anti-ELAM-1-420 Ig fraction was used to capture E-selectin from serum. Nunc microtiter ELISA plates were incubated with 1 μg/well of rabbit Ig in 50 μl of 0.1 M carbonate buffer, pH 9.3, overnight at 4°C, sealed with tape. After rinsing three times with D-PBS, wells were blocked for 2 h at 22°C with 1% BSA in D-PBS. Wells were rinsed three times and left untreated or treated with either 9 ng ELAM-1-420 in 50 μl BSA or 50 μl of serum in sextuplicate from either normal donors or donors with bacteremia/septic shock. Serum was incubated in the plates for 2 h at 22°C and washed three times to remove unbound protein. Ten micrograms per milliliter of the $F(ab)_2$ fragment of the 7A9 antibody were added to some wells as a control. ^{51}Cr-labeled HL60 cells (10^5/well) were added for 30 min at 22°C in a static adhesion assay. Nonadherent cells were removed by three rinses with 1% BSA/D-PBS and the residual radioactivity in the wells was evaluated in a gamma counter.

Results

IL-1 activated endothelial cells release E-selectin

In order to test the fate of E-selectin produced by endothelial cells in response to IL-1, cells were cultured at confluence in the absence or presence of IL-1 for various time intervals and the sEs content of the supernatants or the detergent lysed cell pellets was determined. The results shown in Figure 1 indicate that at 4 h, 38 ng/10^6 cells is detectable in the cell fraction, whereas only 1 ng/10^6 cells is detectable in the supernatant. In control cultures (24 h only) with no IL-1 added, there is 1.25 ng/10^6 cells in the supernatant and 0.45 ng/10^6 cells in the cell fraction. The higher value in the control supernatant fraction is caused by the presence of E-selectin in the human plasma used for cell culture. By 24 h the E-selectin content of the cell fraction has declined by about half to 20 ng/10^6 cells and E-selectin in the supernatant is now detectable at 4 ng/10^6 cells. At 48 h there is a slight increase in the supernatant fraction but another decline in the cell fraction. The viability of replicate cultures harvested at 24 and 48 h was always greater than 98% as assessed by the ethidium bromide-acridine orange technique (13). The data obtained in this and three other assays with similar results indicate that the total E-selectin detectable in this system declines after its peak at 4 h; a proportion of this material can then be detected in supernatants. Compared with supernatants that had not been filtered or centrifuged, identical sEs values were obtained (not shown). Hence the total E-selectin detectable in the system is the sum of the cell and supernatant fractions. In summarizing the results of three experiments, the amount of sEs at 24 h, as a fraction of the total ELAM at 4 h, is 7.6 ± 4.9%. Comparable E-selectin values in supernatants of endothelial cells were detected when cultures were stimulated with TNF-α or LPs (data not shown).

Molecular form of sEs in supernatants

To gain some insight into possible mechanisms of release of E-selectin from IL-1-activated endothelial cells, immunoprecipitates were performed on the cells or the filtered and ultracentrifuged supernatants of [^{35}S]cysteine-labeled cultures. The results shown in Figure 2 indicate that cell pellets have abundant E-selectin at 4 h and considerably less at 24 h, despite the fact that the label is added with the IL-1 at time zero and remains for 24 h. Conversely, the cell supernatants contain no detectable sEs at 4 h, but contain a very distinct E-selectin protein at 24 h. These kinetics are entirely consistent with the ELISA data on the supernatant and cellular fractions.

sEs is detectable in the blood of normal persons

E-selectin values in the serum of 71 normal donors varied from 0.13 to 2.8 ng/ml (mean 0.92, median 0.72, SD 0.66) as determined by the ELISA technique (Table II). sEs values did not correlate with patient age, sex, or smoking status ($p > 0.05$ by Wilcoxon rank-sum test). Twenty-one of the normal donors were retested after several months to see how variable were their sEs values. The mean values ± SD for this group was 1.26 ± 0.31 ng/ml, not significantly different than for the larger group. For 10 of the 21 normal persons retested, their sEs values remained the

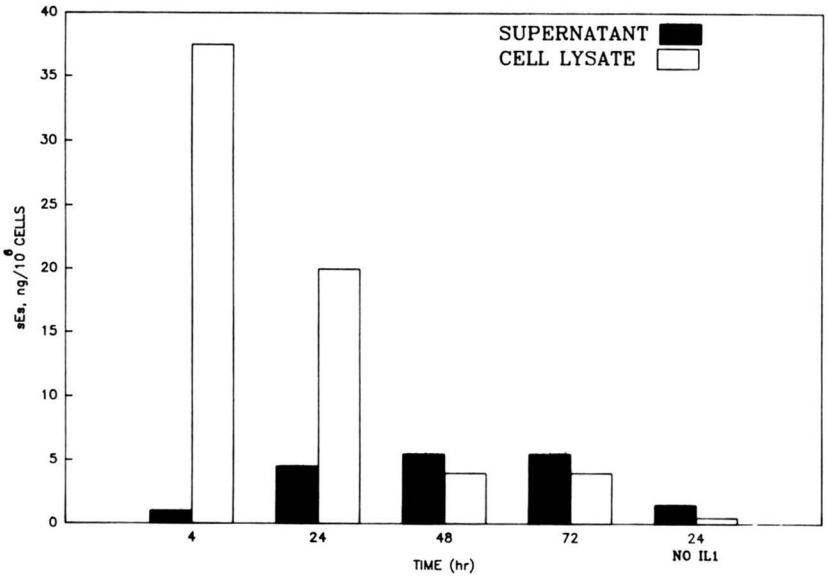

FIGURE 1. sEs values in cell or supernatant fractions of human umbilical vein endothelial cells treated with 1 ng/ml of IL-1 for various time periods, or untreated (24 h). Supernatant samples were centrifuged at 100,000 × *g* for 2 h before assay.

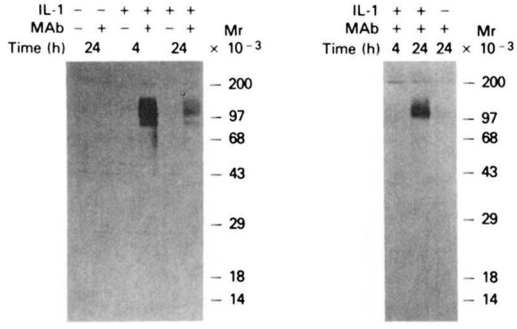

FIGURE 2. E-selectin immunoprecipitates from the cells (*left*) or the supernatants (*right*) after centrifugation at 100,000 × *g*, 2 h at 4 h or 24 h after stimulation with 1 ng/ml of IL-1. SDS-PAGE analysis of [^{35}S]cysteine-labeled immunoprecipitates was performed under reducing conditions.

same (±10%) or increased. For the remainder, the sEs values declined. The maximum increase was 1.4 ng/ml, and the maximum decrease was 0.8 ng/ml. Overall, no significant upward or downward trend in values was noted. Hence sEs levels in normal individuals appears to be a stable trait.

Molecular sieving analysis of E-selectin

In addition to the SDS-PAGE analysis of the molecular form of the supernatant, we have examined serum and supernatant forms by gel-sieving analysis. The reference molecules are the full length E-selectin derived by detergent lysis of IL-1-activated endothelial cells and the truncated ELAM-1-420 molecule. For comparability, supernatant and serum forms were also analyzed by size exclusion chromatography in the presence of a comparable amount (0.5%) of Triton X-100. Addition of detergent, however, did not affect the sEs value of the starting material or the elution volume of the serum and supernatant sEs. The results shown in Figure 3, one of three highly reproducible

Table II
Serum E-selectin levels in bacteremic patients with and without systemic hypotension[a]

Condition	No.	Systolic Hypotension (<100 mm Hg)	sEs (ng/ml)
Bacteremia	10	Yes	23.3 ± 15.9[b]
Bacteremia	7	No	1.1 ± 0.9
Controls	71	No	0.92 ± 0.66

[a] Data as mean ± SD.
[b] $P < 0.05$.

experiments, indicate the cell-derived E-selectin elutes at tube 19 whereas the supernatant material peaks at tube 20 and serum-derived ELAM elutes at peaks 20 to 21. As expected, control lysates from unstimulated endothelial cells show no activity in any column fraction, which likely excludes P-selectin from reactivity in the assay.

sEs is shed from endothelial cells and can be immunoprecipitated from plasma

To distinguish between a shedding and a secreting mechanism for release of E-selectin from IL-1-treated endothelial cells, 4-h activated cells were iodinated on the cell surface with lactoperoxidase. The cells were washed, put back in culture overnight, and then supernatants harvested. Immunoprecipitation of the supernatants and the lysates of 4-h IL-1-activated endothelial cells showed (Fig. 4) that labeled material can be recovered from the supernatant and that this material is approximately 5 kDa smaller than the cell lysate form.

In addition, sEs was purified from fresh pooled human plasma by affinity column chromatography. Plasma was used to avoid the proteases activated in the clotting process. The purified material was iodinated by the iodogen technique and immunoprecipitated in the same experiment. In this case, and in two additional experiments, two

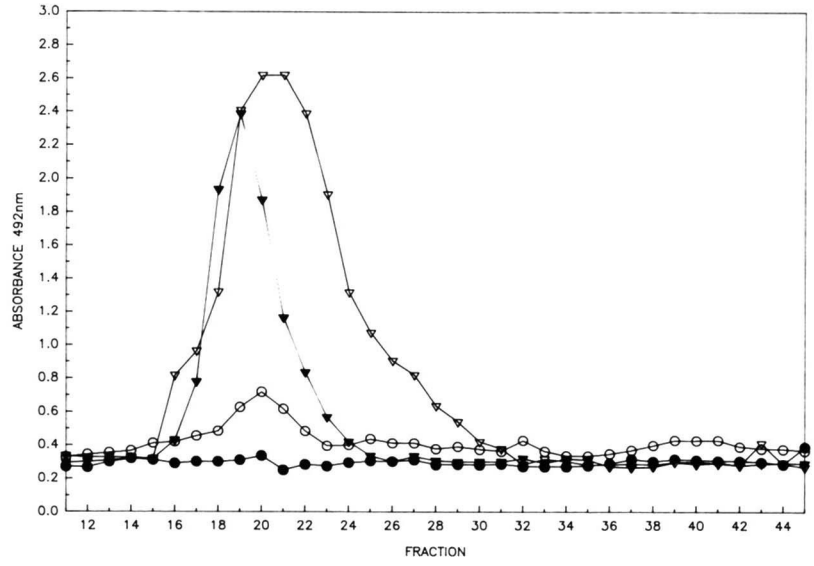

FIGURE 3. Various test samples were fractionated by molecular sieving over Superose-12 (1 cm × 30 cm). Each fraction was assayed immediately by the E-selectin ELISA and results were plotted as absorbance vs fraction number. All fractions were assayed undiluted except the IL-1 cell lysate, which was tested at a 1/10 dilution in running buffer. The column was run at 0.5 ml/minute; 0.5 ml fractions were collected. The elution times of m.w. standards are as follows: thyroglobulin (669 kDa), 16.0 min; ferritin (440 kDa), 18.9 min; catalase (232 kDa), 21.8 min; and aldolase (158 kDa), 22.4 min. Soluble ELAM-1–420 monomer eluted at 20.45 min, giving an estimated m.w. of 338 kDa. (●) Cell lysate, no IL-1; (▼) lysate of 4 h IL-1-treated endothelial cells; (▽) serum; (○) 24 h supernatant of IL-1-treated cells.

bands were identified. The higher one at 105 kDa comigrates with the material from activated endothelial supernatants. In addition, an equally intense band centering around 85 kDa was also identified. These results are shown in Figure 4. The same pattern of 105- and 85-kDa bands was obtained using the polyclonal anti-ELAM-1-420 rabbit serum for immunoprecipitation (data not shown).

sEs lacks an intact cytoplasmic domain

Polyclonal antisera to the 32 mer representing the cytoplasmic domain of E-selectin were made in rabbits and the biotinylated Ig fraction used in an ELISA as follows: Wells coated with 3B7 were incubated with various test samples and two kinds of reporter reagents were used, either the biotinylated 7A9 antibody or the biotinylated Ig fraction of rabbit anti-32 mer of E-selectin. As expected, all sources of E-selectin reacted with the 3B7/7A9 combination. However, the 3B7/anti-32-mer combination, as shown in Figure 5, reacted with the intact E-selectin from IL-1-activated endothelial cells and not with the truncated ELAM-1-420 molecule lacking a cytoplasmic domain. Most interestingly, the 3B7/anti-32-mer combination failed to react with either the endothelial supernatant form of E-selectin or the E-selectin found in the serum of four normal donors.

sEs levels in bacteremic patients

Of the 17 patients with a positive blood culture, 10 patients had evidence of septic shock with persistent systolic hypotension (<100 mm Hg) in spite of volume resuscitation. The sEs levels in these 10 patients were markedly elevated compared with control patients (Table II). The microbiology demonstrated five patients with Gram-negative bacteremia, four patients with Gram-positive bacteremia, and one patient with both Gram-positive and Gram-negative bacteremia. There was no apparent relation between Gram-positive vs Gram-negative bacteremia and the sEs levels in the serum of these 10 patients. In the 7 patients with bacteremia without septic shock, sEs levels were not elevated (Table II). As a control for an increase in cardiac output, two normal individuals had serum drawn within 5 min of completing strenuous excercise (running 5 miles). Their sEs levels were 0.2 and 0.4 ng/ml, within the normal range.

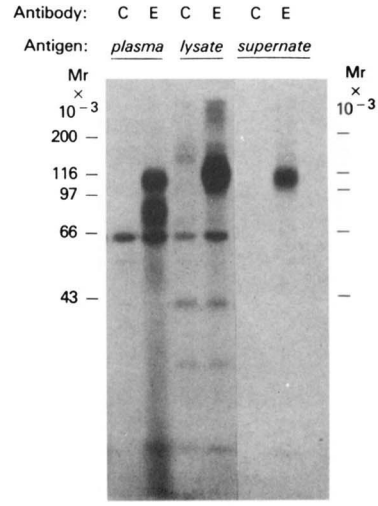

FIGURE 4. E-selectin was immunoprecipitated from several sources by the mAb 3B7 (*E lanes*) or the subclass control antibody MOPC-21 (*C lanes*). The sources of Ag were affinity-purified E-selectin from plasma (see *Materials and Methods*), E-selectin from detergent lysates of endothelial cells activated for 4 h with IL-1 (lysate) or supernatants of endothelial cells labeled with ^{125}I/lactoperoxidase 4 h after IL-1 activation, then cultured overnight (supernatant).

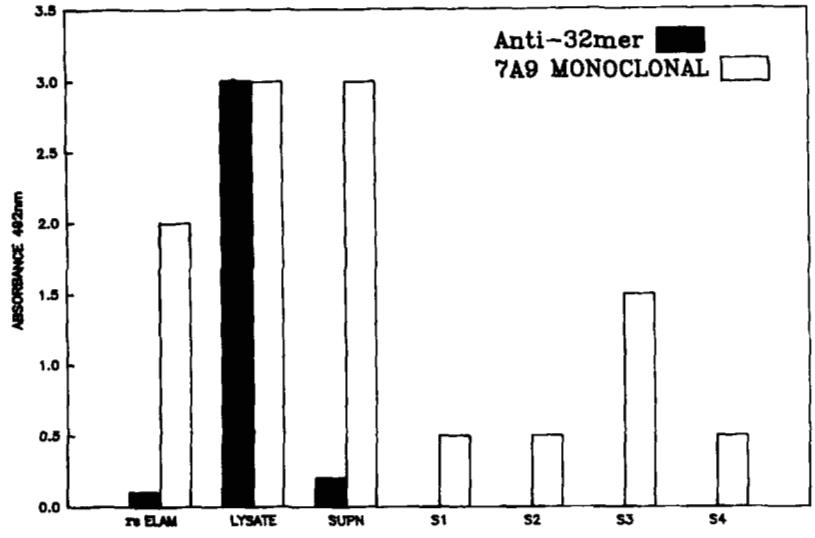

FIGURE 5. Results of an ELISA assay in which test samples were anchored with antibody 3B7 and the reporter molecule was either 7A9-biotin (*open bars*) or the biotin-conjugated polyclonal antibody (*solid bars*) to the 32 mer representing the cytoplasmic domain of E-selectin. Color was developed as described in *Materials and Methods* using streptavidin-peroxidase. S1 through S4 are sera from four normal donors; rsELAM is ELAM-1–420, lysate is a detergent extract of 4-h IL-1-activated endothelial cells and supernatant is from 24-h IL-1 activated endothelial cultures.

sEs in serum of normal and bacteremic patients is biologically active

Taking advantage of the lack of blocking activity of the rabbit anti-E-selectin polyclonal serum, we have asked whether it can be used to anchor recombinant and native soluble E-selectins, which could then be tested for ability to mediate adhesion of the granulocyte-like cell line, HL60, which will adhere to E-selectin via sialyl-Lewis^x (4). Our results shown in Figure 6 indicate that this system can be used to measure the presence of biologically active E-selectin as shown by the adhesion induced by ELAM-1-420. All this adhesion is inhibitable by the blocking anti-E-selectin antibody 7A9, and no adhesion is seen in wells containing only rabbit antibody. Interestingly, the sera from both normal and bacteremic individuals contains biologically active E-selectin as measured in this assay.

Discussion

We have developed a quantitative sandwich ELISA for E-selectin that is linear in the range of 100 pg/ml to 3 ng/ml of fluid and have demonstrated that cytokine-activated endothelial cells in cultures shed a small proportion of the total E-selectin that they synthesize into the culture medium, in the range of a 0.5 to 4 ng/10^6 cells. This amount of sEs is first detectable after overnight incubation of IL-1-stimulated endothelial cells and slowly increases over the next 2 days. Very little material is detectable in the supernatant after 4 h of IL-1 treatment, whereas a dramatic increase in ELAM-1 in the cell fraction is obtained, as expected. This shift in distribution of ELAM from cell to supernatant is mirrored precisely in the immunoprecipitation analysis of [^{35}S]cysteine-labeled and IL-1-stimulated endothelial cells. Hence, no soluble material is detectable in 4-h supernatants whereas 24-h supernatants show a strong band. We have characterized the released E-selectin in several additional assays. Size-exclusion chromatography and analysis of fractions for sEs shows a very reproducible pattern of elution from different sources. The cell lysate-intact form reproducibly emerges at least one fraction ahead of material from either serum or supernatant of IL-1-treated cells. This suggests that the serum and supernatant forms are distinct from the cell lysate form, and may be smaller. The serum form appears to be somewhat heterogeneous judging from the elution profile. The apparent m.w. of the lysate form of E-selectin is 400 kDa by comparison with globular proteins. Anomalous behavior of ICAM-1 on Superose 12 has previously been reported (18).

To try to confirm the reduced mass of sEs in an alternative approach, we have partially purified sEs from a pool of normal plasma by affinity chromatography, iodinated this material, and immunoprecipitated it. SDS-PAGE analysis shows two bands of material precipitated with the 3B7 mAb at 105 kDa and 85 kDa. The same pattern was found (data not shown) by immunoprecipitation with the polyclonal antibody to ELAM-1-420. In addition, immunoprecipitation of the detergent supernatant from surface labeled and cultured E-selectin-bearing endothelial cells show a 105-kDa band that comigrates with the higher band obtained from plasma. Although the 85-kDa form of E-selectin may be an isolation artifact, we have tried to minimize this possibility by using plasma rather than serum, adding a protease inhibitor, and rapidly processing the material in the cold. Alternatively, the 85-kDa material could be a co-precipitated molecule or a further degradation product derived from the 105-kDa form. In any case, these data show that E-selectin is present in a lower m.w. form in supernatant and serum, and that in vivo there may be an additional modification resulting in an 85-kDa circulating form. These data also show that the

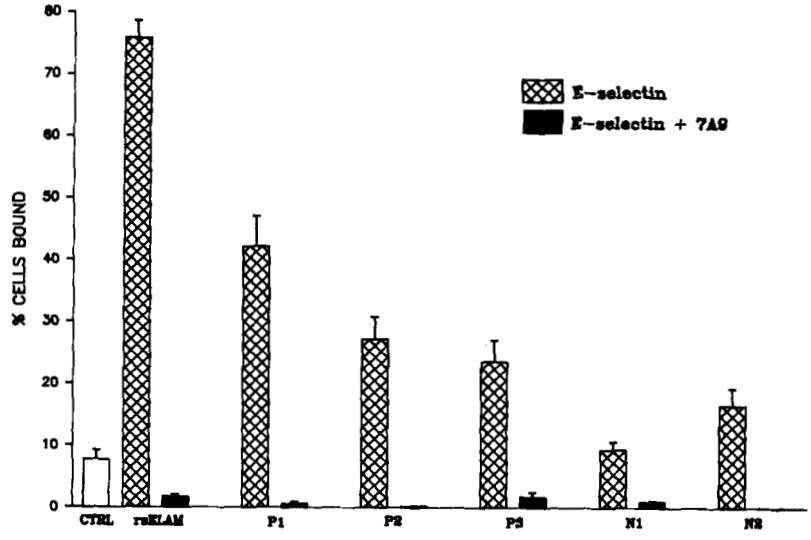

FIGURE 6. The biologic activity of sEs in serum of normal and bacteremic patients was evaluated by anchoring serum E-selectin with the nonblocking polyclonal antibody to ELAM-1–420 and evaluating the percent cells bound using ^{51}Cr-labeled HL60 cells in the absence (*hatched bars*) or presence (*solid bars*) of 10 μg/ml F(ab)'$_2$ 7A9. Control (*CNTL*) shows the percentage of added cells bound to wells coated only with the rabbit anti-ELAM-1–420 serum. P1, P2, and P3 are serum samples from patients with septic shock, and N1 and N2 are serum samples from normal donors. Each bar represents the mean ± SEM of six wells.

supernatant form of E-selectin (105 kDa) is shed from the cell surface.

To further confirm the altered nature of the soluble forms of E-selectin, we have tested whether the cytoplasmic domain is intact using a polyclonal antibody to a 32 mer representing the cytoplasmic portion. Although the cell lysate form of E-selectin reacted well with this reagent, E-selectin from cell supernatants and from the sera of four normal donors failed to react with this antiserum, showing that the cytoplasmic domain of the shed forms of E-selectin is not intact.

However, the amino-terminal lectin domain of E-selectin, from both normal and bacteremic patients, is largely intact as shown by the ability of this sEs, when anchored to plastic via a nonblocking polyclonal serum, to mediate adhesion of HL60 cells in a 7A9-inhibitable manner.

To summarize the immunochemical findings, serum and supernatants of the activated endothelial cells have a shorter form of E-selectin that does have biological activity and a defective cytoplasmic domain. However, we have been unable to determine the precise mechanism of release in culture as cells exposed for 24 h to the maximally tolerated doses of protease inhibitors show undiminished amounts of sEs. The mechanism of release in vivo is also not known and may be different given the presence of the additional 85-kDa form of E-selectin in plasma.

Jung and Daily have shown that treatment of lymphocytes with protein kinase C activators results in a rapid loss of L-selectin from the surface, probably by proteolysis (19), and Kishimoto et al. (20) and Smith et al. (21) have shown that L-selectin is rapidly shed from neutrophils treated with chemotactic factors. Likewise, P-selectin (CD62) mRNA appears to exist in an alternately spliced form lacking the transmembrane domain, suggesting a shed form of this molecule may exist (22). More recently, Dunlop et al. (23) have shown that P-selectin circulates in plasma at high levels compared with the sEs levels reported here, and that it also is biologically active. Hence, the other members of the selectin family appear to be shed from cells, but by distinct mechanisms. In addition, a serum form of ICAM-1 has recently been detected by sandwich ELISA, probably released from B cells (18, 24).

Using the sandwich ELISA assay we have shown that a soluble form of E-selectin is detectable in the serum of normal individuals. This is true for all individuals tested (n = 71). As indicated by the range of values of a battery of diagnostic tests used to monitor ongoing infectious or inflammatory processes, these individuals were normal. When 21 of these individuals were re-evaluated for sEs values several months later, there was no discernible upward or downward trend in overall values. Hence, it is unlikely that the E-selectin present in the serum of normal persons represents a point in time when values are declining from some previous episode of inflammation. This data strongly suggests that E-selectin is synthesized constitutively somewhere in the vasculature. Although this observation does not address whether the mechanism of release of E-selectin from endothelial cells is the same in vivo as in vitro, it does argue against the in vitro release as an artifact of tissue culture. One hypothesis is that this molecule might be found at sites of constant antigenic stimulus such as the skin, lung, or gut. However, Cotran et al. have not found E-selectin to be expressed in any of the following normal tissues: skin, kidney, heart, skeletal muscle, liver, spleen, ovaries, lung, lymph nodes, or s.c. fat (25). Waldorf et al. have also failed to find E-selectin expression in normal skin (26), but two more recent reports suggest skin may have low levels of E-selectin in the microvasculature (27, 28). A survey of other tissues exposed to constant antigenic stimuli such as the gut may help provide an answer to this question.

The objective of these initial studies of plasma E-selectin in patients with bacteremia was to test the hypothesis that this endothelial marker would be released into the plasma of patients with bacteremia because both in vitro (11) and in vivo (29) studies have indicated that there is increased endothelial expression of E-selectin with exposure to cytokines (TNF, IL-1), endotoxin, and live bacteria. The markedly elevated levels of sEs in the plasma of patients with both bacteremia and sustained systemic hypotension, but not in patients with bacteremia alone, indicates that elevated plasma levels of sEs developed in conjunction with the hemodynamic manifestations of advanced septicemia. Because elevated levels of sEs were found in patients with bacteremia complicated by septic shock, it is possible that release of E-selectin in humans is sensitive to the degree of vascular or endothelial injury caused by sepsis. Additional studies in a larger number of patients with multiple samples within a 24-h period after the onset of sepsis will be needed to investigate the relationship between elevated levels of sEs and the development of multiple organ dysfunction and survival in patients with septic shock.

Lastly, the E-selectin ELISA may be useful for monitoring in vivo states of endothelial activation, as in inflammation. It should be especially useful because to date E-selectin has been reported to be expressed only on endothelial cells. Results from analysis of a variety of additional inflammatory states should be available in the near future.

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