P-Selectin Mediates Ca2+-Dependent Adhesion of Activated Platelets to Many Different Types of Leukocytes: Detection by Flow Cytometry

By Lucia G. de Bruijne-Admiraal, 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~ and sialyl-LeX, 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~ on several cell types and their capacity to bind activated platelets. In contrast, the expression of sialyl-LeX on cells was almost perfectly correlated with their ability to bind activated platelets. Thus, while Le~ cannot be a major ligand for P-selectin, a possible role for sialyl-LeX in P-selectin-mediated adhesion 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.

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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 Ca\(^{2+}\) 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 phosphatidyl inositol (PI)-linked proteins.

**MATERIALS AND METHODS**

Reagents. MoAbs used for cell-specific staining are described in Table 1. The MoAbs CLB-thromb/5 and CLB-thromb/6,\(^{22}\) specific for P-selectin, and the MoAbs CLB-gran/2\(^{23}\) and CLB-3B9,\(^{24}\) 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 H1 98), and M161 (clone UL-58) were obtained via Workshop studies.\(^{25}\) The anti-sialyl-Le\(^{a}\) 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 (LNFF III), lacto-N-fucopentaose I (LNFI), and 3-sialyl-Lewis X (sialyl-Le\(^{x}\)) 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/0.2% (wt/vol) bovine serum albumin (PBS/EDTA/BSA). Activated platelets were prepared as follows: platelets were isolated from platelet-rich plasma (PRP) of EDTA-anticoagulated blood, as described by Von dem Borne et al.\(^{26}\) The platelets were washed three times with PBS/EDTA/BSA, resuspended in the same buffer (2 \(\times\) 10\(^{8}\)/mL), and activated with 1 U/mL of human \(\alpha\)-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 \(\kappa\) 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-Isoopaque (\(d = 1.077\) g/cm\(^3\)). 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 NH\(_4\)Cl (155 mmol/L NH\(_4\)Cl; 10 mmol/L KHCO\(_3\), 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, 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 \(\mu\)g/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.

**Table 1. Monoclonal Antibodies Used for Cell-Specific Staining**

<table>
<thead>
<tr>
<th>Cells</th>
<th>MoAb (source)</th>
<th>Directed Against</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelets, neutrophils, U937</td>
<td>W6/32 (Dr S.C. Meuer, Heidelberg)</td>
<td>HLA class I</td>
</tr>
<tr>
<td>Monocytes</td>
<td>CLB-mon/1 (CLB, Amsterdam)</td>
<td>GP55 (CD14)</td>
</tr>
<tr>
<td>Eosinophils, basophils</td>
<td>CLB-thromb/8 (CLB, Amsterdam)</td>
<td>p24 (CD9)</td>
</tr>
<tr>
<td>B lymphocytes</td>
<td>(\gamma 29-55) (Dr H.K. Forster, Basel)</td>
<td>GP40-52 (CD37)</td>
</tr>
<tr>
<td>T lymphocytes</td>
<td>CLB-T11 (CLB, Amsterdam)</td>
<td>GP50 (CD2)</td>
</tr>
<tr>
<td>NK lymphocytes</td>
<td>CLB-FcRgran/1 (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. Griffith, 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>

Antibody inhibition studies were performed by preincubating either the platelets or the leukocytes with ascites fluid (1:50 final dilution) or purified MoAbs (20 \(\mu\)g/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.

**Treatment of cells with enzymes.** Neutrophils (1.0\(\times\)10\(^7\)/mL RPMI) were treated with 10 \(\mu\)L glycosylphosphatidyl inositol-specific phospholipase C (GPI-PLC; a kind gift of Dr M. Low, Columbia University, New York, NY) at a final dilution of 1:10\(^4\) for 15 minutes at 37°C. Thrombin-activated platelets were treated with elastase or trypsin as described.\(^{22}\)

Neuraminidase (Vibrio cholerae, Behringwerke, Marburg, Germany; Arthrobacter ureafaciens, Calbiochem-Behring, La Jolla, CA) treatment of cells was performed by incubating 1.10\(^5\) cells in PBS/0.2% BSA (pH 7.4) with 0.2 U/mL neuraminidase for 60
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.1 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

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Fig 1. Effect of anti-P-selectin MoAbs on platelet adhesion to eosinophils. Fixed isolated eosinophils and platelets were stained with MoAbs CLB-thromb/8 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 testing 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,1 we found that adhesion of activated platelets to lymphocytes was Ca²⁺-dependent (Table 3). After depletion of CD16⁺ cells (<1% NK lymphocytes detectable), using the immunorosette depletion technique,2⁰ in which red blood cells are sensitized with tetrameric complexes containing anti-glycophorin 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 HL60.2 Our data confirm that activated, but not resting, platelets adhere to U937 cells in a P-selectin, Ca²⁺-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-Le⁰ anti-
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

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

<table>
<thead>
<tr>
<th>Lymphocytes Stained With MoAb</th>
<th>% Adhesion to Resting Platelets In Ca^{2+}/Mg^{2+}</th>
<th>% Adhesion to Thrombin-Activated Platelets In Ca^{2+}/Mg^{2+} With CLB-thromb/6</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD2</td>
<td>2.1</td>
<td>24.7 ± 9.1 (7)</td>
</tr>
<tr>
<td>CD4</td>
<td>4.4</td>
<td>20.0 ± 5.3 (4)</td>
</tr>
<tr>
<td>CD8</td>
<td>1.5</td>
<td>33.3 ± 10.4 (4)</td>
</tr>
<tr>
<td>CD37</td>
<td>5.8</td>
<td>8.0 ± 1.1 (2)</td>
</tr>
<tr>
<td>CD16</td>
<td>2.3</td>
<td>55.3 ± 15.2 (6)</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.

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**Fig 2.** Fixed isolated lymphocytes were incubated with CD2, CD4, CD8, or CD16 MoAbs and stained with GAM-FITC. Phycoerythrin-colored platelets were added and, after an incubation period of 30 minutes at room temperature, the adhesion was measured. In this experiment, adhesion of activated platelets was shown to (A) CD3+ cells, (B) CD4+ cells, (C) CD8+ cells, and (D) CD16+ cells; percent adhesion was 27%, 22%, 38%, and 80%, respectively.
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 MoAbs (both directed against PI-linked surface antigens on neutrophils) 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-Le" and Le" are not per se ligands for P-selectin. It has been suggested that the pentasaccharide LNF I (also called CD15 antigen or the sialic acid residue) is the principal ligand for P-selectin on neutrophils and monocytes, since CD15 MoAbs and soluble Le" oligosaccharides block this interaction. We have tested the inhibitory effect of Le" 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/23 and CLB-3B924) 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 Le" 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 were found to express CD15 antigen, with a mean fluorescence of positive cells. Platelet adhesion was measured as described in Materials and Methods.

Table 5. Effect of Neuraminidase Treatment on the Expression of Sialyl-Le" by Various Cells and Binding of Activated Platelets

<table>
<thead>
<tr>
<th>Leukocytes</th>
<th>Not Treated</th>
<th>Neuraminidase-Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antisialyl-Le&quot; Binding (MF)</td>
<td>% Adhesion</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 = 19)</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>103</td>
<td>83.9 (n = 31)</td>
</tr>
<tr>
<td>Basophils</td>
<td>130</td>
<td>83.8 (n = 10)</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-Le" (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.
was assessed as described in the Table 7 notes. The mean fluorescence try. Platelet adhesion was assessed as described in Materials and described in the Methods. Cell surface expression

platelets, but their reactivity with CD15 MoAbs was not corrobore the blocking effect found with the CLB-thromb/6 MoAb. P-selectin belongs to the selectin family of adhesion proteins, which includes the endothelial leukocyte adhesion molecule (ELAM-1 or E-selectin) and the lymphocyte homing receptor (MEL-14/LAM-1 or L-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 structures.

Larsen et al. concluded that P-selectin recognizes the Lea (CD15) antigen, since adhesion of neutrophils to activated platelets was partially inhibited both by MoAbs against Lea (CD15) and by soluble oligosaccharides containing the Lea 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 Lea-BSA conjugates, and (2) treatment of neutrophils with neuraminidase blocked binding of activated platelets, suggesting that sialic acid residues are an essential component of the adhesion site. In our study, we found that neither a series of MoAbs against CD15 nor Lea in high concentrations inhibited the binding of activated platelets to neutrophils. Finally, we found no correlation between expression of Lea (CD15) and the ability of cells to bind activated platelets. The conclusion from our results therefore is that Lea cannot be a major ligand for P-selectin.

Other studies have suggested that as for E-selectin, sialyl-Lea is the ligand for P-selectin. Indeed, we found a good correlation between expression of sialyl-Lea and the ability of cells to bind activated platelets. However, we did not find that an MoAb against sialyl-Lea or, like Aruffo et al, that soluble oligosaccharides containing the sialyl-Lea structure had any inhibitory effect. There is no satisfactory
explanation for the discrepant results obtained with sialyl-Le\textsuperscript{a}, except that the blocking capacity of sialyl-Le\textsuperscript{a} in different preparations might differ or that sialyl-Le\textsuperscript{a} is not the sole ligand for P-selectin. The involvement of other ligands than sialyl-Le\textsuperscript{a} in P-selectin–mediated adhesion is also suggested by our observations that (1) neuraminidase treatment of monocytes, eosinophils, and basophils destroyed the reactivity of the anti–sialyl-Le\textsuperscript{a} 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-Le\textsuperscript{a} 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\textsuperscript{20}, and, moreover, that binding of P-selectin to neutrophils and HL\textsubscript{60} cells could be inhibited by heparin, fucoidin, and dextran sulfate.\textsuperscript{17,18} 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.

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