

Inhibition of adhesion of human neutrophils and eosinophils to P-selectin by the sialyl Lewis^x antagonist TBC1269: Preferential activity against neutrophil adhesion in vitro

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Background: Leukocyte rolling on vascular endothelium is mediated by selectins and their carbohydrate-containing counterligands. The tetrasaccharide sialyl Lewis^x (sLe^x) binds to all 3 selectins, so compounds that mimic sLe^x are potential antagonists.

Objective: Our purpose was to examine the ability of the sLe^x mimetic TBC1269 to inhibit binding of human neutrophils and eosinophils to P-selectin.

Methods: Expression of the primary P-selectin ligand, P-selectin glycoprotein ligand-1 (PSGL-1), was examined on neutrophils and eosinophils, and their adhesion to immobilized P-selectin was examined under both static and dynamic conditions in the presence and absence of TBC1269.

Results: Neutrophils and eosinophils expressed PSGL-1, with eosinophils expressing about twice as much as neutrophils. In the absence of TBC1269, both cell types adhered avidly to P-selectin under static and dynamic conditions. For neutrophils, preincubation of P-selectin-coated plates with TBC1269 (1 to 1000 µg/mL) resulted in concentration-dependent decreases in neutrophil adhesion, with significant inhibition seen at concentrations ≥100 µg/mL. Eosinophil adhesion to P-selectin was more refractory to inhibition by TBC1269 and was only partially inhibited at the highest concentration tested (1000 µg/mL). Two structurally related control compounds, TBC1900 and TBC746, had no effect when tested at similar concentrations.

Conclusion: These data indicate that an sLe^x mimetic can exhibit cell type-specific differences in potencies with respect to antagonism of P-selectin adhesion. Although this may in part be the result of differences in PSGL-1 expression, the discrepancy in potencies may also be due to other differences, including carbohydrate composition and binding affinity of PSGL-1. (*J Allergy Clin Immunol* 2000;105:769-75.)

Key words: Eosinophils, neutrophils, adhesion, P-selectin, PSGL-1, antagonist, glycomimetic, sialyl Lewis^x

Abbreviations used

IC ₅₀ :	Inhibitory concentration of 50%
ICAM:	Intercellular adhesion molecule
MFI:	Mean fluorescence intensity
PSGL-1:	P-selectin glycoprotein ligand-1 (CD162)
s-di-Le ^x :	Sialyl dimeric-Lewis ^x
sLe ^x :	Sialyl Lewis ^x

The movement of leukocytes out of the vasculature into surrounding tissues is crucial to the development of an inflammatory response. Selectively recruited leukocytes secrete inflammatory mediators and assist in the destruction of foreign antigens, as well as the remodeling of injured tissue. Excessive extravasation and activation of leukocytes at sites of inflammation can also be directly injurious to tissues. In these situations, measures that can successfully prevent leukocyte recruitment are likely to have therapeutic value. A primary molecular target for prevention of extravascular leukocyte accumulation has been the adhesion molecules that mediate leukocyte interactions with vascular endothelium and extracellular matrix proteins.¹⁻³

Three families of adhesion molecules (selectins, integrins, and Igs) mediate the multistep process leading to leukocyte emigration.⁴ The first of these families, the selectins, consists of 3 members, L-selectin, P-selectin, and E-selectin, each of which contributes to the earliest phase of leukocyte-endothelial interaction, leukocyte rolling.⁵⁻⁷ Integrins are heterodimers consisting of an α and a β subunit, which interact with structures belonging to the Ig superfamily of adhesion molecules, such as intercellular adhesion molecule (ICAM)-1 and ICAM-2 and vascular cell adhesion molecule-1 to mediate firm adhesion of leukocytes to the endothelial cell surface and migration of leukocytes across the endothelial barrier into the surrounding tissues.⁴

Function-blocking mAbs directed against members of all 3 families of adhesion molecules have been demonstrated to inhibit tissue accumulation of leukocytes, and thus tissue injury, in various inflammatory models.^{2,3,7} Although integrin and Ig superfamily blockade can inhibit leukocyte recruitment, the selectin family of adhesion molecules represents a different type of target for the development of therapeutic agents. Unlike integrin-integrin-ligand interactions, which involve sequence-specific

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protein-protein interactions, the interactions between the selectins and their ligands are carbohydrate based. The counterligands for the selectins share the common feature of being sialylated, fucosylated mucin-like structures⁷⁻⁹ and, although the protein or lipid backbone that present these common carbohydrate structures dictate distinct, high-affinity ligands for each of the selectins, there is substantial overlap between carbohydrate motifs. For example, the tetrasaccharide sialyl Lewis^x (sLe^x), containing α 2,3-linked terminal sialic acid residues and α 1,3-linked fucose, has been demonstrated to bind with low affinity to all 3 selectins.^{6,9,10} The ability of the selectins to bind common carbohydrate moieties allows for the potential development of pan-selectin antagonists, small carbohydrate or noncarbohydrate structures that could block the action of all 3 selectins simultaneously. Indeed, carbohydrate- and non-carbohydrate-based selectin antagonists have been demonstrated to inhibit leukocyte adhesion in vitro and leukocyte recruitment to sites of inflammation in vivo.¹¹⁻¹⁴

The purpose of the studies described here was to examine the ability of a low-molecular-weight, nonoligosaccharide sLe^x antagonist, TBC1269 (Fig 1, molecular weight 862.94),^{15,16} to inhibit human neutrophil and eosinophil adhesion to immobilized P-selectin under static and dynamic conditions in vitro. Human eosinophils and neutrophils both express the high-affinity P-selectin ligand P-selectin glycoprotein ligand-1 (PSGL-1) and have been reported previously to bind avidly to P-selectin under both static and dynamic conditions.¹⁷⁻²⁰ Although sLe^x is incorporated into the PSGL-1 structure, it accounts for less than 1% of the total neutrophil expressed sLe^x, and millimolar concentrations of sLe^x are required to antagonize PSGL-1 interactions with P-selectin.^{21,22} We were therefore interested in determining whether TBC1269, a s-di-Le^x mimetic known to block binding to the lectin domain of all 3 selectins but with greatest potency against P-selectin,¹⁶ could inhibit PSGL-1-mediated binding of neutrophils and eosinophils to immobilized P-selectin. We hypothesized that adhesion of both cell types to P-selectin would be equally inhibited by TBC1269 and that these potencies would be similar to those observed in non-cell-based ligand-binding studies. However, TBC1269 was much more effective at blocking neutrophil adhesion to P-selectin under both static and dynamic conditions.

METHODS

Granulocyte purification

Human neutrophils were isolated from the venous blood of human volunteers by Percoll density gradient centrifugation followed by hypotonic lysis of erythrocytes.²³ Eosinophils were further purified by immunomagnetic bead removal of CD16-positive cells (neutrophils) with use of a CD16 mAb (3G8, generously provided by Dr Jay Unkeless, Mt Sinai School of Medicine, New York, NY) and goat antimouse IgG-coated Dynabeads (Dynal, Great Neck, NY).²⁴ Cell purity, routinely >95%, was determined by light microscopic analysis of Diff-Quik-stained cytocentrifugation preparations. Viability, routinely >95%, was determined by erythrosin B dye exclusion.

Flow cytometric analysis

Flow cytometry was used to quantitate expression of PSGL-1 on granulocyte surfaces with use of methods previously described.²³ The PSGL-1-binding, function-blocking mAb PL-1 (IgG1, Coulter-Immunotech, Miami, Fla) and the non-function-blocking mAb PL-2 (IgG1, Coulter-Immunotech) were used to label cells in PBS containing 2 mg/mL BSA and 4 mg/mL human IgG. Binding of the mAb was detected with use of a saturating concentration of FITC-conjugated goat antimouse IgG/IgM polyclonal antiserum (Coulter-Immunotech) and compared with labeling with an irrelevant IgG1 control mouse mAb. Labeled cells were analyzed with an EPICS Profile II flow cytometer (Coulter) with excitation at 488 nm. The mean fluorescence intensity (MFI) for IgG1 was subtracted from the MFI for PSGL-1 to derive the net MFI.

Granulocyte adhesion to P-selectin-coated microwells under static conditions

Recombinant soluble P-selectin was purchased from R&D Biosystems (Minneapolis, Minn). Ninety-six-well microtiter plates (Nunc Maxi Sorb) were coated for 16 to 20 hours at 4°C with 50 μ L per well of PBS (containing 1 mmol/L Ca⁺⁺ and Mg⁺⁺, PBS) alone or containing 5 μ g/mL recombinant P-selectin.¹⁷ Subsequently, wells were washed and blocked for at least 2 hours with PBS containing 10 mg/mL BSA and then washed with PBS. Wells were then incubated with PBS alone (90 μ L), the anti-P-selectin blocking mAb G1 (F[ab']₂, 25 μ g/mL in 90 μ L PBS), TBC1269 (1-1000 μ g/mL in 90 μ L of PBS), or 2 structurally related control compounds (TBC1900 and TBC746, Fig 1) for 30 minutes at 37°C.¹⁷ Granulocytes were labeled by incubation with chromium 51-labeled sodium chromate for 30 minutes at 37°C. After washing and resuspension in PBS, cells (2.5×10^5 in 10 μ L) were added in triplicate to the BSA control or P-selectin-adsorbed wells and incubated under static conditions for 30 minutes at 4°C. Nonadherent cells were removed by aspiration and washing, and adherent cells were lysed with 1 mol/L ammonium hydroxide for 20 minutes at ambient temperature. Lysates were transferred to test tubes and radiolabel determined by γ -radiation counter. Percent adhesion was determined by comparing radioactivity with that of separate 10 μ L aliquots of ⁵¹Cr-labeled cells.

Cell adhesion and rolling under physiologic flow

Rolling adhesion under physiologic flow was determined essentially as described previously.²⁰ The assembled parallel plate flow assay system consisted of (1) a Plexiglas acrylic plastic (Rohm and Haas, Philadelphia) flow chamber (Glycotech, Rockville, Md) with inlet/outlet ports, vacuum line, and silicone gasket, (2) a Nikon TMS-F inverted-phase contrast microscope with video capacity (Image Systems, Columbia, Md), (3) a high-resolution CCD camera (Hamamatsu, Japan), (4) a black-and-white high-resolution monitor, (5) a videocassette recorder (Sony Corp of America, Park Ridge, NJ), and (6) a Harvard infuse/withdrawal syringe pump (Harvard Apparatus, South Natick, Mass). Before assemblage, the flow chamber was filled with media and all air removed from the system. The flow chamber was then inverted with the gasket in place and media placed on the flow path. The 35-mm plate (ie, coated with immobilized P-selectin, see below) was then placed on top of the chamber and a vacuum created. Once assembled, the chamber and plate were placed upright on the microscope stage and the flow of cells initiated by the syringe pump attached to the outlet port, so that cells were drawn through the chamber at a constant flow through the chamber.

To coat the plate, a demarcated area on the 35-mm polystyrene petri dish was preadsorbed with P-selectin (5 μ g/mL) and blocked

with BSA as described for the static adhesion assay. The demarcated area was then pretreated with the anti-P-selectin mAb G1 (25 $\mu\text{g}/\text{mL}$) or TBC1269 (10–1000 $\mu\text{g}/\text{mL}$) in 75 μL of PBS for 30 minutes at 37°C. In some experiments TBC1269, at the same concentration as was used to pretreat the plate, was also added to the cell suspension before flow was initiated. No additional inhibitory effect of TBC1269 was seen when it was added to the cell suspension, and thus all the data with and without TBC1269 added to the cell suspension have been combined. In some experiments isolated granulocytes were also incubated with the PSGL-1 function-blocking mAb PL-1 or the non-function-blocking mAb PL-2 in PBS-BSA for 30 minutes at 4°C. Cell rolling and adhesion were determined at a shear force of 1 dyn/cm^2 . Video recordings of 1 to 2 minutes' duration were made in 10 different fields, and leukocyte interactions were quantified offline by video playback. The total number of interacting cells (rolling and adherent) were counted per field and the data were expressed as number of cells per field. Within each experiment all conditions were examined in duplicate or triplicate.

Data analysis

All data are presented as the mean \pm SEM. Data were compared by ANOVA with use of post-hoc analysis with the Fisher corrected *t* test. Probabilities of 0.05 or less were considered statistically significant.

RESULTS

Expression of PSGL-1 on human neutrophils and eosinophils

Surface expression of PSGL-1 was examined on isolated human neutrophils and eosinophils by indirect immunofluorescence. Both cell types were found to have significant levels of PSGL-1 on their surface. Eosinophils had almost twice as much PSGL-1 ($P < .01$) than neutrophils (Fig 2). Fig 2, A, shows representative histograms for neutrophil and eosinophil staining with mAb PL-1, which binds the P-selectin-binding site on PSGL-1, and with the isotype-matched control IgG1. Eosinophils consistently showed brighter staining with mAb PL-1, as well as other PSGL-1-binding mAb (mAb PL-2, data not shown), confirming previous observations.^{19,20} The net MFI (MFI for PSGL-1 over IgG1) for neutrophils was 44.3 ± 2.0 ($n = 7$), whereas the net MFI for eosinophils was 78.5 ± 5.5 ($n = 8$) (Fig 2, B).

Effects of TBC1269 on static adhesion of neutrophils and eosinophils to P-selectin

The ability of the selectin antagonist TBC1269 to block neutrophil and eosinophil adhesion to immobilized P-selectin under static conditions was examined. Under control conditions, neutrophils and eosinophils both adhered avidly to immobilized P-selectin (Fig 3), whereas adhesion to immobilized BSA was less than 5% (data not shown). Consistent with the expression of PSGL-1, adhesion of eosinophils to P-selectin was significantly ($P < .03$) greater than that of neutrophils ($63\% \pm 4\%$ adhesion, $n = 5$, vs $50\% \pm 3\%$ adhesion, $n = 4$, respectively). Preincubation of P-selectin-coated wells with the P-selectin function-blocking mAb G1 significantly inhibited adhesion of neutrophils ($86\% \pm 7\%$ inhibition) and eosinophils ($80\% \pm 6\%$ inhibition) to P-selectin. Prein-

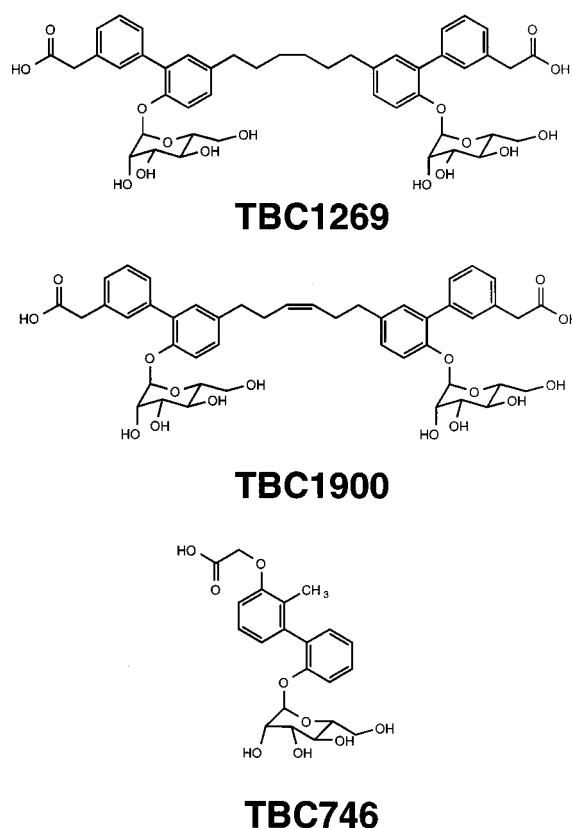


FIG 1. Structures of TBC1269, TBC1900, and TBC746. TBC1269 is an sLe^x mimetic with structural similarities to sialyl dimeric-Lewis^x (s-di-Le^x) (molecular weight 862.94). TBC1900 differs from TBC1269 in the 6-carbon chain that joins the 2 biphenyl units together. In TBC1900 this linker contains a *cis* double bond, which causes 4 of the 6 carbon atoms to lie in the same plane. With a restriction of this sort in the linker, the 2 biphenyl units are limited in the degrees of freedom available, and it is thus thought unable to adopt an optimum "bound conformation." TBC746 has an additional methyl group on the "A" ring, and it was designed to have a limited rotation about the bond that joins the 2 aromatic rings. This restriction is thought to affect the "presentation" of the carboxylic acid to the binding site. Both TBC1900 and TBC746 are inactive in both in vitro and in vivo models involving selectin antagonism (data not shown).

cubation of P-selectin-coated wells with TBC1269 (1 to 1000 $\mu\text{g}/\text{mL}$) resulted in a concentration-dependent decrease in neutrophil adhesion to P-selectin (Fig 3), with an inhibitory concentration of 50% (IC_{50}) of ~ 40 $\mu\text{g}/\text{mL}$. Interestingly, TBC1269 was much less effective in inhibiting eosinophil adhesion to P-selectin over the concentration range examined (Fig 3). Although the highest concentration (1000 $\mu\text{g}/\text{mL}$) of TBC1269 did significantly inhibit adhesion of eosinophils to P-selectin ($\sim 35\%$), an IC_{50} could not be determined. Similar testing with up to 1000 $\mu\text{g}/\text{mL}$ of the control compounds TBC1900 and TBC746 showed no effect on neutrophil or eosinophil adhesion (data not shown). Because we recently reported that granulocyte activation can result in PSGL-1 shedding, we determined whether the differences in potency might be the result of preferential

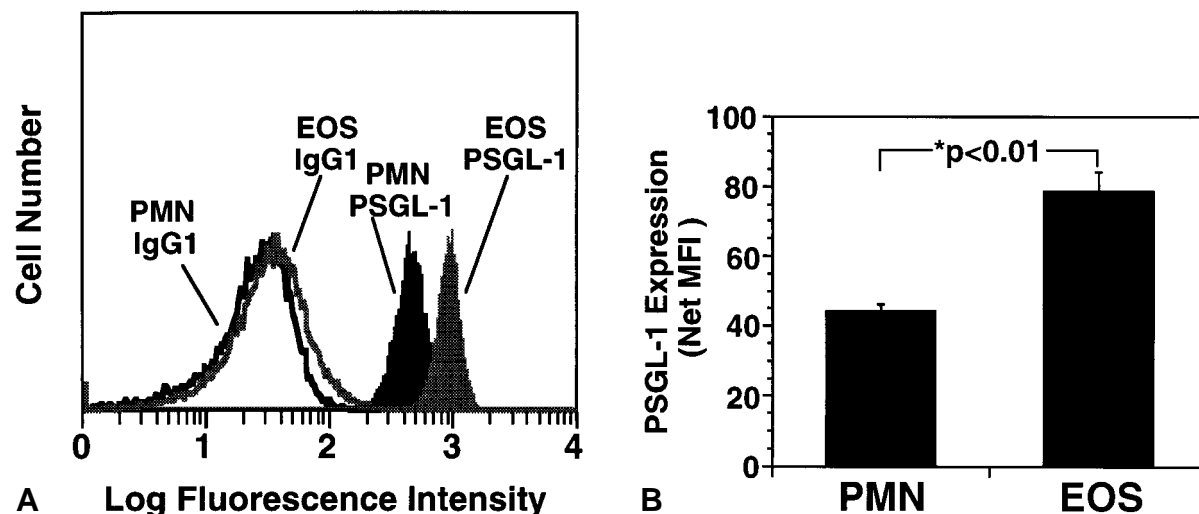


FIG 2. Surface expression of PSGL-1 on human neutrophils and eosinophils. Expression of PSGL-1 was examined on isolated human neutrophils (PMN, $n = 7$) and eosinophils (EOS, $n = 8$) by indirect immunofluorescence. **A**, Representative histograms of neutrophil and eosinophil staining for PSGL-1 (mAb PL-1) and IgG1. Eosinophils were consistently found to have greater expression of PSGL-1 than neutrophils. **B**, Eosinophils express significantly (*asterisk*, $P < .01$) more PSGL-1 than neutrophils.

PSGL-1 shedding from the neutrophil. However, incubation of cells with up to 1000 $\mu\text{g/mL}$ of TBC1269 under identical conditions as in Fig 3 had no effect on PSGL-1 expression as assessed by indirect immunofluorescence and flow cytometry (data not shown).

Effects of TBC1269 on dynamic adhesion of neutrophils and eosinophils to P-selectin

The ability of TBC1269 to block neutrophil and eosinophil adhesion to immobilized P-selectin under conditions of flow at 1 dyn/cm^2 was examined in the parallel plate adhesion assay system. Under flow conditions, both neutrophils and eosinophils rolled on, and adhered to, immobilized P-selectin (Fig 4). Unlike in the static adhesion assay, values for eosinophil adhesion to P-selectin (67 ± 8 cells/field) were not significantly different from neutrophil adhesion values (81 ± 4 cells/field). Preincubation of neutrophils and eosinophils with the PSGL-1 function-blocking mAb PL-1 inhibited adhesion of neutrophils ($90\% \pm 9\%$ inhibition, $n = 3$) and eosinophils ($98\% \pm 0.3\%$ inhibition, $n = 2$) to P-selectin. Preincubation of P-selectin-coated plates with TBC1269 (10 to 1000 $\mu\text{g/mL}$) with or without inclusion of an identical concentration in the buffer along with the perfused cells resulted in decreases in neutrophil adhesion to P-selectin, although compared with the static assay 2- to 3-fold higher concentrations of TBC1269 were required to achieve effective 50% inhibition of neutrophil adhesion. Significant inhibition of neutrophil rolling and adhesion was seen at 100 and 1000 $\mu\text{g/mL}$ of TBC1269. Similar to what was observed in the static adhesion assay system, effects of TBC1269 on eosinophil adhesion were not observed until concentrations of 1000 $\mu\text{g/mL}$ were used.

Correlation between PSGL-1 expression and the effect of TBC1269 on adhesion

To examine whether the reduced potency of TBC1269 in blocking eosinophil adhesion to P-selectin compared with the neutrophil was related to the increased expression of PSGL-1 on the eosinophil surface, the correlation between the net MFI for PSGL-1 expression and the percent inhibition of static and flow adhesion with 100 $\mu\text{g/mL}$ TBC1269 was examined. As seen in Fig 5, there was a significant ($r = 0.59$, $P < .02$) correlation between the amount of PSGL-1 expressed on the cell surface and the percent of inhibition of adhesion achieved with 100 $\mu\text{g/mL}$ of TBC1269 when both were measured on the same cells. Neutrophils had a range of net MFI for PSGL-1 between 33 and 51 and a range of inhibition between 19% and 79%. However, eosinophils had a much higher range of PSGL-1 expression (net MFI 62-111) and a much lower range for inhibition by TBC1269 ($\leq 24\%$ inhibition).

DISCUSSION

Although much is known about the expression and function of the various leukocyte and endothelial adhesion molecules,^{4,25,26} less is known about how these molecules mediate selective recruitment of leukocyte subtypes during inflammation. For instance, despite the fact that neutrophils and eosinophils use both selectins and $\beta 2$ integrins to interact with the vascular endothelium, eosinophils are selectively recruited to skin and airways in allergic disease.^{27,28} Understanding the mechanisms of selective recruitment seems crucial to the development of effective therapeutic agents that can, in turn, selectively inhibit these processes.

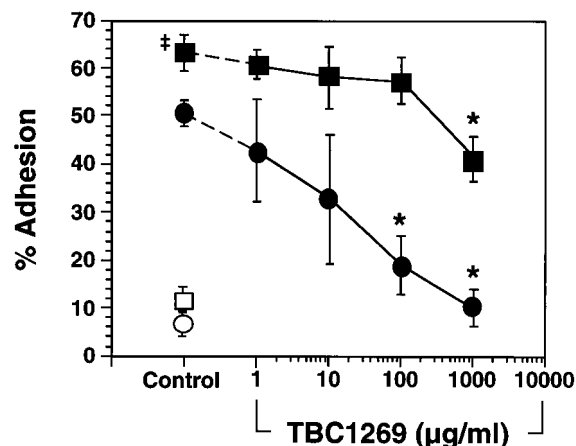


FIG 3. Effects of TBC1269 on static adhesion of neutrophil and eosinophil P-selectin. Neutrophils (closed circles, $n = 4$) and eosinophils (closed squares, $n = 5$) both adhered avidly to immobilized P-selectin. Consistent with the relative expression of PSGL-1, the adhesion of eosinophils to P-selectin was significantly (*double dagger*, $P < .03$) greater than that of neutrophils. Both neutrophil (open circles) and eosinophil (closed circles) adhesion was effectively inhibited by the P-selectin-blocking mAb G1. Preincubation of P-selectin-coated wells with TBC1269 (1 to 1000 µg/mL) resulted in a concentration-dependent decrease in neutrophil adhesion that was significantly (*asterisk*, $P < .05$) decreased with 100 and 1000 µg/mL TBC1269. TBC1269 was not as effective at inhibiting eosinophil adhesion to P-selectin in the concentration range examined, although the highest concentration (1000 µg/mL) of TBC1269 did modestly but significantly (*asterisk*, $P < .05$) inhibit adhesion.

Here we demonstrate that the sLe^x glycomimetic TBC1269 preferentially inhibits neutrophil adhesion to immobilized P-selectin, compared with the eosinophil, under static and dynamic conditions. As previously reported, neutrophils and eosinophils both avidly adhered to immobilized P-selectin, with eosinophil adhesion under static conditions being significantly greater than neutrophil adhesion.^{17,19,20} Under static and dynamic conditions preincubation of P-selectin-coated wells or leukocytes with the anti-P-selectin or anti-PSGL-1 mAb, respectively, completely blocked both neutrophil and eosinophil adhesion to P-selectin. Conversely, preincubation of P-selectin-coated wells with TBC1269 (1 to 1000 µg/mL) resulted in concentration-dependent decreases in neutrophil adhesion to P-selectin ($IC_{50} \sim 40$ µg/mL or ~ 46 µmol/L), whereas this compound showed markedly lower potency in inhibiting eosinophil adhesion to P-selectin over the same concentration range. In fact, the only concentration found to inhibit eosinophil adhesion was the highest concentration tested (1000 µg/mL or ~ 115 mmol/L) and even at this concentration inhibition was minimal. Higher concentrations were not tested, because recent data in animals suggest that higher peak serum levels cannot be readily achieved.²⁹ These data indicate differences in neutrophil and eosinophil interaction with P-selectin, which may significantly affect the ability of sLe^x mimetics to simultaneously inhibit interaction of both cell types with P-selectin.

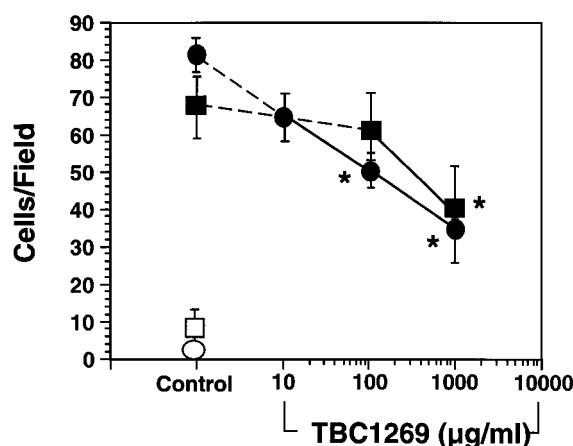


FIG 4. Effects of TBC1269 on dynamic adhesion of neutrophil and eosinophil P-selectin. Both neutrophils (closed circles, $n = 5$) and eosinophils (closed squares, $n = 3$) rolled on and adhered to immobilized P-selectin. Neutrophil (open circles) and eosinophil (open squares) adhesion was effectively inhibited by PSGL-1-blocking mAb PL-1. Significant (*asterisk*, $P < .05$) inhibition was seen with 100 and 1000 µg/mL for neutrophils and 1000 µg/mL for eosinophils.

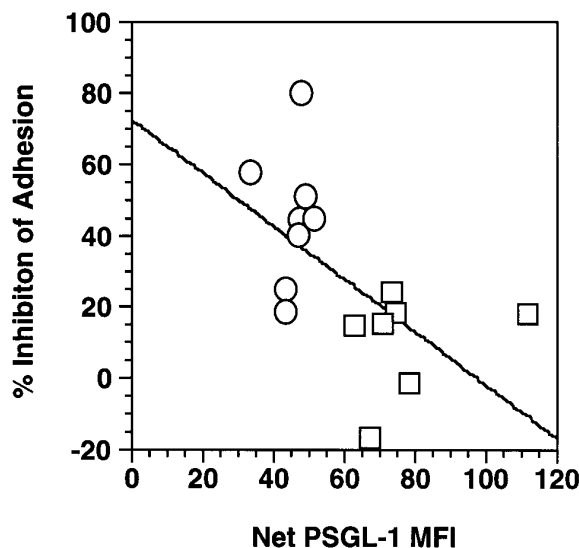


FIG 5. Correlation between PSGL-1 expression and effect of TBC1269 on neutrophil (open circles) and eosinophil (open squares) static and flow adhesion to P-selectin. There was a significant ($r = 0.57$, $P < .03$) correlation between amount of PSGL-1 expressed on cell surface and percent inhibition of adhesion achieved with 100 µg/mL TBC1269.

The glycomimetic TBC1269 is a low-molecular-weight nonoligosaccharide sLe^x antagonist that has been demonstrated to block ligand binding to all 3 selectins.¹⁶ TBC1269 was derived by modification of a biphenyl-based inhibitor to incorporate a mimetic of (s-di-Le^x), a construct that has been demonstrated to be a more effective selectin antagonist than sLe^x alone. This compound comes from a class of compounds in which mannose and carboxylic acid have been used to replace fucose and

sialic acid groups of sLe^x, respectively, and a biphenyl group has been used to replace the lactosamine core.¹⁶ To mimic the s-di-Le^x construct, there are 2 mannose and 2 carboxylic acid groups. In vitro, TBC1269 blocks the interaction sLe^x-expressing HL-60 cells with E-, P-, and L-selectin, with the greatest potency in blocking P-selectin interactions (IC₅₀ ~17 μmol/L).¹⁶ In vivo TBC1269 has been demonstrated to be protective in a rat model of liver ischemia-reperfusion.¹¹ In this model administration of TBC1269 15 minutes before reperfusion significantly decreased neutrophil infiltration into the liver after reperfusion and increased survival time. More recently, TBC1269 has been demonstrated to block leukocyte recruitment to the lung in a sheep model of allergic asthma.²⁹ In this model the neutrophil is the major infiltrating cell, but TBC1269 was also effective in inhibiting eosinophil recruitment to the lung in this model. Our data indicate that inhibition of the eosinophil-P-selectin interaction in the sheep model may not be the predominant mechanism for this effect.

Studies from our laboratories and others have shown that eosinophils use many of the same endothelial adhesion molecules as neutrophils (ie, P-selectin, E-selectin, an L-selectin ligand, ICAM-1) during the recruitment process, but important differences have been found to exist between eosinophil and neutrophil interactions with these molecules.^{1,7,30} For example, eosinophils have been demonstrated to adhere more avidly to P-selectin than neutrophils do.^{17,18} This enhanced adhesivity of eosinophils to P-selectin is thought to be the result of a higher surface expression of PSGL-1, the primary leukocyte counterligand for P-selectin, on eosinophils.¹⁸ First identified as a specific P-selectin ligand, PSGL-1, which is a disulfide-bonded homodimer with two 120-kd subunits, is now known to bind all 3 selectins, but with the highest affinity for P-selectin.^{21,31,32} PSGL-1 has been demonstrated to be the essential ligand for mediating leukocyte rolling on, and adhesion to, P-selectin,²² and as demonstrated here, mAb blockade of PSGL-1 function can completely inhibit neutrophil and eosinophil adhesion to P-selectin (Fig 4). The data presented in Figs 2 and 3 confirm previous findings that eosinophils express higher levels of PSGL-1 than neutrophils and under static conditions, and adhere as well as, if not better than, neutrophils.^{17,19} As shown in Fig 5, the differences in PSGL-1 expression may, at least in part, account for the differences in adhesion blockade seen with TBC1269 because there was a significant correlation between PSGL-1 expression and the percent inhibition of leukocyte adhesion with TBC1269.

Although differences in PSGL-1 expression on eosinophils and neutrophils may account for some of the differences observed in adhesion blockade with TBC1269, it seems unlikely that this is the only explanation because the magnitude of these differences is so dissimilar. For instance, there was a less than 2-fold difference in net MFI for PSGL-1 and adhesion to P-selectin for neutrophils and eosinophils, whereas there was an almost 3 log difference between these cells in the amount

of TBC1269 required to inhibit adhesion to P-selectin. Another possible explanation for the differences in the ability TBC1269 to inhibit neutrophil and eosinophil adhesion to P-selectin may be related to the differences in expression of sLe^x or other sialylated structures on the 2 cell types.³¹ We have previously examined the expression of sLe^x, as well as s-di-Le^x, on both neutrophils and eosinophils by indirect immunofluorescence.²³ Studies using a primary antibody that binds sLe^x revealed that expression of sLe^x on eosinophils was 10-fold lower than expression on neutrophils, whereas expression of s-di-Le^x was only one third that seen on neutrophils. These differences in sLe^x and s-di-Le^x expression, we believe, account for the much lower adhesion of human eosinophils to E-selectin compared with human neutrophils. Because TBC1269 was developed as an sLe^x antagonist, and in particular to mimic s-di-Le^x, our finding that TBC1269 was not as effective an inhibitor of eosinophil adhesion to P-selectin may indicate that some other adhesion-related factor, such as avidity, may be greater for eosinophil adhesion to P-selectin or less susceptible to antagonism by TBC1269. Because PSGL-1-associated sLe^x has been shown to account for less than 1% of the total neutrophil-expressed sLe^x,^{22,31} these data may also indicate that different carbohydrates on PSGL-1 are playing a role in neutrophil versus eosinophil adhesion to P-selectin. Neutrophils and eosinophils have PSGL-1 with differing molecular weights,^{19,20} so even if the core protein structures were identical, differences in glycosylation by these cell types may result in varying adhesive interactions.

In summary, we have examined the ability of the nonligosaccharide sLe^x mimetic TBC1269 to inhibit human eosinophil and neutrophil adhesion to P-selectin. In both static and flow adhesion assay systems, TBC1269 was a much more potent inhibitor of neutrophil adhesion to P-selectin compared with the eosinophil. The most striking implication of the study is that the potency of adhesion molecule antagonists may be markedly different depending on the cell type being studied. This could have a major impact on the range of diseases for which such antagonists would be expected to have efficacy. Whether the observed differences in *in vitro* potency, seen in the P-selectin and perhaps other selectin-based assays, will truly be predictive of efficacy *in vivo* remains to be seen.

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