

## Regulation of human basophil adhesion to endothelium under flow conditions: Different very late antigen 4 regulation on umbilical cord blood-derived and peripheral blood basophils

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### **Abstract:**

**Background:** Although soluble mediators released by basophils in tissue sites contribute to the chronic injury that occurs in hypersensitivity diseases, only limited information is available about how circulating basophils are recruited to tissues. In particular, the interaction of basophils with endothelium under conditions that mimic physiologic flow has not been explored.

**Objective:** We sought to identify adhesion molecules regulating the attachment of human basophils to IL-4-activated human umbilical vein endothelial cells (HUVECs) under flow conditions. **Methods:** A parallel-plate flow chamber and blocking mAbs were used to define the adhesion molecules involved in the interactions of peripheral blood basophils (PBBs) and cord blood-derived basophils (CBDBs) with IL-4-activated HUVECs and with Chinese hamster ovary (CHO) cell transfecants expressing specific adhesion molecules. A fluorescent ligand specific for very late antigen 4 (VLA-4) was used to directly examine the VLA-4 affinity state of basophils. **Results:** Flowing PBBs and CBDBs attached to activated HUVECs and to CHO cells expressing P- or E-selectin. However, only CBDBs attached to vascular cell adhesion molecule 1 (VCAM-1)-transfected CHO cells under flow conditions. The attachment of CBDBs to CHO cells was blocked by mAbs directed against E-selectin, P-selectin, and VCAM-1, whereas attachment of PBBs was blocked by E-selectin and P-selectin mAbs. Activating VLA-4 with Mn<sup>2+</sup> on PBBs resulted in adhesion to the VCAM-1-transfected CHO cells, indicating that VLA-4 activity on PBBs can be regulated, at least in part, through affinity changes. The Mn<sup>2+</sup>-induced upregulation of basophil VLA-4 affinity was demonstrated directly by using a fluorescent ligand for VLA-4 and flow cytometry. **Conclusions:** The interaction of human CBDBs and PBBs with endothelium under flow conditions is mediated in part by both P- and E-selectin. VLA-4 additionally contributes to the adhesion of flowing CBDBs. However, the

affinity of VLA-4 is too low to support the adhesion under flow conditions of unstimulated PBBs.

**Keywords:** Mast cells | basophils | adhesion molecules | cell trafficking | allergy | inflammation

**Article:**

**Abbreviations**

CBDB: Coed blood-derived basophil

CHO: Chinese hamster ovary

HUVEC: Human umbilical vein endothelial cell

PBB: Peripheral blood basophil

PSGL-1: P-selectin glycoprotein ligand 1

VCAM-1: Vascular cell adhesion molecule 1

VLA-4: Very late antigen 4

Leukocytes interact with endothelium and extravasate into tissue through a cascade of molecular events that include (1) attachment and rolling on activated vascular endothelium, (2) activation by chemokines or other chemoattractants, and (3) firm arrest and transmigration through endothelium.<sup>1, 2, 3, 4</sup> Monocytes, lymphocytes, neutrophils, and eosinophils each use a different combination of selectins and integrins to perform these sequential adhesion events. E- and P-selectin are typically expressed on endothelium, whereas L-selectin and the  $\alpha 4\beta 1$  integrin very late antigen 4 (VLA-4) are typically expressed on leukocytes.<sup>3, 4</sup> Tetrasaccharide moieties on leukocyte glycoproteins (eg, P-selectin glycoprotein ligand 1 [PSGL-1] and cutaneous leukocyte antigen) might serve as ligands for endothelial cell selectins.<sup>5</sup> VLA-4 binds to vascular cell adhesion molecule 1 (VCAM-1), the expression of which might be induced on endothelium in response to specific cytokines.<sup>3</sup> After rolling, arrest, and firm adhesion, subsequent transmigration depends on  $\beta 1$  or  $\beta 2$  integrins.<sup>1, 2, 3, 4, 5</sup>

Allergic reactions in the skin and airways are often characterized by the infiltration of peripheral blood basophils (PBBs) during the chronic inflammation phase (the late phase), which occurs between 4 and 48 hours after allergen exposure.<sup>6</sup> Previous static assays have implicated E-selectin, P-selectin, and VLA-4 in the interaction of basophils with endothelium. However, the adhesive receptors that allow basophils to attach and roll under physiologic flow conditions have not been defined, in part because the parallel-plate flow chamber used commonly to make these measurements requires higher cell numbers than can be reasonably obtained from peripheral blood.

We have now produced a miniaturized parallel-plate flow chamber<sup>7</sup> that makes adhesion analyses with flowing PBBs feasible. In addition, techniques have been developed for differentiating basophils from umbilical cord blood; immunophenotyping data revealed a repertoire of adhesion receptors on the surface of these cord blood-derived basophils (CBDBs) that was very similar to that of PBBs.<sup>8</sup> In the studies presented here, we use the well-defined flow field that exists in a parallel-plate flow chamber to determine which adhesion receptors mediate the attachment of PBBs and CBDBs to endothelium activated with IL-4, a key cytokine in allergic disease.

## Methods

### Reagents and mAbs

Anti-Fc $\epsilon$ RI- $\alpha$  subunit mAb 22E7 (IgG1)<sup>9</sup> was a gift from Dr J. Kochan (Hoffman-La Roche, Nutley, NJ). Neutralizing mAbs directed against human E-selectin (IgG1, 7A9) and P-selectin (IgG1, WAPS12.2) were purified from hybridoma lines (ATCC, Rockville, Md), as described previously.<sup>10</sup> Blocking mAbs against VCAM-1 (IgG1, 4B9) were a gift of Dr Tim Carlos (University of Pittsburgh, Pittsburgh, Pa). Recombinant IL-4 was obtained from Gibco (Gaithersburg, Md). Antibodies to PSGL-1 (IgG1, PL1) were purchased from Ancell (Bayport, Mich). Chinese hamster ovary (CHO) cells expressing human E-selectin (a gift of Dr M. Lawrence, Charlottesville, Va), P-selectin (a gift of Dr R. McEver, Oklahoma City, Okla), and VCAM-1 (a gift of D Leavesley, Adelaide, Australia) were maintained as described previously.<sup>11</sup> Human umbilical vein endothelial cells (HUVECs) were a gift from Robert Hoover (Vanderbilt University, Nashville, Tenn). The VLA-4-specific peptide (LDV peptide)<sup>12,13</sup> 4-(N'-2-methylphenyl)ureido-YLDVPAAK-OH and its FITC-conjugated analog were synthesized at Commonwealth Biotechnologies, Inc (Richmond, Va) and prepared as previously described.<sup>13</sup> All reagents used were of the highest grade commercially available to avoid endotoxin contamination.

### Cells

Venous blood (100-300 mL) was collected from normal, healthy, and nonmedicated donors who had given informed consent, as approved by the Human Studies Committee at the University of New Mexico. EDTA (0.01% vol/vol) was added as an anticoagulant. None of the donors were atopic on the basis of history. Basophils were partially purified by means of Percoll gradient centrifugation and negative selection or extensively purified by means of negative selection, as previously described.<sup>14,15,16</sup> Cytocentrifuge preparations were prepared and stained with Wright-Giemsa stain to obtain percentages of basophils (>95%). Suspension cultures of CBDBs were prepared by culturing IL-3-pulsed umbilical cord blood for 21 to 28 days, as described previously.<sup>8</sup>

### Basophil interaction with endothelium or CHO cell transfectants in a parallel-plate flow chamber

Physiologic flow conditions (ranging from 1-4 dynes/cm<sup>2</sup> in postcapillary venules) were reproduced in vitro by using a parallel-plate flow chamber that produces a well-defined laminar flow over confluent HUVEC monolayers grown on coverslips (GlycoTech, Rockville, Md). Our chamber was miniaturized to handle small volumes.<sup>7</sup> In some experiments the HUVECs were incubated in media with or without IL-4 (50 ng/mL) for 24 hours, treated with saturating levels (20  $\mu$ g/mL) of mAbs for 15 minutes at 37°C, or both before being placed in the chamber.<sup>11</sup> A syringe pump (Harvard Apparatus, Natick, Mass) was used to perfuse suspensions of basophils ( $1 \times 10^6$ /mL in RPMI and 10% FBS) through the chamber at a defined flow. All experiments were performed with HUVECs passed fewer than 5 times. Statistical analysis was performed with Graphpad Prism software (San Diego, Calif). Data sets were tested for significant

differences by using the paired *t* test. A *P* value of less than .05 was considered to be statistically significant.

The entire time period of leukocyte perfusion was videotaped under phase-contrast microscopy (Olympus, NY). All experiments were recorded with a Vicon VC240 CCD video camera and Toshiba KV-7168 recorder (ADI, Albuquerque, NM). The total number of attached cells (rolling and arrested) was determined from the videotape at 5 minutes by means of analysis of 8 to 10 random fields (20 $\times$  phase contrast objective). Rolling and arrested cells were easily visualized because they traveled more slowly than free-flowing cells. After experiments, high shear stress (>30 dynes/cm<sup>2</sup>) was applied to wash off attached basophils, and their identity as basophils was confirmed with Wright-Giemsa stain, as described previously.<sup>16</sup>

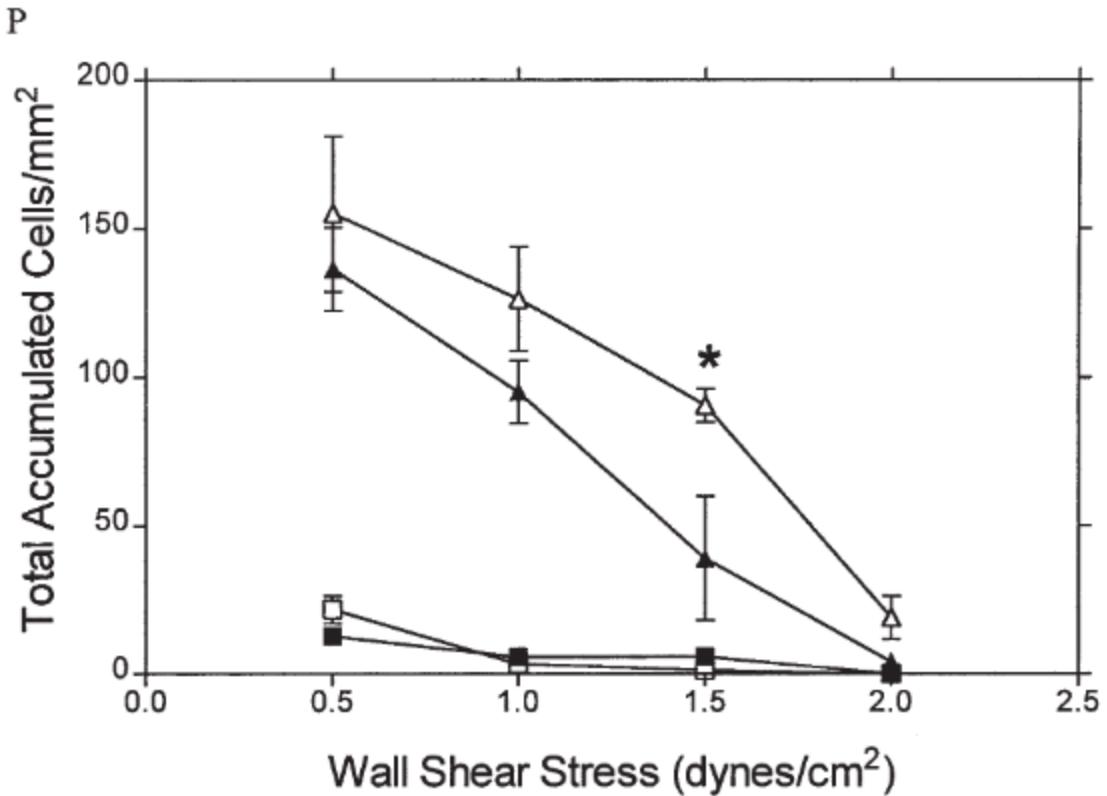
### Measurement of VLA-4 receptor affinity

Equilibrium and kinetic analyses of FITC-labeled LDV peptide binding were performed as described with human leukocytes, including PBBs.<sup>13</sup> For equilibrium-binding studies, PBBs were treated with various concentrations (0.3, 0.6, 1.5, 3.0, and 6.0 nmol/L) of the FITC-labeled LDV peptide for 15 minutes at 37°C (a time point previously shown adequate to reach equilibrium) in the presence or absence of MnCl<sub>2</sub>. Nonspecific binding was determined by competing FITC peptide with a 500-fold excess of unlabeled peptide. Analysis was performed by means of FACS analysis, and 10,000 events were acquired. The resulting data were converted to mean channel fluorescence over time with Tru-Rate software as developed by Seamer and Sklar.<sup>17</sup> To examine the dissociation rate constants ( $K_{off}$ ) of the peptide from VLA-4, cells were preincubated with 6 to 15 nmol/L fluorescent peptide for 10 minutes at 37°C and then treated with 500-fold excess unlabeled peptide, and the dissociation of the fluorescent peptide was followed in a flow cytometer for up to 1000 seconds. Samples were analyzed for 30 to 120 seconds to establish a baseline, the stimulus (Mn<sup>2+</sup>) was added, and FACS acquisition was immediately re-established. The resulting data were converted to mean channel fluorescence over time as above.<sup>17</sup> Affinity constants ( $K_d$ ) were determined as previously described.<sup>13</sup> Data were analyzed by using the Student 2-sample *t* test to calculate statistical significance between different treatments versus control treatment. A *P* value of .05 or less was considered significant. Curve fits and statistics related to LDV peptide binding were performed with GraphPad Prism software.

## Results

### CBDBs and PBBs attach to activated endothelium under physiologic flow conditions

The shear curves in Fig 1 compare the ability of CBDBs and PBBs to interact with resting and IL-4-stimulated HUVEC monolayers over a range of shear stresses (0-10 dynes/cm<sup>2</sup>) that recapitulated flow conditions in postcapillary venules.



**Fig. 1.** Attachment of CBDBs and PBBs to endothelium under flow conditions. CBDBs (*open symbols*) or PBBs (*filled symbols*) were perfused over IL-4-activated (*triangles*) or unactivated (*squares*) endothelium by using a range of shear stresses. The number of cells that attached was determined from videotaped records. Data for 2.5 to 10 dynes is not shown because no attachment occurred. The data represent mean  $\pm$  SD values from 3 separate experiments and donors. \*Indicates statistical significance,  $P = .03$ .

IL-4 was selected as the stimulus because it is a well-documented allergic inflammatory cytokine that activates endothelium.<sup>18, 19</sup> Twenty-four hours of IL-4 incubation was selected because it is consistent with the time of basophil emigration into tissue during the late phase of allergic reactions *in vivo*.<sup>20</sup> CBDBs and PBBs did not interact significantly with unstimulated HUVECs. IL-4 stimulation increased the accumulation of CBDBs and PBBs with all flow rates examined. The number of CBDBs attaching at 1.5 dynes/cm<sup>2</sup> was significantly higher compared with the number of PBBs. However, considerably less or no attachment occurred at higher shear stresses. Therefore all subsequent studies were carried out at 1.5 dynes/cm<sup>2</sup>.

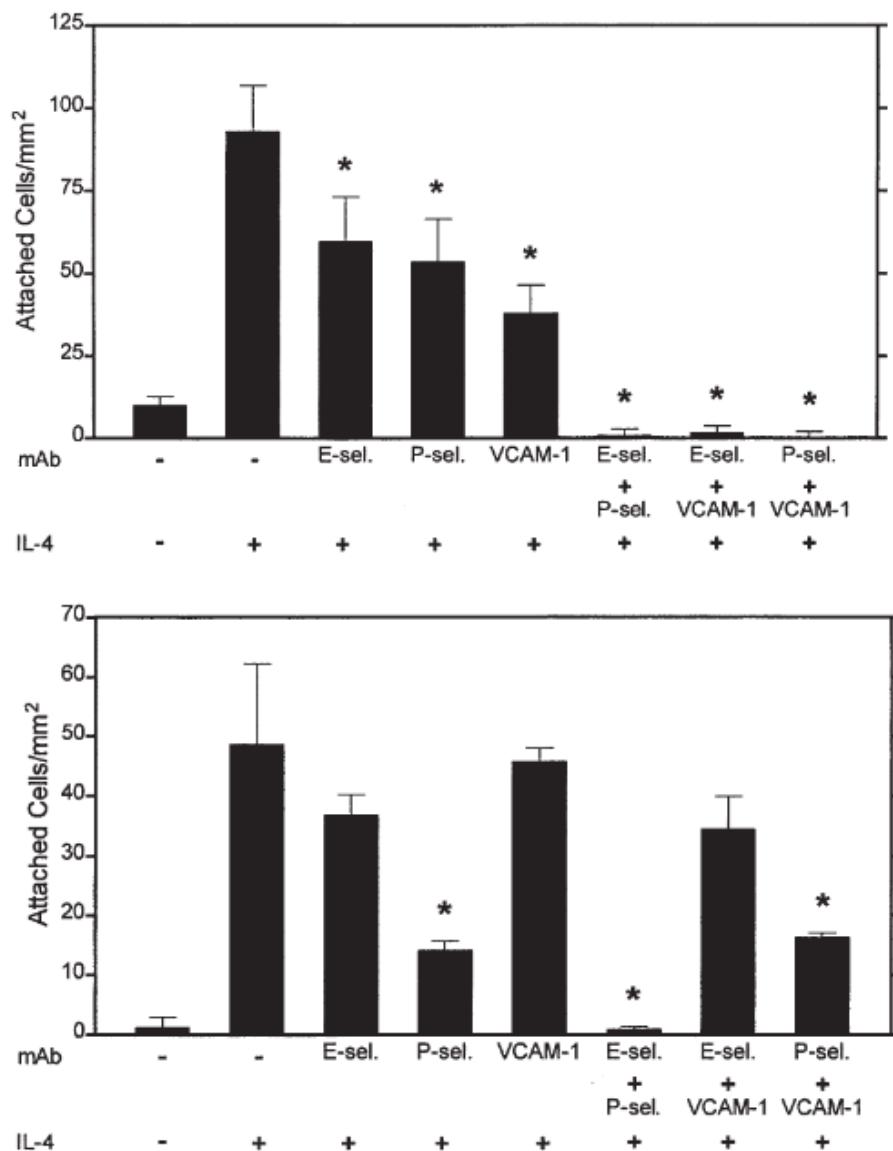
#### VLA-4 mediates initial attachment of CBDBs, but not PBBs, under flow conditions

The mechanisms that mediate initial attachment of CBDBs and PBBs to IL-4-stimulated HUVECs were examined in mAb blocking experiments. As seen in Fig 2, A , mAbs to E-selectin, P-selectin, and VCAM-1 all inhibited binding of CBDBs to IL-4-stimulated HUVEC monolayers under flow conditions.

Individual antibodies reduced adhesion by 41%, 46%, and 57%, respectively. When a combination of any 2 antibodies was used, complete inhibition was seen with less than 2% cell

attachment. Thus CBDBs appear to use a combination of E-selectin, P-selectin, or VLA-4 for attachment to IL-4-stimulated HUVECs.

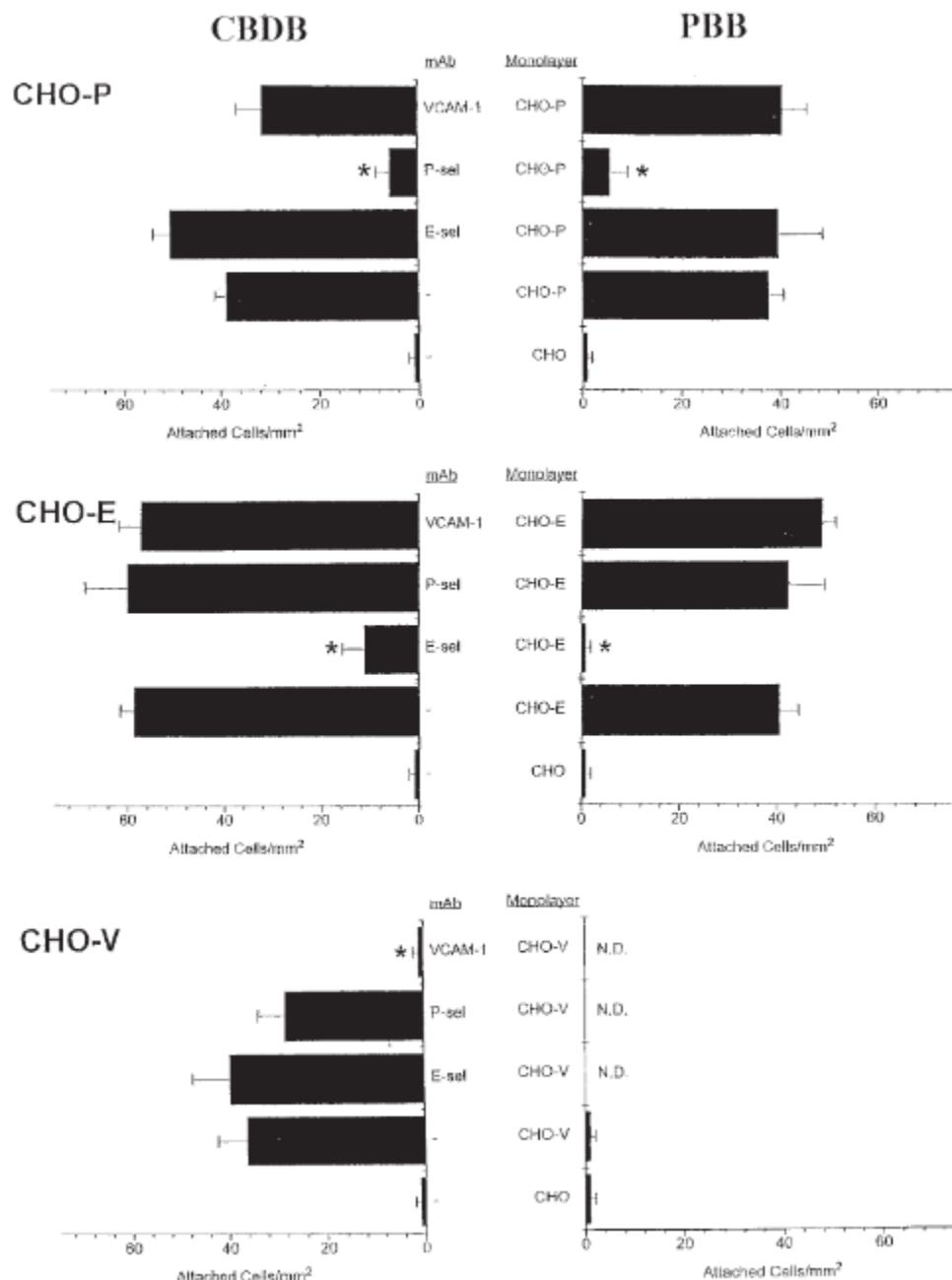
Monoclonal antibodies to P-selectin also significantly inhibited (71%) PBB binding to IL-4-stimulated HUVECs. The contribution of E-selectin became apparent when mAbs against E- and P-selectin were used together, showing greater than 99% inhibition. In contrast, anti-VCAM-1 mAbs did not significantly inhibit PBB attachment unless added with anti-P-selectin mAbs (Fig 2, B). Addition of anti-VCAM-1 and E-selectin mAbs did not decrease basophil attachment. These results imply that the E- and P-selectins, but not VLA/VCAM-1 interactions, mediate attachment of unstimulated PBBs to IL-4-activated endothelium under flow conditions.



**Fig. 2.** Monoclonal antibody inhibition of CBDB and PBB attachment to activated endothelium under physiologic flow conditions. Confluent IL-4-stimulated HUVEC monolayers were incubated with mAbs at saturating concentrations. CBDBs (A) and PBBs (B) were perfused across the monolayers at 1.5 dynes/cm<sup>2</sup>. MOPC was added as a positive control. The number of cells that accumulated was determined from videotaped records. The data represent mean  $\pm$  SD values from 3 separate experiments and donors. \* $P < .05$ .

CBDBs, but not PBBs, bind to VCAM-1-transfected CHO monolayers

The experiments in Fig 3 compare the attachment of CBDBs and PBBs with CHO cells transfected with human E-selectin (CHO-E), P-selectin (CHO-P), or VCAM-1 (CHO-V) under flow conditions.



**Fig. 3.** Attachment of CBDBs and PBBs on CHO monolayers expressing human P-selectin, E-selectin, and VCAM-1. Confluent CHO cell monolayers were incubated with mAbs at saturating concentrations. CBDBs (*left*) and PBBs (*right*) were perfused across the monolayers at 1.5 dynes/cm<sup>2</sup>. The number of cells that accumulated was determined from videotaped records. The data represent mean  $\pm$  SD values from 3 separate experiments and donors. \* $P < .05$ .

As seen in Fig 3 (left panels), CBDBs attached to CHO-E, CHO-P, and CHO-V monolayers at 1.5 dynes/cm<sup>2</sup>, and the addition of mAbs to E- and P-selectins and VCAM-1 inhibited this attachment by approximately 80%, 83%, and 99%, respectively. PBBs also attached to CHO-P and CHO-E monolayers, and blocking mAbs to P- or E-selectin reduced this adhesion by 87% and 99%, respectively (Fig 3, right panels). PBBs did not attach to the CHO-V monolayer.

It is well known that the affinity of VLA-4 for ligand can be upregulated both by signaling molecules that interact with VLA-4 cytoplasmic tail domains and by antibodies and cations, particularly Mn<sup>2+</sup>, that interact with VLA-4 extracellular domain.<sup>21, 22, 23, 24, 25, 26</sup> As shown in Table I, PBBs showed a significant increase in attachment to CHO-V cells under flow conditions after incubation with Mn<sup>2+</sup>.

**Table I. Summary of affinity and off-rate constants on PBBs and CBDBs**

	K <sub>d</sub> (nmol/L)*	K <sub>off</sub> (10 <sup>-4</sup> /s <sup>-1</sup> )†	No. of cells attaching to CHO-V
PBBs (no stimulation)	13 (4)	600 (25)	12 (5)
PBBs (Mn <sup>2+</sup> stimulation)	0.2 (0.1)	25 (5)	57 (7)
CBDBs (no stimulation)	NA	33 (17)	52 (4)

NA, Not applicable (see text for technical details).

\*Equilibrium-binding experiments were conducted with VLA-4-specific peptide by using PBBs with or without Mn<sup>2+</sup> stimulation. The K<sub>d</sub> (affinity constant) of VLA-4 on resting PBBs was determined as previously described.<sup>13</sup> The K<sub>off</sub> (association constant) was determined as described in the “Methods” section. In parallel, flow experiments were conducted to monitor PBB adherence to CHO-V. †Mean (SD) values are shown.

#### Enhanced VLA-4 affinity measured with a VLA-4-binding peptide

Recently, a VLA-4-binding peptide derived from the LDV-binding sequence of VCAM-1 specific for VLA-4 was developed.<sup>26</sup> In its fluorescence-labeled form, this peptide might be used to detect the affinity state of VLA-4 on cells by means of flow cytometry.<sup>13</sup> We used this peptide to measure VLA-4 affinity in resting and Mn<sup>2+</sup>-stimulated PBBs. There was no detectable binding of fluorescent LDV peptide to unstimulated PBBs. In contrast, equilibrium-binding studies demonstrated that the K<sub>d</sub> of VLA-4 for peptide in the presence of Mn<sup>2+</sup> was approximately 0.2 nmol/L, a value similar to that detected on other leukocytes after Mn<sup>2+</sup> stimulation.<sup>12, 13, 24, 25, 26</sup>

Measurements of VLA-4 affinity on resting CBDBs were complicated by the high level of autofluorescence in the cultured cells, and the addition of Mn<sup>2+</sup> to CBDBs caused cell death (data not shown). We therefore compared the VLA-4 activation state on CBDBs and PBBs by using dissociation rate constants. Unstimulated CBDBs and unstimulated and Mn<sup>2+</sup>-stimulated PBBs were incubated with 500-fold excess of nonlabeled LDV peptide to induce dissociation, as described previously.<sup>13</sup> Using this protocol, we determined that VLA-4 on activated CBDBs was in a very similar affinity state as VLA-4 on resting CBDBs (Table I). On the basis of the general principle that the lower the K<sub>off</sub> the higher the affinity constant, VLA-4 on CBDBs is in a higher affinity state than on nonstimulated PBBs.

Immunophenotyping studies<sup>28, 29, 30, 31</sup> confirmed that IL-4 stimulation of HUVECs induced expression of VCAM-1, P-selectin, and E-selectin on IL-4-stimulated HUVECs after 24 hours

(data not shown). Also as expected,<sup>6, 8, 32, 33</sup> both CBDBs and PBBs express the integrins  $\alpha 4\beta 1$  (VLA-4) and PSGL-1 and the E-selectin ligand cutaneous leukocyte antigen.

## Discussion

In this study the attachment of human basophils to IL-4-stimulated endothelial cells under flow conditions was studied by using a miniaturized parallel-plate flow chamber. Wall shear stresses were selected that approximated those in postcapillary venules, where basophil extravasation takes place.<sup>34</sup> We found that human PBBs and CBDBs interact with IL-4-stimulated endothelial cells at levels of wall shear stress of up to 1.5 dynes/cm<sup>2</sup>. The inverse relationship between shear stress and adherence, as well as the magnitude of adhesive interactions, is in agreement with the findings of previous investigators examining *in vitro* flow models with human monocytes, lymphocytes, eosinophils, and neutrophils.<sup>11, 35, 36, 37, 38, 39</sup> Experiments with blocking antibodies established that both PBBs and CBDBs use E- and P-selectin for attachment to endothelium. CBDBs also used VLA-4 to mediate adhesion to endothelium. However, unstimulated PBBs do not use VLA-4 to attach.

VLA-4 has been shown previously to exist in multiple activation states.<sup>26, 40</sup> In particular, constitutive VLA-4 avidity has been described on precursor leukocytes and early lymphocytes, whereas more differentiated cells have regulated VLA-4 avidity.<sup>11, 15, 41, 42, 43, 44</sup> Thus the differences we see in the CBDBs and PBBs might reflect a less differentiated state of CBDBs compared with PBBs. Differences in maturation levels between CBDBs and PBBs were previously suggested by the observation that only a subset of CBDBs react with the PBB-specific mAb 2D7.<sup>8, 16</sup> The switch of VLA-4 affinity on PBBs to a higher-affinity state induced by Mn<sup>2+</sup>, previously shown to induce higher VLA-4 affinity confirmations,<sup>45</sup> corresponds with acquisition of PBB adherence to CHO-V under flow conditions. These findings are consistent with recent results with a conjugate-forming assay, in which it was shown that PBB VLA-4 switches from a lower- to a higher-avidity state after both Mn<sup>2+</sup> and antigen challenge.<sup>46</sup>

Earlier studies of basophil attachment under static conditions implicated E- and P-selectin-mediated interactions in basophil adhesion to endothelium.<sup>19, 32, 33</sup> Extending these studies, our results with CHO transfectants confirm a role for E- and P-selectin in the attachment of CBDBs and PBBs to endothelium under flow conditions. Previous studies also implicated VLA-4 in the static adhesion of unstimulated PBBs to endothelium and endothelial models.<sup>31, 32</sup> In contrast, PBBs show no VLA-4-mediated adhesion to either HUVECs or CHO-V cells under the flow conditions used in our study. Thus static adhesion assays might provide results that are misleading when extrapolated to a physiologic context.

It is becoming evident that basophils play a substantial role in chronic allergic inflammation, including asthma, and, by extension, that understanding how basophils migrate into tissues might have important therapeutic implications. The use of flow conditions to examine basophil adhesion to endothelium is an important step toward the development and testing of specific antagonists of this process.

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