E-Selectin Mediates Leukocyte Rolling in Interleukin-1–Treated Rabbit Mesentery Venules

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The selectins are lectin-like cell surface glycoproteins that have been implicated in playing a crucial role in the initiation of leukocyte adhesion to endothelial cells (ECs) during inflammation. Binding of selectins under conditions of flow mediates leukocyte rolling, which in vivo is almost exclusively observed in venular microvessels. We have shown in previous experiments that intraperitoneal treatment of rabbits with interleukin-1β (IL-1) increases leukocyte rolling in exteriorized mesenteries. In the present study, we used immunohistochemistry of mesenteries and found that IL-1 induced a marked E-selectin immunoreactivity, preferentially in venules. We therefore hypothesized that the increased rolling in response to IL-1 may be related to the induction of E-selectin on venular ECs. Intravital microscopy was used to investigate interactions between leukocytes and ECs after intraperitoneal application of IL-1. The rabbit E-selectin monoclonal antibody (MoAb) 9H9 significantly reduced rolling of leukocytes by approximately 40%. Vehicle alone, class-matched control MoAb or the nonblocking anti–E-selectin MoAb 14G2 had no effect on rolling. These results indicate that leukocytes roll on inflamed venular ECs partly through interactions with E-selectin. Furthermore, we propose that the restricted E-selectin immunoreactivity by venular ECs contributes to the remarkable difference seen between arterioles and venules in exhibiting leukocyte rolling in vivo.

LEUKOCYTE ROLLING is a phenomenon that in vivo is almost exclusively observed in postcapillary and collecting venules. Rheologic differences between venules and arterioles were initially proposed as possible explanations as to why leukocyte rolling occurs in distinct areas of the microvasculature,[1] but this concept has recently been questioned. Instead, differences in surface adhesion receptor expression between arteriolar and venular endothelial cells (ECs) have been proposed.[2,3] Adhesion receptors known to be either constitutively expressed or inducible on ECs by inflammatory cytokines include molecules such as ICAM-2 (CD102), ICAM-1 (CD54), VCAM-1 (CD106), and the vascular selectins, P-selectin (CD62P) and E-selectin (CD62E).[4,5] E-selectin synthesis is induced by inflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor (TNF).[6] In vitro and in vivo studies have shown a transient expression of E-selectin on ECs, with a peak expression 4 to 6 hours postactivation.[6,7] Furthermore, E-selectin was found in vitro to support adhesion of neutrophils, monocytes, eosinophils, a subset of lymphocytes, and certain cancer cells.[8] In the presence of low shear rates, both P- and E-selectin appear to support leukocyte rolling in vitro. Immobilized P-selectin in a lipid bilayer,[9] E-selectin expressed on transfected L cells, or EC monolayers treated with IL-1[10] were used as substrates in flow chambers for neutrophil rolling.

A low frequency of constitutive rolling has been observed in normal skin of anesthetized mice[11,12] and rats,[12] whereas rolling is virtually absent in undisturbed rabbit mesenteries.[13] However, the phenomenon is rapidly induced upon surgical tissue manipulation of the rabbit mesentery.[13] Furthermore, IL-1–induced stimulation of mesenteric venules for several hours leads to an increase in leukocyte rolling,[14] indicating that rolling is mediated in part by one or more inducible structures on venular ECs. Previous animal experiments using intravital microscopy have demonstrated that leukocyte rolling is dependent on the presence of divalent cations in plasma.[15] Rolling is reduced by systemic treatment of animals with neuraminidase,[16] is virtually abolished by certain anionic polysaccharides,[17-20] and is influenced by the action of free oxygen radicals.[21,22] This is suggestive of the participation of lectin-like adhesion molecules in leukocyte rolling.

Recently, it has been shown that engagement of the lectin-like adhesion molecule L-selectin (CD62L), which is constitutively expressed on most circulating leukocytes,[23,24] is of crucial importance for rolling of rabbit,[25] rat,[26] and human[14] leukocytes in vivo. L-selectin on neutrophils is decorated with the fucosylated carbohydrate sialyl-Lewis^x (sLe^x), which may be presented as a ligand to E- and P-selectin on inflamed ECs.[27,28] In fact, it has been proposed that presentation of sLe^x-like carbohydrates by neutrophil L-selectin contributes significantly to rolling in IL-1–treated venules in vivo.[29] In support of this concept is the finding that neutrophils from a patient with an inherited defect in fucose metabolism (leukocyte adhesion deficiency syndrome type 2 [LAD-II]) that cannot express sLe^x or other ligands for E-selectin[30] roll poorly in IL-1–treated venules in vivo despite normal L-selectin expression.[31]

Taken together, these findings suggest that an inducible C-type lectin with specificity for sLe^x-like carbohydrates on

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inflamed venular ECs is involved in leukocyte rolling in IL-1–treated venules. IL-1 treatment of EC is known to induce the expression of E-selectin,[32] which binds sLe^x on leukocytes in a Ca^{2+}-dependent fashion.[8] The purpose of the present study was to test the hypothesis that E-selectin may be involved in leukocyte rolling in acutely inflamed venules in vivo.

MATERIALS AND METHODS

Antibodies. Monoclonal antibody (MoAb) 9H9 (mouse IgM) raised against rabbit E-selectin and functionally blocking[33] and MoAb 14G2 (mouse IgG1) raised against rabbit E-selectin but not functionally blocking[33] were used. Furthermore, purified mouse IgMκ (Pharmingen, San Diego, CA; Ig isotype standard; used in vivo and for immunohistology) and MoAb TEPC 183 (Sigma Chemical Co, St Louis, MO; mouse IgMκ; hapten binding specificity not determined; used exclusively for immunohistology) were used as isotype-matched control antibodies to MoAb 9H9. All antibodies used in vivo were dialyzed against sterile Ca/Mg-free phosphate-buffered saline (PBS) and treated with washed Detoxigel (4× PBS; Pierce Chemical Co, Rockford, IL) to remove endotoxin, before use in vivo. Endotoxin levels determined by the Limulus amebocyte lysate test (BioWhittaker, Inc, Walkersville, MD) were less than 0.5 EU/mL. In addition, MoAb MOPC 21 (Sigma; mouse IgG1κ; hapten binding specificity not determined) and goat-antihuman factor VIII (von Willebrand factor [vWF]; The binding Site Inc, San Diego, CA) were used for immunohistology as were the biotinylated secondary antibodies, horse-antimouse (Vector Laboratories Inc, Burlingame, CA) and swine-antigoat (Boehringer Mannheim Biochemicals, Indianapolis, IN).

Animal preparation. A total of 13 male New Zealand White rabbits weighing 1.1 to 1.5 kg were used. Animals were sedated by intramuscular (IM) injection of 0.1 ml/kg of body weight (BW) of a solution containing 1% fluanison and 0.02% fentanyl (HYPNORM, Janssen, Belgium) or 0.1 mg/kg BW Innovar-vet (Pitman-Moore Inc, Mundelein, IL). Each animal received an intraperitoneal (IP) injection of 5 mL PBS containing IL-1 and a blood sample was taken from an ear artery for evaluation of hematocrit and differential white blood cell counts (WBC) before and after IL-1 treatment. For these studies IL-1 was used from two different sources. The first batch (generous gift from Dr R. Thomas, Genentech, San Francisco, CA) was used at 1,000 U per injection. The second batch (generous gift from Dr D. Witt, Repligen, Cambridge, MA) was used at 3,000 U per injection. Both agents reproducibly increased leukocyte rolling and neither significantly affected body temperature, differential WBC, or hematocrit. To decrease intestinal peristalsis, a single dose of 1 mg loperamide hydrochloride (IMODIUM, Janssen, Belgium) in 1.5 mL PBS was administered orally. Ninety minutes to 2 hours after IP injection of IL-1, animals were once more sedated and a blood sample was taken. Surgical anesthesia was induced by intravenous (IV) injection of 5 to 10 mL of a 20% urethane solution. Animals received a tracheal tube to facilitate breathing. Subsequent blood samples were obtained from a catheter in the left carotid artery. Arterial blood pressure was continuously monitored through a catheter in the left femoral artery. Additional doses of urethane were administered to the animal when needed through an IV line in the left jugular vein, which was also used for continuous volume substitution by infusion of PBS (6 mL/kg/h). Thereafter, animals were prepared for intravital microscopy of the mesenteric microcirculation, as previously described.[14] Briefly, the mesentery was exteriorized and a PE 10 polyethylene catheter was inserted into an arterial side branch upstream from a vascular area chosen for microscopic observation. A balloon catheter was gently wrapped and tied around the main stem of the superior mesentery artery upstream of the cannulated side branch. The balloon catheter was attached to an air-filled syringe. Inflation of the balloon completely blocked blood flow through the mesentery artery. The collateral circulation through the ileum was blocked using occluder clamps. Thus, blood flow in the microvascular segment could be blocked completely and reversibly. Subsequently, the animal was transferred to a Leitz intravital microscope. A setup equipped with transillumination, a silicon intensified tube camera (Dage MTI Inc, Michigan City, IN) connected to a SVHS video recorder (JVC HR-S6600U; Elmwood Park, NJ), and a video-triggered stroboscopic Xenon arc (Chadwick Helmuth, Mountain View, CA) for fluorescent epi-illumination through a Leitz Ploemapak epi-illuminator with an N2 filter block was used. For fluorescent staining of rabbit leukocytes, 2 to 2.5 mL of the nuclear dye acridine red (Chroma, Stuttgart, Germany; 2 mg/mL in Ca/Mg-free PBS) was injected into the left jugular vein before the experiment. Additional small bolusses (0.5 mL) were administered throughout the experiment when needed.

Experimental protocol. A schematic representation of the experimental protocol is given in Fig 1. To determine control period rolling of leukocytes, four recordings of a chosen venular segment were taken at 5-minute intervals using a Leitz 50× saltwater immersion objective (NA 1.00) and stroboscopic fluorescent epi-illumination. A total of 14 venules in 13 animals with diameters of approximately 28.8 ± 7.1 μm (mean ± SD) were analyzed. Each recording lasted approximately 75 seconds. After the 15-minute control period, the mesenteric blood flow was stopped by inflation of the balloon catheter. Immediately after complete cessation of flow, MoAb solution (50 μg/mL) or PBS was slowly injected through the catheter downstream from the occlusion site. Using this procedure, it was possible to completely displace the intravascular blood volume in the venule studied. A maximum of 150 μg MoAb in 3 mL PBS was used per animal (corresponding to 1.4 to 2.0 μg/mL MoAb in blood, depending on weight, assuming a blood volume of 70 mL/kg BW). MoAbs were allowed to react with the endothelium for 10 minutes. Thereafter, the balloon catheter was deflated and blood reentered the microcirculation in the mesentery segment. Within 1 minute after restoration of blood flow, a recording of leukocyte rolling in the venular segment was taken, followed by three further recordings 5, 10, and 15 minutes after restoration of flow. Blood samples for hematocrit and differential WBC counts were taken during the control period as well as within the first 2 minutes after the blood flow had been restored. In all experiments, attempts were made to test at least two MoAbs per animal, one control MoAb (or PBS) and one test MoAb. The order of control and test antibodies was reversed in every second animal. Using this approach, the effects of mIgMκ and MoAb 9H9 were compared in 6 animals, MoAb 14G2 and PBS were studied in 3 animals, and all three MoAb preparations were used in 1 animal. Four more animals received either MoAb 9H9 (1 animal) or MoAb 14G2 (2 animals), or both mIgMκ and 14G2 (1 animal). Thus, the total number of test injections in each group was n = 7 for MoAb 9H9, mIgMκ, and MoAb 14G2 and n = 3 for PBS only.

Image analysis. Video tapes were played back in real time or, when necessary, at reduced speed. Rabbit leukocyte rolling was determined during 30 to 60 seconds from fluorescent recordings at high magnification (300×). First, the total number of fluorescent leukocytes passing through a venular segment (total leukocyte flux [F_t]) was counted. The tape was rewound, and only those leukocytes that were visibly rolling along the venular wall were counted (rolling flux [F_r]). The rolling fraction (Rf), was calculated as $Rf(\%) = F_r/F_t \times 100$. For comparison of MoAb effects, the relative rolling fraction (Rf_{rel}), was calculated as $Rf_{rel}(\%) = Rf_{MoAb}/Rf_{co} \times 100$, where Rf_{MoAb} is Rf measured at a given time point after MoAb application and Rf_{co} depicts the mean of 4 consecutive determinations of Rf during the control period. Mean blood flow velocities

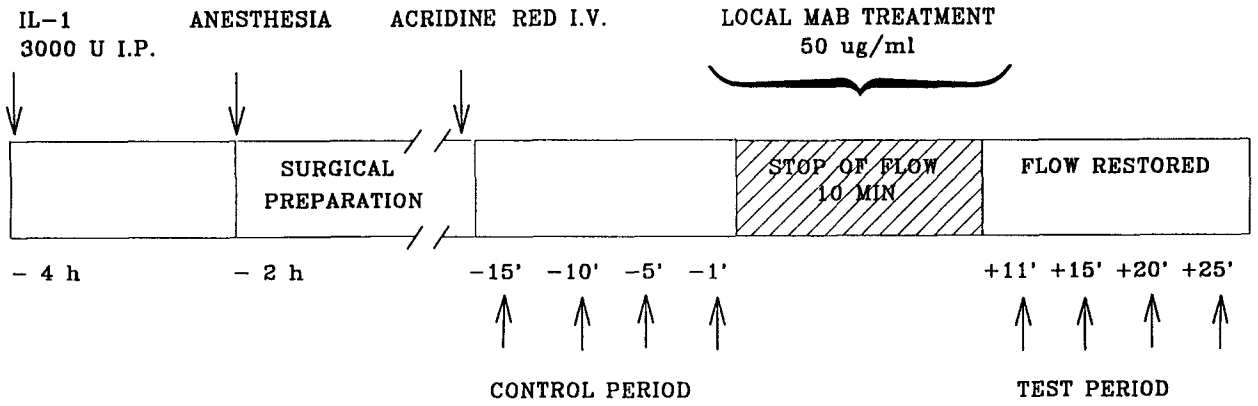

Fig 1. Schematic representation of experimental procedure. IL-1 was injected IP and the animal was prepared for intravital microscopy. The nuclear dye acridine red was injected to stain leukocytes. Four 1-minute video recordings were made in the control period under fluorescent epiillumination (↑). The blood flow was temporarily stopped and MoAb solution or PBS injected thereby, completely displacing the intravascular blood volume. Solutions were allowed to react with the endothelium for 10 minutes. Subsequently, the blood flow was restored and four video recordings of the test period were taken (↑).

(V) were estimated preocclusion and postocclusion by determining individual velocities of 25 consecutive noninteracting leukocytes by measuring the distance traveled between two or more successive video frames as described.[2] For this purpose, a computerized image analysis system designed for microcirculatory research was used.[34]

As a complementary but methodologically different way of analyzing the data, we established a velocity profile for two representative experiments, one from a mIgMκ- and one from an MoAb 9H9-treated venule. We determined individual velocities for approximately 125 consecutive cells, including both noninteracting and interacting cells. The centerline velocity (V_{cl}) was determined from the fastest cell in each sample according to Ley et al.[18] Each cell's individual velocity was divided by V_{cl} yielding a relative velocity. V_{cl} was assigned a value of 1. The cells' relative velocities were assigned to 8 classes, with 0.125 increments from 0 to 1. The critical velocity (V_{crit}) was calculated as described previously[2] and expressed relative to V_{cl}. Cells with a relative velocity greater than relative V_{crit} were assumed to be free cells, whereas cells with a relative velocity less than relative V_{crit} were assumed to be rolling.

Immunohistochemistry. Untreated rabbit mesenteries (n = 4) and mesenteries treated with IL-1 (1,000 U in 5 mL Dulbecco's PBS; Genentech) for 4 (n = 3) or 6 hours (n = 3) were rapidly removed from the animal's abdomen and frozen in OCT-media (Baxter Healthcare Corp, McGaw Park, IL). Sections (6 μm) were prepared and fixed in cold acetone for 5 minutes. Primary antibodies were used at 5 μg/mL, except for goat-antihuman factor VIII (vWF), which was used at a dilution of 1:250. Biotinylated secondary antibodies, horse-antimouse, was used as per instructions from the manufacturer (Vector Laboratories Inc) and swine-antigoat (Boehringer Mannheim Biochemicals) was diluted 1:250. After incubation with biotinylated secondary antibodies, sections were incubated with avidin-biotin-horseradish peroxidase complex (Vector Laboratories Inc) and subsequently developed with diaminobenzidine (DAB; Vector Laboratories Inc) to yield a brown complex. Immunoperoxidase-stained sections were counter-stained with 2% methyl green (Sigma Chemical Co) in dH_2O. In all immunohistochemical experiments, controls were performed without primary and/or secondary antibodies to exclude nonspecific staining.

Evaluation of staining for E-selectin was performed by counting the number of positively stained sections. Because it was not possible in this study to distinguish surface-expressed versus intracellular E-selectin, positive staining was considered representative of the total endothelial E-selectin pool present at a given time point. The staining intensity was subjectively graded by light microscopy as either 0 (absent), I (weak), II (moderate), or III (strong). Operator accuracy was verified by reading 100 slides in a blinded manner. The counterstain, showing smooth muscle cell nuclei surrounding arterioles, and the localization of vWF allowed morphologic distinction between arterioles and venules. Previous studies have shown that vWF density and staining pattern varies between different parts of the microvascular bed and between different species.[35] vWF staining is localized to Weibel-Palade bodies[36-38] and exhibits the characteristic granular appearance in both vessel types.[39] The number of E-selectin–positive versus –negative arterioles and venules was determined.

Statistics. Paired data were analyzed using Student's *t*-test for comparison of paired samples. Two-sided *t*-test was used for testing the difference between unpaired data. The Friedmann test was used for multiple comparisons on ranks of paired data. Arithmetic means ± SD are given when applicable.

RESULTS

Hematocrit, WBC counts, and blood pressure. Hematocrit or blood pressure were not altered by IP injection of IL-1, which is consistent with our previous observations.[14] These parameters were also not affected by MoAb injections. Hematocrit remained constant throughout the entire experiments. Before IL-1 treatment, the leukocyte counts were 8.5 ± 3.1 × 10^6 leukocytes/mL (29.2% ± 8.7% polymorphonuclear leukocytes [PMN]). The surgical procedure increased systemic leukocyte counts to 13.5 ± 6.7 × 10^6 leukocytes/mL and resulted in an increase of percentage PMN to 64.6% ± 15.4%, as seen previously.[14] These parameters remained elevated throughout the experiments. Neither the temporary occlusion of the superior mesentery artery nor treatment of animals with MoAb was found to have significant effects on systemic or differential WBC counts in these experiments.

Effect of temporary arterial occlusion on microhemodynamics. The experimental protocol required a short-term period without perfusion of the mesentery and the adjacent gut. Therefore, ischemia-induced damage to mesenteric tis-

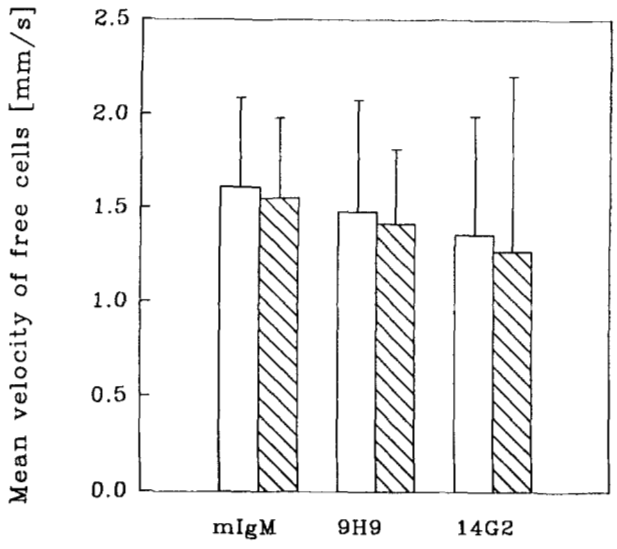

Fig 2. Mean velocities of free cells were not altered by the experimental procedures. Individual velocities of 20 to 25 consecutive noninteracting leukocytes passing a venular segment before arterial occlusion and during the first video recording after restoration of blood flow were determined using an image analysis system for microcirculatory research. No statistically significant differences in mean blood flow velocities could be detected in the reperfusion periods (▨) as compared with their control periods (□), irrespective of MoAb injected. Mean ± SD of cell velocities determined in 7 (mIgMκ and 9H9) and 4 (14G2) venules are shown.

sue could possibly occur as an undesired side effect. However, atmospheric oxygen from the surrounding room air is likely to diffuse through the superfusion buffer in quantities that may be sufficient to prevent hypoxic damage. Furthermore, our experiments showed no indication pointing to acute ischemic effects. There was no change in rolling or sticking after the no-flow period and there were no changes during the 10-minute occlusion or after restoration of flow in systemic parameters such as arterial blood pressure, hematocrit, or leukocyte counts. Mean blood flow velocities (V) were estimated by determining individual velocities of approximately 25 (24 ± 2; range, 13 to 25) consecutive noninteracting leukocytes passing a venular segment immediately before arterial occlusion and during the first recording after restoration of blood flow. Seven experiments were analyzed before and after application of mIgMκ or MoAb 9H9 (diameter, 26.9 ± 5.1 μm; range, 21.5 to 37.4 μm). The effect of MoAb 14G2 was studied in 4 venules (diameter, 29.0 ± 6.5 μm; range, 23.4 to 37.5 μm). There was no statistically significant alteration in vessel diameter observed before and after occlusion. Figure 2 shows that mean blood flow velocities were not significantly altered by the experimental procedure, indicating that a posthypoxic arterial vasodilation did not occur. Similar results were observed in two venules when vehicle alone was injected (data not shown).

Inhibition of rolling in vivo by rabbit E-selectin–specific MoAb 9H9. Mean rolling fractions during control periods (Rf_{co}) for each experimental group were determined as 51.8% ± 18.0% for MoAb 9H9 (n = 7); 45.4% ± 21.2% for mIgMκ (n = 7); 69.6% ± 18.8% for MoAb 14G2 (n = 7); and 64.3% ± 17.5% for PBS (n = 3). Mean rolling fractions before MoAb injection were not statistically significantly different between groups. MoAb 9H9 significantly reduced rolling of leukocytes by 40.1% ± 20.7% during the first minute after restoration of blood flow, irrespective of whether MoAb 9H9 was injected before or after a control antibody ($P < .001$ *v* control period, Fig 3). At 5 and 10 minutes after restoration of blood flow, the reduction of leukocyte rolling was still significant, ie, 39.3% ± 28.7% ($P < .01$, n = 7) and 24.8% ± 25.6% ($P < .05$, n = 7), respectively. Rolling returned to control level after 15 minutes. In contrast, an isotype-matched nonendothelial binding control antibody, mIgMκ (n = 7), had no statistically significant effect on rolling. Similarly, no change in rolling versus control period could be detected on injection of PBS (n = 3) or when the nonblocking anti–E-selectin MoAb 14G2 (*n* = 7) was used.

As a complement to determining Rf, velocity profiles were established in two separate and representative experiments. Velocities of individual consecutive cells (123 to 128 cells) passing the observed venular segments were determined before and immediately after MoAb treatment. Cells travelling with a relative velocity greater than the relative V_{crit} (0.20 and 0.25 for Fig 4A and B, respectively) were assumed to be freely flowing, whereas cells with a relative velocity less than the relative V_{crit} were assumed to be rolling. Profiles of leukocyte velocities determined after MoAb 9H9 or mIgMκ treatment were compared with their respective control period recording and are shown in Fig 4A and B. In the presence of MoAb 9H9, more leukocytes were seen to be freely flowing and less cells were rolling, illustrated graphically by the shift to the right in the velocity profile. In this particular experiment, the rolling fraction was reduced from 49.2% in the control period to 27.2% after MoAb 9H9 treatment, corresponding to a reduction of 45%. Mean absolute velocity of rolling cells showed a tendency to increase in this particular experiment, albeit statistically not significant ($P < .134$), when comparing control period (mean ± SD = 0.20 ± 0.12 mm/s; median, 0.19 mm/s) and the first minute after MoAb 9H9 treatment (0.24 ± 0.12 mm/s; 0.25 mm/s). In contrast, the velocities of free cells remained the same before (1.20 ± 0.41 mm/s; 1.24 mm/s) and after (1.18 ± 0.30 mm/s; 1.26 mm/s) MoAb 9H9 treatment, indicating that the decrease in rolling frequency was not due to a change in local microhemodynamics. After mIgMκ treatment, more cells with a relative velocity less than V_{crit} were found in this experiment; Rf before and after mIgMκ treatment was 43.7% and 52.8%, respectively. There were no statistical differences in mean velocities for rolling leukocytes before and after mIgMκ treatment (0.21 ± 0.11 mm/s, 0.21 mm/s *v* 0.18 ± 0.15 mm/s, 0.13 mm/s). Velocities of free cells in control and test period increased in this particular experiment from 1.15 ± 0.45 mm/s (median, 1.275 mm/s) to 2.23 ± 0.74 mm/s (median, 2.09 mm/s). However, no statistically significant differences attributable to mIgMκ treatment could be found in the average mean velocities of free cells measured in seven experiments (Fig 2).

Immunohistology. E-selectin–specific staining of EC was detected with both antirabbit–E-selectin antibodies

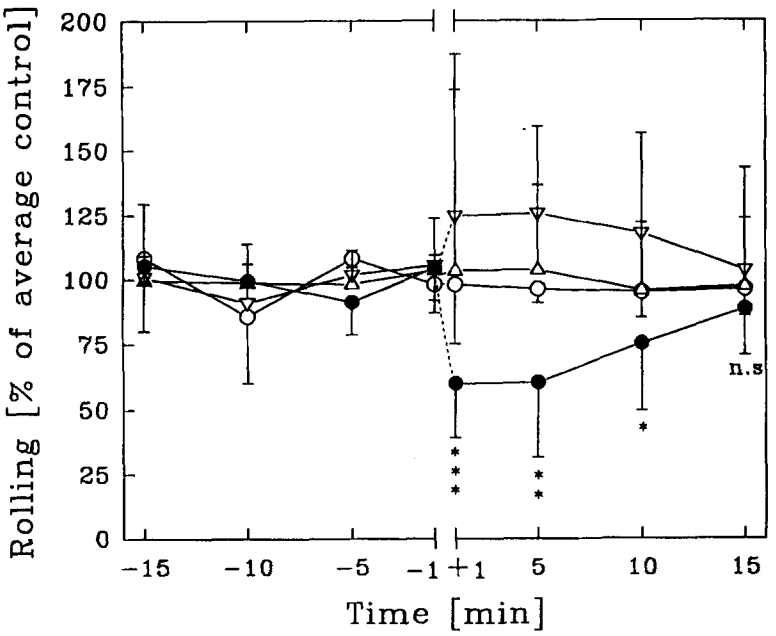

Fig 3. Inhibition of leukocyte rolling by rabbit E-selectin–specific MoAb 9H9. MoAb was allowed to react with the endothelium for 10 minutes, as graphically illustrated by a break in the x-axis. Rolling after MoAb injection (RfMoAb) is expressed as percentage of $Rf_{\text{of average control period}}$ ± SD. MoAb 9H9 significantly reduced rolling of leukocytes by 40.1% ± 20.7% at the first video recording (n = 7; ●). At 5 and 10 minutes after restoration of blood flow, the reduction of rolling was still significant, whereas at 15 minutes, rolling had returned to control level. An isotype-matched control antibody, mIgM$_\kappa$, did not statistically significantly affect rolling (n = 7; ▽). Vehicle alone (PBS without Ca/Mg, n = 3; ○) or an E-selectin binding but not functionally blocking MoAb, 14G2 (n = 7; △), did not affect rolling. n.s., not significant; *$P < .05$; **$P < .01$; and ***$P < .001$.

(14G2 and 9H9) in IL-1–treated tissue. In contrast, MoAb 14G2 in concentrations of up to 100 μg/mL failed to stain untreated tissue, indicating a complete absence of E-selectin in noninflamed mesenteric microvessels. As shown in Table 1, 29 sections from 3 animals treated with IL-1 for 4 hours and 31 sections from 3 animals treated for 6 hours were examined for E-selectin immunoreactivity using MoAb 14G2. In 62% and 71% of those sections E-selectin immunoreactivity was detected. Due to variable orientation of mesenteric tissue upon embedding and sectioning, both cross-sectioned and longitudinally sectioned vessels were obtained. More intense vWF staining was observed localized to venules. Based on nuclear counterstain and vWF staining pattern, it was possible to make a distinction between arterioles and venules in 16 of 40 IL-1–treated sections (identified in 3 of 6 animals in juxtaposed serial sections). A total of 80% and 96% of the vessels identified as venules stained positive for E-selectin after 4 or 6 hours of IL-1 treatment, respectively. Only 5 of 14 (36%) identified arterioles were immunoreactive after IL-1 treatment for 4 hours, whereas 0 of 16 (0%) was found to be positive after 6 hours (Table 1 and Fig 5). In contrast to vWF, E-selectin exhibited an evenly distributed staining pattern, whereas immunohistochemical analysis with isotype-matched control antibodies showed no specific staining of ECs, neither in untreated nor in IL-1–activated rabbit mesenteric tissue.

DISCUSSION

Leukocyte rolling is commonly regarded as a feature of early inflammation depicting the initial step in a cascade of events eventually leading to transendothelial leukocyte migration into the extravascular space.[5] However, it has also been reported to occur constitutively at a low frequency in the dermal microvasculature of mice and rats without apparent signs of tissue inflammation.[11,12] In contrast, rolling appears to be absent in other microvascular networks, such as in the rabbit mesentery, in which it is induced within minutes after surgical tissue manipulation[13] and can be dramatically enhanced within hours after IL-1–induced inflammation.[14] This inducibility of rolling in mesenteric venules suggests that venular EC surface properties can change with distinct kinetics involving either rapid activation and/or redistribution of preformed endothelial adhesion molecules or cytokine-triggered de novo synthesis of inducible surface receptors. To date, the identity of such inducible endothelial receptors that mediate in vivo rolling in response to proinflammatory cytokines has remained unknown.

In vitro studies of neutrophil interactions with endothelial monolayers or endothelial adhesion molecules presented in lipid bilayers or by transfected cells, respectively, have shown that both P- and E-selectin can support rolling under conditions of shear flow.[9,10,40] P-selectin is found in α-granules of resting platelets and in Weibel-Palade bodies of ECs and gets rapidly translocated to the cell surface on activation through histamine, thrombin, or superoxide.[41,42] Thus, P-selectin may be a possible candidate for the mediation of early rolling seen in exteriorized mesenteries, as recently reported for canine[43] and murine[44] mesenteries.

In contrast, E-selectin expression on ECs is induced within several hours after stimulation with inflammatory agents such as bacterial endotoxin or the cytokines IL-1, TNF, and, possibly, IL-3.[6,45] Several in vivo studies have shown a correlation between E-selectin expression and the accumulation of neutrophils and eosinophils in acutely inflamed tissues,[7,46-48] but these studies were unable to identify specific mechanisms through which E-selectin determines leukocyte behavior.

A number of observations by us and others consistently point to a direct correlation between E-selectin expression and leukocyte rolling in vivo. Firstly, our immunohistologic studies reported here demonstrate that topical administration

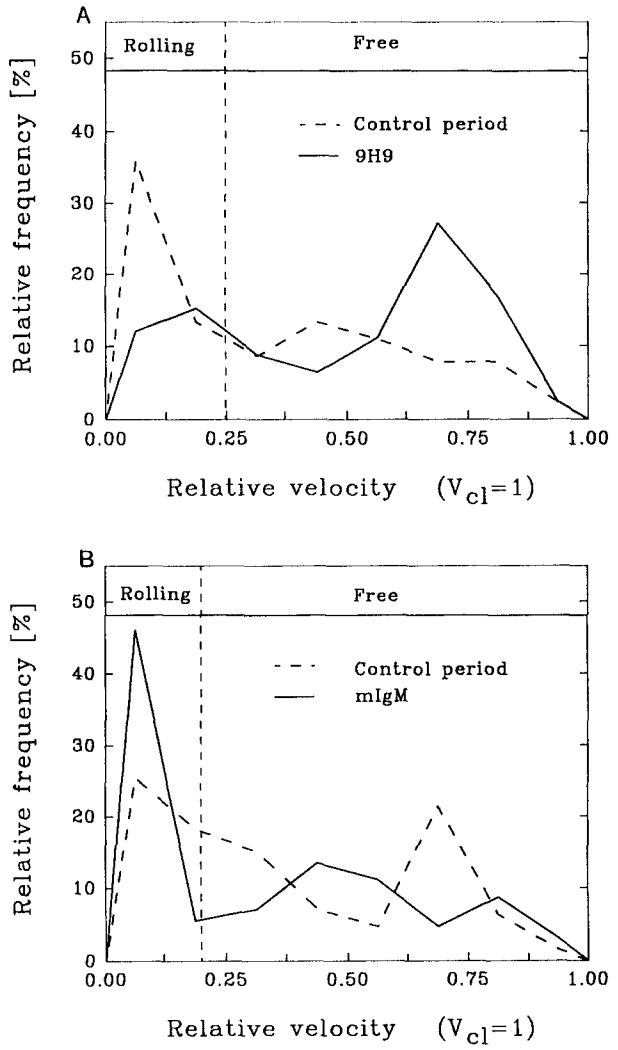

Fig 4. Velocity profile during control recordings and after MoAb treatment. Velocities of approximately 125 consecutive leukocytes, both noninteracting and interacting, passing venular segments were determined in two separate vessels in two representative experiments. The relative V_{crit}, shown as vertical broken lines, were 0.20 and 0.25, respectively. (A) In the presence of MoAb 9H9, more freely flowing leukocytes and less rolling cells were observed illustrated graphically by the shift to the right in the velocity profile. The rolling fraction was reduced from 49.2% to 27.2% (ie, 45% reduction) after MoAb 9H9 treatment. Mean velocities ± SD for rolling cells showed a tendency to increase, albeit statistically not significant ($P < .134$), when comparing the control period and the first minute after MoAb 9H9 treatment (0.20 ± 0.12 mm/s and 0.24 ± 0.12 mm/s). Mean velocities for free cells were not affected by MoAb 9H9 treatment: 1.20 ± 0.41 mm/s and 1.18 ± 0.30 mm/s, respectively. (B) After mIgMκ treatment, more cells with a relative velocity less than V_{crit} were found. In this particular experiment, Rf increased from 43.7% to 52.8% before and after mIgM treatment. Mean velocities of rolling leukocytes before mIgMκ treatment of 0.21 ± 0.11 mm/s were comparable to 0.18 ± 0.15 mm/s after mIgMκ treatment. Velocities of free cells in control and test period were increased from 1.15 ± 0.45 mm/s to 2.23 ± 0.74 mm/s.

of IL-1 in the peritoneal cavity of rabbits induced detectable E-selectin synthesis by microvascular EC. Secondly, coinciding with E-selectin expression, rolling was enhanced by deliberate induction of an inflammatory reaction in the microvasculature. In earlier studies, we found that IL-1 treatment of rabbit mesenteries stimulated rolling of rabbit leukocytes as well as locally injected human neutrophils.[14] This effect appeared to be due to a localized inflammatory reaction of venular EC. Thirdly, the initiation of IL-1–induced interactions of neutrophils with inflamed EC requires the presence of intact L-selectin.[14] In vitro experiments have shown that L-selectin on neutrophils can present carbohydrate ligands to E-selectin[27] and neutrophil binding to E-selectin expressed on activated human umbilical vein ECs (HUVECs) was shown to involve an adhesion pathway that is shared by L- and E-selectin.[28] Consistent with these in vitro studies, we have found that L-selectin can mediate neutrophil rolling in vivo by binding distinct EC ligands through direct engagement of its lectin domain and, simultaneously, through presentation of fucosylated carbohydrates such as sLe^x and related structures that may serve as ligands for E-selectin.[29] Finally, we have recently demonstrated a direct involvement of sLe^x in rolling by treating neutrophils with anti-sLe^x MoAbs or with neuraminidase, which in both cases reduced rolling by nearly 50%.[29] Thus, the destruction of sLe^x on neutrophils that was shown to abrogate binding to E-selectin in vitro, reduced rolling in vivo to a similar extent as treatment with anti–E-selectin MoAb 9H9 did in the present study. The importance of fucosylated sLe^x-like ligands for selectins is further emphasized by the discovery of LAD-II. Patients with LAD-II have a genetic defect in fucose metabolism that prevents them from synthesizing detectable amounts of fucosylated carbohydrates such as sLe^x.[30] Neutrophils from LAD-II patients fail to interact with E-selectin in vitro[30] and roll poorly in IL-1–treated rabbit mesenteries in vivo.[31] Consequently, the affected individuals suffer from severe recurrent bacterial infections that are characterized by the absence of pus formation due to the inability of neutrophils to accumulate in inflamed tissues. Taken together, these various observations provide strong support for the hypothesis that the increase in leukocyte rolling in response to IL-1 involves E-selectin expressed by stimulated venular ECs.

This study provides direct evidence for this hypothesis. Local treatment of IL-1–stimulated microvessels with anti–E-selectin MoAb 9H9 reduced rolling by 40% and had a tendency to increase the velocity of rolling cells that were not detached after MoAb treatment. In contrast, neither a nonblocking anti–E-selectin MoAb nor a class-matched control MoAb had any statistically significant effect on rolling. These results indicate that blocking of endothelial surface E-selectin decreases the adhesive force that allows marginated leukocytes to withstand the shear stress exerted by the blood stream. Our findings are in agreement with recent in vitro flow chamber studies that demonstrate E-selectin–mediated rolling of human neutrophils on transfected L cells and also a partial contribution of E-selectin to neutrophil rolling on cytokine-stimulated HUVECs.[10] In these studies, blockade of E-selectin expressed on activated HUVECs in vitro also

Table 1. Summary of Immunohistochemical Staining

IL-1	Total No. of Sections/ Total No. of Animals Mesenteries	Positive Sections (% of total)	Staining Intensity (no. of mesenteries in class)				Positive Microvessels (% of total identified)	
			0	I	II	III	Venules	Arterioles
None	16/4	0	4	0	0	0	0	0
4 h	29/3	62	0	1	2	0	80	36
6 h	31/3	71	0	1	0	2	96	0

E-selectin antigen could not be identified in untreated tissue. E-selectin–specific staining was found in IL-1–treated tissue and was preferentially localized to venules.

reduced rolling of human neutrophils by about half, a finding that is remarkably similar to our in vivo observations.

MoAb 9H9, the antibody used here to block E-selectin–dependent adhesion in these experiments, is a murine IgM raised against recombinant rabbit E-selectin.[33] In preliminary studies, we found that adhesion of HL60 cells to COS or CHO cells transfected with rabbit E-selectin cDNA was effectively decreased when E-selectin was blocked with 10 μg/mL of MoAb 9H9 (B.W., unpublished observation). However, MoAb 9H9 had no significant effect on leukocyte rolling in IL-1–treated rabbit mesenteries when administered IV at a dose of up to 2 mg/kg BW (U.H.v.A., unpublished observation). We speculated that the avidity of MoAb 9H9, as reported for other IgM antibodies, may be rather low.

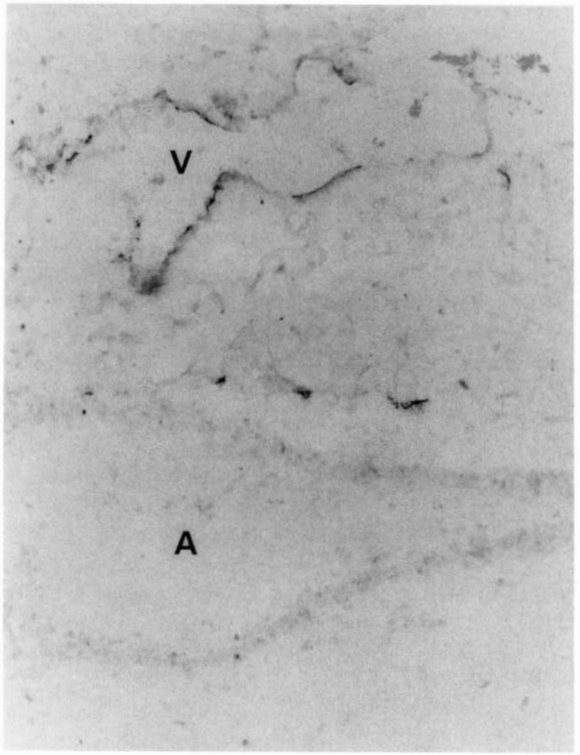

Fig 5. E-selectin localization to venules 6 hours after IL-1 treatment. Brown reaction product (DAB) depicting MoAb 14G2 binding to E-selectin is localized to the venule (V), whereas no specific staining could be detected in the adjacent arteriole (A). Methyl green was used as nuclear counterstain. (original magnification × 140).

This could be especially true in vivo, in which a constant washing effect exerted by the blood stream on the endothelial lining may prevent sufficient binding of MoAb 9H9 to its antigen. Therefore, we developed a novel approach that allowed more time for intravascular MoAb binding without interference by the blood flow and local administration of MoAb at a higher concentration than achievable by systemic treatment.

We supplemented our previously published model[14] by developing a method that allowed us to completely and reversibly block blood flow in a segment of the mesentery. Subsequently, unperfused venules could be treated locally with antibody solution. This technique made it possible to target small quantities of MoAb in high concentration and for prolonged periods of time to a selected microvascular network. As an additional advantage, the effect on leukocyte adhesion of more than one agent could be tested and compared in one preparation. In these experiments, the maximum theoretical systemic concentration of any given antibody in whole blood after local injection did not exceed 2 μg/mL. As mentioned above, IV injection of up to 2 mg/kg BW of MoAb 9H9 resulting in a more than 10-fold higher systemic concentration did not affect leukocyte rolling, probably due to insufficient binding of freely circulating MoAb. We could interchange the order of MoAb application and saw significant reduction of rolling only when MoAb 9H9 was used, irrespective of whether it was administered before or after control agents. Thus, this modification enabled us to analyze treatment effects by using paired data comparisons and to reduce the number of test animals needed.

The assumption that MoAb 9H9 may only have moderate affinity for venular E-selectin is consistent with the observation that its effect on rolling was highest within the first minutes after restoration of blood flow. Rolling returned to baseline levels within 15 minutes after reperfusion. This may indicate that the MoAb was progressively washed off the EC by the blood stream and supports the hypothesis that the affinity of MoAb 9H9 is too low for it to be able to bind to E-selectin expressed in physiologically perfused venules. However, we cannot exclude that the progressive loss of effect of MoAb 9H9 may be due in part to internalization of surface-bound MoAb.

Although the reduction in rolling after treatment with MoAb 9H9 was statistically highly significant during the early test period, it was not complete but only amounted to 40% compared with rolling under control conditions. This could partly be due to insufficient blocking of E-selectin–

mediated rolling by MoAb 9H9. In static in vitro binding assays, leukocyte adhesion to E-selectin expressed on transfected cells was maximally reduced by MoAb 9H9 to approximately 25% of control using 10 μg/mL (B.W., unpublished data). Therefore, it is likely that the concentration of 50 μg/mL used here was at least initially sufficiently high to allow maximum inhibition achievable with this MoAb. However, the in vitro data also indicate that MoAb 9H9 did not abolish leukocyte binding completely. Thus, we cannot exclude the possibility that the actual contribution of E-selectin in our experiments was even higher than indicated by the degree of inhibition achieved with MoAb 9H9.

Nevertheless, it appears likely that E-selectin is not the sole EC adhesion molecule involved in IL-1–induced leukocyte rolling in rabbit mesenteries. Several other adhesion molecules, such as ICAM-1, VCAM-1, and a putative ligand for L-selectin, have been reported to be induced by IL-1. ICAM-1 is a ligand for β2 integrins.[49] Both ICAM-1 and β2 integrins are likely not involved in the initiation of leukocyte rolling.[9,25,50] VCAM-1 is a ligand for α4β1 integrin.[51] It is currently unknown whether VCAM-1 or α4 integrins can initiate leukocyte adhesion under conditions of flow in the absence of selectins. Other investigators have reported that L-selectin–mediated adhesion to cultured HUVECs can be induced by cytokines, suggesting the existence of a putative inducible L-selectin ligand.[52] Although no such ligand for L-selectin has been identified in nonlymphoid tissues in vivo, recent evidence suggests that such molecules do exist. Pre-B cells expressing human or mouse L-selectin, but not vector-transfected control cells, roll in mesenteric venules.[29,53] The fact that these cells do not interact with vascular selectins in vitro suggests a direct interaction of L-selectin, presumably through its lectin domain, with an as yet unidentified ligand. This interaction is unlikely to be affected by treatment with MoAb 9H9 and could significantly contribute to the residual rolling seen after MoAb treatment.

Endothelial P-selectin could also play a role. In vitro results provide evidence that this molecule can support rolling under low shear.[9,40] Furthermore, P-selectin has been shown to support leukocyte rolling in exteriorized canine[43] and murine mesenteries.[44] Although originally reported to be expressed only transiently (less than 1 hour) in cultured ECs on stimulation,[41] P-selectin has recently been found to be induced on ECs exposed to cytokines as well.[54] Therefore, it cannot be excluded that P-selectin may be expressed for prolonged periods of time in vivo and, thus, may also play a role in the present experiments. However, it appears unlikely that the 10-minute ligation of the mesentery artery induced a significant upregulation of P-selectin in the present study, because this would likely result in increased rolling immediately after restoration of flow; this effect was not observed.

Leukocyte rolling is a feature characteristic of and largely restricted to venules; it is mostly absent in arterioles.[1,55] Recent experiments have provided evidence that this distinction of leukocyte behavior is not primarily determined by microvascular fluid dynamics, but is likely based on differences in endothelial cell phenotypes.[2,3] In agreement with this model, our data obtained from immunohistologic analysis of IL-1–treated mesenteries show that E-selectin immunoreactivity was not equally observed in all segments of the microvascular network, but was preferentially detected in venules, whereas arterioles rarely showed E-selectin–specific staining. This finding is in agreement with several previous reports.[56,57] A predominant immunoreactivity of venules with anti–E-selectin MoAb has been demonstrated in skin organ cultures treated with IL-1, TNF, LPS,[58] or substance P,[59] in chronically inflamed skin[60] and in inflamed synovia from patients with rheumatoid arthritis and osteoarthritis.[61] However, in other experimental settings, E-selectin was reported to be expressed in a less selective manner. For instance, induction of septic shock in baboons caused E-selectin expression in all parts of the renal microvasculature and in venules, capillaries, and occasional arterioles in the lung.[62] Furthermore, E-selectin expression in various human liver diseases was reported to involve veins and arteries in the portal tract as well as central veins, whereas sinusoidal endothelia remained negative even during severe inflammation.[63] Thus, the regulation of E-selectin expression may be dependent on tissue- or stimulus-specific mechanisms.

The role of E-selectin in inflammation, although clearly a mediator of initial leukocyte attachment and rolling, may not be limited to this function. In vitro reports suggest that binding of E-selectin to neutrophils can induce functional upregulation of β2-integrins,[64,65] which themselves do not appear to be capable of initiating rolling interactions in the absence of selectins.[9,14] However, E-selectin is not required for transendothelial leukocyte migration[10,66] and it is currently unclear whether E-selectin can have a signaling function in vivo. Undoubtedly, further research is required to elucidate these questions.

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