

The Association between α_4 -Integrin, P-Selectin, and E-Selectin in an Allergic Model of Inflammation

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Summary

In this study, we examined the relationship between the endothelial selectins (P-selectin and E-selectin) and whether they are critical for α_4 -integrin-dependent leukocyte recruitment in inflamed (late phase response), cremasteric postcapillary venules. Animals were systemically sensitized and 2 wk later challenged intrascrotally with chicken ovalbumin. Leukocyte rolling flux, adhesion, and emigration were assessed at baseline and 4 and 8 h postantigen challenge. There was a significant increase in leukocyte rolling flux, adhesion, and emigration in sensitized and challenged mice at both 4 and 8 h. At 8 h, the increase in leukocyte rolling flux was $\sim 50\%$ inhibitable by an anti- α_4 -integrin antibody, 98% inhibitable by fucoidin (a selectin-binding carbohydrate), and 100% inhibitable by an anti-P-selectin antibody. P-selectin-deficient animals displayed no leukocyte rolling or adhesion at 8 h after challenge. However, at 8 h there were many emigrated leukocytes in the perivascular space suggesting P-selectin-independent rolling at an earlier time point. Indeed, at 4 h postantigen challenge in P-selectin-deficient mice, there was increased leukocyte rolling, adhesion, and emigration. The rolling in the P-selectin-deficient mice at 4 h was largely α_4 -integrin dependent. However, there was an essential E-selectin-dependent component inasmuch as an anti-E-selectin antibody completely reversed the rolling, and in E-selectin and P-selectin double deficient mice rolling, adhesion and emigration were completely absent. These results illustrate that P-selectin underlies all of the antigen-induced rolling with a brief transient contribution from E-selectin in the P-selectin-deficient animals. Finally, the antigen-induced α_4 -integrin-mediated leukocyte recruitment is entirely dependent upon endothelial selectins.

The movement of leukocytes from the intravascular compartment to the extravascular space is mediated by three sequential events. First, circulating leukocytes in the flowing stream of blood make initial contact with the endothelium that is manifest as a tethering and rolling motion along the length of the venule. Second, rolling leukocytes are activated to firmly adhere to the endothelium by various proinflammatory mediators. Once firmly adherent, the cells are able to perform the third and final motion, emigration out of the vasculature. Each of these events are sequential inasmuch as inhibition of leukocyte tethering and rolling inhibits further adhesion and emigration. In addition, each event is mediated by distinct adhesion molecules. It is generally accepted that two endothelial selectins are responsible for leukocyte tethering and rolling, P-selectin

mediating early rolling events and E-selectin overlapping with P-selectin with time of inflammation. However, the exact temporal relationship between the selectins remains unclear. The integrins and members of the immunoglobulin superfamily are responsible for leukocyte adhesion and emigration (1–3). More recently, however, a number of reports have suggested that the α_4 -integrin is not only capable of mediating leukocyte adhesion, but also initiating the rolling interactions (4–6).

Luscinskas et al. (5) recently reported that under flow conditions in vitro, monocytes firmly adhere via the α_4 -integrin to activated endothelial cells. In that study, however, the authors illustrated that the initial tethering and rolling interaction was entirely dependent upon selectins (L-selectin and P-selectin). Neutralizing selectin function in that sys-

tem prevented the α_4 -dependent adhesion regardless of the shear stress (0.8–4.0 dynes/cm²). These observations suggested that in the presence of selectins the α_4 -integrin was capable of mediating only the adhesion event. In another *in vitro* study, however, Alon et al. (4) illustrated that the α_4 -integrin on lymphocytes was capable of mediating tethering, rolling, and firm adhesion on its ligand vascular cell adhesion molecule 1 (VCAM-1). It should be noted, however, that the entire sequence of events occurred at a shear stress of <1 dyn/cm². At higher shear stresses where selectins can clearly tether leukocytes and support rolling, the α_4 -integrin could not support tethering to VCAM-1.

At sites of leukocyte–endothelial cell interactions *in vivo*, the shear stresses are usually much higher than 1 dyn/cm², ranging between 2–16 dyn/cm², where 2 dyn/cm² is at the very low end of physiologic shear (7). One might predict, therefore, that *in vivo*, the α_4 -integrin would not be likely to tether cells to the endothelial surface. However, *in vivo*, there are other features that may promote leukocyte–endothelial cell interactions and allow for α_4 -integrin-dependent tethering and rolling. For example, circulating red blood cells displace larger cells (leukocytes) from the mainstream of blood to the periphery of blood vessels, forcing them to make initial contact with vascular endothelial cells (7). It is conceivable, therefore, that in the presence of red blood cells, α_4 -integrins may be sufficient to recruit circulating leukocytes independent of selectins. Indeed, introduction of red blood cells into flow chambers increased leukocyte–endothelial cell interactions (8). Another critical difference between *in vitro* and *in vivo* systems may be the site density of ligands for the α_4 -integrin. The use of various *in vitro* substrata instead of microvascular endothelium may greatly underestimate the site density of ligands (e.g., VCAM-1) for α_4 -integrins. Therefore, it is conceivable that under inflammatory conditions, α_4 -integrin ligands may be expressed in sufficient numbers to gain the capacity to mediate leukocyte tethering, rolling, and adhesion independent of selectins.

In this study, we developed an allergen model of inflammation, and using intravital microscopy we studied the contribution of P-selectin, E-selectin, and α_4 -integrin to leukocyte recruitment in post-allergen-treated cremaster muscle. Moreover, this approach permitted us to examine the interrelationship between each of these three adhesion molecules with particular emphasis on establishing whether α_4 -integrin-mediated leukocyte recruitment was dependent upon P-selectin and/or E-selectin. Additionally, it permitted examination of the time profiles for P-selectin and E-selectin in mediating leukocyte rolling during a late phase allergic response.

Materials and Methods

Mice deficient in P-selectin, E-selectin, or both P-selectin and E-selectin were generated by gene targeting in embryonic stem

cells as previously described (9–11). The mutant mice used for these experiments were from a 129/Sv × C57Bl/6 mixed background, whereas both 129/Sv × C57Bl/6 and C57Bl/6 nonmutant mice were used as wild-type controls. The results from these two sets of control animals were not significantly different from each other and therefore, were grouped together. Only E-selectin–P-selectin double mutant mice that did not show obvious signs of disease were used for these studies (10). All animals used weighed between 20–35 g.

Immunization Protocol. To develop a model of chronic inflammation, a type I hypersensitivity reaction was elicited by systemically (intraperitoneal injection) sensitizing animals with 10 μ g of chicken ovalbumin (Sigma Chemical Co., St. Louis, MO) and 10 mg grade V AIOH (Sigma Chemical Co.) in a total volume of 0.2 ml saline. 2 wk later, animals were challenged locally (intrascrotal injection) with the sensitizing antigen. Sham sensitization and sham challenge involved systemic and local injection of 0.2 ml saline, respectively. The animals were prepared for intravital microscopy and leukocyte–endothelial cell interactions were examined during the late phase response, at 4 or 8 h after saline or ovalbumin challenge.

Intravital Microscopy. Animals were anesthetized by intraperitoneal injection of a cocktail of 10 mg/kg Xylazine (MTC Pharmaceuticals, Cambridge, Ontario, Canada) and 200 mg/kg ketamine hydrochloride (Rogar/STB Inc., Montreal, Quebec, Canada). The left jugular vein was cannulated to administer anesthetic and various drugs. An incision was made in the scrotal skin to expose the left cremaster muscle, which was then carefully removed from the associated fascia. A lengthwise incision was made on the ventral surface of the cremaster muscle. The testicle and epididymis were separated from the underlying muscle and reintroduced into the abdominal cavity. The muscle was then spread out over an optically clear viewing pedestal, and secured along the edges with 5-0 suture. The exposed tissue was suffused with warm bicarbonate-buffered saline (pH 7.4). The cremasteric microcirculation was observed through an intravital microscope (Nikon Inc., Mississauga, Canada) with a 25× objective lens (L25/0.35; Wetzlar E. Leitz Inc., Munich, Germany) and a 10× eyepiece. The image of the microcirculatory bed (magnification of 1400 on the video monitor) was recorded using a video camera (Panasonic-Digital 5100; Panasonic, Osaka, Japan) and a video recorder (NV8950, Panasonic). This preparation has previously been used to visualize the microcirculation (12, 13). Images of the microcirculation were recorded at 0, 15, and 30 min and all experimental parameters were measured at these time points.

Single, unbranched cremasteric venules (20–40 μ m diam) were selected for each study. Venular diameter (D_v) was measured using a video caliper (Microcirculation Research Institute, Texas A & M University, College Station, TX). Rolling leukocytes were defined as those leukocytes that rolled at a velocity slower than that of red blood cells. Leukocyte rolling velocity was measured for the first 20 leukocytes entering the field of view at the time of recording, and determined as the time required for a leukocyte to traverse a given length of venule. Leukocyte adhesion was quantified as the number of leukocytes that adhered to the vessel wall for 30 s or more within a given segment of the vessel. The number of emigrated leukocytes was quantified by counting cells in the extravascular space within the field of view (a region of $\sim 200 \times 300 \mu$ m). Red blood cell velocity (V_{RBC}) was measured online using an optical Doppler velocimeter (Microcirculation Research Institute, Texas A & M University). Venular blood flow was calculated from the product of cross-sectional area and mean red blood cell velocity ($V_{mean} = V_{RBC}/1.6$),

¹Abbreviations used in this paper: D_v , venular diameter; dyn, dynes; PCA, passive cutaneous anaphylaxis; VCAM-1, vascular cell adhesion molecule 1.

assuming cylindrical geometry. Venular wall shear rate ($\dot{\gamma}$) was calculated based on the Newtonian definition: $\dot{\gamma} = 8(V_{\text{mean}}/D_v)$, and venular wall shear stress was $\dot{\gamma} \times \text{blood viscosity}$, where blood viscosity was assumed to be 0.025 poise (14).

Passive Cutaneous Anaphylaxis Reaction. Serum was obtained from all OVA-sensitized animals at the end of the experiment by intracardiac puncture. Serial dilutions (1:8–1:64) of the serum samples were prepared and 200 μl of each sample was injected intradermally into the shaved backs of control, untreated mice and Sprague-Dawley rats. Serum from immunized mice elicited the same response in Sprague-Dawley rats as it did in C57Bl/6 mice. Therefore, rats were used for all subsequent passive cutaneous anaphylaxis (PCA) reactions primarily because detection of PCA was easier to record. After 72 h, animals were challenged with an intracardiac injection of a solution containing 2.5 mg Evan's blue dye and 5 mg chicken OVA in a total volume of 1.5 ml (saline). The final reaction was read 60 min later as the highest dilution that produced a distinct blue region (Evan's blue dye extravasation) at the center of the injection site (15). Sensitized animals had serum anti-OVA antibody titres of at least 1:64, whereas sham-sensitized animals had no anti-OVA antibodies. To determine if the serum-induced PCA reaction was mediated by anti-OVA IgG, the serum samples were heated for 30 min at 56°C in another series of experiments. This protocol denatures IgE leaving IgG immunoglobulins intact (15). A PCA reaction was then carried out with heat-treated serum samples as described above.

Drug Administration and Experimental Protocol. All drugs were administered intravenously, at 5 min of the experimental protocol: 10 mg/kg of fucoidin (Sigma Chemical Co.); 75 or 150 μg /animal of a monoclonal anti- α_4 -integrin antibody (R1-2; PharMingen, San Diego, CA); 20 μg /animal of a monoclonal anti-P-selectin antibody (RB40.34; PharMingen); and 100 μg /animal of a monoclonal anti-E-selectin antibody (9A9) (12, 13, 176–19). Some animals received an isotype-matched (IgG) nonbinding control antibody.

In the first set of experiments, sham-sensitized and OVA-sensitized animals were challenged with either saline or OVA. Leukocyte rolling, adhesion, and emigration were examined at 4 or 8 h after challenge. To determine if the α_4 -integrin was involved in the OVA-induced leukocyte recruitment, OVA-sensitized and challenged animals were treated with an anti- α_4 antibody. To study the role of selectins in this model of leukocyte recruitment, we used three different strategies. First, animals were treated with fucoidin, a lectin-binding carbohydrate, and leukocyte rolling, adhesion, and emigration were assessed over a 30-min period. Second, to specifically study a role for either P-selectin or E-selectin in this response, another series of animals received either an anti-P-selectin antibody or an anti-E-selectin antibody. In a third series of experiments, inflammation was elicited in animals which were genetically deficient in P-selectin, E-selectin, or both E-selectin and P-selectin. In addition, some sensitized and challenged P-selectin-deficient mice received an anti-E-selectin antibody at 4 h. Both baseline and antigen-induced leukocyte rolling, adhesion, and emigration were examined in these mutant animals and compared to wild-type OVA-sensitized and challenged animals.

Circulating Leukocyte Counts. At the end of each experiment, whole blood was drawn via cardiac puncture. Total leukocyte counts were performed using a hemocytometer (Bright-line; Hausser Scientific, Horsham, PA) in untreated, wild-type, and the various mutant animals.

Statistical Analysis. Data are presented as mean \pm SEM. A Student's *t* test with bonferroni correction was used for multiple comparisons. Statistical significance was set at $P < 0.05$.

Results

The OVA-induced Late Phase Response Was Mediated Primarily by Anti-OVA IgE Antibodies. All sensitized animals used in this study had serum anti-OVA antibody titres of at least 1/64 as assessed by a PCA reaction. There was no evidence of anti-OVA antibodies in sham-sensitized animals. Approximately 10% of the heat-treated samples were also able to induce a dermal hypersensitivity response (data not shown) suggesting that at least 90% of the OVA-induced late phase responses were mediated by anti-IgE antibodies (heating inactivates IgE, but leaves IgG intact [15]).

Allergen Increases Leukocyte-Endothelial Cell Interactions in Sensitized Animals. Fig. 1 illustrates the flux of rolling leukocytes (*top*), leukocyte adhesion (*middle*), and leukocyte emigration (*bottom*) in untreated animals at baseline, and animals that were sham sensitized and sham challenged (SS),

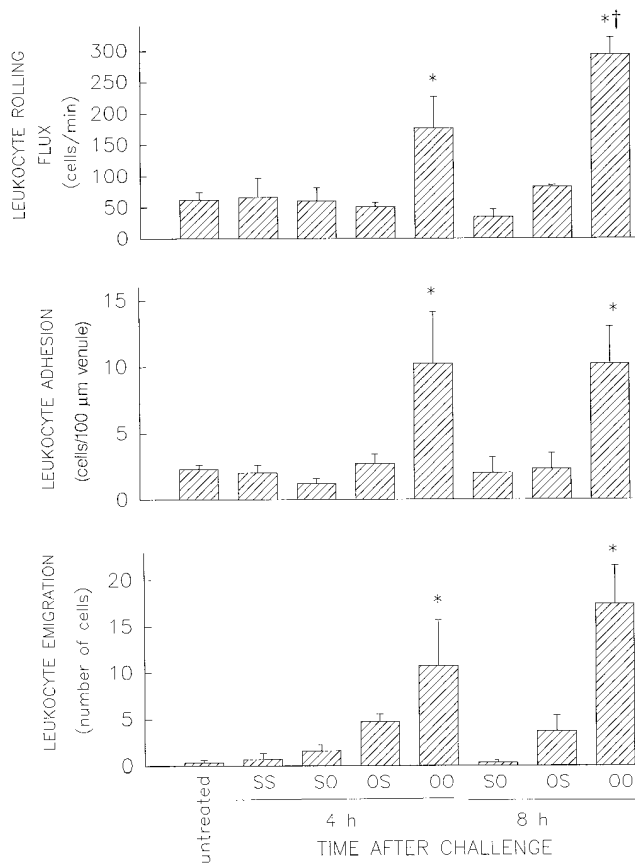


Figure 1. The flux of rolling leukocytes (*top*), leukocyte adhesion (*middle*), and leukocyte emigration (*bottom*) in untreated animals ($n = 3$) at baseline; sham-sensitized, sham-challenged (SS; $n = 3$); sham-sensitized, OVA-challenged (SO; $n = 8$); OVA-sensitized, sham-challenged (OS; $n = 6$); and OVA-sensitized and OVA-challenged (OO; $n = 9$) animals at 4 h after antigen challenge, and SO, OS, and OO animals at 8 h after challenge. In sham- or antigen-sensitized animals, the cremaster muscle was exteriorized after 4 or 8 h of challenge and values were obtained 30 min after exteriorization. Flux, adhesion, and emigration did not change over the 30 min experimental protocol. * $P < 0.05$ relative to respective SO and OS values. † $P < 0.05$ relative to OO at 4 h after challenge.

Table 1. Hemodynamic Parameters in OVA-sensitized and Challenged (8 h) Wild-type Animals

Time	Leukocyte rolling velocity	V_{RBC}	D_v	Venular shear stress
min	$\mu m/s$	mm	μm	dyn/cm^2
0	32.6 ± 5.0	3.3 ± 0.4	33.6 ± 1.9	12.4 ± 1.6
15	47.1 ± 7.0	4.2 ± 0.5	33.6 ± 1.9	$15.9 \pm 1.9^*$
30	52.6 ± 4.8	3.5 ± 0.5	33.6 ± 1.9	13.2 ± 1.6

* $P < 0.05$ relative to time 0 min value.

sham sensitized and OVA-challenged (SO), OVA sensitized and sham challenged (OS), and OVA sensitized and OVA challenged (OO) at 4 and 8 h after challenge. In untreated animals, the flux of rolling leukocytes was ~ 60 cells/min. In sham-sensitized, OVA challenged and OVA-sensitized, sham-challenged animals, leukocyte rolling flux was not different from untreated animals. In OVA-sensitized animals at 4 h after OVA challenge, however, there was a very dramatic increase in leukocyte rolling flux compared to the respective control groups. Furthermore, after 8 h of challenge, leukocyte rolling was even further increased to ~ 300 cells/min, a value above that observed after 4 h of challenge ($P < 0.05$). We have not seen this degree of leukocyte rolling in any of our previous models including rolling induced by histamine (20), oxidants (21), or leukotriene C_4 (22) in the rat, ischemia/reperfusion in the cat (23), or cytokine-induced rolling in the mouse (our unpublished observations). Fig. 1 also illustrates that there was very little leukocyte adhesion (*middle*) and leukocyte emigration (*bottom*) in untreated and sham animals at 4 and 8 h after challenge. However, in sensitized animals exposed to allergen, there was a significant increase in both leukocyte adhesion (*middle*) and emigration (*bottom*) after 4 and 8 h of challenge. The adhesion and emigration was not unlike values observed after exposure to potent chemoattractants (24–26). All leukocyte parameters were stable over the 30 min experimental protocol. Finally, at the end of the 8-h treatment with OVA, histological analysis (hematoxylin and eosin) revealed that at least 50% of the leukocytes were PMN, whereas the remaining cells were eosinophils or mononuclear cells.

Table 1 summarizes leukocyte rolling velocity, V_{RBC} , D_v , and venular shear stress in sensitized mice at 8 h after allergen challenge. There was no difference in leukocyte rolling velocity, red blood cell velocity, or venular diameter over the course of the experiment or when compared to untreated controls. Venular shear stress increased at 15 min, but was back down to control levels by 30 min. Leukocyte rolling velocity, V_{RBC} , D_v or shear stress did not differ in any of the experimental groups (data not shown).

α_4 -Integrin Mediates Leukocyte Rolling in the Late Phase Reaction. To ensure that each animal tested had a late phase

leukocytic response, antibodies were added during the course of the experiment. Administration of a monoclonal antibody against murine α_4 -integrin (75 μg /animal) reduced leukocyte rolling by 50% (Fig. 2). Nevertheless, there still remained more than twice the number of rolling cells as that observed in any of the sham groups of animals (~ 60 cells/min) suggesting a population of leukocytes that rolled independent of α_4 -integrin. In every case, there was a short delay (15 min) before the α_4 -integrin antibody blocked leukocyte rolling. Therefore, in some animals a higher dose of the α_4 -integrin antibody was used (150 μg /animal). Although this concentration immediately reduced leukocyte rolling, the reduction was identical to the magnitude of response observed with the lower concentration. Therefore, the data are pooled. Administration of an isotype-matched (IgG) control antibody had no effect on the antigen-induced leukocyte rolling. Neither antibody affected systemic leukocyte counts.

P-selectin Is Critical to All Leukocyte Rolling at 8 h After Allergen Challenge. A role for P-selectin at 8 h after antigen challenge was established in three different ways. In the first instance, we treated animals with a selectin-binding carbohydrate, fucoidin (Fig. 3; *top*), which reduced the antigen-induced increase in leukocyte rolling by $\sim 98\%$ at 8 h after allergen challenge. An anti-P-selectin antibody (Fig. 3; *middle*) completely reversed the OVA-induced increase in leukocyte rolling, suggesting that P-selectin is critical for the leukocyte recruitment in this model. The leukocytes that were already adherent were not affected by either the P-selectin antibody or fucoidin treatment, consistent with the view that the firm adhesion per se was due to other adhesive

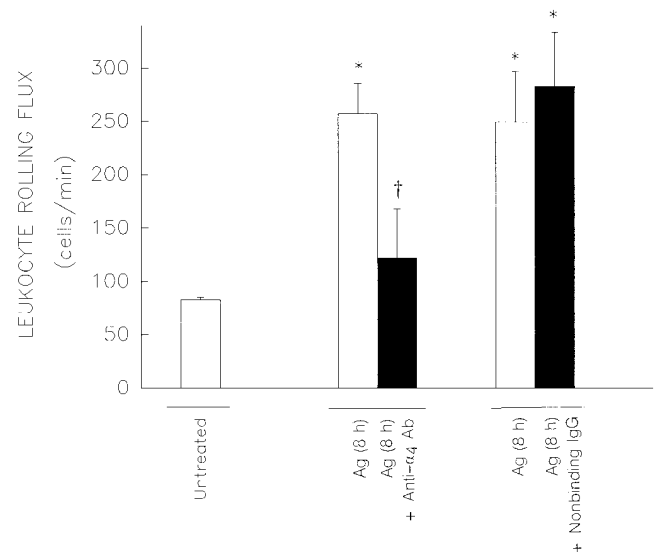


Figure 2. Leukocyte rolling flux in untreated animals ($n = 3$) and sensitized wild-type animals at 8 h after antigen challenge, before and after treatment with either an anti- α_4 -integrin antibody ($n = 5$) or a nonbinding, isotype-matched control antibody ($n = 3$). At 8 h after antigen challenge, administration of an anti- α_4 -integrin antibody reduced leukocyte rolling flux by $\sim 50\%$, whereas a nonbinding IgG had no effect. * $P < 0.05$ relative to untreated value. † $P < 0.05$ relative to respective Ag 8 h value.

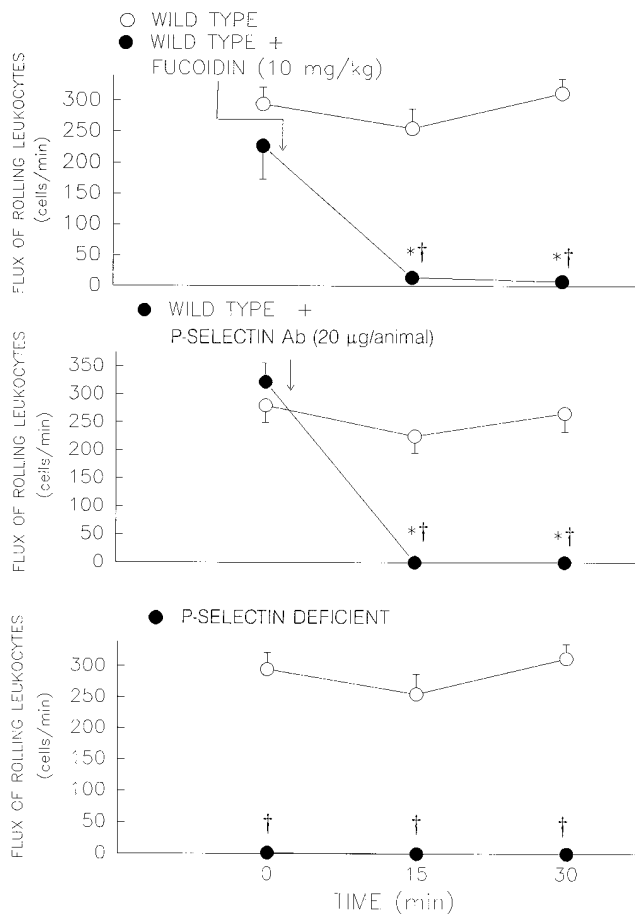


Figure 3. Leukocyte rolling flux in sensitized wild-type animals that received fucoidin (*top*; $n = 4$), an anti-P-selectin antibody (*middle*; $n = 3$), or in sensitized P-selectin-deficient animals (*bottom*; $n = 3$) at 8 h post-OVA challenge. Fucoidin and the P-selectin antibody were each administered at 5 min of the experimental protocol. * $P < 0.05$ relative to 0 min value. † $P < 0.05$ relative to respective wild-type value.

mechanisms (data not shown). In the third series of experiments, we elicited the late phase response in animals genetically deficient in P-selectin. The bottom panel of Fig. 3 illustrates that in P-selectin-deficient mice there was absolutely no leukocyte rolling at 8 h after antigen challenge. This three-pronged approach suggests that both the α_4 -integrin-dependent and α_4 -integrin-independent leukocyte rolling was contingent upon functional P-selectin.

Leukocyte-Endothelial Interactions Do Occur in P-selectin-deficient Mice. Despite the lack of rolling at 8 h after antigen challenge in the P-selectin-deficient animals, there was always a very significant increase in leukocyte emigration. To further investigate this observation, some P-selectin-deficient animals were examined at 0 and 4 h after antigen challenge (Fig. 4). No leukocyte rolling (*top*), adhesion (*middle*), or emigration (*bottom*) was noted in the P-selectin-deficient mice immediately after challenge (0 h). At 4 h after antigen challenge, however, the P-selectin-deficient mice displayed significant leukocyte rolling (*top*). Although there was some leukocyte rolling in every P-selectin-deficient

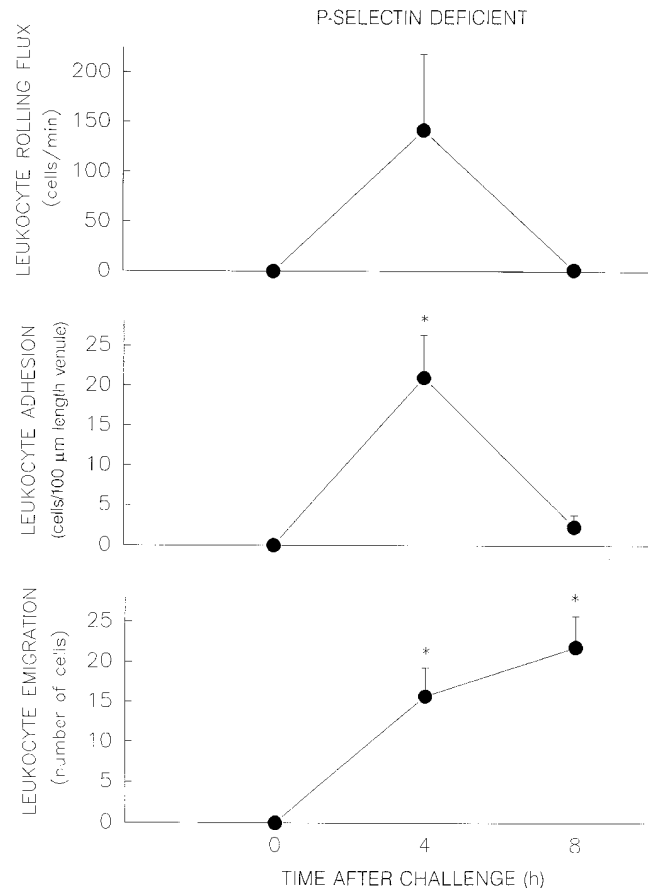


Figure 4. Alterations in leukocyte rolling flux (*top*), leukocyte adhesion (*middle*), and leukocyte emigration (*bottom*) in sensitized P-selectin-deficient animals at 0 ($n = 4$), 4 ($n = 6$), and 8 ($n = 3$) h after OVA challenge. * $P < 0.05$ relative to 0 h.

vessel examined at 4 h, there was a tremendous amount of variability between vessels ranging from as few as 4 cells/min to as many as 421 cells/min. On average, ~100–150 cells/min rolled in the P-selectin-deficient vessels. Associated with the increased rolling of leukocytes at this time point, leukocyte adhesion (*middle*) and emigration (*bottom*) were also noted. In fact, more than twice as many cells adhered at 4 h in P-selectin-deficient vessels compared to their wild-type counterparts (Fig. 1 versus Fig. 4). At 8 h after antigen challenge, leukocyte rolling disappeared (*top*) and there was very little leukocyte adhesion in the P-selectin-deficient mice compared to the wild-type controls at the 8 h time point (Fig. 1 versus Fig. 4). However, there was a significantly elevated number of emigrated leukocytes. In fact, the amount of emigrated cells did not differ between the P-selectin-deficient mice and their wild-type counterparts (Fig. 1 versus Fig. 4). It is unlikely that the cells were emigrating from other vessels or other regions of the microcirculation since an examination of all other vessels in the general area revealed no rolling cells at 8 h after antigen challenge. Clearly, the only explanation is that at ~4 h after challenge, a P-selectin-independent mechanism

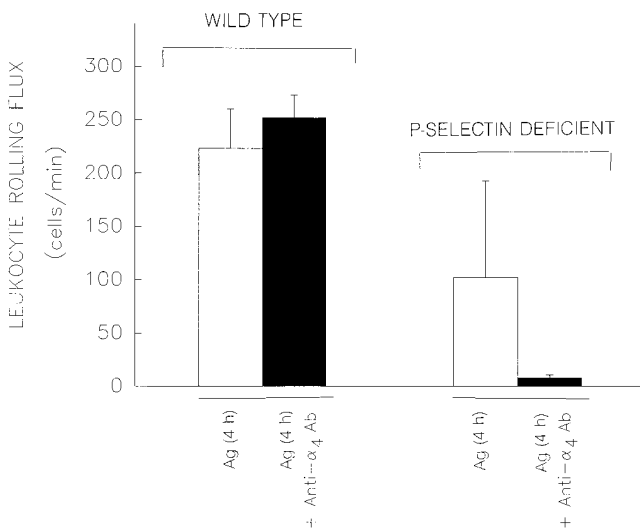


Figure 5. Leukocyte rolling flux in sensitized wild-type ($n = 5$) and P-selectin-deficient animals ($n = 3$) at 4 h after antigen challenge, before and after administration of an anti- α_4 -integrin antibody. Administration of the antibody had no effect on leukocyte rolling in wild-type animals at this time point, however, there was an almost complete reversal of leukocyte rolling in the P-selectin-deficient animals.

was transiently induced which allowed leukocytes to roll, adhere, and emigrate into the interstitium.

Leukocyte Rolling in P-selectin-deficient Vessels Is Dependent on α_4 -Integrin. As there was significant α_4 -integrin-dependent leukocyte rolling at 8 h after allergen challenge in wild-type animals, we examined whether this mechanism was responsible for leukocyte rolling at 4 h in the P-selectin-deficient vessels. Fig. 5 illustrates that when the anti- α_4 -antibody was administered to sensitized and challenged P-selectin-deficient mice, the rolling dissipated by 90% to fewer than 15 rolling cells/min. This was, however, specific to the P-selectin-deficient vessels as addition of the same anti- α_4 -integrin antibody 4 h after challenge in wild-type mice had no detectable effect on the flux of rolling leukocytes. Clearly a much greater proportion of rolling cells were dependent upon α_4 -integrin in P-selectin-deficient mice (>90%) at 4 h than in the wild-type mice at either 4 h (0%) or 8 h (50%, Fig. 2) after allergen challenge. The anti- α_4 -antibody had no effect on leukocyte adhesion or emigration in either wild-type animals or the P-selectin-deficient animals at this time point (data not shown).

α_4 -Integrin-Associated Rolling Is Dependent upon E-selectin in P-selectin-deficient Vessels. To determine if the other endothelial selectin, E-selectin, contributed to the α_4 -integrin-dependent rolling pathway at 4 h in the P-selectin-deficient vessels, P-selectin-deficient mice were treated with an antimurine E-selectin antibody at 4 h after antigen challenge. Fig. 6 (top) illustrates that administration of the anti-E-selectin antibody completely reversed the antigen-induced leukocyte rolling at 4 h after challenge in the P-selectin-deficient mice. In an additional series of experiments, we sensitized and challenged E-selectin- and P-selectin-deficient mice and examined the leukocyte recruitment profiles at 0,

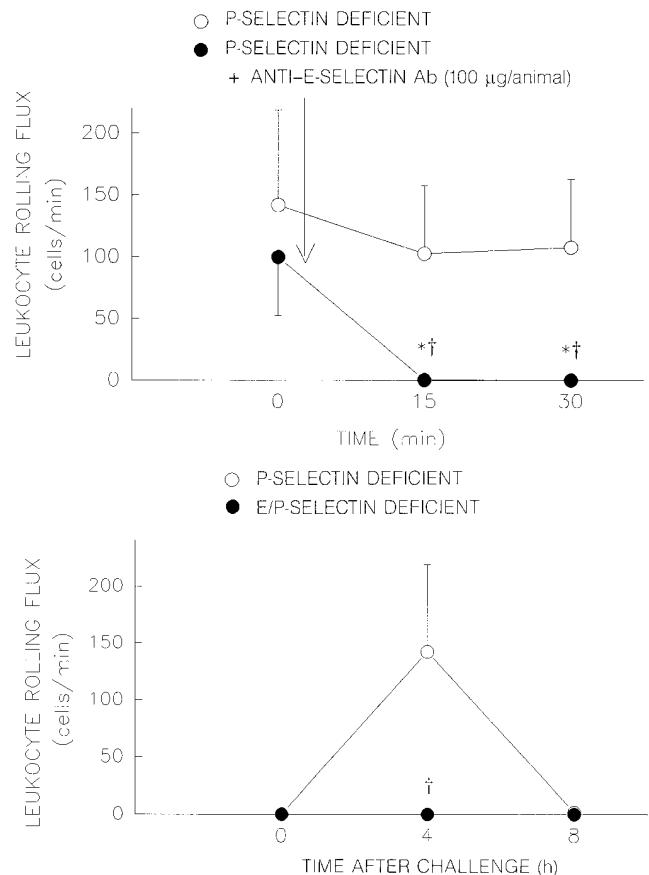


Figure 6. Leukocyte rolling flux at 4 h after antigen challenge in P-selectin-deficient mice (top) with ($n = 6$) or without ($n = 6$) an anti-E-selectin antibody. Administration of an anti-E-selectin antibody completely reversed leukocyte rolling flux. The anti-E-selectin antibody had no effect in wild-type animals at the same time point (data not included). The bottom panel compares the leukocyte rolling flux at 0, 4, and 8 h after antigen challenge in P-selectin-deficient mice ($n = 6$) and E-selectin- and P-selectin-deficient mice ($n = 6$). In contrast to the transient increase in leukocyte rolling in P-selectin-deficient mice, the E-selectin- and P-selectin-deficient animals displayed no leukocyte rolling at either 0, 4, or 8 h after antigen challenge. *Relative to 0 min value. †Relative to respective P-selectin-deficient value.

4, and 8 h after challenge. Fig. 6 (bottom) also illustrates that there was absolutely no leukocyte rolling in the E-selectin- and P-selectin-deficient vessels at 0, 4, or 8 h after allergen challenge. Consistent with this observation is the lack of leukocyte adhesion and emigration at 0, 4, or 8 h of OVA exposure (data not shown). The lack of emigration at 8 h in the E-selectin- and P-selectin-deficient animals is consistent with no leukocyte-endothelial cell interactions throughout the 8 h time point.

For completeness, the same experiments were also carried out in E-selectin-deficient mice (Fig. 7). After 4 or 8 h of antigen challenge in these animals, leukocyte rolling (top), adhesion (middle), and emigration (bottom) were indistinguishable from wild-type controls.

Fig. 8 illustrates the rolling velocity profiles for wild-type animals under control conditions and wild-type animals,

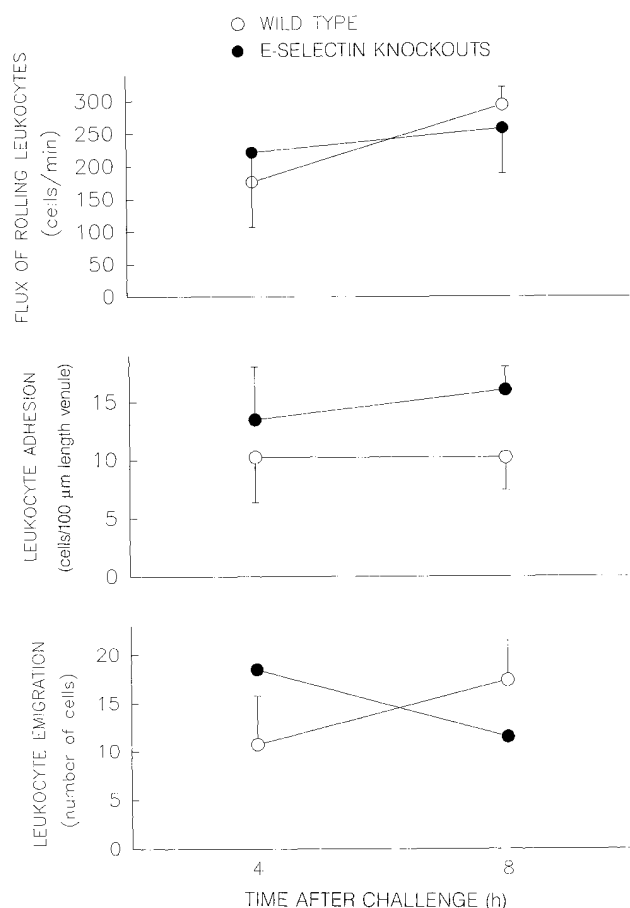


Figure 7. This figure illustrates the flux of rolling leukocytes (*top*), leukocyte adhesion (*middle*), and leukocyte emigration (*bottom*) in sensitized wild-type controls ($n = 9$) and sensitized E-selectin-deficient mice ($n = 6$) at 4 and 8 h after antigen challenge. There is no difference in either leukocyte rolling flux, adhesion, or emigration between the two groups.

P-selectin-deficient, and E-selectin-deficient animals at 4 h after antigen challenge. On average leukocyte rolling velocity was not different between wild-type animals and any of the experimental groups at either 0 or 4 h. In each series of animals, >90% of the leukocytes rolled between 30–60 μ m/sec, regardless of whether they were rolling on P-selectin (A, B, and C) or E-selectin (D) with a contribution from α_4 -integrin (D).

Total circulating leukocyte counts were significantly elevated in P-selectin-deficient mice ($13.7 \pm 1.5 \times 10^6/\text{ml}$) and E-selectin- and P-selectin-deficient mice ($16.5 \pm 1.4 \times 10^6/\text{ml}$) when compared to control animals ($3.3 \pm 0.6 \times 10^6/\text{ml}$). Leukocyte counts in the E-selectin-deficient mice ($2.9 \pm 1.4 \times 10^6/\text{ml}$) were not significantly different from control animals. All leukocyte counts were performed at the end of each experiment. Administration of the anti- α_4 -integrin antibody, the anti-P-selectin antibody, or the anti-E-selectin antibody had no effect on blood leukocyte counts in either the control animals or the P-selectin-deficient animals. All animals used were properly sensitized as assessed by anti-OVA antibody titres of at least 1:64.

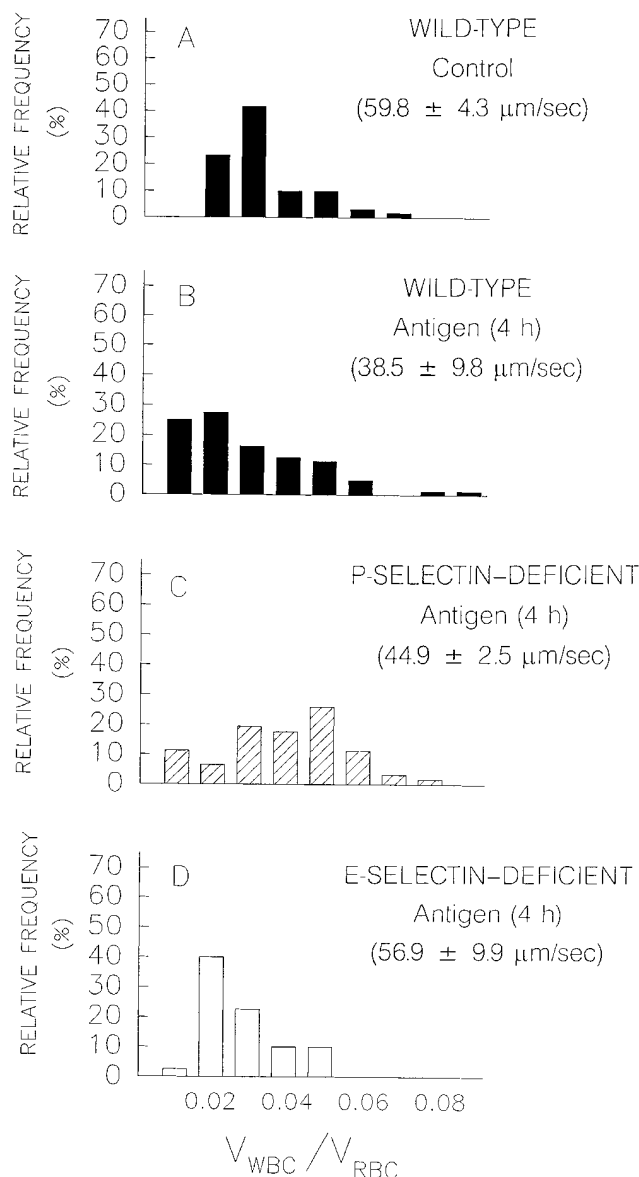


Figure 8. These data illustrate the leukocyte rolling velocity profiles in sensitized wild-type animals under control conditions and sensitized wild-type animals, P-selectin-deficient, and E-selectin-deficient animals at 4 h after antigen challenge. The velocity of each leukocyte (V_{WBC}) was expressed relative to centerline V_{RBC} . The numbers in parentheses indicate the average leukocyte rolling velocity in each series of experiments. Leukocyte rolling velocity did not differ between any of the experimental groups.

Discussion

The late phase reaction in response to allergens is characterized by the recruitment of numerous leukocyte populations that are thought to ultimately cause the tissue injury associated with various pathological conditions including asthma and various skin disorders (15, 27–30). It is becoming apparent that the leukocytic infiltrate is dependent upon various adhesion molecules including the selectins and various integrins. Antibodies directed against P-selectin, E-selectin, and α_4 -integrin have all been reported to attenuate leu-

kocyte infiltration into various tissues or have prevented tissue injury (edema formation) in allergic models (19, 29, 31, 32). Although these experiments suggest an important role for each of these adhesion molecules to the recruitment of leukocytes and ultimate tissue injury, a major limitation of these studies is the inability to determine the actual mechanism(s) and times at which the different adhesion molecules contribute to the multi-step recruitment process. We report herein the development of an allergic model of inflammation which permits us to visualize leukocyte recruitment into regions undergoing a late phase response associated with type I hypersensitivity. We define a role for P-selectin, E-selectin, and the α_4 -integrin in antigen-induced leukocyte recruitment, and also highlight the subtle interrelationships that exist between these adhesive molecules.

In this study, we report that P-selectin is essential for all of the leukocyte rolling at 8 h after allergen challenge in sensitized tissues. This contention is based on the view that addition of a P-selectin antibody or a selectin-binding carbohydrate completely prevented leukocyte rolling at this time point. Moreover, the postcapillary venules of P-selectin-deficient mice also displayed a lack of leukocyte-endothelial cell interactions at the 8 h time point. This suggests that leukocyte recruitment at 8 h after allergen challenge is unequivocally P-selectin mediated. Although initial baseline rolling (11, 33, 34) and acute mediator-induced leukocyte rolling (20–22) are thought to be P-selectin-dependent, more prolonged leukocyte recruitment associated with cytokines or use of intraperitoneal thioglycollate have been thought to be E-selectin-dependent, based on the original work in the P-selectin-deficient mice (11). However, Subramaniam et al. (31) recently observed reduced leukocyte recruitment in a delayed-type hypersensitivity response (24 h) in P-selectin-deficient mice. Although the authors postulated that the late effect could have reflected an early defect, i.e., the sensitization phase, or defective infiltration in the first few hours of the response, our data clearly demonstrate that P-selectin plays a critical role as late as 8 h after antigen challenge. Therefore, the profound reduction in leukocyte recruitment into challenged tissues observed by Subramaniam et al. (31), could be partially related to P-selectin-dependent leukocyte recruitment certainly as late as 8 h, and perhaps even at 24 h after allergen challenge. Indeed, our observation that P-selectin is very important in the long-term effect of leukocyte recruitment is consistent with transcriptional regulation of P-selectin by various cytokines and perhaps reuse of previously endocytosed P-selectin (5, 35–37), and challenges the view that E-selectin completely replaces P-selectin with time of inflammation.

Unlike P-selectin-deficient mice, the E-selectin-deficient animals failed to show any reduction in rolling at 8 h or at a time (4 h) when its upregulation is predicted. These data are consistent with the work of Labow et al. (38), who have reported a lack of phenotype for E-selectin-deficient mice in numerous inflammatory models including a delayed-type hypersensitivity reaction. Data from E-selectin antibody studies have also failed to provide significant pro-

tection against leukocyte-mediated injury in several inflammatory models including indomethacin-induced gastric mucosal injury in the rat (39), a primate model of spontaneous chronic colitis (40), skeletal muscle ischemia/reperfusion injury (41), and immune complex-mediated nephritis in the rat (42). By contrast a handful of studies in skin and lung have revealed a role for E-selectin in leukocyte recruitment, raising the possibility that E-selectin may recruit leukocytes to only a few tissues (32, 43, 44). This latter statement must be tempered by our observation of a critical role for E-selectin in recruiting a significant number of leukocytes in P-selectin-deficient vessels during a late phase response. Nevertheless, the expression and rolling was transient inasmuch as it was never observed at the 8 h time point. Interestingly, however, an E-selectin-dependent pathway was sufficient to gather as many leukocytes into P-selectin-deficient tissue as all of the adhesion molecules recruited in wild-type tissue. This highlights the importance of being able to visualize leukocyte behavior in inflamed vessels inasmuch as histology would have derived the wrong conclusion that leukocyte influx or emigration into postantigen challenged tissues at 8 h was not dependent on P-selectin based on the results from P-selectin-deficient mice.

Interestingly, the P-selectin- and E-selectin-mediated rolling in this *in vivo* study did not differ in the magnitude of the rolling velocity despite observations *in vitro* that E-selectin and P-selectin may differ in this regard (45, 46). Rather than the type of selectin, we feel that it is more likely that the leukocyte rolling velocity is dictated by the number of binding sites between the leukocyte and endothelium and perhaps the activation state of the cells. Indeed, numerous site density studies (45, 47, 48) have revealed that high binding site density for any selectin will increase the number of rolling cells as well as decrease the rolling velocity. Additionally, we have demonstrated *in vivo* (16, 49) and *in vitro* (50, 51) that inhibiting CD18 directly, or inactivating chemotactic agents that activate CD18, will reverse the reduction in leukocyte rolling velocity. For example, P-selectin-dependent rolling velocity *in vivo* can be very slow in response to leukotriene C_4 (22) but not histamine (20), suggesting that the mediator may also be important. It is conceivable that particular proinflammatory mediators induce the rapid synthesis of chemotactic agents including platelet-activating factor, which may then reduce leukocyte rolling velocity. Although a direct comparison between E-selectin- and P-selectin-dependent rolling velocity is impossible in our study without knowing the specific number of receptors expressed, we report that the rolling velocity did not differ when cells rolled on E-selectin in P-selectin-deficient mice or on P-selectin in E-selectin-deficient mice. A direct comparison of leukocyte rolling velocity *in vitro* on P-selectin versus E-selectin incorporated into lipid bilayers at similar densities suggested that cells rolled more slowly on P-selectin (45).

In this study, we clearly demonstrate a role for α_4 -integrin as a mediator of leukocyte rolling at 8 h (not 4 h) after allergen challenge. This is consistent with an important role

for this adhesion molecule in the recruitment of leukocytes in late phase reactions (18, 19, 29, 52). Moreover, the observation is also consistent with previous reports that α_4 -integrin is capable of supporting leukocyte rolling in vivo (6, 53). The novelty of our study was the fact that we could examine the interplay between α_4 -integrin and the selectins. This revealed that the α_4 -integrin-mediated leukocyte rolling was in turn entirely dependent upon endothelial selectins. This conclusion is supported by the fact that α_4 -integrin-mediated rolling was eliminated in animals deficient in P-selectin or in animals treated with fucoidin or an anti-P-selectin antibody. We also observed α_4 -integrin-dependent leukocyte rolling in the P-selectin-deficient animals at 4 h after antigen challenge. However, this interaction was entirely dependent upon E-selectin inasmuch as deleting E-selectin in the P-selectin-deficient animals i.e., by immunoneutralization or E-selectin and P-selectin deletion at the genetic level, again eliminated the α_4 -integrin-dependent rolling. Therefore, this study clearly demonstrates that α_4 -integrin-dependent rolling will not occur independent of the selectins in postcapillary venules of the inflamed cremaster muscle.

At first glance it may be difficult to explain how two different adhesion molecules (a selectin and α_4 -integrin) might support the same event i.e., leukocyte rolling. However, there is a growing body of evidence that leukocyte rolling per se can be divided into at least two mechanistically separate components; the initial capturing of the leukocyte is referred to as tethering and the second event is thought to be rolling (1). To date, this has been demonstrated for L-selectin and E-selectin in flow chambers in vitro i.e., the capturing or tethering of leukocytes to E-selectin was dependent on L-selectin (47). However, if L-selectin-deficient cells

were permitted to settle on E-selectin thereby bypassing the initial tethering event and flow in the chamber was resumed, these cells could then roll at very high shear rate. We propose the same mechanism for the selectins and the α_4 -integrin. We would predict that the selectins tether or capture leukocytes because (a) α_4 -integrin is far less effective at tethering leukocytes to its ligand VCAM-1 (0.7 dyn/cm²) than selectins are to their ligands (>1 dyn/cm²), and (b) α_4 -integrin can support rolling at even very high shear forces despite its inability to capture at these shear conditions (4). As the shear stress was much higher than 1 dyn/cm² in the inflamed cremasteric postcapillary venules, the view that it was the selectins rather than α_4 -integrin that tethered the cells to the endothelium permitting the integrin to then mediate rolling is supported. Although the possibility cannot be excluded that selectins and α_4 -integrin work cooperatively to tether and then allow leukocytes to roll, our data are conclusive that selectins are essential for α_4 -dependent rolling in the late phase reaction.

In conclusion, we illustrate that endothelial selectins, primarily P-selectin, are essential for α_4 -dependent leukocyte rolling in chronically inflamed venules in vivo. In P-selectin-deficient mice, E-selectin is induced transiently to recruit leukocytes. Nevertheless, a critical role for P-selectin resumes at 8 h. This study unequivocally illustrates that selectins are critical for the initiation of events leading to leukocyte recruitment regardless of the induction of subsequent rolling via other routes, including the α_4 -integrin pathway. Clearly, despite the importance of α_4 -integrin in asthma and associated pathologies, targeting the selectins may still be an efficient means of controlling leukocyte infiltration during a late phase reaction.

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