

Expression and function of P-selectin glycoprotein ligand 1 (CD162) on human basophils

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Background: The endothelial cell adhesion molecule P-selectin may contribute to selective leukocyte migration in allergic diseases by binding to its ligand, P-selectin glycoprotein ligand 1 (PSGL-1), on eosinophils and other leukocytes. Although expression of PSGL-1 on basophils has been detected in leukocyte typing workshops, its function on basophils has not been explored.

Objective: We sought to characterize the expression and function of PSGL-1 on human basophils and a basophil-like cell line (KU812) and to compare these characteristics with those for PSGL-1 on eosinophils and neutrophils.

Methods: Basophils, eosinophils, and neutrophils were enriched from peripheral blood by using density gradient centrifugation and immunomagnetic negative selection. KU812 cells were cultured by using standard techniques. Indirect immunofluorescence and flow cytometry were used to determine surface PSGL-1 expression under various conditions, and Western blotting was used to analyze the molecular forms of PSGL-1 on each cell type. Static adhesion assays were performed by using immobilized recombinant P-selectin and relevant blocking antibodies. Histamine release assays were done by using adherent and nonadherent basophils to determine whether adhesion by means of PSGL-1 altered basophil releasability.

Results: The expression of PSGL-1 on basophils was similar to that on neutrophils but was approximately 30% less bright than levels on eosinophils. Levels on basophils were 10-fold higher than on KU812 cells. Basophil activation by means of IgE cross-linking resulted in reductions in surface expression of PSGL-1 and L-selectin, as well as increased CD11b expression. Western blot analysis of PSGL-1 revealed that the molecular weights of the bands for neutrophils and basophils were similar, whereas those for eosinophils were of greater molecular weights. Static adhesion assays demonstrated that basophils bound well to P-selectin, whereas KU812 cells bound poorly. Adhesion of basophils to P-selectin was completely blocked by antibodies to either P-selectin or PSGL-1. Finally, adhesion to P-selectin did not alter the magnitude or kinetics of anti-IgE-induced histamine release.

Conclusion: Expression of PSGL-1 on basophils is more similar to that on neutrophils than that on eosinophils. KU812 cells express much lower levels of this molecule but, like basophils and other cells, bind to P-selectin by means of PSGL-1. P-selectin expression at sites of allergic inflammation is likely to play an important role in human basophil recruitment, but adhesion by means of PSGL-1 does not alter IgE-dependent basophil histamine release. (*J Allergy Clin Immunol* 2000;106:918-24.)

Key words: Basophils, eosinophils, neutrophils, KU812 cells, P-selectin glycoprotein ligand 1, P-selectin, endothelial cells, adhesion, histamine release

Basophils and their mediators are primary effectors of allergic inflammation.¹ Basophilic granulocytes have been demonstrated in lungs and sputum of allergic asthmatic subjects, in nasal mucosa and secretions of patients with allergic rhinitis, and in skin lesions of patients with atopic dermatitis.²⁻⁴ After antigen challenge, basophil numbers in the skin and airways also increase, often more than 100-fold, and can account for as many as 3% of the cells.⁴⁻⁸ Although the precise role of basophils in the pathophysiology of chronic allergic diseases is not clearly defined, the presence of basophils and basophil-derived mediators during allergic and other inflammatory responses suggests that mechanisms exist favoring their recruitment to these sites.

Adhesion molecules are essential for each step of cell migration into tissues, including rolling, tethering, firm adhesion, and transendothelial migration.⁹ Human basophils express a number of adhesion molecules on their surface, including selectins, integrins, sialyl-Lewis^x-containing carbohydrates, and Ig superfamily members, which they use for binding various endothelial counterligands.^{1,10-12} Previous studies suggest that in many respects basophil adhesive function and phenotype resemble those seen with neutrophils.¹³ Exceptions include IgE- and IL-3-dependent regulation of basophil adhesion^{10,14,15} and, along with eosinophils, the use of integrins $\alpha 4\beta 1$ and $\alpha 4\beta 7$.^{10,16,17} Defining the roles of adhesion molecule interactions among basophils, endothelial cells, and matrix proteins is important to determine how these cells are selectively recruited to the skin and airways of patients with asthma and other allergic diseases.¹⁸

Previous studies support the possibility that P-selectin expressed on activated endothelial cells contributes to leukocyte recruitment in vivo.¹⁹ Furthermore, it has been proposed that P-selectin glycoprotein ligand 1 (PSGL-1),

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Abbreviations used

PIPES: Piperazine-N,N'-bis-2-ethanesulfonic acid
PSGL-1: P-selectin glycoprotein ligand 1 (CD162)

a homodimeric surface protein found on eosinophils and other leukocytes that functions as a ligand for P-selectin, might contribute to selective eosinophil migration in allergic disease.²⁰⁻²² It also appears that during adhesion, PSGL-1 can mediate outside-in signaling and activation of neutrophils.²³ For basophils, phenotyping studies demonstrated what appeared to be modest levels of expression of PSGL-1 on basophils,^{12,24,25} but no biochemical or functional characterization of basophil PSGL-1 has been reported. Therefore the aims of this study were to characterize the expression of PSGL-1 on basophils and the basophilic cell line KU812, to determine whether basophils use PSGL-1 as a ligand for P-selectin, and to assess whether adhesion to P-selectin alters IgE-dependent basophil histamine release responses.

METHODS

Reagents and antibodies

Reagents used were as follows: Dulbecco's PBS (Biofluids, Rockville, Md); piperazine-N,N'-bis-2-ethanesulfonic acid (PIPES), EDTA, paraformaldehyde, BSA, erythrosin B dye, Triton X-100, alcian blue, phenylmethylsulfonylfluoride, aprotinin, sodium orthovanadate, leupeptin, pepstatin, β_2 -mercaptoethanol, and human IgG (Sigma Chemical Company, St Louis, Mo); Percoll (Pharmacia, Uppsala, Sweden); RPMI-1640 medium with 25 mmol/L HEPES, glutamine, penicillin, streptomycin, and fungizone (Life Technologies, Rockville, Md); and soluble P-selectin (R&D Systems, Minneapolis, Minn). Buffers used included PIPES buffer (25 mmol/L PIPES, 100 mmol/L NaCl, 6 mmol/L KCl, and 40 mmol/L NaOH [pH 7.4]); PAG buffer (PIPES buffer containing 0.003% human serum albumin and 0.1% glucose); PAGCM buffer (PAG buffer containing 1 mmol/L CaCl_2 and MgCl_2); PAG-EDTA (PAG buffer containing 1 mmol/L EDTA); and saline-EDTA (saline containing 1 mmol/L EDTA).

Antibodies used were as follows: polyclonal goat IgG anti-human IgE (generously provided by Dr Donald MacGlashan Jr, Johns Hopkins University School of Medicine, Baltimore, Md); FITC-conjugated polyclonal goat IgG anti-human IgE and control FITC-conjugated polyclonal normal goat IgG (Kirkegaard and Perry Labs, Gaithersburg, Md); PSGL-1 blocking murine mAb PL1²⁶ (Ancell, Bayport, Minn); CD11a and CD11b murine mAb MHM24 and H5A4²⁷ (generously provided by Dr James Hildreth, Johns Hopkins University School of Medicine); L-selectin murine mAb LAM1-116²⁸ (generously provided by Drs Thomas Tedder and Douglas Steeber, Duke University Medical Center, Durham, NC); P-selectin blocking murine mAb G1 and nonblocking control murine mAb S12²⁹ (both generously provided as F(ab')₂ reagents by Centocor, Malvern, Pa); and murine IgG1 control mAb and phycoerythrin-conjugated F(ab')₂ goat anti-mouse IgG (Caltag Laboratories, Inc, South San Francisco, Calif).

Basophil, eosinophil, and neutrophil purification and culture of KU812 cells

Human basophils were enriched from EDTA-anticoagulated venous blood with PIPES-diluted Percoll (specific density, 1.065 g/mL) layered over a single layer of Percoll (specific density, 1.079

g/mL).³⁰ Viability after purification was determined by using erythrosin B exclusion and always exceeded 95%. Basophil counts were performed after staining with alcian blue.³¹ This yielded basophils with purities that ranged from 4% to 15%. For experiments using basophils of enhanced purity, cells were further purified by using immunomagnetic negative selection with a basophil purification kit (containing a mixture of mAbs recognizing CD3, CD7, CD14, CD15, CD16, CD36, CD45RA, and HLA-DR) and a MidiMACS column (both from Miltenyi Biotec Inc, Auburn, Calif). Viability after this purification was consistently greater than 90%, and enriched cell purity ranged up to 99%.

For some experiments, eosinophils and neutrophils were also purified (sometimes simultaneously with basophils from the same donor) by using separate aliquots of EDTA-anticoagulated venous blood and density gradient centrifugation with (for eosinophils) or without (for neutrophils) CD16 immunomagnetic negative selection, as previously described.¹⁶ Viability and purity always exceeded 95%. The basophil-like KU812 cell line, which expresses low levels of surface Fc ϵ RI and contains intracellular markers found in either basophils or mast cells,³² was obtained from the American Type Culture Collection (ATCC, Manassas, Va) and cultured in RPMI-1640 medium with 10% FCS and 1% L-glutamine.

Indirect immunofluorescence and flow cytometric analyses

Basophils were specifically labeled with saturating concentrations of FITC-conjugated polyclonal goat IgG anti-human IgE together with saturating concentrations of primary murine mAb directed against a specific adhesion molecule or an isotype-matched control mAb.³³ Cells were then washed and incubated with the secondary antibody, phycoerythrin-conjugated F(ab')₂ goat anti-mouse IgG. Labeling of KU812 cells, and purified eosinophils and neutrophils, was identical, except that the FITC anti-human IgE reagent was omitted. The labeled cells were then fixed with 1% paraformaldehyde and stored at 4°C for up to 2 days before being analyzed by means of flow cytometry with an EPICS Profile II flow cytometer with a 488-nm argon laser (Coulter Corporation, Hialeah, Fla). Basophils were identified by gating on FITC-positive (IgE bearing) cells. Forward- and side-scatter gating was also used for each cell type to identify a uniform population of single cells for analysis. Fluorescence histograms were generated by using a 4 log scale and were expressed in units of mean fluorescence intensity or net mean fluorescence intensity (with IgG control values subtracted). At least 500 cells were analyzed to generate each histogram.

In some experiments designed to determine the effect of IgE cross-linking on surface expression of PSGL-1 and other adhesion molecules, basophils were preincubated with the FITC-conjugated polyclonal goat IgG anti-human IgE at subsaturating concentrations found to be optimal for histamine release, as previously reported (0.1 $\mu\text{g/mL}$, 45 minutes, 37°C in PAGCM buffer),¹⁴ before being subjected to the labeling protocol mentioned above. Histamine release, determined as the percentage of total release, was monitored in supernatants.³⁴

Basophil and KU812 cell adhesion

Basophil adherence was assessed by using the histamine content of perchloric acid lysates of adherent cells, as previously described.¹³ Briefly, 96-well plates were precoated with optimal concentrations of soluble P-selectin (5 $\mu\text{g/mL}$) or buffer alone overnight and then blocked with PBS and 1% BSA to reduce background adherence.²⁹ In some experiments mAbs S12 and G1 recognizing P-selectin were added to the appropriate wells and incubated at 4°C for 30 minutes. In other experiments basophil cell suspensions were preincubated with the PSGL-1 mAb PL1 and then

added to the appropriate wells and incubated for 30 minutes at 4°C. After rewarming to 37°C, basophils were added to the plates and allowed to adhere for 30 minutes. Next, the supernatants were harvested, centrifuged to remove cells, and saved for histamine analysis. Wells were then washed to remove any remaining nonadherent cells. Perchloric acid was then added to each well to lyse adherent basophils. The histamine content of supernatants and lysed adherent cells was quantitated as mentioned above. The percentage of histamine release and the percentage of basophil adherence were then calculated by comparison with the histamine content of separate aliquots of starting basophil suspension. Under these conditions, no histamine release during the adhesion assay was ever detected (data not shown). All experiments were performed in triplicate with a coefficient of variation of less than 15%. KU812 cell adherence was assessed exactly as for basophils, except that cells were labeled with chromium 51 and adhesion quantified by using a gamma counter using methods identical to those previously reported for purified eosinophils and neutrophils.²⁹

To determine the effect of adhesion on the kinetics and magnitude of basophil histamine release, separate experiments were performed in which cells were first allowed to adhere to P-selectin or BSA control-coated wells for 30 minutes at 37°C. Without rinsing, 10 μ L of additional buffer or buffer containing polyclonal goat anti-human IgE (final concentration, 1-1000 ng/mL; see text) was then added, and cells on the plate were allowed to incubate for up to an additional 45 minutes (see text). For these experiments, the histamine content of supernatants and lysed adherent cells were separately quantitated, as mentioned above, to monitor for effects on histamine release and to verify effective P-selectin adhesion, respectively.

Western blot analysis

Cells (10^6 basophils, eosinophils, or neutrophils; purity for each, >95%) were lysed for 5 minutes with lysis buffer (10 mmol/L TRIS [pH 7.4], 150 mmol/L NaCl, 5 mmol/L EDTA, 10% [vol/vol] glycerol, 1% Triton X-100, 1 mmol/L phenylmethylsulfonylfluoride, 10 mg/mL aprotinin, 1 mmol/L sodium orthovanadate, 1 mmol/L leupeptin, and 1 mmol/L pepstatin A) on ice. Insoluble cell debris was removed by centrifugation (14,000 rpm for 5 minutes). The lysates were boiled for 5 minutes in SDS sample buffer (10% SDS, 500 mmol/L TRIS [pH 6.8], 100 mmol/L β_2 -mercaptoethanol, 0.1% bromophenol blue, and 10% glycerol). To ensure equal protein loading, total protein was measured by using the BCA protein assay (Pierce, Rockford, Ill). Protein extracts (10 μ g per lane) were separated on a 6% SDS polyacrylamide minigel by electrophoresis for 1.5 hours at 100 V and then blotted for 1.5 hours with a Hoefer eletrophoretic transfer cell (Pharmacia Biotech Inc, San Francisco, Calif) to Trans-Blot Transfer Medium PVDF membranes (BIO-RAD, Hercules, Calif). Membranes were blocked in PBS plus 5% BSA and 0.1% Tween-20 for 1 hour at room temperature. The membranes were then incubated with 2 μ g/mL mAb PL1 in PBS and 0.1% Tween-20 for 1 hour at room temperature with continuous shaking. After two washes with PBS and 0.1% Tween (5 minutes at room temperature), membranes were incubated for 1 hour with the secondary antibody, a horseradish peroxidase-conjugated goat antimouse antibody (Amersham Corp, Arlington Heights, Ill). After a final washing step (3 times with PBS and 0.1% Tween for 10 minutes at room temperature), immunoreactive bands were visualized by using enhanced chemiluminescence (Amersham).

Statistical analysis

Values were presented as means $\pm$ SEM. Comparative analysis was achieved by using the unpaired two-tailed *t* test with statistical software (Statview; Abacus Concepts, Berkeley, Calif). A *P* value of .05 or less was considered statistically significant.

RESULTS

Expression of PSGL-1 on human basophils and KU812 cells compared with that on eosinophils and neutrophils

In initial experiments indirect immunofluorescence and flow cytometry were used to compare surface expression of PSGL-1 on basophils, eosinophils, neutrophils, and KU812 cells (Fig 1, *n* = 4). Levels of PSGL-1 were comparable on basophils (116 ± 10 net mean fluorescence) and neutrophils (113 ± 10.1 net mean fluorescence) but higher on eosinophils (158 ± 10 net mean fluorescence, *P* < .01). Levels of PSGL-1 on KU812 cells were much lower (10 ± 1 net mean fluorescence or $17\% \pm 5\%$ positive). Western blot analysis revealed two bands for basophils, eosinophils, and neutrophils, representing the monomer and incompletely dissociated homodimer. Both PSGL-1 bands for eosinophils were consistently greater in molecular mass than those for neutrophils and basophils (Fig 2 and data not shown, *n* = 4).

Regulation of PSGL-1 expression on basophils

To determine whether activation alters surface expression of PSGL-1, enriched basophils were incubated with or without 0.1 μ g/mL FITC anti-IgE in PAGCM buffer for 45 minutes at 37°C. Supernatants were harvested and analyzed for histamine release, and the cells were labeled and analyzed by using flow cytometry. Anti-IgE exposure caused histamine release ($81\% \pm 2.4\%$) and significantly decreased PSGL-1 expression (from an average mean fluorescence of 117 ± 5 to 86 ± 2 , *P* < .001, Fig 3). This treatment also reduced L-selectin expression (from an average mean fluorescence of 28 ± 4 to 8 ± 1 , *P* < .001) and increased CD11b expression (from an average mean fluorescence of 41 ± 5 to 53 ± 4 , *P* < .001) while having no effect on CD11a expression (Fig 3), which is consistent with data from a previous report.³⁵

PSGL-1 on basophils is a ligand for P-selectin

Static adhesion assays demonstrated that basophils bound well to P-selectin ($46.0\% \pm 2.3\%$ adhesion). In contrast, the KU812 cell line bound poorly ($5.7\% \pm 0.8\%$ adhesion, Fig 4), which is consistent with their markedly lower levels of PSGL-1 expression (Fig 1). Adhesion of basophils and KU812 cells to P-selectin by means of PSGL-1 was confirmed (Fig 4) by experiments in which adhesion was completely inhibited by blocking mAb to either P-selectin (G1) and PSGL-1 (PL1) but not by using nonblocking control mAb to P-selectin (S12) or CD11a as a control mAb (*n* = 1, data not shown).

Effect of adhesion to P-selectin on basophil releasability

To determine whether adhesion to P-selectin altered the magnitude or kinetics of histamine release, basophils were allowed to adhere to P-selectin or BSA control-coated wells before being exposed to various concentra-

tions of anti-IgE, and effects on histamine release were monitored at several time points. As shown in Fig 5, A and B, neither the magnitude nor the kinetics of histamine release was different between cells allowed to settle on BSA or P-selectin over a range of anti-IgE concentrations and time points ($n = 2$).

DISCUSSION

In the present studies the expression and function of PSGL-1 on human basophils were explored. Expression of PSGL-1 on basophils was found to be very similar to that on neutrophils with respect to surface levels and relative molecular weight, both of which were less than that seen on eosinophils. Levels on basophils were approximately 10-fold higher than on the basophilic cell line KU812. Activation of basophils by means of IgE cross-linking resulted in a statistically significant 30% reduction in cell surface expression of PSGL-1. Basophils adhered well to P-selectin and, consistent with the much lower levels of PSGL-1, KU812 cells adhered poorly. Levels of adhesion seen with basophils were similar to that reported previously for neutrophils and eosinophils. It has been suggested that there may be slightly greater attachment of eosinophils, perhaps because of their higher levels of PSGL-1,^{20,21,29,36} although not all studies support this notion.²² Basophil adhesion to P-selectin was completely blocked by antibodies to P-selectin or PSGL-1. Finally, adhesion to P-selectin did not affect the magnitude or kinetics of IgE-dependent histamine release. Taken together, these data suggest that like other leukocytes, PSGL-1 functions as a major if not exclusive ligand on human basophils for P-selectin.

The reduced surface expression of PSGL-1 seen after basophil activation was initially an unexpected finding, although additional studies with other cell types (eg, neutrophils, monocytes, and eosinophils) have yielded similar findings.³⁷ Before this, it was thought that cellular activation simply facilitated redistribution of PSGL-1 on the cell surface to microvilli,^{19,25,38} but more recent data suggest that activation and reduced P-selectin adhesion may be the result of loss of surface PSGL-1. Whether PSGL-1 shedding occurs from basophils *in vivo* during recruitment responses, including those after allergen challenge, as well as the identities of the enzymes involved in this phenomenon, have not yet been determined.

The molecular species present on basophils appear to be more similar to that found on the neutrophil compared with that found on the eosinophil. Given the fact that the predicted core polypeptide molecular weight for PSGL-1 monomer is only 38,608 d, the vast majority of its molecular mass is due to posttranslational glycosylation. Although the PSGL-1 carbohydrates on eosinophils, neutrophils, and basophils have not been directly compared, it is clear that the species on all 3 granulocyte subtypes mediate adhesion to P-selectin. It has been suggested that both carbohydrates (sialylated, fucosylated O-glycans) and specific protein epitopes containing sulphytyrosine (such as those recognized by mAb PL1) are functionally

Relative cell number

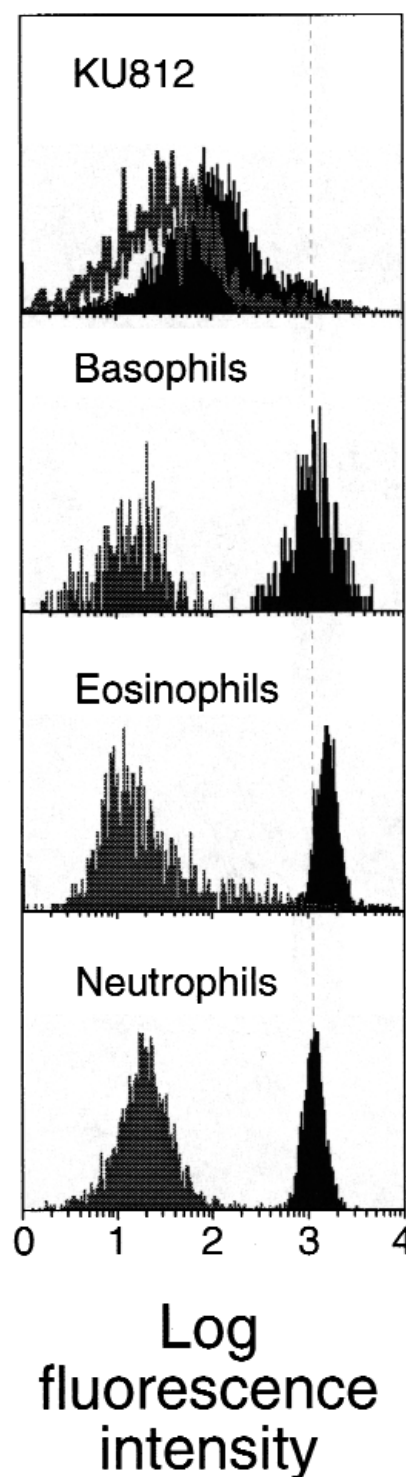


FIG 1. Surface expression of PSGL-1 on KU812 cells and human basophils, eosinophils, and neutrophils. IgG1 control labeling is shown in gray, and PSGL-1 labeling (with mAb PL1) is shown in black. The vertical dashed line was added to aid in comparing expression among cell types. Data are representative of 4 experiments with similar results.

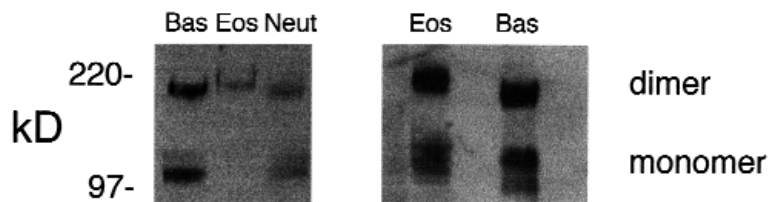


FIG 2. Western blot analysis of PSGL-1 from human basophils (*Bas*), eosinophils (*Eos*), and neutrophils (*Neut*). Shown are representative results from two separate experiments. In the *left panel* basophils, eosinophils, and neutrophils were isolated simultaneously from the same donor, and in the *right panel* basophils and eosinophils were isolated simultaneously from a different donor.

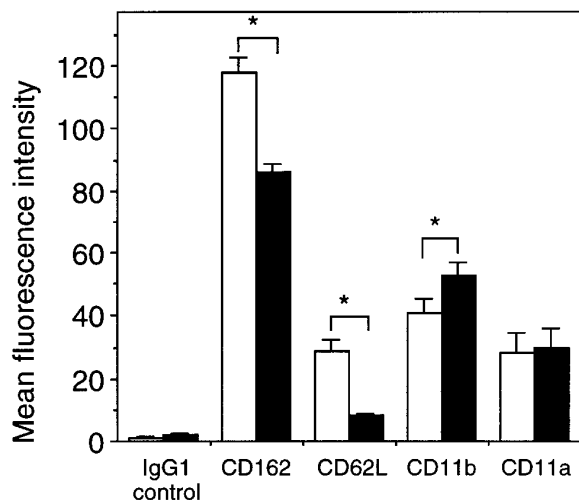


FIG 3. Effect of IgE cross-linking (0.1 μ g/mL for 45 minutes at 37°C) on basophil surface expression of various adhesion molecules. Shown are values for average mean fluorescence intensity $\pm$ SEM with an isotype-matched control mAb, as well as mAbs to CD162 (PSGL-1), CD62L (L-selectin), CD11b (Mac-1), and CD11a (LFA-1) after exposure to 0.1 μ g/mL anti-IgE (filled bars) or buffer alone (open bars, $n = 3$; * $P < .001$).

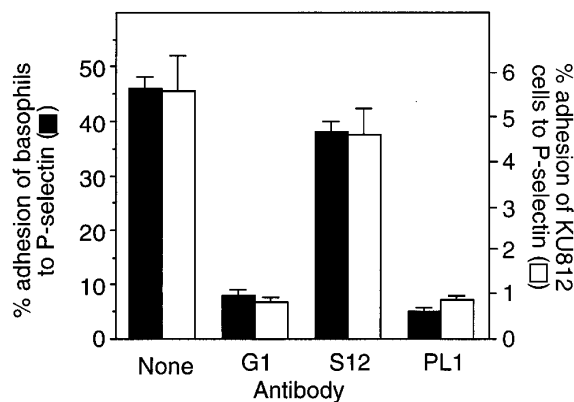


FIG 4. Adhesion of human basophils (filled bars) and KU812 cells (open bars) to immobilized P-selectin in the presence or absence of mAbs G1 (P-selectin blocking), S12 (P-selectin nonblocking), and PL1 (PSGL-1 blocking). Note the differences in scales on the two vertical axes. Data are means $\pm$ SEM for 3 separate experiments. Background adhesion to BSA-coated wells was less than 5% (data not shown).

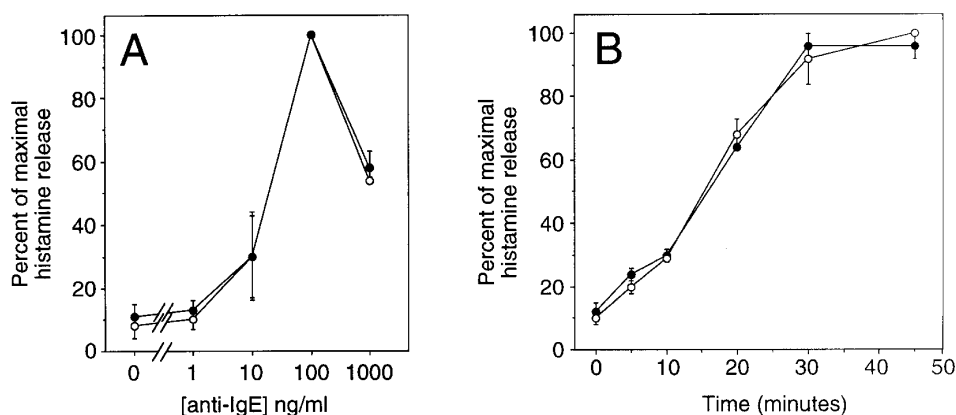


FIG 5. A, Effect of adhesion to BSA (open circles) or P-selectin (filled circles) on the magnitude of anti-IgE-induced basophil histamine release. In these experiments maximal histamine release at 0.1 μ g/mL averaged 84%, and adhesion to BSA and P-selectin, monitored in separate wells simultaneously, averaged 8% and 75%, respectively ($n = 2$). **B,** Effect of adhesion to BSA (open circles) or P-selectin (filled circles) on the kinetics of anti-IgE-induced (0.1 μ g/mL) basophil histamine release. After cells were allowed to adhere to BSA or P-selectin for 30 minutes, 0.1 μ g/mL anti-IgE was added, and then supernatants were harvested at the indicated time points. In these experiments maximal histamine release averaged 73%, and adhesion to BSA and P-selectin, monitored in separate wells simultaneously, averaged 20% and 74%, respectively ($n = 2$).

important.¹⁹ However, glycomimetics for selectin antagonism have already been used to show differences among eosinophils and neutrophils for inhibition of P-selectin adhesion,³⁶ and therefore there may be additional variations among forms of PSGL-1 on leukocytes that remain to be determined.

It has been suggested that ligation of PSGL-1 on neutrophils and monocytes can alter their function and cause tyrosine phosphorylation of several intracytoplasmic proteins.^{19,23,39} This suggests that PSGL-1 may serve as a signal-transducing molecule, mediating outside-in signaling. Other studies, especially those involving attachment by means of $\beta 1$ integrins, have shown that adhesion to matrix proteins can alter basophil and mast cell function.⁴⁰⁻⁴² We therefore examined whether adhesion by means of PSGL-1 on basophils was affected by histamine release responses. Although no effect on IgE-mediated histamine release was seen, it is still possible that release of other mediators, such as cytokines, or responses to other secretagogues, such as those acting by means of 7 spanner receptors, may be altered. However, our data suggest that adhesion to P-selectin does not directly contribute to basophil priming or alter releasability.

Finally, it is currently believed that the main function of PSGL-1 is to mediate interactions with P-selectin under conditions of blood flow and shear forces. Although the studies performed here were done under static conditions, many studies with other granulocyte types studied under both static and flow conditions have generated similar findings and conclusions.^{22,29,43,44} Given the difficulties in generating sufficient numbers of highly purified basophils, we have not as yet been able to study the function of selectins and selectin ligands using human basophils in vitro under flow conditions. Our results suggest that KU812 cells would not be reliable surrogates for these studies. Nevertheless, it appears highly likely that PSGL-1 is an important functional adhesion molecule on human basophils that can participate in the early events associated with basophil recruitment, such as that seen during the late phase after allergen challenge. P-selectin antagonists used in vivo⁴⁵ are therefore likely to have effects on basophil trafficking as well.

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