P-Selectin Mediates Spontaneous Leukocyte Rolling In Vivo

By Monique Doré, Ronald J. Korthuis, D. Neil Granger, Mark L. Entman, and C. Wayne Smith

Rolling represents the initial step leading to leukocyte extravasation from blood vessels during an inflammatory reaction. In vitro studies indicate that P-selectin could be one of the ligands on endothelium involved in the rolling phenomenon, although the molecular determinants responsible for this transient attachment in vivo are still undefined. Our objectives were to develop a blocking monoclonal antibody against canine P-selectin and to use it to investigate the role of P-selectin in leukocyte rolling in vivo using the technique of intravital microscopy. P-selectin was immunoaffinity purified from canine platelets and used for the production of monoclonal antibodies. One of the hybridomas generated, MD6, was shown by enzyme-linked immunosorbent assay and by flow cytometry to bind preferentially to stimulated platelets and to completely prevent binding of stimulated platelets to neutrophils. Visualization of canine mesenteric venules by intravital microscopy showed that administration of MD6 resulted in a marked inhibition in the number of rolling leukocytes (18.96 ± 9.92 vs. 166.40 ± 19.50 leukocytes/min, P < .05; 88.3% ± 6.0% inhibition). Control antibody MD3 (which recognizes a nonfunctional epitope of canine P-selectin) had no effect on the number of rolling leukocytes or on their rolling velocity. These results show for the first time that P-selectin plays an essential role in leukocyte rolling in vivo, and therefore may be a key participant of the inflammatory response.

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LOCALIZATION of neutrophils to inflammatory sites involves a series of precisely regulated events ultimately leading to their emigration from the circulation to the tissues. Rolling of neutrophils along the lining of postcapillary venules, a phenomenon commonly observed in tissues prepared for intravital microscopy, might represent the first event bringing in close contact neutrophils and endothelial cells during an inflammatory reaction. This initial interaction of neutrophils with the blood vessel is probably necessary before a more complete attachment and extravascular migration can occur. Recent evidence indicates that adhesion molecules present on both neutrophils and endothelial cells participate in the rolling process.

Members of the β2 integrin family have been shown to support binding of neutrophils to endothelium and transmigration under static conditions, but several studies have shown that these molecules do not contribute to the process of rolling. However, increasing evidence supports an important role for the members of the selectin family of adhesion molecules in rolling. Reports of inhibition of leukocyte rolling in rabbit venules by sulfated polysaccharides are consistent with a selectin-mediated interaction. L-selectin, present on the neutrophil surface and downregulated by activation, has been shown to participate in neutrophil rolling. For example, Smith et al showed that monoclonal antibodies (MoAbs) to L-selectin were able to reduce by 50% the adherence of unstimulated neutrophils to cytokine-activated endothelial cells at a wall shear stress of 1.85 dyn/cm².

The molecular ligands on endothelial cells involved in rolling are still unknown. Recent evidence from our laboratory showed that cytokine-induced E-selectin expression on endothelium can support neutrophil rolling in an in vitro model. However, E-selectin is not constitutively expressed, and therefore cannot account for the rolling observed at the beginning of an inflammatory reaction. In contrast, P-selectin is stored in the Weibel-Palade bodies of endothelial cells and can be rapidly expressed on the cell surface. It could therefore represent a prime candidate for the initial phase of leukocyte rolling. Rapid surface expression of P-selectin on endothelial cells follows stimulation with inflammatory agents such as histamine or thrombin. A more prolonged expression of P-selectin on the cell surface can be induced by exposure to oxygen radicals. Under static conditions, P-selectin has been shown to be able to mediate binding of neutrophils. For example, Geng et al showed that neutrophils adhere to COS cells transfected with the full-length cDNA of human P-selectin and that MoAbs to P-selectin inhibit neutrophil binding to histamine-stimulated endothelial cells.

Although P-selectin is believed to participate in transient neutrophil attachment to endothelium under conditions of flow, no studies confirming this role have yet been reported. The absence of MoAbs recognizing a functional epitope of P-selectin in an animal model has precluded the demonstration of a role for P-selectin in rolling in vivo. We have recently reported the production of an MoAb (MD3) that recognizes a nonfunctional epitope of canine P-selectin. Therefore, the first objective of the present study was to develop and characterize a blocking MoAb against canine P-selectin (ie, that would inhibit platelet-neutrophil or neutrophil-endothelial cell interactions). Second, this MoAb was used to investigate the role of P-selectin in spontaneous leukocyte rolling in vivo in dog mesenteric venules using the technique of intravital microscopy.
Fig 1. Immunoadfinity purification of P-selectin from canine platelets. (A) Proteins in total platelet extracts (2.5 μg) and proteins eluted from the immunoadfinity column of Sepharose-MD3 (1 μg) were electrophoretically separated by SDS-PAGE under nonreducing conditions and visualized using silver nitrate staining. Eluted proteins from two different preparations are shown. A major protein band of approximately 140 Kd is present in both preparations. (B) Western blotting analysis of the eluted proteins was performed using MoAb MD3 (which recognizes a nonfunctional epitope of canine P-selectin). MD3 recognized a protein of approximately 140 Kd in canine platelet extracts (right lane) as well as in the eluted preparations (left lane).

MATERIALS AND METHODS

Purification and amino-terminal amino acid sequencing of canine P-selectin. P-selectin was purified from canine platelets by affinity chromatography using MD3, an MoAb recognizing a nonfunctional epitope of canine P-selectin. The column was prepared by coupling 2 mg of affinity-purified MD3 to cyanogen bromide-activated Sepharose 4B according to the manufacturer’s instructions. Platelet-rich plasma was obtained from several units of canine blood and the platelets were pelleted down by centrifugation at 1,800g for 20 minutes. The platelets were then sonicated on ice in Tris-buffered saline (TBS) containing 1 mmol/L phenylmethylsulfonyl fluoride (PMSF) for 4 cycles of 20 seconds. Intact cells were removed by low-speed centrifugation (900g for 15 minutes) and membranes were pelleted by centrifugation at 100,000g for 60 minutes. The pellet was solubilized in TBS, 1% Triton-X, and 1 mmol/L PMSF and sonicated as above. The solubilized extract was centrifuged at 100,000g for 60 minutes and the supernatant was used for affinity chromatography. The affinity-column was equilibrated with TBS, 1% Triton-X before the solubilized platelet extract was applied. After washing the column with TBS, 1% Triton-X, followed by 1 mmol/L NaCl, 0.1% Triton-X, and with TBS, 0.1% Triton-X, bound proteins were eluted with 0.1 mol/L glycine NaOH, pH 11.5, 0.5 mmol/L PMSF, 0.1% Triton-X in a tube containing 1 mol/L Tris-HCl, pH 7.4. Protein concentration was determined by the method of Bradford (Bio-Rad protein assay; BioRad, Richmond, CA). Proteins present in the eluted fraction were resolved by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing conditions and were visualized by staining with silver nitrate. For amino-terminal amino acid sequence analysis, proteins eluted from the column were resolved by SDS-PAGE and electrophoretically transferred to a polyvinylidene difluoride membrane (ProBlott; Applied Biosystems, Foster City, CA) in 3-cyclohexylamino-1-propane-sulfonic acid buffer. The membrane was then stained with Coomassie blue and the band of interest was excised. Amino-terminal microsequencing analysis was performed by Dr Richard G. Cook (Department of Immunology, Baylor College of Medicine, Houston, TX) using an Applied Biosystem 473A gas-phase sequencer following the methodology provided by the manufacturer.

Production of MoAbs. Balb/c mice were immunized with purified canine P-selectin (15 μg/mouse) over a period of 2 months at 3-week intervals with a final boost administered 3 days before the fusion. Splenocytes were fused with P3 × 653.Ag8 myeloma cells according to the method described by Köhler and Milstein. Hybridomas were tested for the production of anti-P-selectin antibo-

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<th>Table 1. Antibody Binding to Platelets in Unstimulated and PAF-Stimulated Dog Blood Assessed by Flow Cytometry</th>
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<td>Positive control (MD3)</td>
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<td>Negative control</td>
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PAF was administered at 100 ng/mL for 15 minutes at 37°C.
Fig 2. Visual assay of platelets-neutrophils binding. Isolated canine platelets were stimulated for 15 minutes at 37°C with 0.02 U/mL of human thrombin, fixed with 1% paraformaldehyde, and incubated with tissue culture supernatant (MD3 or MD6) or with PBS for 15 minutes, and isolated canine neutrophils were added. After 30 minutes, the cell suspension was examined under phase contrast to determine binding of platelets to neutrophils. Results showed that (A) unstimulated platelets did not bind to neutrophils; (B) thrombin-stimulated platelets bound to neutrophils, resulting in the formation of multicellular neutrophil aggregates; (C) clone 3A10 (MD6) completely prevented binding of stimulated platelets to neutrophils; and (D) MD3 (directed against a nonfunctional epitope of canine P-selectin) did not inhibit the binding of stimulated platelets to neutrophils.
The mesentery was draped over an optically clear viewing pedestal that allowed for transillumination. The pedestal was maintained at 37°C with a constant temperature circulator (model 80; Fisher Scientific Co, Pittsburg, PA). The exposed bowel was draped with Saran wrap (Dow Chemical Co, Indianapolis, IN). The exposed mesentery was suffused with a warmed (37°C) bicarbonate-buffered salt solution (pH 7.4) at a rate of 2.0 mL/min. The oxygen tension of the suffusion solution was reduced to 40 mm Hg by bubbling with a mixture of 5% CO₂-95% N₂.

The mesenteric microcirculation was observed through an intravital video microscope (Ortholux II; Ernst Leitz, Wetzlar, Germany) with a × 20 objective lens (Leitz Wetzlar L 20/0.32; Carl Zeiss, Inc, Thornwood, NY) and a × 10 eye piece. The mesentery was transilluminated with a 12 V-100 W DC-stabilized light source. A video camera (Javalin; MOS) mounted on the microscope projected the image onto a color monitor, and the images were recorded using a video cassette recorder.

Single unbranched venules with a diameter of 30 to 40 μm and a length of ~175 μm were studied. Vessel diameter was measured with a video caliper (Microcirculation Research Institute, Texas A & M University, College Station, TX). The flux of rolling leukocytes (cells/min) was determined off-line during playback of videotaped images. Rolling leukocytes were defined as white blood cells that continued to move but at a velocity less than that of the erythrocytes and were in contact with the endothelial cell surface, as previously described.18 The flux of rolling leukocytes was determined from the number of cells crossing a fixed region of venule per minute. Leukocyte rolling velocity was calculated from the time required for a leukocyte to traverse 50 μm length of microvessel.

Each vessel was videotaped for 30 minutes under baseline conditions. An MoAb directed against either a functional (MD6; n = 6 dogs) or a nonfunctional (MD3; n = 2 dogs) epitope of canine P-selectin was then administered intravenously (0.8 to 1.0 mg/kg) and leukocyte rolling was monitored for an additional 30 minutes. Blood was drawn from the jugular vein before injection of the MoAb and 5 minutes after injection to determine total white blood cell count.

The data were analyzed by use of one-way analysis of variance followed by multiple comparisons of means with the Fisher’s least significant difference (LSD) procedure. All values are given as mean ± standard error mean (SEM), and statistical significance was set at P < .05.

RESULTS

Purification and N-terminal amino acid sequencing of canine P-selectin. Canine P-selectin was purified from solubilized platelet extracts by affinity chromatography using the MoAb MD3 coupled to sepharose. Proteins present in the eluted fraction were electrophoretically resolved by SDS-PAGE gel under nonreducing conditions and visualized by staining with silver nitrate. Among the proteins eluted from the MD3 column, a major protein of 140 Kd was detected (Fig 1A). Immunoblot analysis showed that MoAb MD3, which is directed against a nonfunctional epitope of canine P-selectin, recognized the same 140-Kd band (Fig 1B). The higher molecular weight protein present on the silver stained gel was not recognized by MD3.

Amino-terminal amino acid sequencing was performed to confirm the identity of the 140-Kd band. Results showed that the amino-terminus of the purified protein (Thr-Tyr-Asn-Tyr-Ser-Thr-Lys-Ala-Tyr-Ser) was highly homologous to the amino-terminus of human P-selectin (Trp-Thr-Tyr-His-Tyr-Ser-Thr-Lys-Ala-Tyr-Ser).29 with 9 of 10 aligned residues being identical. Purified canine P-selectin was subsequently used in an immunization protocol in an attempt to generate functional MoAbs (ie, that would inhibit platelet-neutrophil or neutrophil-endothelial cell interactions).

Production of a blocking MoAb against canine P-selectin. From a fusion performed with a mouse immunized for 2 months with affinity-purified canine P-selectin, 248 clones were screened using an ELISA on unstimulated and thrombin-stimulated gel-filtered canine platelets. This first screening procedure showed that 14 clones showed preferential binding to PAF-stimulated platelets, as compared with resting platelets. These 14 clones were subsequently re-screened using a second ELISA on microtiter plates that had been coated with affinity-purified canine P-selectin. In this assay, 9 of the 14 clones showed high levels of binding to the purified protein (range of values, 0.763 to 1.332 arbitrary units for positive clones v 0.085 units for negative control).
Flow cytometry was then used to evaluate the ability of the antibodies produced by these 9 clones to bind to platelets in whole blood. Results showed that the antibody from 7 of the 9 clones bound preferentially to activated platelets in PAF-stimulated blood compared with resting platelets in unstimulated blood (Table 1). To determine if these 7 antibodies were recognizing a functional epitope of P-selectin, a functional assay of platelets-neutrophils binding was used, as binding of activated platelets to neutrophils has previously been shown to be mediated by P-selectin. Results showed that, whereas unstimulated canine platelets did not bind to neutrophils (Fig 2A), activation of platelets with thrombin induced marked platelet-neutrophil binding (Fig 2B). Moreover, the presence of numerous large neutrophil aggregates was probably the result of heterotypic (platelets-neutrophils) as well as homotypic (platelets-platelets) interactions. When clones identified by ELISA to recognize canine P-selectin were tested in this functional assay, the clone 3A10 was able to completely prevent binding of activated platelets to isolated neutrophils (Fig 2C). In contrast, preincubation of stimulated platelets with MD3 did not inhibit their binding to neutrophils (Fig 2D). Clone 3A10 was subsequently designated MD6, and the antibody was shown to belong to the IgG1 subclass. Figure 3 shows the flow cytometric analysis of binding of MoAb MD6 to PAF-stimulated platelets in canine whole blood.

**Leukocyte rolling in canine mesenteric venules.** Spontaneous leukocyte rolling can be observed in vessels of exteriorized mesentery prepared for intravital microscopy. Immunohistochemical staining of canine mesentery using MoAb MD3 showed that P-selectin was present in the endothelial cells of blood vessels (Fig 4). To investigate the contribution of endothelial cell P-selectin to the rolling process, the effect of the newly developed functional anti-P-selectin antibody MD6 on leukocyte rolling was studied using intravital microscopy on venules of the canine mesentery. MoAb MD3 was used as control because it binds to canine P-selectin but does not block its function. Mesenteric venules ranging in diameter between 30 and 40 μm were analyzed for a period of 30 minutes before injection of the antibody. Under baseline conditions, several leukocytes were observed rolling along the wall of the venules in all 8 animals (Fig 5A and C) with an average flux of 146.10 ± 17.90 rolling leukocytes/min and an average velocity of 109.00 ± 10.90 μm/s.

Intravenous administration of MD6 resulted in a rapid (within 15 to 20 seconds) and marked decrease in the flux of rolling leukocytes (Fig 5B). Figure 6A shows the kinetic of the response for each individual. In the six animals treated with MD6, the flux of rolling leukocytes decreased to 11.73% ± 6.02% of preinjection levels (18.96 ± 9.92 v 156.40 ± 19.50 rolling leukocytes/min, P < .05) during the first 10 minutes after injection of the antibody (Fig 5B). At 20 minutes postinjection, some cells started to roll again, although the flux of rolling leukocytes was still significantly decreased (40.1% ± 17.9%, P < .05) when compared with levels before administration of MD6. After 30 minutes, two different kinetics were observed. In three subjects, the marked inhibition of rolling leukocytes lasted until the end of the study (30 minutes), whereas in the other three animals the number of rolling cells continued to increase. However, when all six animals were combined, the flux of rolling cells at 30 minutes was still significantly lower (51.40% ± 14.70%, P < .05) than preinjection levels. Administration of MD6 had no effect on the rolling velocity of the leukocytes that continued to roll at all the time points studied (Fig 7), on systemic arterial pressure, or on heart rate (data not shown).

In contrast to the marked inhibition after injection of MD6, administration of antibody MD3 had no effect on the flux of rolling leukocytes (Fig 6C), with several leukocytes observed rolling along the wall of the venules at all times after injection of the antibody (Fig 5D). MD3 also did not affect the rolling velocity of the cells (Fig 7), systemic arterial pressure, or heart rate (data not shown).

Finally, to verify that the almost complete disappearance
Fig 5. Intravital microscopy on canine mesenteric venules. Mesenteric segments were prepared for intravital microscopy as described under Materials and Methods. Shown are examples from two different dogs injected with either MoAb MD3 or MD6. Direction of flow is from right to left. Leukocytes were observed rolling along the wall of the vessel in both dogs before injection of antibody MD6 (A; arrow indicates 1 of 5 rolling leukocytes) or antibody MD3 (C; arrow indicates 1 of 4 rolling leukocytes). Very rapidly after administration of the blocking anti-P-selectin antibody MD6, rolling leukocytes disappeared (B), whereas administration of antibody MD3 had no effect on the flux of rolling leukocytes (D; arrow indicates a leukocyte still rolling after administration of antibody MD3).

DISCUSSION

The present study provides for the first time evidence supporting the role of the adhesion molecule P-selectin in the process of spontaneous leukocyte rolling in vivo. Our results show that administration of an MoAb directed against a functional epitope of P-selectin results in a rapid and almost complete inhibition of the flux of rolling leukocytes. In contrast, administration of an MoAb recognizing a nonfunctional epitope of the molecule had no effect on leukocyte rolling. The canine P-selectin molecule appears highly homologous to human P-selectin, with a molecular weight of 140 Kd under nonreducing conditions and an amino-terminal amino acid sequence almost identical to human P-selectin (9 of 10 residues similar). Recent cloning studies also confirmed the microsequencing analysis, with all 10 amino acid residues being identical to the deduced amino acid sequence of a canine P-selectin cDNA.

We have previously shown by flow cytometry that the molecule is found on the surface of activated but not resting canine platelets, and have documented by immunohistochemistry its localization to vascular endothelial cells of most tissues in the dog. The complete inhibition of leukocyte rolling observed after administration of the anti-P-selectin antibody lasted on average 10 to 15 minutes, after which time rolling was progressively reinitiated. Studies by Hattori et al of the surface expression of P-selectin on histamine-treated endo-
Fig 6. Effect of the administration of the anti–P-selectin MoAbs MD6 and MD3 on the flux of leukocyte rolling in canine mesenteric venules. Rolling leukocytes were defined as those white blood cells that continued to move but at a velocity less than that of the erythrocytes and were in contact with the endothelial cell surface. Rolling cells were expressed either as the number of cells per minute (A and C) or as percent of preinjection levels (B). The number of rolling cells/minute was determined before (Pre), and 10, 20, and 30 minutes after administration of the antibody MD6 (A, B) or MD3 (C). Different symbols represent different animals. Results are expressed as mean ± SEM. *Significantly different from preinjection levels, P < .05.

Fig 7. Effect of the administration of the anti–P-selectin MoAbs MD6 and MD3 on the velocity of rolling leukocytes in canine mesenteric venules. The velocity of rolling leukocytes was determined before (Pre) and 10, 20, and 30 minutes after administration of the antibody MD6 (A) or MD3 (B). Rolling velocity was calculated from the time required for a leukocyte to traverse 50 μm length of microvessel. Results are expressed as mean ± SEM (n = 6 for MD6 experiment, n = 2 for MD3 experiment). *Could not be determined due to the low number of rolling cells at 10 minutes.

Table 2. Peripheral White Blood Cell Counts Before and After Administration of the Anti–P-Selectin MoAbs MD3 and MD6

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<th>MoAb</th>
<th>Before (cells/μL)</th>
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<tr>
<td>MD3</td>
<td>9.125 ± 175</td>
<td>9.175 ± 125</td>
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<tr>
<td>MD6</td>
<td>9.125 ± 927</td>
<td>8.312 ± 1.850</td>
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Values are mean ± SEM.
Participation of P-selectin in transient neutrophil attachment to endothelium under conditions of flow was previously suggested by in vitro studies by Lawrence and Springer, who showed that neutrophils could roll on artificial lipid bilayers containing purified P-selectin, but not on bilayers containing ICAM-1, a member of the Ig superfamily. Recent in vivo studies have implicated another member of the selectin family, L-selectin, in the rolling process. Using intravital microscopy on rat mesenteric venules, Ley et al showed a marked inhibition in the number of rolling leukocytes after microinfusion of an anti-L-selectin polyclonal serum or a recombinant soluble chimera (LEC-IgG), and proposed that rolling results from the interaction of L-selectin on leukocytes with a constitutively expressed or rapidly induced endothelial ligand. However, the exact nature of the endothelial ligand(s) interacting with L-selectin is still undetermined.

Specific polysaccharides such as the yeast-derived phosphomannan monoester (PPME) have been shown to block the interactions of L-selectin with its ligand(s) on both high endothelial venules (HEV) and cultured human umbilical vein endothelial cells, suggesting that the lectin-like domain of L-selectin is involved in this interaction. Biochemical characterization of the ligand on HEV showed that it is at least partially carbohydrate in nature. Ley et al also recently identified a 50-Kd glycoprotein as an HEV ligand for L-selectin. The newly described molecule, a highly glycosylated molecule localized to the apical surface of lymph node HEV, was designated GlyCAM-1 (glycosylation-dependent cell adhesion molecule).

Results from the present study clearly indicate that P-selectin is involved during spontaneous rolling in vivo, and could therefore be one of the ligands interacting with L-selectin to mediate leukocyte rolling. Suggestion of an interaction between L-selectin and P-selectin was presented by Picker et al, who showed that an MoAb antibody to L-selectin could block the binding of neutrophils to P-selectin-transfected COS cells. The exact nature of the ligand(s) for P-selectin on leukocytes is still undetermined. A recent study reports the identification of a glycoprotein with molecular weight of 250 Kd under nonreducing conditions as a major ligand for P-selectin in myeloid cells.

Evidence for the involvement of P-selectin in the initial contact of leukocytes with the vascular endothelium in vivo suggests that P-selectin likely participates in a variety of disease processes in which an acute inflammatory response takes place. However, few studies have yet addressed the possible role played by P-selectin in the pathogenesis of such pathologic conditions. Recently, Mulligan et al provided data supporting the existence in the rat of a P-selectin-like molecule reacting with an antibody to human P-selectin, and used a model of acute lung injury after systemic activation of complement with cobra venom factor to show a role for P-selectin in the pathogenesis of the pulmonary damage. In their model, administration of an anti-P-selectin MoAb together with the cobra venom factor had protective effects on the pulmonary injury as assessed by permeability changes, hemorrhages, and lung myeloperoxidase content. However, the investigators could not determine if the protective effect observed after administration of the antibody was attributable to its binding to P-selectin that had been released from activated platelets and subsequently became associated with the surface of endothelial cells, or to P-selectin present in the endothelium membrane.

In conclusion, we have presented evidence that P-selectin is directly involved in leukocyte rolling in vivo in venules of tissues prepared for intravital microscopy. This rolling process might result from a localized inflammatory reaction. It is likely that future studies will substantiate the role for P-selectin in the pathogenesis of inflammatory processes in vivo. For that purpose, the newly developed blocking MoAb against canine P-selectin represents a useful reagent to further investigate the functions of P-selectin in canine models of hemostasis and inflammatory diseases.

**ACKNOWLEDGMENT**

The authors are grateful to Janice Russell and Amy Welch for their expert technical assistance.

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P-selectin mediates spontaneous leukocyte rolling in vivo

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