Fucoidin, But Not Yeast Polyphosphomannan PPME, Inhibits Leukocyte Rolling in Venules of the Rat Mesentery

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Leukocyte rolling in venules is inhibited by several sulfated polysaccharides, by antibodies to the leukocyte adhesion receptor L-selectin (LECAM-1), and by recombinant soluble L-selectin. The sulfated fucose polymer fucoidin and the polyphosphomannan PPME bind to L-selectin and inhibit L-selectin-mediated lymphocyte adhesion to lymph node high endothelial venules (LN-HEV). We investigated whether fucoidin and PPME also inhibit leukocyte rolling. Rolling leukocyte flux was determined by intravital microscopy in 47 venules (diameter 21 to 50 μm) of the rat mesentery with and without micro-infusion of each reagent through 8-μm glass micropipettes. Micro-infusion (1 mg/mL) or intravenous (IV) injection (25 mg/kg) of fucoidin, but not vehicle, reduced leukocyte rolling by greater than 90%. The half-effective concentration was approximately 2.5 μg/mL. Stroboscopic fluorescence video microscopy showed that fucoidin decreased the fraction of rolling leukocytes from 44% of all leukocytes passing the venules in control to less than 1%.

Before leukocytes adhere to and emigrate from venules during the acute inflammatory response, they marginate and roll along the venular endothelium. Leukocyte rolling is known to be absent in undisturbed tissues such as the bat wing, which does not require surgery for visualization of the microcirculation, but it has been reported to be present in dermal microvessels of anesthetized mice. In the exposed mesentery, rolling is routinely seen without intentional stimulation and is restricted to venules. Leukocyte rolling is not present in undisturbed mesenteric venules, but is rapidly induced by tissue handling associated with exteriorization. Rolling leukocytes are predominantly, but not exclusively, granulocytes.

In postcapillary venules, leukocytes are displaced from the axial flow toward the vessel wall because of interactions with erythrocytes and erythrocyte aggregates in the flowing blood. As the leukocyte approaches the wall, its velocity decreases because of the shape of the velocity profile and drag forces in the vicinity of the wall. The velocity of a spherical object of leukocyte dimensions (7-μm diameter) ranges well above 300 μm/s in the absence of adhesion when traveling at molecular distances (approximately 20 nm) from the wall at flow conditions typical for postcapillary venules. Therefore, even a fully marginated leukocyte in, eg, a 30-μm venule will travel much faster than a rolling cell, whose typical velocity is about 50 μm/s. Hence, leukocyte rolling as typically observed in postcapillary venules requires a transient adhesive interaction between the leukocyte and the endothelial cells. This adhesive interaction has been shown to be inhibited by dextran sulfate, to some extent by heparin and chondroitin sulfate type C, but not by carboxymethyl dextran, chondroitin sulfate type A and B, and keratan sulfate. These inhibitors of leukocyte rolling share a hexose backbone with a varying amount of sulfate groups attached. The absence of an inhibitory effect of some of the sulfated polysaccharides has been interpreted to indicate some structural specificity of the interaction of these inhibitors with the adhesive molecules underlying leukocyte rolling. Recently, we have shown that leukocyte rolling in rat mesenteric venules is inhibited by an antiseraum raised in rabbits against the murine cell adhesion lectin L-selectin (LECAM-1), and by soluble recombinant L-selectin, indicating that L-selectin is involved in mediating leukocyte rolling in vivo. This is confirmed by the finding that a murine monoclonal antibody (MoAb) recognizing human and rabbit L-selectin blocks leukocyte rolling in rabbit mesenteric venules.

L-selectin, originally described as MEL-14 antigen (gp90Mel), is involved in recirculation and homing of lymphocytes to lymph nodes. The MoAb MEL-14 inhibits lymphocyte attachment to lymph node HEV in the Stamper-Woodruff in vitro assay using cryostat sections of lymph node tissue. The algal sulfated fucose polymer fucoidin has been shown to also inhibit this adhesion. This suggested that an association between a protein and a carbohydrate moiety, ie, a lectin-like interaction, was involved in lymphocyte-lymph node high endothelial venule (LN-HEV) recognition. Subsequently, the primary structure of L-selectin showed a lectin-like domain, and the adhesion-blocking MEL-14 antibody was shown to bind to this lectin portion of the molecule. MEL-14 inhibits binding of the mannose-6-phosphate-rich yeast polyphosphomannan PPME to murine lymphocytes, while another antibody (Ly22) mapping to the epidermal growth factor (EGF) domain of the L-selectin molecule does not. MEL-14 was also shown to block binding of murine lymphocytes to micro-beads coated with PPME. Like fucoidin, soluble PPME was able to block the interaction of murine lymphocytes and granulocytes with LN-HEV. Similar findings have been reported in the rat: In Stamper-Woodruff adhesion assays, PPME (100 to 200 μg/mL) blocked 60% to 80% of rat lymphocyte attach-
ment to rat lymph node cryostat sections, compared with 80% inhibition produced by fucoidin (10 μg/mL) in the same system,28 suggesting that fucoidin may be about 10 to 20 times more potent than PPME in this assay. In the mouse, both PPME and fucoidin have also been shown to block lymphocyte entry into peripheral lymph nodes and other lymphatic organs in vivo.29

L-selectin is abundantly expressed not only on lymphocytes, but also on monocytes and granulocytes.30-33 Anti-L-selectin antibodies30,32 or soluble recombinant L-selectin32 as well as removal of L-selectin from the granulocyte surface by phorbol ester stimulation33 or mild chymotrypsin treatment34 have been shown to attenuate granulocyte recruitment to inflammatory sites. These findings advocate a pivotal role for L-selectin and hence for leukocyte rolling in the inflammatory process.

The present study was undertaken to investigate the effects of fucoidin and PPME on leukocyte rolling in vivo. In view of independent evidence suggesting a role for L-selectin in leukocyte rolling,7,8,26 we aimed at establishing further parallels and possible differences between the lectin interactions underlying lymphocyte homing and leukocyte rolling. To this end, we examined the effect of fucoidin and PPME on leukocyte rolling in venules of the exposed rat mesentery. We chose to use a micro-infusion technique7,8 because PPME is available in small quantities only. Moreover, systemic side effects of intravenous application could influence the outcome of the experiments. Minute volumes (typically less than 0.5 μL) were infused via 8-μm glass micropipettes into upstream side branches of the venules to be studied, and the effect on the number of rolling leukocytes per time (rolling leukocyte flux) was measured.

MATERIALS AND METHODS

Animals. Twenty-three female Sprague-Dawley rats (body mass 250 to 300 g) were anesthetized with ketamine (Ketavet, Parke-Davis, Berlin, Germany; 75 mg/kg intramuscularly [i.m.]) after premedication with pentobarbital (Nembutal, Sanofi, Hannover, Germany; 20 mg/kg i.m.). Trachea, right carotid artery, and right jugular vein were cannulated, and arterial blood pressure and heart rate were recorded continuously. Leukocyte concentration in carotid blood samples (20 μL) was determined at approximately 45-minute intervals using an electronic cell counter (Coulter Dx, Herts, UK). The animals were thermo-controlled to 36.5°C to 37°C via a heating pad and a rectal thermostor. The peritoneal cavity was opened using a thomocauter to prevent local bleeding. A few loops of ileum proximal to the appendix were exteriorized onto a lucite stage and superfused with thermostated (37°C) bicarbonate-buffered saline (composition, mmol/L: NaCl 132, KCl 4.7, CaCl2 2, MgCl2 1.2, NaHCO3 18, equilibrated with 5% CO2 in N2 to adjust pH to 7.35). The animals received a continuous intravenous (IV) infusion of physiologic saline containing pentobarbital (0.2 mg/mL) at 40 mL·kg⁻¹·h⁻¹ to maintain anesthesia.

Reagents. The superfusion solution was prepared fresh daily from 10× stock solutions. The fluorescent dye acridine red (Chroma, Stuttgart, Germany) was dissolved in phosphate-buffered saline (PBS) at 1 mg/mL, and undissolved material was removed by centrifugation. Fucoidin, an algal fucose and fucose-4-sulfate polymer with a molecular mass of about 100 Kd (78 to 133 Kd, depending on method of determination), was obtained from Sigma (Deisenhofen, Germany; F-5631, lot no. 01 F-3860). Two different batches of the mannose-6-phosphate-rich polysaccharoman from Hansenula holstii, PPME, were generously supplied by Dr S.D. Rosen (Department of Anatomy, University of California, San Francisco) and by Dr M.E. Slodki (US Department of Agriculture, Peoria, IL), respectively. The batch of PPME received from Dr Rosen has previously been tested in an in vitro assay of lymphocyte adhesion and was found to completely inhibit binding of Jurkat T cells to high endothelial venules in cryostat sections of peripheral lymph nodes at 10 μg/mL (S.D. Rosen, personal communication, November 1991). MNN2, a yeast mannann containing less phosphate, was also a gift from Dr Rosen. All reagents were dissolved freshly in Dulbecco’s PBS (GIBCO, Berlin, Germany).

Micro-application. Glass micropipettes were drawn from standard borosilicate glass (outer diameter 0.9 mm; Hilgenberg, Malsfeld, Germany) on a vertical micropipette puller (Getra, Munich, Germany) and beveled to a tip diameter of 7 to 10 μm using a revolving abrasive plate with a 0.2-μm abrasive foil (World Precision Instruments, New Haven, CT). The pipettes were filled with approximately 15 μL of filtered (0.45 μm, Millex HV4; Millipore, Bedford, MA) reagent solution. An air-filled tubing and syringe system was attached to the pipette holder and served as a pressure reservoir controlled by a 10-μL syringe that permitted stable, continuous micro-infusions. An upstream side branch (approximately 20-μm diameter) of the venule to be investigated was impaled with the micropipette using a piezo-driven micromanipulator (Märzhäuser PM 10, Wetzlar, Germany). Micro-infusion was started and maintained for 60 seconds by adjusting pressure under microscopic control as described,7,8 allowing for admixture of blood from tributaries of the investigated venule. The infusate replaced approximately one fifth to one half of the blood in the investigated vessel.

Intravital microscopy. After a brief resting period, the mesenteric microcirculation was observed using a Leitz (Wetzlar, Germany) intravaltral microscope (objective SW 25/0.60, projection eyepiece 1.25×) modified for telescopic imaging,35 and recorded on 1/4-inch videotape (Sony U-matic; Sony, Berlin, Germany) via a 1/4-inch videocamera (RCA, Lancaster, PA). Rolling leukocyte flux was determined off-line by counting the number of leukocytes distinguishable from the blood stream passing a line perpendicular to the vessel axis. For this purpose, the videotapes were replayed at one half to one fifth of their original speed, and the time intervals between rolling leukocytes recorded on a personal computer. For analysis of leukocyte rolling, counting visibly distinguishable cells is widely used and sufficiently reliable and accurate at blood flow velocities above approximately 0.5 mm/s.4,8,9 We determined the number of rolling leukocytes passing each venule per 2-second time interval and converted it to rolling leukocyte flux. For comparison between observations made in different venules, all flux values were normalized with respect to the average rolling leukocyte flux during the control period following each micro-infusion.

In some experiments, all passing leukocytes, both freely flowing and rolling, were visualized by fluorescent labeling with acridine red (Chroma, Stuttgart, Germany; approximately 5 mL/kg in saline, IV). The resulting concentration of acridine red was sufficient for fluorescent staining of leukocytes, but did not interfere with systemic leukocyte concentration or leukocyte rolling, as verified in independent transillumination experiments (data not shown). To obtain a clear image of even the fast-moving cells, stroboscopic (50 s⁻¹, Strobex 236, Chadwick Helmuth, Mountain View, CA) epi-illumination through a Leitz Ploemopak adapter (filter cube N 2.1) was used. Fluorescent scenes were recorded via a silicon intensified target camera (Bosch TYC 9A, Stuttgart, Germany), yielding a field of view of approximately 300 by 250 μm with the 25× objective used. Venules with diameters of approximately 30 μm were selected, because earlier in vivo studies had shown that in these vessels all leukocytes can
be detected with acridine red labeling and epi-fluorescence. Video scenes of 2 to 5 minutes duration were recorded during and after micro-infusion of fucoidin and vehicle, and were evaluated using a personal computer (PC)-based interactive digital image processing system developed for microcirculatory applications.  

Within each scene, the velocities of about 30 consecutive leukocytes were determined by measuring the distance traveled between two or more successive video frames. Using the leukocyte velocity data, mean blood flow velocity $v_b$ and a critical velocity $v_{cn}$ were determined as described previously.  

$\text{Critical velocity is defined as the minimum velocity a freely flowing leukocyte can assume: } v_{cn} = \frac{v_b}{2 - \epsilon}$ 

where $\epsilon$ is the ratio of leukocyte ($7 \mu m$) to vessel diameter. This estimate of critical velocity represents one-half of the velocity a fluid particle would have if at the distance of one leukocyte radius from the wall in undisturbed parabolic flow. This corresponds to the velocity of a non-interacting sphere with $7 \mu m$ diameter would have when traveling at a distance of 20 nm from the vessel wall.  

Leukocyte discharge concentration in each investigated venule was calculated by dividing total leukocyte flux by estimated blood flow as described earlier.

Data are presented as mean $\pm$ SEM of n applications, with the range indicated where appropriate.

**Flow cytometry.** Granulocytes were isolated from freshly drawn, heparinized (10 U/mL) human blood as described. Briefly, erythrocyte sedimentation was accelerated by adding approximately 40 vol/vol of autologous plasma to the blood samples. The leukocyte-rich plasma (2 mL per 16-mm tube) was layered onto a discontinuous (3 mL of 55% on 4 mL of 74%) isotonic Percoll (Sigma, Deisenhafen, Germany) gradient and centrifuged at approximately 600 g. The polymorphonuclear granulocyte (PMN) suspension (lower band) was washed in isotonic saline and suspended in M 199 (GIBCO, Berlin, Germany) containing 1% fetal bovine serum. Leukocyte content of the suspension as evaluated by counting 100 cells stained with Turk's solution was greater than 95% granulocytes. PMN viability as evaluated by the Trypan Blue exclusion test (15 minutes at room temperature) was greater than 97% in all experiments. PMN suspensions were used within three hours of isolation. Isolation of rat granulocytes was attempted using gradients made from Percoll at different densities, Ficoll 1077 over Ficoll 119 (3 mL each; Sigma), or hypotonic and/or detergent lysis. The preparations obtained were highly contaminated with erythrocytes and were not suitable for flow cytometric analysis of carbohydrate binding to rat granulocytes.

Fluorescein isothiocyanate (FITC)-fucoidin and FITC-PPME were prepared as described and incubated with the granulocytes at 3 μg/mL on ice. Specificity of binding was assessed by running parallel samples in calcium-free media in the presence of 10 mM/L EDTA (pH adjusted), because both PPME and fucoidin binding to L-selectin is known to be calcium-dependent. In some experiments, granulocytes were pre-incubated with a 100-fold excess (300 μg/mL) of fucoidin or PPME for 30 minutes before adding the fluoresceinated reagents. Also, granulocytes were activated by adding the chemotactic peptide, formyl-methionyl-leucyl-phenylalanine (FMLP, Sigma; final concentration, 100 nM/L) at 25°C for 1, 2, and 10 minutes before incubation with FITC-PPME or fucoidin on ice. In addition, granulocytes were pre-incubated with the anti-L-selectin MoAb, TQ-1 (clone 5FC128T17G6; Coulter Immunocytochemicals, Krefeld, Germany). TQ-1 binding was visualized using FITC-conjugated polyclonal rabbit anti-mouse IgG (F 261, lot 059; Dakopatts, Hamburg, Germany).

Measurements were done using a Becton-Dickinson (Heidelberg, Germany) FACScan flow cytometer and the LYSIS II 1.0 software (Becton Dickinson). Five thousand to 10,000 events were analyzed per sample. Remaining erythrocytes and debris were gated out based on forward and side scatter. Data shown are representative of two to three independent experiments.

**RESULTS**

A total of 270 micro-infusions were made into 47 venules (diameter 39 ± 2 μm, range 21 to 50 μm) of 23 rats, whose blood pressure and heart rate remained stable at normal values throughout the experiments. Systemic leukocyte concentration increased from 6,500 ± 500 μL$^{-1}$ to 9,300 ± 1,200 μL$^{-1}$ during the 90-minute experimental protocol.

Fucoidin inhibits leukocyte rolling. Micro-infusion of vehicle (PBS) did not significantly alter rolling leukocyte flux during 45 applications in five animals, which is in agreement with earlier observations with the same technique. By contrast, fucoidin at 1 mg/mL reduced rolling leukocyte flux by 94% ± 6% (range 68% to 99%) during 31 applications in five animals (Fig 1). Rolling leukocytes disappeared within 10 to 20 seconds of micro-infusion of fucoidin, and rolling remained suppressed throughout the injection period. Rolling leukocytes returned within 15 seconds on termination of micro-infusion and reached control values within 20 to 30 seconds. Beyond this period of transition, fucoidin had no sustained effect on blood flow or leukocyte rolling, which did not change further during a 3-minute postinfusion control period (data not shown). Rolling leukocytes entering the fucoidin-perfused venule from tributaries typically continued to roll for 50 to 100 μm (1 to 2 seconds) before becoming detached from the endothelium.

To investigate the concentration-response relationship, fucoidin was micro-infused at concentrations between 0.1 μg/mL and 10 mg/mL. The half-effective concentration was found to be about 10 μg/mL in the micropipette (Fig 2), which corresponds to approximately 2.5 μg/mL in the venule because of the dilution effect of the admixed blood. The dilution effect was estimated by comparing leukocyte concentrations in microvessels during and after micro-infusion using fluorescence video microscopy. The discharge concentration of leukocytes averaged 8,100 ± 1,200 μL$^{-1}$ (95% ± 10% of systemic concentration, n = 13) during the control period,
and 5,900 ± 900 μL⁻¹ (71% ± 9% of systemic) during micro-infusion. This indicates that the micro-infused fluid was diluted with blood by an average ratio of 1:4.

In five animals, fucoidin was injected IV at a dose of 25 mg/kg body weight corresponding to approximately 300 μg/mL blood. This reduced rolling leukocyte flux by 91% ± 4% (Fig 3), which corresponds well with the effect of micro-infusion of fucoidin. IV fucoidin did not alter systemic leukocyte concentration; it was 6,500 ± 1,500 μL⁻¹ immediately before and 6,300 ± 1,400 μL⁻¹ 10 minutes after the injection.

**Fucoidin leads to detachment of rolling leukocytes.** Effective inhibition of leukocyte rolling by fucoidin was confirmed in three separate experiments using fluorescent labeling of leukocytes by intravenous acridine red and stroboscopic video microscopy. Cell velocities were measured for a total of 777 leukocytes during and after seven micro-infusions of fucoidin (1 mg/mL) and six micro-infusions of PBS. While during the control period 44% ± 5% of all leukocytes passing the investigated venules were traveling at subcritical velocities, ie, rolling, this fraction was reduced to 1% ± 0.6% by micro-infusion of fucoidin (Fig 4). By contrast, PBS had no effect on the fraction of rolling leukocytes: it remained at 46% ± 6% of total leukocyte flux during micro-infusion of PBS. Fucoidin and PBS were injected in hemodynamically similar and sometimes identical venules of equal size (29 ± 2 μm). Blood flow velocity increased to 161% ± 31% of control in the fucoidin group and to 176% ± 24% in the PBS group.

Additional insight into the time course of the return of rolling leukocytes after fucoidin micro-infusion was obtained by measuring the velocities of approximately 100 consecutive leukocytes during and after each of five micro-infusions. While the velocities of freely flowing leukocytes and hence blood flow velocity returned to control values immediately on termination of micro-infusion, the first rolling cells did not appear until 12 seconds later (Fig 5). This agrees with the direct measurements of rolling leukocyte flux. Hence, the delayed return of rolling leukocytes cannot be attributed to a sustained velocity increase after micro-infusion.

**PPME has no effect on leukocyte rolling.** The yeast-polysaccharidic PPME was micro-infused 49 times at different doses between 1 μg/mL and 1 mg/mL in five animals. No inhibitory effect on leukocyte rolling was seen even at the highest concentration used (1 mg/mL, Fig 6). In these experiments, both sources of PPME were used and found to be equally ineffective. The control polysaccharide, MNN2, did not inhibit leukocyte rolling either. In one experiment, PPME was injected IV at a dose of 14 mg/kg, yielding a calculated blood concentration of 170 μg/mL, which also failed to reduce rolling leukocyte flux. This indicates that PPME does not affect leukocyte rolling in rat mesenteric venules.

**PPME and fucoidin specifically bind to L-selectin.** Both PPME and fucoidin have previously been shown to bind to rat and human lymphocytes in a calcium-dependent fashion. To investigate the extent and quality of PPME and fucoidin binding to granulocytes, flow cytometry experiments were done using FITC-labeled polysaccharides. Both PPME and fucoidin (3 μg/mL) bound to human granulocytes in a calcium-dependent fashion (Table 1, and data not shown). PPME binding was downregulated by activation of the PMNs with fMLP (100 nM/L, 1 to 10 minutes); this was paralleled by a reduced binding of the MoAb TQ-1 recognizing L-selectin. Pre-incubation of human granulocytes with TQ-1 resulted in greatly diminished binding of FITC-PPME and FITC-fucoidin. This pattern of binding indicates that both PPME and fucoidin recognize L-selectin. However, it should be noted that fucoidin shows less specificity for L-selectin than PPME. As shown in Table 1, 20% to 40% of the binding sites on human neutrophils for FITC-fucoidin at 3 μg/mL are not inhibited by either EDTA or TQ-1. Cation-independent binding increases at higher concentrations of fucoidin, suggesting the presence of high capacity, low affinity binding sites (data not shown).
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Fig 4. Velocity histograms (logarithmic bins) of normalized leukocyte velocities in 7 venules during (left) and after (right) micro-infusion of fucoidin (1 mg/mL in micropipette, approximately 0.25 mg/mL in venule; top panels) and in six venules during and after micro-infusion of vehicle (PBS, lower panels).

DISCUSSION

At a half-effective concentration of about 2.5 μg/mL, fucoidin is among the most effective carbohydrate inhibitors of leukocyte rolling known, matched only by dextran sulfate.8,17 Fucoidin is known to interact with blood lymphocytes, blocking their adhesion to LN-HEV in the Stamper-Woodruff in vitro adhesion assay.20,21 and their entry into lymphatic organs in vivo.29

Both fucoidin and PPME are known to bind to the leukocyte adhesion receptor, L-selectin (LECAM-1).21,22,43 L-selectin is abundant not only on lymphocytes, but also on monocytes31 and granulocytes,27,30 where its glycosylation pattern is different.44,45 Because L-selectin has been shown to be important in mediating leukocyte rolling in mesenteric venules,18 it appears plausible that the inhibitory effect of fucoidin is caused by its binding to L-selectin. Soluble fucoidin binds to lymphocytes readily and blocks binding of the anti-L-selectin antibody MEL-14,22 which is known to recognize the lectin portion of the L-selectin molecule.25 The binding of both PPME and fucoidin is calcium-dependent,46,47 which is consistent with the C-type lectin domain found in L-selec-
tin.23,24

PPME, like fucoidin, is functional in suppressing lymphocyte and granulocyte binding to LN-HEV in cryostat sections.1,27,46 On the other hand, PPME binds poorly to murine lymphocytes31 and does not block MEL-14 binding.22 Another difference between the two polysaccharides is their divergent ability to block binding of a soluble recombinant L-selectin-IgG chimera.25 While fucoidin (1 to 10 μg/mL) completely inhibits binding of L-selectin-IgG to high endothelial venules of cryostat sections of peripheral lymph nodes, PPME at the same concentration has little effect.46 (S. Watson, Genentech Inc, South San Francisco, CA; personal communication November 1991). Moreover, fucoidin completely blocks precipitation of a ligand for L-selectin on LN-HEV by the L-selectin–IgG chimera, while PPME is only marginally effective.47 The requirements for HEV binding and PPME binding by L-selectin are not identical, because certain MoAbs to L-selectin exclusively block one function, but not the other, while other antibodies block both.48

In the present study, PPME at a concentration 100 times greater than the half-effective concentration of fucoidin consistently had no effect on leukocyte rolling. In view of the similar inhibitory effects reported for PPME (100 μg/mL) and fucoidin (10 μg/mL) on rat lymphocyte binding to LN-HEV in cryostat sections of peripheral lymph nodes in vitro, the present results appear to indicate a differential effect of PPME on leukocyte rolling and lymphocyte binding to LN-HEV. However, in the absence of in vivo studies addressing the effect of PPME and fucoidin on lymphocyte homing in the rat, it remains formally possible that the divergent effects of fucoidin and PPME on leukocyte rolling may be caused by their different affinities for the ligand-binding site on rat granulocyte L-selectin.

Alternatively, the two polysaccharides may interact with partially distinct sites on L-selectin. The shared site may be essential for the binding of lymphocytes to HEV while the fucoidin–only site may be necessary for leukocyte rolling. If this view is correct, then the ligands for L-selectin on LN-HEV and nonlymphoid venules may differ structurally. Recently, a putative ligand for L-selectin has been described on cytokine-activated human umbilical vein.49 This ligand is reported to be sialidase-sensitive and binding of both granulocytes and lymphocytes to cytokine-activated human umbilical vein endothelial cells is inhibited by PPME (3 μg/mL). At present, it is unclear whether this ligand may be similar or identical with that isolated from peripheral lymph nodes.

Alternatively, granulocyte and lymphocyte L-selectin may be influenced by PPME in a different way or to a different extent, which could distinguish the effect of PPME on rolling and LN-HEV attachment, because the majority of rolling cells are granulocytes.2,4,8,10 Although the protein backbones of human granulocyte and lymphocyte L-selectin are identical,44 different glycosylation may leave room for such functional differences. Specifically, only granulocyte L-selectin
The observation of calcium-independent fucoidin binding at higher concentrations raises the possibility that fucoidin inhibits rolling through a nonspecific increase in charge density on the leukocyte surface. However, previous studies in rat lymphocytes showed that PPME (200 μg/mL) and heparin (25 μg/mL) increased charge density to a greater degree than fucoidin at 10 μg/mL. In the current study, fucoidin at 1 to 10 μg/mL inhibited leukocyte rolling, while PPME at 1,000 μg/mL did not. Thus, a non-specific increase in charge density on the leukocyte is an unlikely cause of inhibition.

As a further alternative, fucoidin may be able to also interact with another member of the selectin family of adhesion receptors, P-selectin (GMP-140, PADGEM, CD62). P-selectin is an endothelial cell-leukocyte adhesion molecule that was originally identified in activated platelets and later found to be contained in Weibel-Palade bodies of cultured human endothelial cells. In a recent study, fucoidin has been reported to inhibit binding of a P-selectin-IgG chimera to the monocytic cell line U937 by about 50% at 100 μg/mL. Also, fucoidin has been shown to block binding of purified soluble P-selectin to the promyelocytic cell line HL-60 at 400 μg/mL. Notably, however, the doses of fucoidin necessary to

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**Table 1. Flow Cytometry of Isolated Human Granulocytes**

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<th>Median Value % of Control</th>
<th>Positive Cells %</th>
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<tr>
<td></td>
<td>PPME</td>
<td>Fucoidin</td>
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<tr>
<td>Binding of PPME and fucoidin to granulocyte L-selectin</td>
<td></td>
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</tr>
<tr>
<td>Control</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>10 mmol/L EDTA</td>
<td>18</td>
<td>38</td>
</tr>
<tr>
<td>100-fold excess*</td>
<td>4</td>
<td>20</td>
</tr>
<tr>
<td>TQ-1</td>
<td>15</td>
<td>25</td>
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Parallel loss of PPME and TQ-1 binding on granulocyte activation with fMLP (100 nmol/L), median values, % of control.

<table>
<thead>
<tr>
<th></th>
<th>PPME</th>
<th>TQ-1</th>
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<tr>
<td>Control</td>
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<td>100</td>
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<tr>
<td>1 min</td>
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<td>41</td>
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<tr>
<td>2 min</td>
<td>28</td>
<td>40</td>
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<tr>
<td>10 min</td>
<td>23</td>
<td>41</td>
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* Samples were preincubated with 300 μg/mL of unlabeled PPME or fucoidin, respectively, before incubation with FITC-PPME or FITC-fucoidin.

† TQ-1: Specific binding (total binding minus binding seen with FITC-labeled secondary antibody only).
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Affect P-selectin-mediated binding appear to be one to two orders of magnitude larger than those required for blocking leukocyte rolling. At lower doses (3 μg/mL), fucoidin has been reported to specifically bind to the lectin domain of L-, but not to that of P-selectin. Moreover, circumstantial evidence suggests that fucoidin, like some other sulfated polysaccharides, influences the circulating leukocytes rather than acting on the endothelial cells. In the present study, micro-infusion of fucoidin had no sustained effect on leukocyte rolling after termination of the actual infusion, and rolling leukocytes entering the perfused venule from tributaries continued to roll along the venular endothelium for a few revolutions before becoming detached. The 10- to 20-second delay seen before the return of rolling leukocytes after termination of the micro-infusion does not compromise this interpretation, because it can readily be explained by the time taken for a rolling leukocyte to proceed from the site of micro-infusion to the site of observation.

Recently, a rolling-like movement of isolated granulocytes has been observed in an artificial flow chamber coated with P-selectin, but not with CD54 (inter-cellular adhesion molecule-1, ICAM-1). Because the selectins are heavily glycosylated, they may contain carbohydrate structures that could serve as ligands for other selectins. This possibility has recently been proposed for the interaction of E-selectin (ELAM-1) with granulocyte, but not lymphocyte L-selectin. Plastic-bound L-selectin purified from granulocytes, but not material purified from lymphocytes, supported binding of the mouse pre-B-cell line L1-2 transfected with E-selectin cDNA. This ability was correlated by the investigators with the presence of the putative ligand for E-selectin, sialyl-Lewis^ antigen (sialyl-CD15)^ on granulocyte, but not on lymphocyte L-selectin. Moreover, binding of isolated granulocytes to COS-cells transfected with E- or P-selectin cDNA was reduced by approximately 50% by an MoAb to L-selectin. Whether the possible presentation of sialyl-Lewis^ tetrasaccharide by L-selectin is indeed significant for adhesion mediated by endothelial E- or P-selectin remains to be determined. In a recent study, Spertini et al. report that MoAbs to L- and E-selectin have a clearly separable and additive effect on granulocyte and lymphocyte binding to cytokine-stimulated endothelial cells. By contrast, Kishimoto et al. did not see additive effects of a different set of antibodies to L- and E-selectin in a similar experimental system, suggesting that L- and E-selectin may be elements of a common adhesion pathway. In the same report, an additional component of E-selectin-dependent, but L-selectin-independent granulocyte adhesion to cytokine-stimulated endothelial cells was shown using CD18-deficient granulocytes. Taken together, these results suggest that specific ligands exist for both L- and E-selectin. A novel, mucin-like ligand for L-selectin has recently been identified on LN-HEV. Its distribution is restricted to lymph nodes and the lung as determined by Northern blot analysis.

The data presented here are consistent with the notion of L-selectin on rolling leukocytes recognizing a carbohydrate-containing ligand on endothelial cells: Both the dynamics of inhibition of leukocyte rolling by fucoidin as discussed, and previous results with soluble recombinant L-selectin and blocking antibodies appear to support this idea. Any possible contribution of endothelial P-selectin and/or E-selectin in mediating leukocyte rolling in vivo remains to be determined.

In view of the crucial role L-selectin-mediated granulocyte adhesion plays in the inflammatory process, the present findings represent a significant step on the way to a more complete understanding of this adhesion mechanism. Inhibiting leukocyte rolling either with soluble L-selectin or anti-L-selectin antibodies reduces subsequent firm leukocyte adhesion and hence their recruitment to inflammatory sites. Pharmacologic interference with L-selectin-mediated adhesion may represent a useful approach to an anti-inflammatory therapy of inflammatory and ischemic diseases. The present study suggests that carbohydrate compounds could be engineered to specifically inhibit L-selectin-mediated leukocyte-endothelial interaction.

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REFERENCES


Fucoidin, but not yeast polyphosphomannan PPME, inhibits leukocyte rolling in venules of the rat mesentery

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