Two-step model of leukocyte–endothelial cell interaction in inflammation: Distinct roles for LECAM-1 and the leukocyte β2 integrins in vivo


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ABSTRACT The lectin homing receptor LECAM-1 (LAM-1, Leu8) and the β2 integrins, particularly Mac-1 (CD11b/CD18), participate in leukocyte–endothelial cell interactions in inflammation. LECAM-1 is rapidly shed while Mac-1 expression is dramatically increased upon neutrophil activation, suggesting functionally distinct roles for these molecules. Using intravital video microscopy, we have compared the effect of antibodies against LECAM-1 and CD18 on leukocyte interactions with rabbit mesenteric venules. Anti-LECAM-1 monoclonal antibody and its Fab fragments inhibited initial reversible leukocyte rolling along the vascular wall. Anti-CD18 monoclonal antibody had no effect on rolling but prevented subsequent firm attachment of leukocytes to venular endothelium. These results support a two-step model of leukocyte–endothelial cell interactions: reversible rolling mediated in part by LECAM-1 facilitates leukocyte recruitment by the local microenvironment and precedes activation-dependent firm attachment involving β2 integrins.

Leukocyte influx into tissues is one of the hallmarks of acute inflammation. It is an important feature of physiological reactions to inflammatory stimuli and of the pathophysiology of inflammatory disease. Leukocyte extravasation is initiated by interactions between circulating leukocytes and activated endothelial cells lining venules of inflamed tissues. The earliest visible leukocyte–endothelial cell interaction in vivo involves slow rolling or sliding of leukocytes along the wall of postcapillary and collecting venules. Rolling is a reversible event that can be followed by release of the leukocyte back into the bloodstream or by arrest and firm adhesion to endothelium leading to subsequent diapedesis. These phenomena are both amenable to analysis in the microcirculation of the rabbit mesentery, an excellent model for videomicroscopic examination of leukocyte–endothelial cell interactions in situ.

Antibodies to CD18, the leukocyte integrin β2 chain, block firm attachment and diapedesis of neutrophils but have no effect on leukocyte rolling at normal venular shear rates (1). The surface molecules on leukocytes and endothelial cells that mediate rolling have not been defined. Rolling is attenuated by superoxide dismutase and action of oxygen-derived free radicals on endothelial cells may play a role in its induction (2, 3). This unique event is calcium dependent (4) and can be inhibited by intravenous injection of neuraminidase but not sialic acid (5) and by some (but not all) polyvalent polysaccharides, such as dextran sulfate or heparin (6–9). These findings suggest a possible role for cell-surface adhesion receptors with affinity for charged carbohydrates.

The leukocyte surface selectin/LECAM-1 (LAM-1, Leu8), the peripheral lymph node homing receptor (10, 11), has been implicated in leukocyte interactions with activated endothelium in inflammation. The lectin domain in the extracellular portion of LECAM-1 contains many positively charged amino acids (12–14) and can interact with certain anionic carbohydrates (15). Anti-LECAM-1 monoclonal antibodies (mAbs) block neutrophil binding to cytokine-activated endothelial cells in vitro, inhibiting an adhesion pathway that is independent of β2 integrin function (16, 17). Administration of anti-LECAM-1 mAbs or a soluble homing receptor–IgG chimeric molecule as well as removal of LECAM-1 from the cell surface dramatically reduces leukocyte extravasation into sites of acute inflammation in various inflammatory models (18–21). The finding that LECAM-1 is rapidly shed from the neutrophil surface in response to several cytokines in vivo (20, 21) and in vitro (22, 23) in conjunction with observations of a parallel increase in β2 integrin expression has led to the hypothesis that LECAM-1 might mediate early neutrophil adhesive events, preceding the role of activation-triggered β2 integrins (20–23). Such early events might be involved in leukocyte rolling. The present studies were undertaken to examine the role of LECAM-1 in in situ leukocyte interactions with venular endothelium in the rabbit mesentery.

MATERIALS AND METHODS

Antibodies. mAb DREG-200, which reacts with a surface molecule on rabbit leukocytes as well as with human LECAM-1 (24), was affinity purified from tissue culture supernatants on a protein A-Sepharose column (Pharmacia). Purified anti-CD18 mAb IB4 (25) was used as a control. Endotoxin contamination of antibody solutions was assessed by the limulus amoebocyte assay and maximum endotoxin doses an animal received during an experiment were found to be <1 ng. DREG-200 Fab fragments were produced from purified mAb with a commercial kit (ImmunoPure, Pierce) and endotoxin contamination was removed (26). The amount of residual whole IgG as assessed by electrophoresis of the concentrated Fab preparation was found to be <1%.

Flow Cytometry. Flow cytometric kinetic analysis of the modulation of surface LECAM-1 and Mac-1 expression on rabbit neutrophils was performed after activation with platelet-activating factor (PAF). Heparinized rabbit blood (1 ml) was incubated at 37°C and PAF was added at t = 0 to a final

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Abbreviations: mAb, monoclonal antibody; PAF, platelet-activating factor; FITC, fluorescein isothiocyanate.
concentration of $10^{-6}$ M. Immediately before and at defined time points after activation with PAF, a 50-μl aliquot of blood was transferred to 50 μl of ice-cold Heps buffer containing a saturating concentration of fluorescein isothiocyanate (FITC)-labeled mAb DREG-200 or mAb IB4 (5 μg/ml). Further reaction of leukocytes was immediately stopped by the rapid cooling to 0°C. Cells were incubated on ice for 40 min to allow antibody binding. Erythrocytes were lysed by adding a 50-μl aliquot to 50 μl of 10 μg/ml saponin solution in phosphate-buffered saline (PBS) (pH 7.4) followed by incubation for 30 sec on ice. FITC mAb binding to neutrophils was determined on a FACScan flow cytometer (Becton Dickinson) after gating for neutrophils by characteristic forward and side light scatter.

Animal Preparation. Male New Zealand White rabbits (0.9–1.3 kg) were sedated with 0.1 ml of Hypnorm i.m. and anesthetized with 20% urethane (5–10 ml per kg of body weight i.v.). The animals were allowed to breathe spontaneously through a tracheal tube and their body temperature was maintained at 37°C by a heating pad controlled by a rectal thermometer. A continuous infusion of physiological saline (6 ml/kg·h) was applied via a jugular vein catheter through which supplementary doses of anesthesia were given when needed. The mean arterial blood pressure was continuously recorded through a femoral artery line. Blood samples for determination of systemic leukocyte counts, hematocrit, and arterial blood gas analysis were obtained from a carotid artery catheter.

Intravital Microscopy. For intravital microscopy of the mesenteric microcirculation a midline abdominal incision was made. The terminal ileum was exteriorized from the abdomen and carefully spread over a heated (37°C) glass window on the microscopic stage. During the preparation and throughout the experiment, the exteriorized intestines were superfused (5–10 ml/min) with a freshly prepared, endotoxin-free, bicarbonate-buffered saline solution heated to 36.5°C and equilibrated with 5% CO₂ in N₂. A segment of a postcapillary or small collecting venule (diameter, 20–40 μm) in a mesentery section proximally 10–20 cm from the appendix veriformis was chosen for microscopic observation using a Leitz intravital microscope with a ×25 salt water immersion objective (numerical aperture, 1.00). The exteriorized mesentery was allowed to stabilize for at least 30 min prior to a 15-min control period, after which time 1 ml of antibody solution (DREG-200 or IB4) was given in a single dose of 1 mg per kg of body weight followed by continuous antibody infusion of 5 μg/min. Some animals received instead DREG-200 Fab fragments (3 mg) or 3.3 mg per kg of body weight followed by continuous infusion of 1 μg/min, 1 mg per kg of body weight and 3.3 μg/min, and 3.0 mg per kg of body weight and 10 μg/min). Control animals were treated with PBS.

Freely flowing or fast rolling leukocytes in mesenteric venules are virtually invisible in normal transmitted light due to relatively high velocities. Therefore, acridine red solution (7 mg/kg·h⁻¹; Boehringer, Mannheim, Germany) was continuously infused; this fluorescent dye stains nuclei of leukocytes and endothelial cells but had no detectable effect on leukocyte–endothelial cell interaction or hematomatological parameters in our hands. Aided by a Leitz Plinomak epi-illuminator equipped with an N2.1 filter block (excitation wavelength, 540 nm) and video-triggered stroboscopic illumination from a xenon arc (Schott, Mainz, Germany) it was possible to clearly visualize every leukocyte passing through the venular segment. Exposure to fluorescent light was restricted to 1-min intervals in order to avoid possible tissue alteration due to strong epi-illumination. Continuous video recordings of venular segments in fluorescent and low transmitted light were obtained from a silicon-intensified target camera (Dage–MTI 65 or SIT68; Michigan City, IN) and stored on tape using a SVHS video recorder (JVC HR-S6600U; Elmwod Park, NJ).

Analysis of Leukocyte–Endothelial Cell Interaction. Analysis of leukocyte–endothelial cell interaction was performed on fluorescent recordings of mesentery venules. This allows investigation of the total population of leukocytes—freely flowing or interacting—which pass the venular segment under observation. All cells that traveled (rolled) visibly slower than the main bloodstream were assumed to be interacting with the vascular endothelium. The rolling leukocyte flux was determined by counting the number of interacting leukocytes in a segment of a selected mesentery venule (diameter, 20–40 μm) for 1 min and was assessed at four reference points before and at seven time points after injection of antibodies or saline. Results are expressed as percentage of average rolling leukocyte flux in each corresponding control period.

Video recordings of some experiments were further analyzed by using a PC-based image analysis system (27). For determination of individual leukocyte velocities video recordings from fluorescent scenes were replayed frame-by-frame, and the distance a leukocyte traveled between a certain number of frames was measured. Fast rolling (as well as freely flowing) leukocytes escape detection when normal transillumination is used. They do not appear as diffusive spheres in the bloodstream but disappear among fast-rolling erythrocytes as their image becomes blurred when conventional video frame rates are used. The velocities presented here were determined by using fluorescent techniques combined with stroboscopic epi-illumination. As reported previously, maximum rolling velocities obtained by this method are considerably higher than those obtained from recordings under transmitted light (8). Some rolling leukocytes were observed as they detached from or attached to the wall of the venular segment. The velocity of such cells was only measured while they were continuously interacting.

Total leukocyte flux, mean blood flow velocity, as well as maximum flow velocities in the centerline of the bloodstream were determined as described (8). A leukocyte velocity profile was established by measuring the velocity of each leukocyte passing through a venular segment during 1 min. The critical velocity (VCRIT)—i.e., the minimum velocity a freely flowing leukocyte can assume in a microvessel with a paraboloid velocity profile—was determined according to refs. 8 and 28. Velocities of individual leukocytes were normalized to VCRIT, which was given a value of 1. Cells with a normalized velocity >1 were defined as freely flowing cells; a leukocyte was assumed to be interacting with the venular wall if its normalized velocity was <1.

For quantification of vasoactive effects of Fab fragments on firm leukocyte arrest, five postcapillary or small collecting venules were randomly chosen in each preparation 90 min after bolus injection and subsequent continuous infusion of either saline or mAbs IB4 or DREG-200 (1 mg per kg of body weight and 5 μg/min) or DREG-200 Fab fragments (3 mg per kg of body weight and 10 μg/min). A segment of each venule was observed in transmitted light with a Leitz ×25 salt water immersion objective (n.a., 0.6) and length and diameter were measured with the help of a calibrated reticle in one of the eyepieces. The number of intraluminally adherent leukocytes per mm of vessel length was counted.

Statistics. Statistical analysis of paired data was performed by using Friedman’s nonparametric test for two-way analysis of variance and multiple comparisons on ranks. Unpaired data were examined by the Kruskal–Wallis test for one-way analysis of variance followed by multiple comparisons on ranks using the Bonferroni correction of P.

RESULTS

Characterization of the DREG-200 Antigen on Rabbit Leukocytes. Preliminary studies were carried out to characterize...
the expression and regulation of rabbit neutrophil LECAM-1 using anti-human LECAM-1 mAb DREG-200 (24). Quantitative flow cytometric analysis (29) of unactivated rabbit neutrophils using FITC-labeled Fab fragments revealed a mean frequency of \( \approx 70,000 \) binding sites per cell. The molecule detected is similar to human and murine LECAM-1; an 80- to 90-kDa glycoprotein was identified by immunoprecipitation of DREG-200-specific cell-surface determinants performed on lysates from isolated rabbit bone marrow cells (data not shown). Immunohistology as well as flow cytometric determination of DREG-200 binding to various rabbit lymphoid tissues revealed a staining pattern typical for a peripheral lymph node homing receptor (R.F.B. and E. Resurreccion, unpublished data). Although the mAb cross-reacted weakly with smooth muscle cells in sensitive immunofluorescence histology, no antibody binding to normal or inflamed rabbit endothelial cells was detectable. These results strongly indicate that mAb DREG-200 reacts with rabbit LECAM-1.

A flow cytometric kinetic analysis of the modulation of surface LECAM-1 and CD18 expression on rabbit neutrophils after activation with PAF was performed. As described for murine (20–22) and human (23, 24) leukocytes, inverse regulation of LECAM-1 (DREG-200 antigen) and \( \beta_2 \) integrins (CD18, IB4 antigen) upon activation of rabbit leukocytes was demonstrated (Fig. 1). The cytokine-induced downregulation of the DREG-200 antigen on rabbit neutrophils is paralleled by dramatic upregulation of \( \beta_2 \) integrin expression as monitored by increased binding of the anti-\( \beta_2 \) mAb IB4. The increase in \( \beta_2 \) expression preceded downregulation of the DREG-200 determinant. The time for 50% of maximum response was 43 sec for IB4 binding sites compared with 78 sec for DREG-200 sites. Similar results were found after stimulation of rabbit neutrophils with formylmethionylleucylphenylalanine (fMet-Leu-Phe) and leukotriene B4 and on isolated human neutrophils after stimulation with fMet-Leu-Phe (data not shown).

**Effect of i.v. Injection of Anti-LECAM-1 and Anti-CD18 on Leukocyte–Endothelial Cell Interactions.** Mesentery venules (diameter, 20–40 \( \mu \)m) of 24 animals were analyzed. A large variability in spontaneous rolling leukocyte flux was found when different venules were compared. Control values in a range from 13.6 to 169.4 rolling cells per min (mean \( \pm \) SD: 51.4 \( \pm \) 31.8) were measured. In contrast to the interindividual variability, the rolling leukocyte flux in each single venule was found to be stable throughout the 15-min control period. Intravenous administration of mAb DREG-200 resulted in a rapid and sustained decrease of rolling leukocyte flux (Fig. 2). In contrast, in agreement with previous reports (1), injection of mAb IB4 or saline had no effect on leukocyte rolling. Neither treatment had significant influence on mean arterial blood pressure, arterial blood gases, or hematocrit. A slight drop in systemic leukocyte counts of 24.5% \( \pm \) 15.6% (mean \( \pm \) SD) was observed 15 min after injection of mAb DREG-200 (but not Fab fragments; see below), but cell counts were not significantly different from control values when averaged over a period of 2–90 min after injection. Therefore, the rapid and dramatic reduction in rolling leukocyte flux to levels as low as 21% \( \pm \) 12% (mean \( \pm \) SD) of control values 90 min after DREG-200 injection cannot be attributed to a mere reduction in total leukocyte flux.

PC-based off-line video analysis (27) of the velocity of rolling and freely flowing cells further confirmed the effects of mAb DREG-200. Fig. 3 shows the normalized leukocyte velocity distribution in a typical experiment 1 min before and 30 min after treatment with mAb DREG-200. Venular diameter (28 \( \mu \)m) was not affected by the antibody injection. Total leukocyte flux was 78 cells per min before and 88 cells per min after mAb application. Maximum flow velocities in the centerline of the bloodstream were determined as 2.25 mm/sec and 2.34 mm/sec; assuming a paraboloid velocity profile, the mean blood flow velocity can be calculated as 1.13 mm/sec and 1.17 mm/sec, respectively (8). \( \mathbf{v}_{\text{mean}} \) was 0.982 mm/sec in the control period and 1.019 mm/sec 30 min after treatment. The fraction of interacting (i.e., rolling) leukocytes decreased from 40/78 (51.3%) in the control period to 16/88 (18.2%) 30 min after injection of mAb DREG-200, resulting in a marked shift of the leukocyte velocity profile. The mean velocity of rolling cells increased from 0.194 mm/sec (17.2% of mean blood flow velocity) in the control period to 0.309 mm/sec (26.4% of mean blood flow velocity) after antibody application.

The effect of DREG-200 cannot be attributed to crosslinking of antigen or Fc interaction since DREG-200 Fab fragments used in the same protocol also decreased rolling in a dose-dependent fashion (Fig. 4) and had no effect on systemic

![Fig. 1.](image1.png)  
**Fig. 1.** Inverse regulation of LECAM-1 (DREG-200 antigen) and \( \beta_2 \) integrins (CD18, IB4 antigen) upon activation of rabbit leukocytes with PAF. FITC mAb binding to neutrophils was determined on a FACScan flow cytometer (Becton Dickinson). Data are expressed as percent of the median fluorescence channel number at \( t = 0 \) of DREG-200 (○) or IB4 (■) binding to neutrophils. Each point represents mean \( \pm \) SD of six replicate determinations from two independent experiments. Curves were calculated using a curve-fitting computer program (\( r^2 = 0.993 \) for DREG-200 and 0.981 for IB4).

![Fig. 2.](image2.png)  
**Fig. 2.** Rolling leukocyte flux determined from fluorescence recordings by counting leukocytes visibly interacting with the vascular wall in a segment of a selected mesentery venule (diameter, 20–40 \( \mu \)m) for 1 min. Results are expressed as % average rolling leukocyte flux in each corresponding control period (\( \approx 15 \) to 0 min). Data points and error bars represent mean \( \pm \) SD of six animals in each group treated with saline (○), IB4 (■), or DREG-200 (●). Antibodies were injected i.v. at a dose of 1 mg per kg of body weight at \( t = 0 \), followed by continuous infusion of 5 mg/kg/min. The decrease in rolling leukocyte flux 2–90 min after treatment with DREG-200 was highly significant as compared with control values (\( P < 0.001 \)); injection of IB4 or saline had no effect on rolling leukocyte flux.
Fig. 3. Leukocyte velocity profile in a typical experiment 1 min before (broken line) and 30 min after (solid line) treatment with mAb DREG-200. Velocities of individual leukocytes were normalized to $V_{crit}$, which was given a value of 1. Cells with a normalized velocity $\geq 1$ were defined as freely flowing cells; a leukocyte was assumed to be interacting with the venular wall if its normalized velocity was $< 1$. Lines were drawn after counting the number of cells in classes from 0 to $<0.25$, 0.25 to $<0.5$, and so on.

granulocyte or mononuclear cell counts (data not shown). During the first 15 min after bolus injection, the average reduction in rolling leukocyte flux in the two animals treated with the highest dose of Fab fragments (3 mg per kg of body weight followed by 10 $\mu$g/min) was 71% $\pm$ 12% and 65% $\pm$ 15%, respectively. This is very similar to the average reduction seen in rabbits treated with whole antibody (1 mg per kg of body weight) (65% $\pm$ 17% during the first 15 min; 70% $\pm$ 17% during 90 min). The effect of Fab fragments weakened at later time points particularly in animals that received low doses (0.3 mg per kg of body weight and 1 $\mu$g/min). This was probably due to a higher dissociation rate and faster clearance of Fab fragments from the circulation.

Firmly adhered leukocytes, which are not dislodged by shear stress exerted by the flowing blood, as well as slow rolling leukocytes, are absent in most normal tissues but rapidly accumulate in surgically prepared tissues such as the exteriorized rabbit mesentery (Fig. 5A; ref. 9). Importantly, the decrease in rolling induced by mAb DREG-200 was paralleled by a dramatic decrease in the number of leukocytes firmly adhering to the endothelium of randomly chosen venules (diameter, 15–40 $\mu$m) in mesenteries treated with continuous infusion of DREG-200 IgG (Fig. 5C) or Fab fragments for 90 min (Fig. 6). In contrast, the anti-$\beta_2$ integrin mAb IB4 inhibited arrest (Fig. 5B) but had no effect on rolling, as described (1).

**DISCUSSION**

Here we report that intravenous administration of murine anti-LECAM-1 mAb DREG-200 or its Fab fragments blocks leukocyte rolling in rabbit mesentery venules. Rabbit LECAM-1, like its human and murine counterparts, has an apparent molecular mass of 80–90 kDa and shares with them a similar tissue distribution by immunohistology as well as flow cytometric analysis of various rabbit lymphoid organs. Furthermore, rabbit LECAM-1 is rapidly downregulated on the surface of activated neutrophils and this is paralleled by upregulation of $\beta_2$ integrins. The finding that CD18 upregulation immediately precedes downregulation of LECAM-1 is consistent with the hypothesis that shedding of LECAM-1 may result from enzymatic action of exteriorized granule contents such as chymotrypsin-like proteases (21), an event coincident with externalization of $\beta_2$ integrins and other adhesive elements stored in granule compartments (30).

Our findings support a two-step model of *in vivo* leukocyte-endothelial cell interaction in acute inflammation in which initial rolling and subsequent attachment represent separate processes mediated by different molecular mecha-

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**Fig. 4.** Effect of three different doses of DREG-200 Fab fragments on the rolling leukocyte flux in mesentery venules. Fab fragments were injected i.v. at $t = 0$ in three different doses; $\bigcirc$, 0.33 mg per kg of body weight followed by continuous infusion of 1 $\mu$g/min; $\triangle$, 1 mg per kg of body weight and 3.3 $\mu$g/min; $\blacksquare$, 3 mg per kg of body weight and 10 $\mu$g/min. Each curve represents the mean of two independent experiments.

**Fig. 5.** Photomicrographs of representative mesentery venules taken 90 min after treatment of animals with either saline (A), mAb IB4 (B), or mAb DREG-200 (C). Spontaneous intravascular attachment of leukocytes was apparent in untreated control animals but was dramatically decreased after administration of mAbs IB4 or DREG-200. (Bar = 50 $\mu$m.)
Our results imply that interference with either LECAM-1 or CD18-mediated adhesion events may be effective in therapeutic reduction of neutrophil-endothelial cell interactions in such life-threatening conditions as reperfusion injury, adult respiratory distress syndrome, and other diseases in which neutrophil-mediated tissue damage is thought to play a role.

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