P-Selectin Mediates Ca$^{2+}$-Dependent Adhesion of Activated Platelets to Many Different Types of Leukocytes: Detection by Flow Cytometry

By Lucia G. de Bruijne-Adirmaal, Piet W. Modderman, Albert E.G.Kr. Von dem Borne, and Arnoud Sonnenberg

Previous studies have shown that thrombin-activated platelets interact through the P-selectin with neutrophils and monocytes. To identify other types of leukocytes capable of such an interaction, eosinophils, basophils, and lymphocytes were isolated from whole blood. Binding of these cells to activated platelets was examined in a double immunofluorescence assay and the results show that activated platelets not only bind to neutrophils and monocytes, but also to eosinophils, basophils, and subpopulations of T lymphocytes. Using monoclonal antibodies (MoAbs) specific for subsets of T cells, we could further demonstrate that the T cells which bind activated platelets are natural killer (NK) cells and an undefined subpopulation of CD4$^+$ and CD8$^+$ cells. All these interactions were dependent on divalent cations and were completely inhibited by an MoAb against P-selectin. Thus, P-selectin mediates the binding of activated platelets to many different types of leukocytes. Studies with leukocytes treated with proteases or neuraminidase have shown that the structures recognized by P-selectin are glycoproteins carrying sialic acid residues. Because the loss of binding of activated platelets to neuraminidase-treated neutrophils was almost complete, but only partial to treated eosinophils, basophils, and monocytes, the latter cell types may have different P-selectin ligands in addition to those present on neutrophils. We found that two previously identified ligands for P-selectin, the oligosaccharides Le$^a$ and sialyl-Le$^a$, had little or no inhibitory effect on adhesion of activated platelets to leukocytes and that binding was not inhibited by MoAbs against these oligosaccharides. In addition, there was no correlation between the expression of Le$^a$ on several cell types and their capacity to bind activated platelets. In contrast, the expression of sialyl-Le$^a$ on cells was almost perfectly correlated with their ability to bind activated platelets. Thus, while Le$^a$ cannot be a major ligand for P-selectin, a possible role for sialyl-Le$^a$ in P-selectin-mediated adhesion processes cannot be dismissed. Finally, activated platelets were found to bind normally to monocytes and neutrophils of patients with paroxysmal nocturnal hemoglobinuria (PNH) and to neutrophils from which phosphatidyl inositol (PI)-linked proteins had been removed by glycosylphosphatidylinositol–specific phospholipase C (GPI-PLC) digestion. This suggests that at least part of the P-selectin ligands on these cells are not GPI-anchored.

© 1992 by The American Society of Hematology.

From the Department of Immunohematology, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, and Laboratory for Experimental and Clinical Immunology, University of Amsterdam, Amsterdam, The Netherlands; and the Department of Haematology, Academic Medical Centre, University of Amsterdam, The Netherlands.


Address reprint requests to Arnoud Sonnenberg, PhD, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, PO Box 9190, 1006 AD Amsterdam, The Netherlands.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1992 by The American Society of Hematology.

0006-4971/92/8001-0032$3.00/0

Blood, Vol 80, No 1 (July 1), 1992: pp 134-142
anti-P-selectin monoclonal antibodies (MoAbs). Our studies show that platelets not only bind to neutrophils and monocytes, but also to eosinophils, basophils, natural killer (NK) cells, and a second as yet undefined population of lymphocytes. This adhesion is dependent on Ca²⁺ and is mediated by P-selectin. Furthermore, we present evidence that there is more than one ligand for P-selectin and that these ligands are not phosphatidylinositol (PI)-linked proteins.

MATERIALS AND METHODS

Reagents. MoAbs used for cell-specific staining are described in Table 1. The MoAbs CLB-thromb/6 and CLB-thromb/5, specific for P-selectin, and the MoAbs CLB-gran/2,23 and CLB-B9,24 specific for CD15, were produced in our laboratory. The anti-P-selectin MoAbs RUU 1.18, RUU 2.15, and RUU 2.17 were kindly provided by Dr H.K. Nieuwenhuis (University Hospital, Utrecht, The Netherlands). MoAbs M29 (clone L16), M50 (clone Vim 10), M55 (clone 6F3), M56 (clone BRA4F1), M68 (clone Vim D5), M133 (clone 7ML H15), M141 (clone HI 98), and M161 (clone UL-58) were obtained via Workshop studies.25 The anti-sialyl-Lea MoAb CSLEX-1 (mouse IgM, high performance liquid chromatography [HPLC]-purified) was a gift of Dr P. Terasaki (University of California Medical School, Los Angeles, CA). The oligosaccharides lacto-N-fucopentaose III (LNF III), lacto-N-fucopentaose I (LNF I), and 3-sialyl-Lewis X (sialyl-Lea) were purchased from Oxford Glycosystems (Oxon, UK).

Isolation and staining of platelets. Resting platelets were prepared from EDTA-anticoagulated whole blood by direct fixation with paraformaldehyde (PFA) at a final concentration of 1% (wt/vol), for 10 minutes at room temperature. Platelets were washed twice with phosphate-buffered saline/10 mmol/L EDTA (PBS/EDTA/BSA) to remove the PFA. Activated platelets were prepared as follows: platelets were isolated from platelet-rich plasma (PRP) of EDTA-anticoagulated blood, washed three times with PBS/EDTA/BSA, resuspended in the same buffer (2 × 10⁸/mL), and activated with 1 U/mL of human α-thrombin (Sigma Chemical, St Louis, MO) for 10 minutes at 37°C. The platelets were then washed once, fixed with PFA, and washed two additional times. For analysis by flow cytometer, platelets were incubated for 30 minutes at 22°C with W6/32, a mouse MoAb against HLA class I (see Table 1), washed, and stained with a phycoerythrin-labeled rat MoAb against the κ light chain of mouse immunoglobulin (Becton Dickinson, Mountain View, CA). Then, the platelets were washed and incubated with a mixture of irrelevant mouse MoAbs of the IgG1 and IgG2a subclasses to block unoccupied binding sites of rat anti-mouse MoAb. The platelets were washed once more before use in adhesion assays.

Isolation and staining of peripheral blood cells and cell line cells. Lymphocytes were prepared by density centrifugation on Ficoll-Isopaque (d = 1.077 g/cm³). The lymphocyte preparation contained approximately 20% of contaminating monocytes, detected with MoAb CLB-mon/1 in the immunofluorescence test. Neutrophils were isolated from the Ficoll-pellet, in which red blood cells were lysed with isotonic NH₄Cl (155 mmol/L NH₄Cl; 10 mmol/L KHCO₃; 0.1 mmol/L EDTA in aqua dest, pH 7.4) at 4°C for 12 minutes. This fraction was contaminated with approximately 5% to 10% lymphocytes (as detected using a mixture of CD2 and CD37 MoAbs). Eosinophils were prepared from the granulocyte fraction according to Koenderman et al.27 The purity of the eosinophil preparation was approximately 90%. Basophils and monocytes were isolated by means of elutriation centrifugation. The purity of the monocyte and basophil suspensions was approximately 90% and 80%, respectively. The majority of the contaminating cells of the basophil fraction consisted of lymphocytes and a small percentage of monocytes and neutrophils. The monocyte fraction was contaminated with lymphocytes.28 Cell lines U937, ML-1, KG1a, K562, HEL, ROS, and B-ALL were cultured in RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY) supplemented with penicillin G (100 U/mL), streptomycin sulphate (100 μg/mL), and 10% fetal calf serum. All cell types were fixed with 1% PFA, incubated with appropriate MoAbs (Table 1), stained with fluorescein-isothiocyanate goat anti-mouse Ig (GAM-FITC, CLB, Amsterdam, The Netherlands), and treated with irrelevant mouse MoAbs as described above for staining of platelets.

Cell adhesion assay. Fixed immunostained cells and platelets were resuspended in 25 mL/L Tris-HCl, pH 7.0/150 mmol/L NaCl/0.2% BSA, supplemented with either 1 mmol/L CaCl₂ and 1 mmol/L MgCl₂ or 5 mmol/L EDTA (incubation buffer). Fifty microliters of the platelet suspension (1 × 10⁷/mL) was mixed with 50 μL of the cell suspension (1 × 10⁷/mL), giving a platelet to cell ratio of 10:1. After incubation for 30 minutes at room temperature, platelet-cell adhesion was quantitated by measuring red and green fluorescence on a FACScan cytometer (Becton Dickinson) using the Consort C30 double fluorescence program. The forward scatter-channel (FSC) was used as system trigger. The threshold (FSC) was set on a level high enough to exclude single platelets from data acquisition. The FL-1:FL-2 histogram was divided into quadrants using three control samples. The first contained cells incubated with a control antibody and stained with GAM-FITC as a negative control. The other two control samples were platelets and cells separately incubated with MoAb W6/32 and specific MoAbs (see Table 1), respectively, and each stained by fluorescent anti-IgG conjugates of a different color.

Antibody inhibition studies were performed by preincubating either the platelets or the leukocytes with ascites fluid (1:50 final dilution) or purified MoAbs (20 μg/mL). Then, without washing, platelets and cells were gently mixed and the incubation was continued for an additional 30 minutes. Adhesion was measured as described above.

Table 1. Monoclonal Antibodies Used for Cell-Specific Staining

<table>
<thead>
<tr>
<th>Cells</th>
<th>MoAbs (source)</th>
<th>Directed Against</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelets</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophils,</td>
<td>W6/32 (Dr S.C. Meuer, Heidelberg)</td>
<td>HLA class I</td>
</tr>
<tr>
<td>U937</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monocytes</td>
<td>CLB-mon/1 (CLB, Amsterdam)</td>
<td>GP55 (CD14)</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>CLB-Thromb/8 (CLB, Amsterdam)</td>
<td>p24 (CD9)</td>
</tr>
<tr>
<td>Basophils</td>
<td>γ29-55 (Dr H.K. Forster, Basel)</td>
<td>GP40-52 (CD37)</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>CLB-T11 (CLB, Amsterdam)</td>
<td>GP50 (CD2)</td>
</tr>
<tr>
<td>NK lymphocytes</td>
<td>CLB-FCR-GR (CLB, Amsterdam)</td>
<td>FcRII (CD16)</td>
</tr>
<tr>
<td>K562</td>
<td>CLB-mon-gran/2 (CLB, Amsterdam)</td>
<td>MAC-1 (CD11b)</td>
</tr>
<tr>
<td>HEL</td>
<td>My-9 (Dr J.D. Griffin, Boston)</td>
<td>GP67 (CD33)</td>
</tr>
<tr>
<td>B-ALL, ROS</td>
<td>CLB-HLA DR/2 (CLB, Amsterdam)</td>
<td>HLA class II</td>
</tr>
</tbody>
</table>

PLATELET ADHESION MEDIATED BY P-SELECTIN

From www.bloodjournal.org by guest on September 15, 2017. For personal use only.
minutes at 37°C. After treatment with enzymes, the cells were washed twice with PBS/0.2% BSA, fixed with PFA, and stained for detection by flow cytometry as described above.

RESULTS

P-selectin-mediated adhesion of thrombin-activated platelets to monocytes, neutrophils, eosinophils, and basophils, measured by flow cytometry. In our initial experiments, monocytes, neutrophils, eosinophils, and basophils were prepared and stained as described in the Methods and their interaction with fixed thrombin-activated platelets was determined by light microscopy. It appeared that activated, but not resting, platelets adhere to monocytes and neutrophils, confirming previous results of Jungi et al., and furthermore, that activated platelets also adhere to eosinophils and basophils.

To quantify the binding of activated platelets to eosinophils and to basophils, we have developed a double immunofluorescence assay, which involves the separate labeling of platelets and blood cells with specific MoAbs (Table 1) and with phycoerythrin-labeled rat MoAb against mouse IgG and GAM-FITC antibodies, respectively. After platelets and blood cells had interacted in a buffer containing Ca²⁺ and Mg²⁺, each at 1 mmol/L, binding was assessed by flow cytometry without additional washings. For reference, we also analyzed the binding of activated platelets to neutrophils and monocytes.

Figure 1A shows the analysis of the interaction of activated platelets and eosinophils. The intensity of the red (platelets) and green fluorescence (eosinophils) is displayed on the abscissa and ordinate, respectively. Four subpopulations can be distinguished: quadrant 1, cells that only emit green fluorescence representing single eosinophils; quadrant 4, cells that only emit red fluorescence representing platelets (only small aggregates were detected in this quadrant); quadrant 2, platelet-eosinophil aggregates with both red and green fluorescence; and quadrant 3, nonfluorescent other cells. In this experiment, 85% of the eosinophils were complexed with platelets. When in the same sample adhesion was assessed by light microscopy, 79% of the eosinophils appeared to have bound two or more platelets. If directly fixed nonactivated platelets were used, which do not express P-selectin, only 8% of the eosinophils bound platelets. The binding of activated platelets to eosinophils was inhibited by chelation of divalent cations with EDTA (not shown) and was blocked by some MoAbs against P-selectin (CLB-thromb/6 and 5), but not by others (RUU 1.18, RUU 2.15, and RUU 2.17). Figure 1B shows that antibody CLB-thromb/6 almost completely blocked adhesion of activated platelets to eosinophils; only 5% of the eosinophils had bound platelets. A partial

---

**Fig 1.** Effect of anti-P-selectin MoAbs on platelet adhesion to eosinophils. Fixed isolated eosinophils and platelets were stained with MoAbs CLB-thromb/6 and W6/32, respectively (see Table 1) and subsequently with GAM-FITC and phycoerythrin-rat anti-mouse IgG. Eosinophils and platelets were mixed in a ratio of 1:10 and incubated for 30 minutes. The effects of the MoAbs were studied by preincubating the platelets with ascites fluid containing anti-P-selectin MoAbs (1:20, final dilution), for 30 minutes at room temperature, before adding the eosinophils. Adhesion was then measured by flow cytometry. (A) Adhesion of thrombin-activated platelets to eosinophils in a medium containing 1 mmol/L CaCl₂ and 1 mmol/L MgCl₂. (B) Essentially complete inhibition of adhesion with MoAb CLB-thromb/6. A similar fluorescence pattern is observed when resting platelets were used or when adhesion was determined in a medium containing 5 mmol/L EDTA. (C) Partial inhibition of adhesion with MoAb CLB-thromb/5.
blocking effect on platelet-eosinophil interaction was observed with CLB-thromb/5 (Fig 1C). Although platelets still bound to 64% of the eosinophils, a reduction in the mean immunofluorescence intensity indicates that fewer platelets had bound per eosinophil.

From Table 2 it can be seen that similar results were obtained when basophils were used and that adhesion of activated platelets to eosinophils and basophils seems to occur by the same mechanism as to neutrophils and monocytes, because (1) adhesion of activated platelets to eosinophils and basophils and to neutrophils and monocytes was dependent on added divalent cations, and (2) anti-P-selectin antibodies blocked binding of activated platelets to eosinophils and basophils and to neutrophils and monocytes.

P-selectin-mediated adhesion of thrombin-activated platelets to lymphocytes. Adhesion of lymphocytes to activated platelets has been noted previously, but nothing is known about the receptors involved and the subpopulation(s) of lymphocytes which in fact bind the platelets. To study this interaction, we have sensitized lymphocytes with specific MoAbs detecting either peripheral T lymphocytes (CD2), B lymphocytes (CD37), NK cells (CD16), T-helper cells (CD4), or T-suppressor cells (CD8), and quantified the interaction of activated platelets with these subpopulations using the double immunofluorescence assay. Because in these experiments the various T-cell subpopulations had not been further purified, aggregates of red fluorescent platelets and nonfluorescent T cells appeared in quadrant 4. This is an essential difference from the experiments described in Fig 1, in which the interaction of activated platelets with a single isolated cell population was determined.

Of the cells stained with either CD2, CD4, or CD8 MoAbs, 20% to 30% adhered to activated platelets (quadrants 2 in Fig 2). Approximately 50% of the cells stained with a CD16 MoAb (a marker of NK cells) adhered to thrombin-activated platelets. Resting, directly fixed platelets, did not adhere to either the T-cell subsets or the NK cells. A summary of these experiments is given in Table 3.

In contrast to the results of Jungi et al, we found that adhesion of activated platelets to lymphocytes was Ca\(^{2+}\)-dependent (Table 3). After depletion of CD16\(^+\) cells (<1% NK lymphocytes detectable), using the immunorosette depletion technique, in which red blood cells are sensitized with tetrameric complexes containing antilymphocytin A and CD16 MoAbs, the percentage of CD2\(^+-\), CD4\(^+-\), and CD8\(^+-\) cells that bound to activated platelets was unaltered. After preincubating the platelets with MoAb CLB-thromb/6, no adhesion was demonstrated (Table 3), indicating that platelets also adhere to T cells and NK cells by P-selectin.

Adhesion of thrombin-activated platelets to different cell lines. To further study the distribution of the ligand for P-selectin, we have investigated the adhesion of activated platelets to a number of in vitro cell lines. It has been reported that platelets can interact via P-selectin with the cell lines U937 and HL60. Our data confirm that activated, but not resting, platelets adhere to U937 cells in a P-selectin, Ca\(^{2+}\)-dependent manner (Table 4). In addition, we found that activated, but not resting, platelets adhere to the cell line KG1a (myeloid origin). Again, the adhesion to KG1a cells was dependent on divalent cations, since it was strongly inhibited by EDTA. All other cell lines tested, ML-1 (myeloid origin), K562, and HEL (both megakaryocytic, erythroid origin), ROS (Burkitt type cell line), and B-ALL (B cell line), failed to adhere to both resting and thrombin-activated platelets (Table 4).

Characterization of the ligand for P-selectin. Treatment of neutrophils, monocytes, eosinophils, and basophils with neuraminidase from Vibrio cholerae decreased binding of all four cell types to activated platelets, but the degree of reduction in binding appeared to vary (Table 5). While binding to neutrophils was decreased to 15%, when compared with sham-treated controls, the effect on binding to monocytes, eosinophils, and basophils appeared to be less pronounced: approximately 60% of these cells still bound activated platelets. Similar results were observed when a fivefold higher concentration of neuraminidase or when neuraminidase from Arthrobacter ureafaciens was used. Furthermore, longer incubations of the cells with neuraminidase did not alter the percentage of binding. The efficacy of the treatment with neuraminidase was demonstrated by the complete loss of the reactivity of the anti-sialyl-Le\(^a\) anti-

---

Table 2. Effect of EDTA and Anti-P-Selectin MoAbs on Platelet Adhesion to Neutrophils, Monocytes, Eosinophils, and Basophils

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Neutrophils</th>
<th>Monocytes</th>
<th>Eosinophils</th>
<th>Basophils</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Adhesion</td>
<td>MF</td>
<td>% Adhesion</td>
<td>MF</td>
</tr>
<tr>
<td>Resting platelets</td>
<td>3.6</td>
<td>100</td>
<td>11.4</td>
<td>12.9</td>
</tr>
<tr>
<td>Activated platelets</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 mmol/L EDTA</td>
<td>86.4</td>
<td>100</td>
<td>79.1</td>
<td>100</td>
</tr>
<tr>
<td>RUU 1.18</td>
<td>4.4</td>
<td>9.1</td>
<td>12.9</td>
<td>12.9</td>
</tr>
<tr>
<td>RUU 2.15</td>
<td>84.2</td>
<td>112</td>
<td>79.5</td>
<td>112</td>
</tr>
<tr>
<td>RUU 2.17</td>
<td>84.9</td>
<td>120</td>
<td>78.5</td>
<td>126</td>
</tr>
<tr>
<td>CLB-thromb/6</td>
<td>8.3</td>
<td>53</td>
<td>9.6</td>
<td>57</td>
</tr>
<tr>
<td>CLB-thromb/5</td>
<td>62.4</td>
<td>70</td>
<td>58.6</td>
<td>75</td>
</tr>
</tbody>
</table>

The effect of EDTA and anti-P-selectin MoAbs on adhesion of platelets to neutrophils, monocytes, eosinophils, and basophils was studied as described in the legend to Fig 1. The mean fluorescence (MF) of bound platelets is expressed as a percentage of the value in the absence of antibody (100%). The values for neutrophils, monocytes, eosinophils, and basophils are the mean of the results obtained in 7, 6, 2, and 2 experiments, respectively.
body CSLEX-1 with all four different types of leukocytes (Table 5).

It was already shown above that the binding of platelets to all four cell types could be prevented by MoAb CLB-thromb/6 against P-selectin. Because the residual binding after neuraminidase could also be fully inhibited by MoAb CLB-thromb/6 (not shown), the recognition structure for P-selectin may include, in addition to sialic acid residues, other determinants. Alternatively, P-selectin may bind to two distinct ligands, one sensitive to neuraminidase treatment and present predominantly on neutrophils, and a second, insensitive to neuraminidase treatment and primarily expressed by monocytes, eosinophils, and basophils.

To investigate whether the ligand for P-selectin might be PI-linked, we used neutrophils and monocytes from a patient with paroxysmal nocturnal hemoglobinuria (PNH), which are deficient in PI-linked proteins. Neutrophils and monocytes of a patient with PNH and of a healthy volunteer were tested with a panel of MoAbs directed against PI-linked proteins (Table 6). Because the monocytes of the patient with PNH failed to react with the anti-CD14 MoAb (directed against a PI-linked surface antigen), we used a

---

**Table 3. Adhesion of Platelets to Subpopulations of Lymphocytes**

<table>
<thead>
<tr>
<th>Lymphocytes Stained With MoAb</th>
<th>% Adhesion to Resting Platelets in Ca²⁺/Mg²⁺</th>
<th>% Adhesion to Thrombin-Activated Platelets In Ca²⁺/Mg²⁺</th>
<th>% Adhesion to EDTA</th>
<th>In Ca²⁺/Mg²⁺ With CLB-thromb/6</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD2</td>
<td>2.1</td>
<td>24.7 ± 9.1 (7)</td>
<td>11.7 ± 9.5 (3)</td>
<td>3.6 ± 1.2 (2)</td>
</tr>
<tr>
<td>CD4</td>
<td>4.4</td>
<td>20.0 ± 5.3 (4)</td>
<td>2.8</td>
<td>4.6</td>
</tr>
<tr>
<td>CD9</td>
<td>1.5</td>
<td>33.3 ± 10.4 (4)</td>
<td>1.7</td>
<td>1.7</td>
</tr>
<tr>
<td>CD37</td>
<td>5.8</td>
<td>8.0 ± 1.1 (2)</td>
<td>6.9</td>
<td>6.0 ± 5.1 (2)</td>
</tr>
<tr>
<td>CD16</td>
<td>2.3</td>
<td>55.3 ± 15.2 (6)</td>
<td>13.6 ± 10.9 (4)</td>
<td>12.0 ± 9.0 (2)</td>
</tr>
</tbody>
</table>

Subpopulations of lymphocytes were stained with specific MoAbs (see Table 1), mixed with resting or activated platelets, and incubated for 30 minutes under the conditions indicated. Adhesion was quantitated by flow cytometry as described in Materials and Methods. The number of experiments is shown in parentheses and the values are means ± SD of the results of the indicated number of experiments.
CD36 MoAb to sensitize the monocytes for the adhesion assay. The adhesion of activated platelets to neutrophils and monocytes, missing several PI-linked proteins, appeared to be normal (Table 6). We also studied the effect of treatment of normal neutrophils with GPI-PLC, an enzyme that cleaves the PI-linkage. The binding of CD24 and CD16 was reduced to 71% and 31%, respectively, on GPI-PLC-treated neutrophils. Also, their capacity to adhere to thrombin-activated platelets was normal (not shown). Taken together, these results suggest that at least part of the ligands for P-selectin on neutrophils and monocytes are not PI-linked.

*Sialyl-Lea and Lea are not per se ligands for P-selectin.* It has been suggested that the pentasaccharide LNF II (also called CD15 antigen or Lea) is the principal ligand for P-selectin on neutrophils and monocytes, since CD15 MoAbs and soluble Lea oligosaccharides block this interaction.14 We have tested the inhibitory effect of Lea and LNF I (a control oligosaccharide) in a concentration range from 25 to 200 µg/mL on the binding of activated platelets to neutrophils and monocytes, but failed to detect any inhibition. The involvement of CD15 in P-selectin recognition was also tested by studying the effects of a panel of CD15 MoAbs from our laboratory (CLB-gran/2 and CLB-3B9) and of CD15 MoAbs obtained via the Workshop on Leukocyte Typing IV.25 No effect of any of these reagents on binding could be demonstrated. To further examine whether Lea is involved in P-selectin recognition, we studied whether the expression of CD15 on the cell lines used above is correlated with their ability to bind activated platelets. As shown in Table 7, monocytes, basophils, and eosinophils express CD15 at levels similar to those of neutrophils, whereas red blood cells and B lymphocytes do not express CD15.

**Table 4. Adhesion of Platelets to Various Cell Lines**

<table>
<thead>
<tr>
<th>Cell Lines Tested</th>
<th>% Adhesion to Resting Platelets</th>
<th>% Adhesion to Thrombin-Activated Platelets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In Ca2+/Mg2+</td>
<td>In Ca2+/Mg2+</td>
</tr>
<tr>
<td>U937</td>
<td>2.7</td>
<td>70.5</td>
</tr>
<tr>
<td>K562</td>
<td>2.4</td>
<td>1.9</td>
</tr>
<tr>
<td>HEL</td>
<td>1.8</td>
<td>6.4</td>
</tr>
<tr>
<td>ML-1</td>
<td>0.4</td>
<td>0.1</td>
</tr>
<tr>
<td>KG1a</td>
<td>3.4</td>
<td>58.3</td>
</tr>
<tr>
<td>ROS</td>
<td>3.7</td>
<td>3.0</td>
</tr>
<tr>
<td>B-ALL</td>
<td>3.1</td>
<td>2.8</td>
</tr>
</tbody>
</table>

Different types of cell lines were stained with specific MoAbs (see Table 1), mixed with resting or activated platelets, and incubated for 30 minutes under the conditions indicated. The adhesion was measured by flow cytometry as described in Materials and Methods. Values for U937 and KG1a cells are the means of three and two measurements, respectively.

**Table 5. Effect of Neuraminidase Treatment on the Expression of Sialyl-Lea by Different Types of Leukocytes and Platelet Adhesion**

<table>
<thead>
<tr>
<th>Leukocytes</th>
<th>Not Treated</th>
<th>Neuraminidase-Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anti-Sialyl-Lea Binding (MF)</td>
<td>% Adhesion</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>1.012</td>
<td>86.5 (n = 5)</td>
</tr>
<tr>
<td>Monocytes</td>
<td>667</td>
<td>76.5 (n = 4)</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>103</td>
<td>83.7 (n = 3)</td>
</tr>
<tr>
<td>Basophils</td>
<td>130</td>
<td>83.6 (n = 3)</td>
</tr>
</tbody>
</table>

Leukocytes were incubated with 0.2 U/mL of *Vibrio cholerae* neuraminidase for 1 hour at 37°C and control leukocytes for the same period of time without enzyme. Treated and untreated cells were then incubated with anti-sialyl-Lea (CSLEX-1) MoAb and the bound antibody was detected by reaction with GAM-FITC. The mean fluorescence (of the gated population) was measured by flow cytometry. Adhesion of thrombin-activated platelets to neuraminidase-treated and -untreated leukocytes was determined as described in Materials and Methods. The number of experiments is shown in parentheses and the results are the means of the results of these experiments.

**Table 6. Cell Surface Expression of PI-Linked Antigens on Neutrophils and Monocytes From a Patient With PNH and a Control Donor, and Platelet Adhesion**

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Control</th>
<th>PNH</th>
<th>Antibodies</th>
<th>Control</th>
<th>PNH</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD24</td>
<td>449</td>
<td>37</td>
<td>CD14</td>
<td>544</td>
<td>87</td>
</tr>
<tr>
<td>CD16</td>
<td>466</td>
<td>85</td>
<td>CD36</td>
<td>243</td>
<td>558</td>
</tr>
<tr>
<td>CD67</td>
<td>61</td>
<td>43</td>
<td>CD15</td>
<td>709</td>
<td>649</td>
</tr>
</tbody>
</table>

% Platelet adhesion

77 72 86 75

Antibody binding to neutrophils and monocytes from a control donor and a patient with PNH was detected by reaction with GAM-FITC, followed by flow cytometry. MoAbs CD24, CD16, CD67, and CD14 are directed against PI-linked antigens. MoAbs CD15 and CD36 were used as markers for neutrophils and monocytes, respectively. The mean fluorescence (MF) of the gated population was measured by flow cytometry. Adhesion assays were performed as described in Materials and Methods.

**Table 7. Comparison of Lea (CD15) and Sialyl-Lea Expression by Various Cells and Binding of Activated Platelets**

<table>
<thead>
<tr>
<th>Cells and Cell Lines Tested</th>
<th>Anti-Lea Binding</th>
<th>Anti-Sialyl-Lea Binding</th>
<th>% Adhesion to Activated Platelets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophils</td>
<td>&gt; 95</td>
<td>706</td>
<td>90 1,041 86</td>
</tr>
<tr>
<td>Monocytes</td>
<td>&lt; 5</td>
<td>—</td>
<td>76 901 79</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>&gt; 95</td>
<td>634</td>
<td>65 238 89</td>
</tr>
<tr>
<td>Basophils</td>
<td>10</td>
<td>24</td>
<td>85 202 90</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>&lt; 5</td>
<td>—</td>
<td>25 524 22</td>
</tr>
</tbody>
</table>

**Cell line cells**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Anti-Lea Binding</th>
<th>Anti-Sialyl-Lea Binding</th>
<th>% Adhesion to Activated Platelets</th>
</tr>
</thead>
<tbody>
<tr>
<td>U937</td>
<td>&gt; 95</td>
<td>1,118</td>
<td>85 1,367 70</td>
</tr>
<tr>
<td>ML-1</td>
<td>&gt; 95</td>
<td>3,897</td>
<td>&lt; 5 — &lt; 5</td>
</tr>
<tr>
<td>KG1a</td>
<td>12</td>
<td>106</td>
<td>52 1,041 58</td>
</tr>
<tr>
<td>K562</td>
<td>35</td>
<td>710</td>
<td>8 — &lt; 5</td>
</tr>
<tr>
<td>HEL</td>
<td>19</td>
<td>226</td>
<td>&lt; 5 — &lt; 5</td>
</tr>
<tr>
<td>ROS</td>
<td>&lt; 5</td>
<td>—</td>
<td>&lt; 5 — &lt; 5</td>
</tr>
<tr>
<td>B-ALL</td>
<td>&lt; 5</td>
<td>—</td>
<td>&lt; 5 — &lt; 5</td>
</tr>
</tbody>
</table>

Expression of Lea (CD15) and sialyl-Lea by cells was determined by flow cytometry, using the MoAb CLB-gran/2, directed against the Lea antigen, and the MoAb CSLEX-1, directed against the sialyl-Lea. MF is mean fluorescence of positive cells. Platelet adhesion was measured as described in Materials and Methods.
was assessed as described in the Table 7 notes. The mean fluorescence
try. Platelet adhesion was assessed as described in Materials and
platelets, but their reactivity with CD15 MoAbs was not
cate that CD15 per se can not be the ligand for P-selectin.

diminished (Table 8). Taken together, these results indi-
neutrophils treated with neuraminidase, elastase, or trypsin
ML-1 cells, which strongly express CD15, do not bind

Methods.
not or only weakly express the CD15 antigen. Furthermore,
neutrophils treated with neuraminidase, elastase, or trypsin
had almost completely lost the capacity to bind activated
platelets, but their reactivity with CD15 MoAbs was not
diminished (Table 8). Taken together, these results indi-
cate that CD15 per se can not be the ligand for P-selectin.

Similar to the experiments described above for Le 
, experiments were performed with sialyl-Le 
 oligosaccha-
rades (final concentrations, 0.5 to 50 µg/mL) and anti-sialyl-
Le 
 MoAb CSLEX-1 (final concentrations, 0.5 to 100
µg/mL). None of these reagents affected the binding of
activated platelets to monocytes and neutrophils. However,
there was a close correlation between the expression of
sialyl-Le 
 on cells and their ability to bind activated plate-
lets (Table 8). Therefore, sialyl-Le 
 might still be involved in
P-selectin recognition, probably not as a simple tetrascara-
chide, but as part of a more complex oligosaccharide.
Furthermore, P-selectin may additionally recognize protein
components, since treatment of neutrophils and monocytes
with elastase and trypsin markedly diminished the binding
of activated platelets, while the reactivity of the MoAb
CSLEX-1 remained unaltered (Table 8).

DISCUSSION

The results of the present study show that the double
immunofluorescence technique is a useful assay to quantify
the adhesion of activated platelets to different types of
peripheral blood cells. Particularly, the interaction of plate-
lets with subpopulations of cells for which specific MoAbs
are available, can be studied without isolating the subpopu-
lations.

Our results show that activated platelets adhere not only
to neutrophils and monocytes, but also to eosinophils,
basophils, NK cells, and a second, undefined subpopulation
of peripheral T lymphocytes in both CD4 and CD8 subsets.
Only thrombin-stimulated platelets, but not resting plate-
lets, adhered. Adhesion of activated platelets to eosinophils
and basophils is dependent on divalent cations, since no
adhesion was observed in a medium containing 5 mmol/L
EDTA. In contrast to the results of Jungi et al,1 we found
that the adhesion of platelets to lymphocytes (including NK
cells) is also Ca 
-dependent.

The receptor on activated platelets responsible for the
interactions with the different leukocyte subpopulations
appeared to be P-selectin in all cases. The MoAb CLB-
thromb/6 completely blocked the interaction of activated
platelets with leukocytes, whereas MoAb CLB-thromb/5
blocked only partially. The MoAbs RUU 1.18, RUU 2.15,
and RUU 2.17 had no effect on binding. In a previous
study,22 we have shown that there are several distinct epitopes on P-selectin. The epitopes detected by the
MoAbs CLB-thromb/5 and CLB-thromb/6 are sensitive to
treatment with elastase and trypsin, whereas the epitopes
recognized by RUU 1.18, RUU 2.15, and RUU 2.17 are
not. Moreover, treatment of activated platelets with neur-
amidase reduced the expression of the epitope recog-
nized by CLB-thromb/6 by 50%. Thus, both protein and
sialic acid residues may be part of the CLB-thromb/6-
defined epitope. The expression of this epitope is closely
associated with the function of P-selectin as an adhesion
receptor, since its destruction by either protease or neura-
imidase treatment diminished the ability of activated
platelets to bind to neutrophils (data not shown). This
 corroborates the blocking effect found with the CLB-
thromb/6 MoAb.

P-selectin belongs to the selectin family4 of adhesion
proteins, which includes the endothelial leukocyte adhesion
molecule (ELAM-1 or E-selectin) and the lymphocyte
homing receptor (ELAM-1 or E-selectin). The members of this family are characterized by a unique
primary structure, containing a lectin domain, that is
thought to recognize carbohydrates on their ligand struc-
tures.

Larsen et al14 concluded that P-selectin recognizes the
Le 
 (CD15) antigen, since adhesion of neutrophils to
activated platelets was partially inhibited both by MoAbs
against Le 
 (CD15) and by soluble oligosaccharides contain-
ing the Le 
 structure. On the contrary, other investigators
found that (1) the binding of P-selectin to neutrophils was
not blocked by an anti-CD15 antibody or by multivalent
Le 
-BSA conjugates21 and (2) treatment of neutrophils
with neuraminidase blocked binding of activated platelets,
suggesting that sialic acid residues are an essential compo-
nent of the adhesion site.20,21 In our study, we found that
neither a series of MoAbs against CD15 nor Le 
 in high
concentrations inhibited the binding of activated platelets
to neutrophils. Finally, we found no correlation between
expression of Le 
 (CD15) and the ability of cells to bind
activated platelets. The conclusion from our results there-
fore is that Le 
 cannot be a major ligand for P-selectin.

Other studies have suggested that as for E-selectin,33
sialyl-Le 
 is the ligand for P-selectin.15,16 Indeed, we found a
good correlation between expression of sialyl-Le 
 and the
ability of cells to bind activated platelets. However, we did
not find that an MoAb against sialyl-Le 
 or, like Aruffo et
al,19 that soluble oligosaccharides containing the sialyl-Le 

structure had any inhibitory effect. There is no satisfactory

| Table 8. Expression Le 
(CD15) and Sialyl-Le 
 by Neutrophils After Treatment With Neuraminidase, Trypsin, or Elastase, and Adhesion of Activated Platelets |
|---|---|---|---|
| Neutrophils Treated With: | % Platelet Adhesion | MF Le 
 | MF Sialyl-Le 
 |
| — | 70 | 1,058 | 1,077 |
| Neuraminidase | 16 | 1,403 | 24 |
| Trypsin | 14 | 1,182 | 1,023 |
| Elastase | 9 | 1,224 | 996 |

Neutrophils were treated with proteases or neuraminidase as de-
scribed in the Methods. Cell surface expression of Le 
 and sialyl-Le 
 was assessed as described in the Table 7 notes. The mean fluorescence (MF) of the total neutrophil population was measured by flow cytome-
try. Platelet adhesion was assessed as described in Materials and
Methods.
the sole ligand for P-selectin. The involvement of other ligands than sialyl-LeX in P-selectin-mediated adhesion is also suggested by our observations that (1) neuraminidase treatment of monocytes, eosinophils, and basophils destroys the reactivity of the anti-sialyl-LeX antibody CSLEX-1, but only partially inhibited the binding of activated platelets to these cells, and (2) protease treatment of myeloid cells markedly diminished adhesion of activated platelets, although a normal amount of sialyl-LeX could still be detected. In fact, two other groups have obtained evidence for the existence of multiple ligands for P-selectin. They found that P-selectin binds to sulfatides and, moreover, that binding of P-selectin to neutrophils and HL60 cells could be inhibited by heparin, fucoidin, and dextran sulfate. All these molecules are negatively charged and, therefore, apart from sialylation, other types of anionic modification of oligosaccharides, such as sulfation and phosphorylation, may contribute to P-selectin recognition. Our finding that the P-selectin-mediated adhesion is sensitive to proteases would argue against the possibility that sulfatides themselves are essential components of the ligand for P-selectin.

It is not clear from our data whether the protein components are directly recognized by P-selectin or whether they are important for the proper exposure of the oligosaccharide structures. Binding studies performed with neutrophils and monocytes from a patient with PNH and with normal cells treated with GPI-PLC indicated that the ligands for P-selectin are probably not PI-linked proteins.

ACKNOWLEDGMENT

We thank C. Paul Englert and Taco Kuipers for carefully reading the manuscript, Erik Mul and Anton Tool for preparing basophils and eosinophils, and Caroline Snethlage and Wanda Winkel for their secretarial assistance.

REFERENCES


P-selectin mediates Ca(2+)-dependent adhesion of activated platelets to many different types of leukocytes: detection by flow cytometry

LG de Bruijne-Admiraal, PW Modderman, AE Von dem Borne and A Sonnenberg