

The α_4 -Integrin Supports Leukocyte Rolling and Adhesion in Chronically Inflamed Postcapillary Venules In Vivo

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Summary

A role for the α_4 -integrin ($\alpha_4\beta_1$ or $\alpha_4\beta_7$), has been implicated in the recruitment of peripheral blood mononuclear cells (PBMCs) to sites of inflammation. However, the adhesive interactions (i.e., tethering, rolling, and adhesion) mediated by the α_4 -integrin have not been characterized in vivo. The objective of this study was to establish a model wherein postcapillary venules were chronically inflamed, and then use intravital microscopy to identify the adhesive interactions mediated by the α_4 -integrin in vivo. Between 4 and 20 d after immunization with *Mycobacterium butyricum*, animals developed a systemic vasculitis characterized by large increases in the numbers of rolling and adhering leukocytes within mesenteric venules. The selectins could only account for $\sim 50\%$ of the leukocyte rolling whereas the remaining cells rolled exclusively via the α_4 -integrin. Anti- α_4 therapy also eliminated the increase in leukocyte adhesion observed in this model, whereas selectin therapies and an anti-CD18 (β_2 -integrin) monoclonal antibody (mAb) did not reduce adhesion. A serum against polymorphonuclear leukocytes (PMNs) was used to confirm that a significant proportion of rolling cells, and most of the adhering cells were PBMCs. Sequential treatment with anti-PMN serum and the anti- α_4 mAb demonstrated that α_4 -dependent rolling was distinct from PMN rolling populations. Initial leukocyte tethering via the α_4 -integrin could not be demonstrated in this model, whereas L-selectin did support leukocyte tethering. These data suggest that the α_4 -integrin can mediate both rolling and adhesion in the multistep recruitment of PMBCs in vivo, and these interactions occur independently of the selectins and β_2 -integrins.

There is a wealth of information describing the recruitment of PMNs from the mainstream of blood to sites of extravascular injury. This multistep process is initiated by the tethering of PMNs to the endothelium, followed by weak transient adhesive interactions manifested as leukocyte rolling, leading ultimately to firm adhesion of PMNs to the vessel wall (1–4). If an appropriate chemotactic stimulus is present, firm adhesion then allows leukocytes to transmigrate across the vessel wall to sites of potential injury (5, 6). Different mechanisms appear to mediate PMN rolling and adhesion; rolling is dependent on selectins expressed on endothelium (P-selectin, E-selectin) and leukocytes (L-selectin), whereas firm adhesion is dependent on the binding of leukocyte integrins (CD11/CD18) to their ligands (intercellular adhesion molecule 1 [ICAM-1], etc.) found on endothelial cells (3, 4, 7, 8). Intravital microscopy has played a critical role in establishing the molecular mechanisms underlying PMN rolling and adhesion as it provides a window for the direct observation of PMN behavior within intact postcapillary venules. However, because of a lack of chronic inflammatory models amenable to intravital microscopy, there is very little information regarding the adhesive

mechanisms that regulate the influx of other leukocytes such as PBMCs into postcapillary venules.

There is a growing body of evidence implicating an important role for α_4 -integrin complexes ($\alpha_4\beta_1$ or $\alpha_4\beta_7$) in the recruitment of PBMCs into sites of inflammation. $\alpha_4\beta_1$ is expressed on a large proportion of PBMCs, and mediates adhesion of these cells to cytokine-activated endothelium in vitro via the counterreceptor vascular cell adhesion molecule 1 (VCAM-1)¹ (9–12). In vivo studies have demonstrated that ¹¹¹indium-labeled rat lymphocyte subpopulations migrate via α_4 -integrins to inflammatory sites in the skin in response to cytokines or delayed-type hypersensitivity reactions (13, 14), and to arthritic joints of rats treated with complete Freund's adjuvant (14). Numerous in vivo and in vitro studies have demonstrated that dual anti- α_4 and anti-CD18 therapy provided an additive inhibition of PBMC infiltration, raising the possibility that the two adhesion molecules function in tandem for optimal move-

¹Abbreviations used in this paper: fMLP, N-formyl-met-leu-phe; VCAM-1, vascular cell adhesion molecule 1.

ment of PBMC into tissues (11, 15–17). Assuming that PBMCs, like PMNs, infiltrate tissues via a multi-step process that may engage numerous adhesive mechanisms, an obvious shortcoming of the aforementioned studies is the inability to determine at which point in the cascade (tethering, rolling, or adhesion) the α_4 -integrin intervenes. This may be particularly important in that in vitro studies using flow conditions raise the possibility of an unexpected versatility for the α_4 -integrins; PBMCs have been shown to tether, roll, and adhere via α_4 -integrins to isolated VCAM-1 or VCAM-1-transfected L cells (18–20). Increasing the complexity of the substratum to cytokine-treated endothelium gave more ambiguous results; antibodies against the α_4 -integrins inhibited firm adhesion of PBMCs but failed to affect the initial tethering and/or rolling interaction in some but not all studies (19–21). These somewhat discrepant observations make it difficult to extend these in vitro studies to the in vivo setting wherein the α_4 -integrin may or may not function as a tethering, rolling, and/or adhesion molecule.

Clearly, there is a need to assess the role of α_4 -integrins in postcapillary venules at potential sites of inflammation. Since acutely inflamed vessels support primarily PMN-endothelial cell interactions (22, 23), the visualization of α_4 -dependent interactions (tethering, rolling, and adhesion) has not been documented using existing models of intravital microscopy. Therefore, the first objective was to establish a model wherein postcapillary vessels would support α_4 -dependent interactions, and then to determine whether this adhesion molecule contributes to leukocyte tethering, rolling, and/or adhesion in vivo. In this study, we describe the development of a systemic vasculitis 4–20 d after animals are immunized with complete Freund's adjuvant. This condition is hallmarked by a tremendous increase in the number of tethering, rolling, and adhering leukocytes within mesenteric postcapillary venules. Approximately 50% of these cells were PBMCs, and interacted with the venular endothelium independently of the selectins or the β_2 -integrin. Antibody studies revealed that the α_4 -integrin mediated at least two pathways in the multi-step recruitment of PBMCs in vivo; both rolling and adhesion (but not tethering) were mediated by the α_4 -integrin, and these interactions could occur independently of selectins or the β_2 -integrins, respectively.

Materials and Methods

Adjuvant Immunization. Under light anesthetic (diethyl ether; BDH Inc., Toronto, ON, Canada), male Sprague-Dawley rats (175–275 g) were injected subcutaneously at the base of the tail with a solution of heat killed *Mycobacterium butyricum* in Freund's mineral oil adjuvant (0.75 mg *M. butyricum* in 0.1 ml adjuvant; both from Difco Laboratories Inc., Detroit, MI). This protocol has been used as a model of arthritis in other laboratories and is described in detail elsewhere (14, 16, 24). The animals were monitored closely and weighed at 4-d intervals. Preliminary experiments using intravital microscopy (described below) revealed a tremendous increase in leukocyte trafficking through postcapillary venules of the mesenteric connective tissue at 4, 8, 12, and 20 d after immunization. One group of animals was immunized with

0.1 ml of mineral oil without *M. butyricum*. Leukocyte trafficking in this group was indistinguishable from untreated animals, therefore immunized animals were compared directly with untreated controls.

Intravital Microscopy. Rats were maintained on a purified laboratory diet and fasted for 18–24 h before surgery. Animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (65 mg/kg body weight). The right carotid artery and jugular vein were cannulated to measure systemic arterial blood pressure (pressure transducer model P23XL; Viggo-Spectramed, Oxnard, CA; and model 7 physiologic recorder; Grass Instruments Co., Quincy, MA) and administer drugs and antibodies, respectively. After laparotomy, rats were placed in a supine position on an adjustable Plexiglass™ microscope stage and a segment of the mid-jejunum was exteriorized and prepared for intravital microscopy. Briefly, the mesentery was placed over an optically clear viewing pedestal and exposed tissues were covered with saline-soaked gauze to minimize dehydration. The temperature of the pedestal was maintained at 37°C with a constant temperature water circulator (Haake Fisons, Karlsruhe, Germany) and the rats were kept at 37°C using an infrared heat lamp. The exposed mesentery was suffused with warmed bicarbonate-buffered saline (pH 7.4) using a peristaltic pump (Minipuls3; Villiers Le Bel, Gilson, France), while excess fluid was removed via a suction pump. This preparation has been used extensively by us and others to study leukocyte-endothelial cell interactions within the mesenteric circulation (1, 25–28).

The mesenteric preparation was observed through an intravital microscope (Optiphot-2; Nikon Inc., Mississauga, Canada) with a $\times 25$ objective lens (Wetzlar L25/0.35; E. Leitz Inc., Munich, Germany) and a $\times 10$ eyepiece. A video camera (model 5100 HS; Panasonic, Osaka, Japan) mounted on the microscope projected the image onto a color monitor (model PVM 2030; Sony, Tokyo, Japan), and the images were recorded using a videocassette recorder (model AG-1790; Panasonic, Osaka, Japan) for subsequent playback analysis. The final magnification of the image on the monitor was $\times 1800$. Single unbranched mesenteric venules (25–50 μm in diameter) were selected for study. The same section of venule was observed throughout the experiment to control for variations between different regions. Venular diameter (D_v) was measured on-line using a video caliper (Microcirculation Research Institute, Texas A&M University, College Station, TX). Centerline red blood cell velocity (V_{rbc}) was also measured on-line 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 ($V_{\text{mean}} = V_{rbc}/1.6$) 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 $\dot{\gamma} \times$ blood viscosity (η), where η was assumed to be 0.025 poise (29).

The number of rolling and adherent leukocytes was determined off-line during video playback analysis. Leukocytes were considered adherent to the venular endothelium if they remained stationary for a period of time equal to or exceeding 30 s. Rolling leukocytes were defined as those white blood cells that moved at a velocity less than that of erythrocytes within a given vessel. The flux of rolling leukocytes was determined as the number of white blood cells that rolled past a fixed point in the venule during a 1-min interval using frame-by-frame analysis. Leukocyte rolling velocity was calculated from the time required for a given leukocyte to travel a fixed distance along the length of the venule. Leukocyte tethering was measured as the number of new endothelial interactions that were initiated within a given segment of venule during a 1-min time interval.

Experimental Protocols. Upon locating a mesenteric venule 25–50 μm in diameter, the image was recorded for 5 min. Additional 5-min recordings were made at 15-min intervals over a 60-min period and revealed little or no change in hemodynamic parameters or leukocyte kinetics throughout the experiment. Therefore, antiadhesion molecule therapies were administered after two baseline recordings (at the 20-min time point) so that each animal served as its own control. Initially, we examined leukocyte trafficking through mesenteric postcapillary venules at 4, 8, 12, or 20 d after immunization. No animals were allowed to survive past 20 d due to the development of severe arthritis and other systemic complications. Leukocyte kinetics appeared to be identical at each of the days after treatment with *M. butyricum*, and therefore we undertook to examine the adhesive mechanisms involved at day 4 to avoid unnecessary discomfort related to the development of arthritis between days 8 and 20.

Initially, we characterized the importance of L- and P-selectin in the increased leukocyte influx. The anti-P-selectin antibody, PB1.3 (30) (Cytel Corp., San Diego, CA) was administered at 2 mg/kg i.v. at 20 min. We have previously demonstrated that this concentration of PB1.3 was most effective at preventing P-selectin-dependent leukocyte rolling in vivo (27). To study a role for L-selectin, animals were treated with an anti-L-selectin antibody, HRL-3 (31) (Upjohn Company, Kalamazoo, MI) at 1 mg/kg i.v. at 20 min (32). An isotype-matched control antibody had no effect on leukocyte rolling in the rat mesentery (27). Additionally, we examined the role of a selectin-binding polysaccharide, fucoidin (25 mg/kg; Sigma Chemical Co., St. Louis, MO), that targets L- and P-selectin (1, 33, 34). To confirm that this concentration of fucoidin prevented selectin-dependent rolling, untreated (no *M. butyricum* immunization) animals were also given the carbohydrate moiety. This regimen has previously been shown to inhibit >85% of rolling interactions under baseline conditions (1, 25, 26).

M. butyricum-immunized animals responded only partially to anti-selectin therapy, suggesting the possibility that other rolling pathways exist in vivo. To date, in addition to selectins, only the α_4 -integrin has been proposed to support leukocyte rolling on endothelium (20). Therefore, in another series of experiments, immunized animals received an antibody directed against the α_4 -integrin chain (TA-2, 4 mg/kg). This antibody has been shown to block lymphocyte adhesion to cytokine-stimulated rat endothelial cells in vitro (14, 35), and attenuate lymphocyte accumulation in rat dermal sites during delayed-type hypersensitivity reactions, without causing leukocytopenia or lymphocyte aggregation (13). Evidence that TA-2 binds the α_4 -integrin has been documented extensively elsewhere (35). To ensure that this antibody did not affect leukocyte trafficking nonspecifically, untreated animals received identical doses of TA-2. To determine whether the β_2 -integrin was contributing to α_4 -dependent adhesion, a rat anti-CD18 antibody (WT-3, 2 mg/kg) was given to animals (36,

37). At this concentration, WT-3 inhibits leukocyte adhesion induced by *N*-formyl-met-leu-phe (fMLP) (37).

Since α_4 -integrins are not found on circulating neutrophils (8), it was likely that a nonneutrophil leukocyte population was interacting with endothelium in *M. butyricum*-immunized animals. To confirm this possibility, animals were given an anti-PMN serum (0.1 ml/rat; Accurate Chemical & Scientific Corp., Westbury, NY) that depletes circulating PMN populations, without affecting other leukocyte subpopulations. To confirm that the α_4 -dependent population of leukocytes was distinct from the PMNs, some animals were treated with the anti-PMN serum and then received the anti- α_4 antibody after depletion of PMNs. In a final experiment, animals were treated with fucoidin, and once a new baseline flux was achieved, the animals received the anti- α_4 antibody.

Systemic blood samples were obtained at the beginning and end of each experiment for determination of total cell counts and population distributions.

Statistical Analysis. All values are reported as means \pm SEM. The data within groups were compared using a paired Student's *t* test with Bonferroni corrections for multiple comparisons where appropriate. An unpaired Student's *t* test was used to compare between groups. Statistical significance was set at $P < 0.05$.

Results

Hemodynamic measurements, including red blood cell velocity, calculated venular shear stress, and shear rates of control and *M. butyricum*-immunized rats are summarized in Table 1. These parameters remained stable throughout the 60-min protocol in both control and immunized rats. Additionally, venular diameters did not change throughout the 60-min protocols (data not shown). The red blood cell velocities and shear measurements did not differ significantly between any of the groups. Immunized animals did not show any macroscopic signs of disease until day 8, at which point tissue necrosis was observed around the injection site. By day 12, some joint swelling was apparent and increased by day 20. Immunized animals also exhibited reduced weight gain compared with controls (data not shown).

Despite the lack of any clinical evidence for disease 4 d after immunization, the flux of rolling leukocytes through the mesenteric venules was profoundly elevated from less than 30 cells/min in untreated animals to more than 150 cells/min in *M. butyricum*-treated animals (Fig. 1 A). This value varied between ~ 120 and 230 cells/min with different lots of *M. butyricum* and mineral oil adjuvant. The increased flux of rolling leukocytes was maintained at elevated

Table 1. Hemodynamic Parameters of Untreated and *M. butyricum*-immunized animals

Parameter	Control ($n = 7$)	<i>M. butyricum</i> immunized			
		Day 4 ($n = 10$)	Day 8 ($n = 4$)	Day 12 ($n = 4$)	Day 20 ($n = 6$)
RBC velocity (mm/s)	2.4 \pm 0.3	2.1 \pm 0.3	2.1 \pm 0.3	2.0 \pm 0.2	1.9 \pm 0.3
Shear stress (dyn/cm ²)	9.6 \pm 1.4	7.0 \pm 1.1	8.0 \pm 1.9	7.0 \pm 1.0	6.8 \pm 1.1
Shear rate (s ⁻¹)	383 \pm 57	281 \pm 44	320 \pm 74	279 \pm 40	270 \pm 45

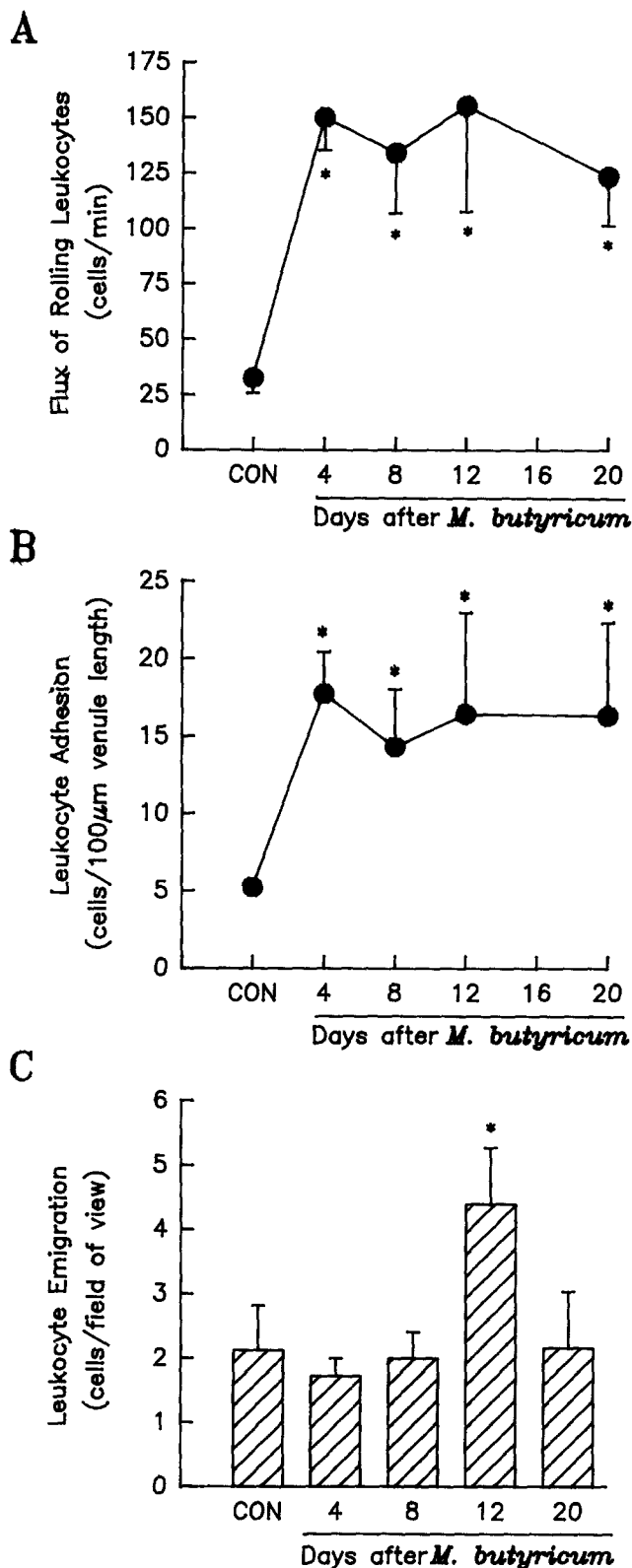


Figure 1. Leukocyte (A) rolling flux, (B) adhesion, and (C) emigration in mesenteric postcapillary venules of untreated (CON, $n = 7$) and *M. butyricum*-immunized (4–20 d) animals. Leukocyte trafficking in immunized animals was examined 4 ($n = 10$), 8 ($n = 4$), 12 ($n = 4$), or 20 ($n = 6$) d after treatment with heat-killed *M. butyricum* in Freund's mineral oil adjuvant. Values represent the average leukocyte flux and adhesion

levels until at least 20 d after immunization. *M. butyricum* treatment appears to be a very potent inducer of leukocyte rolling inasmuch as we have not observed more than 70 rolling cells/min in the rat mesenteric vasculature under control or stimulated (leukotrienes, histamine, oxidants, mast cell activation) conditions (27, 28, 38, 39). Fig. 1 B also demonstrates that 4, 8, 12, and 20 d after *M. butyricum* treatment, a significant number of rolling cells stopped and adhered firmly to the venular endothelium relative to very few adherent cells (~ 5 cell/100- μm -length venule) in untreated animals. The peak number of adherent cells was observed at day 4, reaching an average of 18 cells/100- μm -length venule. This is comparable to values previously observed after acute exposure of the mesentery to proinflammatory molecules such as PAF or LTB₄ (40, 41). It is interesting to note that very few of the adherent cells emigrated into the surrounding interstitium in immunized animals at any time point during the 20-d period after treatment (Fig. 1 C). At day 12, a slight increase in emigration was observed (4–5 cells), nevertheless, this was of much lower magnitude than the 20–40 cells that transmigrated after administration of inflammatory mediators such as platelet activating factor, leukotriene B₄, or fMLP (40, 41).

Table 2 summarizes circulating leukocyte numbers in control and *M. butyricum*-immunized animals. In untreated animals, ~ 30 and 70% of circulating leukocytes were PMNs and PBMCs, respectively. 4 d after immunization with *M. butyricum*, the total number of circulating leukocytes was increased by 53%. The increase could be entirely attributed to PMNs inasmuch as there was a four-fold increase in these cells and a reduction in the number of circulating mononuclear cells. This reduction in PBMCs may be related to an increased marginated pool inasmuch as the anti- α_4 mAb did increase circulating mononuclear cell numbers (data not shown). Differential analysis revealed a change in the distribution of cells in *M. butyricum*-immunized animals; $\sim 70\%$ of the leukocytes were PMNs whereas PBMCs accounted for 30% of the population. This distribution was maintained 20 d after treatment.

As leukocyte and hemodynamic parameters reached new baseline levels by 4 d after immunization, and remained constant for the next 20 d, we chose to focus the remainder of this study on the adhesive mechanisms underlying leukocyte trafficking 4 d after immunization with *M. butyricum*. Fig. 2 is a photomicrograph illustrating the high level of leukocyte rolling and adhesion observed in *M. butyricum*-immunized animals after exteriorization of the rat mesenteric microcirculation (A) compared with the few rolling or adhering leukocytes observed in mesenteric venules of untreated control preparations (B). Fig. 3 shows that over a 60-min experiment, the mesenteric microcirculation in *M. butyricum*-treated (4 d) rats supported significantly elevated numbers

observed during the first 60 min after exteriorization of the mesentery on the indicated day after immunization. Leukocyte emigration reflects the initial emigration observed upon exteriorization of the mesentery on the indicated day after immunization. (*) $P < 0.05$ relative to untreated group.

Table 2. Cell Counts and Distributions in Untreated and *M. butyricum*-immunized Animals

	Total count ($\times 10^5$ /ml)	% PMN distribution	PMN count ($\times 10^5$ /ml)	% PBMC distribution	PBMC count ($\times 10^5$ /ml)
Control ($n = 7$)	71.7 \pm 4.8	28.2 \pm 1.8	20.2 \pm 1.4	71.8 \pm 1.8	51.5 \pm 3.4
<i>M. butyricum</i>					
Day 4 ($n = 10$)	110.3 \pm 7.6*	71.8 \pm 3.3*	79.2 \pm 5.5*	28.2 \pm 3.3*	31.1 \pm 2.1*
Day 20 ($n = 6$)	146.1 \pm 8.6*	67.0 \pm 1.7*	97.9 \pm 5.8*	33.0 \pm 1.7*	48.2 \pm 2.8

* $P < 0.05$ with respect to control.

of rolling (A), and adherent leukocytes (B), but no increase in the number of emigrated leukocytes (C), relative to untreated control animals. Elevated leukocyte rolling was stable over 60 min in immunized animals, whereas elevated leukocyte adhesion increased further by 15 min and then remained stable for the remainder of the experiment. These observations are important inasmuch as stability in leukocyte kinetics by 15 min permitted the use of each animal as its own control in subsequent experiments where the effects of various antiadhesive molecules were examined.

Fig. 4 summarizes the role of selectins in the leukocyte rolling observed in *M. butyricum*-treated animals. An anti-P-selectin antibody (PB1.3) reduced leukocyte rolling by <25%, whereas an anti-L-selectin antibody (HRL-3) was more effective, reducing leukocyte rolling by 43% (from 150 to 85 cells/min). The inset illustrates the pattern of inhibition of leukocyte rolling; after steady elevated levels of rolling (time 0 and 15 min), HRL-3 was administered (20 min) and the number of rolling cells remaining was determined at 30 and 60 min of the protocol. The data reveal that a rapid reduction in leukocyte rolling flux could be achieved with HRL-3, and that this reduction was maintained for the subsequent 30 min. This pattern was consistent for each of the anti-selectin therapies used. Fucoidin, a P- and L-selectin binding carbohydrate, reduced leukocyte rolling by 52% (Fig. 4). It is important to note that the same concentration of fucoidin reduced leukocyte rolling in untreated animals by >90%, to a flux of 3 cells/min (Table 3). In immunized animals treated with fucoidin, 70 cells/min still rolled through the mesenteric venules, suggesting that 4 d of exposure to heat-killed *M. butyricum* evokes new pathways of leukocyte rolling in vivo.

Qualitatively, after fucoidin administration, there was a noticeable decrease in the size of the rolling cells, suggesting that the remaining rolling leukocytes were a distinct population of white cells. To test this hypothesis, immunized animals were treated with an anti-PMN serum. Fig. 5 demonstrates that the anti-PMN serum reduced leukocyte rolling to the same degree as fucoidin (~50%) and again revealed a greater proportion of smaller cells rolling. Confirmation that the anti-PMN serum was only affecting PMNs is illustrated in Table 4. The anti-PMN serum reduced the number of circulating PMNs by ~88%, without affecting the PBMC population. Higher doses of the anti-PMN serum

caused reductions in systemic blood pressure and blood flow and therefore were not used (data not shown). In untreated animals, the same regimen reduced leukocyte rolling by ~75%, to less than 10 cells/min (Table 3).

Based on recent in vitro work by numerous investigators, there is growing evidence that the α_4 -integrin can support non-PMN (i.e., PBMC) rolling and adhesion (18–21). Indeed, administration of an anti- α_4 antibody (TA-2) to *M. butyricum*-immunized animals caused a 50% reduction in the flux of rolling leukocytes within minutes of administration, and this rolling continued to wane until at 60 min >65% of the leukocyte rolling was eliminated (Fig. 6). These data illustrate for the first time the importance of the α_4 -integrin as an adhesive mechanism that can mediate the rolling of endogenous leukocytes along the venular endothelium in a chronic inflammatory model. This observation again differed significantly from untreated animals in which baseline leukocyte rolling was unaffected by the anti- α_4 mAb (Table 3).

These data raise the possibility that PMNs use primarily selectins to roll in postcapillary venules, whereas PBMCs roll primarily via the α_4 -integrin in vivo. To provide further evidence that there were two distinct populations of cells rolling via two distinct mechanisms, tandem therapy was administered to some animals. Fig. 7 demonstrates a 57% reduction in leukocyte rolling with the anti-PMN serum (given at 20 min), which was inhibited further to >88% when the anti- α_4 mAb was given at 65 min. Although the data do not add up to exactly 100%, an explanation for this may be related to the incomplete (~88%) removal of circulating PMNs with the antiserum. Tandem therapy with fucoidin plus TA-2 (Fig. 8) eliminated leukocyte rolling in *M. butyricum*-treated animals. After fucoidin treatment, ~70 cells/min rolled past a fixed point in the venule, however subsequent addition of the anti- α_4 antibody completely eliminated leukocyte rolling. These data demonstrate that some leukocytes can roll in vivo via a selectin-independent, α_4 -dependent mechanism, and that selectins are not obligatory to maintain rolling.

In addition to rolling, it is now well appreciated that the α_4 -integrin can support firm adhesion in vitro (18–21). Fig. 9 demonstrates that the increased number of adherent leukocytes observed in *M. butyricum*-immunized animals was eliminated by the anti- α_4 mAb, consistent with previous in vitro data (35). Administration of the anti-PMN serum or

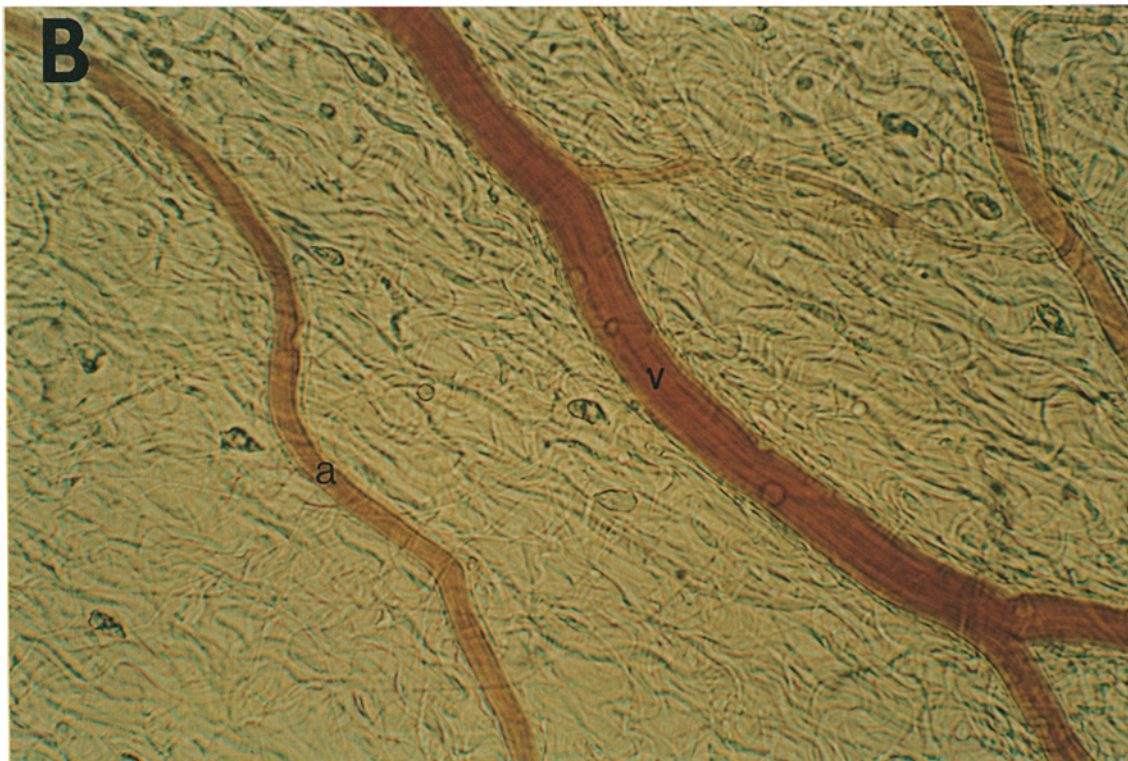
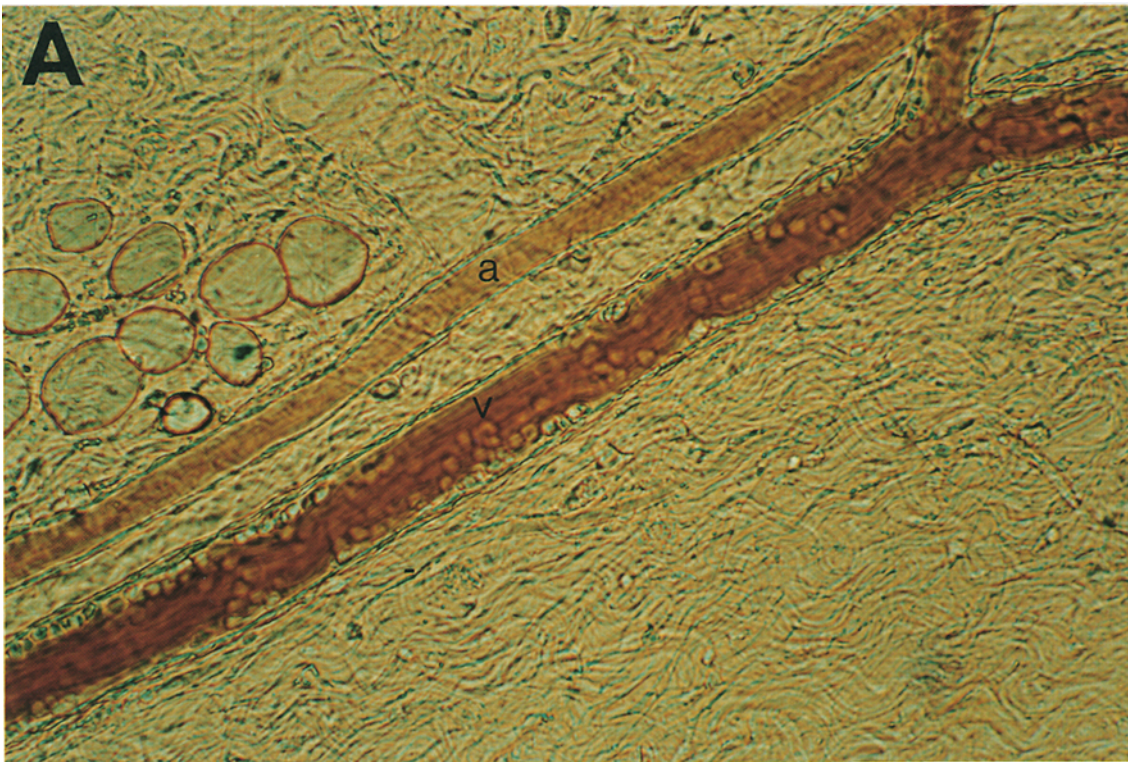


Figure 2. Representative photomicrographs of mesenteric preparations in (A) *M. butyricum*-immunized (4 d after treatment), and (B) untreated animals. In the immunized animal, >150 cells rolled or adhered to the venular wall. 11 leukocytes can be seen rolling through the venule from the untreated preparation. Playback analysis (frame-by-frame) is necessary to quantitate the number of rolling and adherent leukocytes within a 100- μ m-length vessel segment. Leukocytic influx is observed in venules (*v*) but not arterioles (*a*). $\times 315$.

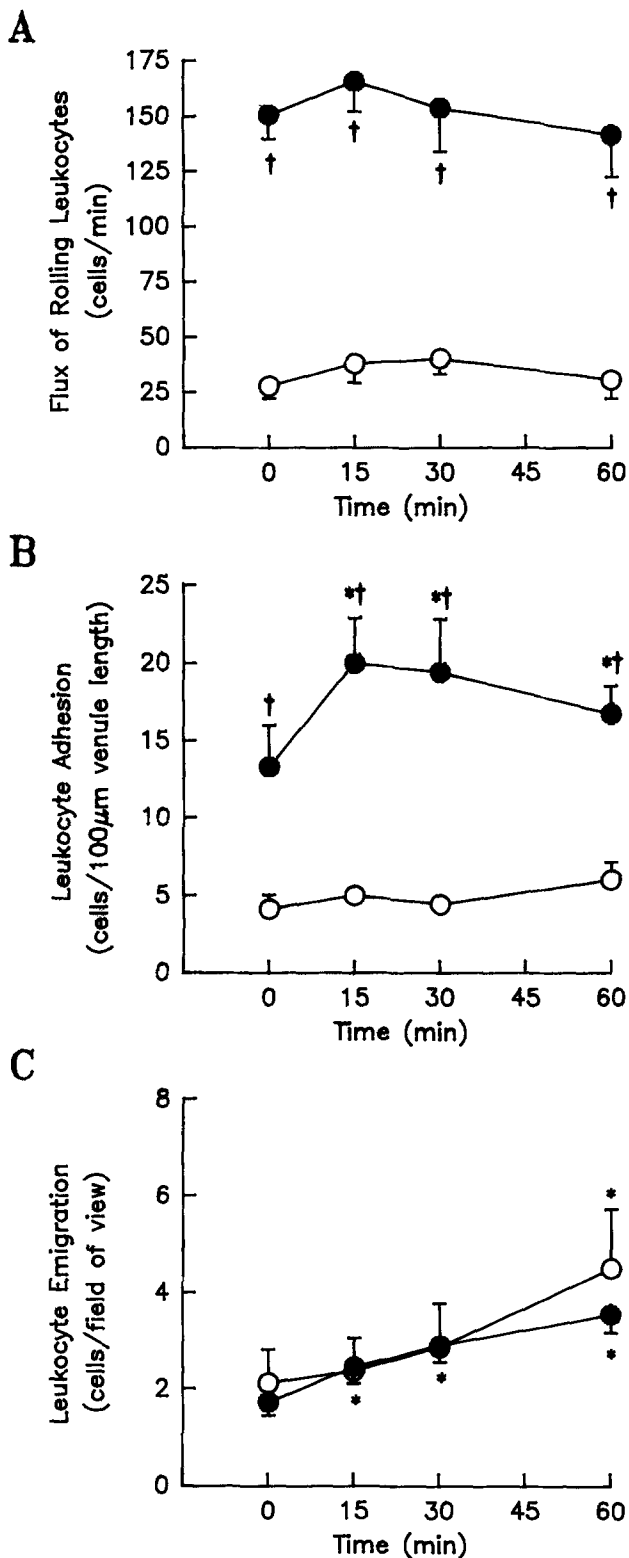


Figure 3. Leukocyte (A) rolling flux, (B) adhesion, and (C) emigration in mesenteric postcapillary venules of untreated (open circles, $n = 7$) and *M. butyricum*-immunized (closed circles, $n = 10$) animals 4 d after treatment. Leukocyte rolling flux in *M. butyricum*-immunized animals remained stable over the 60-min protocol, and was elevated relative to untreated animals. Leukocyte adhesion was elevated relative to untreated animals and

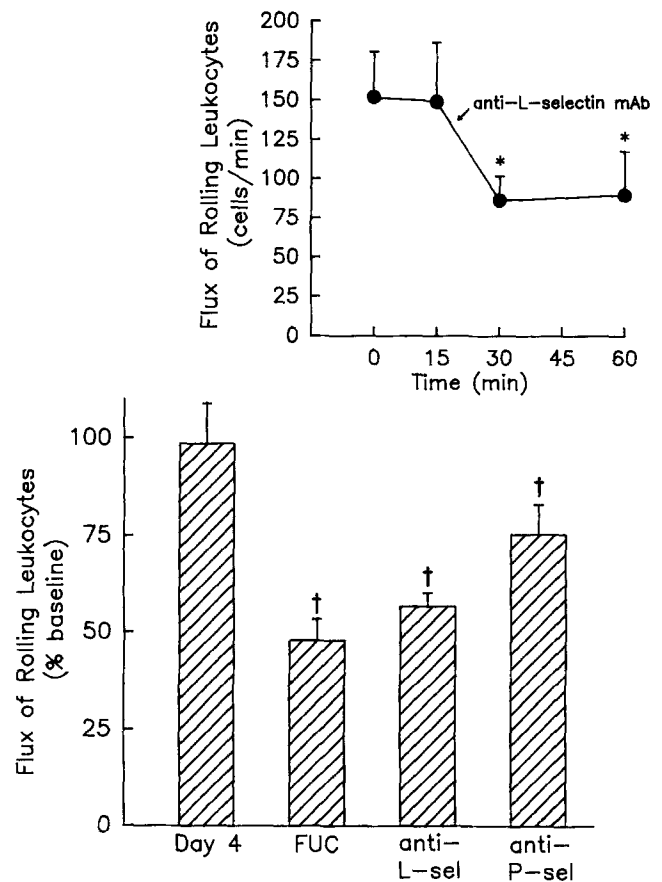


Figure 4. Effect of anti-selectin therapy on leukocyte rolling flux in mesenteric venules of *M. butyricum*-immunized (4 d) animals. Immunized animals were treated with fucoidin (FUC, 25 mg/kg; $n = 8$), an anti-L-selectin mAb (HRL-3, 1 mg/kg; $n = 5$), or an anti-P-selectin mAb (PB1.3, 2 mg/kg; $n = 4$) 20 min into a 60-min protocol. (Inset) Administration protocol used for all treatments. In this case, administration of HRL-3 at 20 min caused a rapid and stable reduction in rolling flux, allowing the treatment time points to be compared with the initial baseline time points within each animal. The other treatments produced similar response patterns (not shown). (*) $P < 0.05$ relative to time 0 min. (†) $P < 0.05$ relative to immunized animals not receiving therapy.

an anti-CD18 mAb (WT-3) at 20 min had no effect on leukocyte adhesion in this chronic model (Fig. 9). Leukocyte adhesion was not affected by any of the three different anti-selectin treatments (data not shown).

Leukocyte rolling has recently been demonstrated to consist of two distinct events (42), the first step, the initial attachment or tethering of leukocytes to the endothelium and the second step, the actual rolling event. Both L-selectin and the α_4 -integrin have been postulated to play a critical role in leukocyte tethering in vitro (20, 42). Fig. 10 demonstrates a role for L-selectin, but not the α_4 -integrin in leukocyte tethering in vivo. Treatment with an anti-L-selectin mAb reduced the number of tethering interactions

increased further by 15 min before remaining stable. Emigration increased slightly in both groups during the 60-min experiments. (*) $P < 0.05$ relative to time 0 min. (†) $P < 0.05$ relative to untreated group.

Table 3. Leukocyte Rolling Flux in Control Animals Receiving Antiadhesion Molecule Therapy

	Flux of rolling leukocytes (cells/min)
Control (<i>n</i> = 7)	34.5 ± 7.5
plus fucoidin (25 mg/kg; <i>n</i> = 4)	3.0 ± 1.0*
plus anti-PMN serum (0.1 ml/rat; <i>n</i> = 5)	9.8 ± 2.4*
plus anti- α_4 mAb (4 mg/kg; <i>n</i> = 3)	33.0 ± 4.5

* *P* < 0.05 with respect to control.

by ~50%. Treatment with the anti- α_4 mAb however, did not cause a significant reduction in the number of tethering interactions. After the administration of the anti- α_4 mAb, a very distinct pattern of leukocyte-endothelial cell interactions was observed. A certain proportion of leukocytes made very brief (<1 s) contacts with the vessel wall but then detached instead of rolling, giving the appearance of a glancing, stop-and-go interaction. Both PMNs and PBMCs tethered in the postcapillary venules inasmuch as the anti-PMN serum reduced the number of tethering interactions by only 60% (Fig. 10).

The velocity of rolling leukocytes was unaffected by any of the treatments administered in this study (Fig. 11), suggesting that PMNs and PBMCs roll at similar velocities despite the differences in adhesive mechanisms employed. Additionally, the behavior of PBMCs was very similar to that previously described for PMNs. Cells always appeared to roll along the endothelium before adhering firmly.

Discussion

In this manuscript we describe for the first time, the importance of the α_4 -integrin as an adhesion molecule that

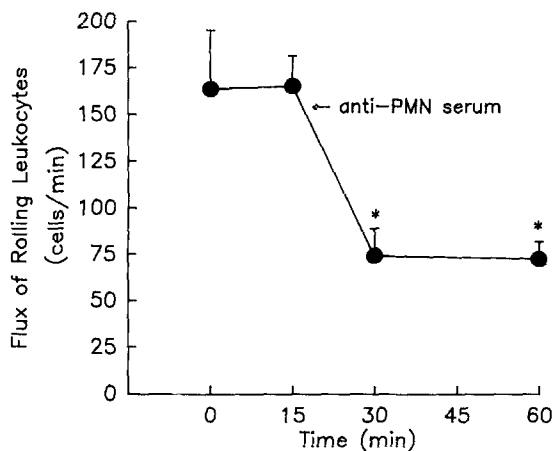


Figure 5. Leukocyte rolling flux in *M. butyricum*-immunized animals treated at 20 min with an anti-PMN serum (0.1 ml/rat; *n* = 7). This serum reduced the number of circulating PMNs by 88% without depleting PBMC populations (see Table 4). (*) *P* < 0.05 relative to time 0 min.

Table 4. Cell Counts in *M. butyricum*-immunized Animals Treated with Anti-PMN Serum (*n* = 7)

	Total count ($\times 10^5$ /ml)	PMN count ($\times 10^5$ /ml)	PBMC count ($\times 10^5$ /ml)
Day 4 <i>M. butyricum</i>	102.9 ± 9.0	72.9 ± 6.4	30.0 ± 2.6
plus anti-PMN serum (0.1 ml/rat)	40.6 ± 3.8*	8.4 ± 0.8*	32.2 ± 3.0

* *P* < 0.05 with respect to day 4 *M. butyricum* treatment.

supports both PBMC rolling and adhesion under physiologic shear stress (4–16 dyn/cm²) in a model of chronic inflammation. The study also documents that the α_4 -integrin can support leukocyte rolling independent of selectins, inasmuch as fucoidin did not inhibit the α_4 -integrin-dependent leukocyte rolling. These observations question the obligatory requirement for selectins in the recruitment of leukocytes to sites of inflammation. A similar conclusion is reached with respect to adhesion inasmuch as an anti- α_4 antibody entirely prevented the adhesion of cells without the necessity of further reinforcement from other adhesion molecules such as the β_2 -integrins. Clearly unlike selectins or other integrins, the α_4 -integrin was sufficiently versatile to independently support two distinguishable steps of the multi-step recruitment paradigm i.e., rolling and adhesion. Finally, our study demonstrates that the level of α_4 -integrin expression present on endogenous cells is sufficient to support PBMC rolling and adhesion at physiological shear rates, but was not sufficient to support the initial tethering interaction in vivo.

It is well established that virtually all of the leukocyte rolling observed under baseline or acute inflammatory conditions can be eliminated by anti-L-selectin or anti-P-selectin antibodies or by the selectin-binding polysaccharide fucoidin (1, 27, 43). In contrast, a very significant number of

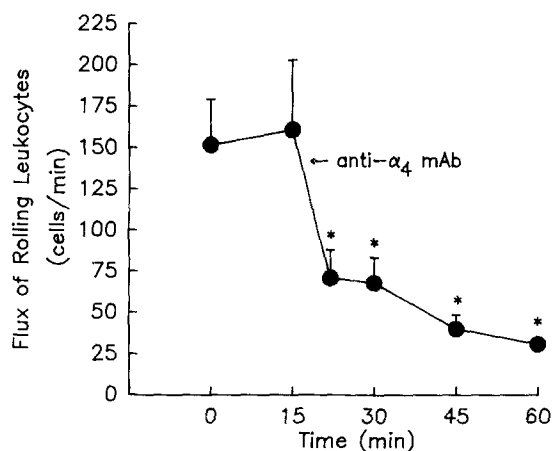


Figure 6. Leukocyte rolling flux in *M. butyricum*-immunized animals treated at 20 min with an anti- α_4 mAb (TA-2, 4 mg/kg; *n* = 4). (*) *P* < 0.05 relative to time 0 min.

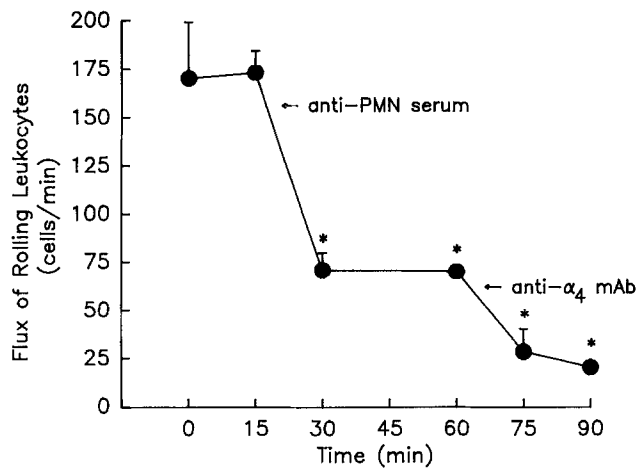


Figure 7. Leukocyte rolling flux in *M. butyricum*-immunized animals treated at 20 min with an anti-PMN serum (0.1 ml/rat) followed at 65 min with an anti- α_4 mAb (TA-2, 4 μ g/kg; $n = 3$). (*) $P < 0.05$ relative to time 0 min.

rolling cells in postcapillary venules of *M. butyricum*-immunized animals were resistant to these therapies and dependent entirely on the α_4 -integrin. These α_4 -dependent rolling cells were also insensitive to an anti-PMN serum, whereas $>85\%$ of all circulating PMNs and $\sim 75\%$ of leukocyte rolling could be eliminated by this antiserum under baseline conditions. These data suggest that the α_4 -integrin-dependent rolling cells were not PMNs but more likely PBMCs.

Presently, it remains technically impossible to more specifically identify the type of PBMC that was rolling via α_4 -integrins in vivo. Circulating monocytes, eosinophils, and T cells (but not neutrophils) all are known to express α_4 -integrins (35, 44, 45), with memory T cells expressing higher levels than naive CD4⁺ T cells (35). However, it does not appear that α_4 -integrins necessarily support identical behavior in each of these cell populations. Luscinskas et al. (21) reported that monocytes that express both L-selectin and

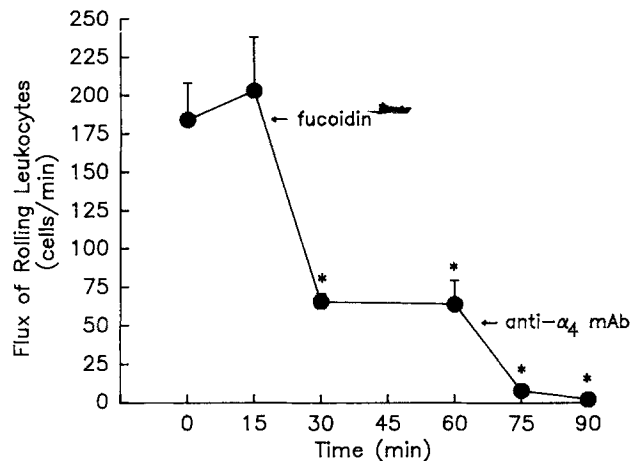


Figure 8. Leukocyte rolling flux in *M. butyricum*-immunized animals treated at 20 min with fucoidin (25 mg/kg) followed at 65 min with an anti- α_4 mAb (TA-2, 4 mg/kg; $n = 4$). (*) $P < 0.05$ relative to time 0 min.

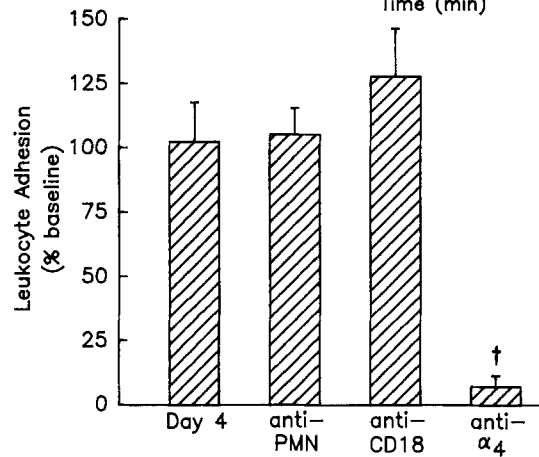
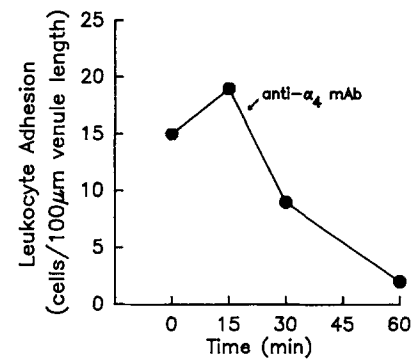


Figure 9. Leukocyte adhesion in *M. butyricum*-immunized animals treated at 20 min with an anti-PMN serum (0.1 ml/rat; $n = 7$), an anti-CD18 mAb (WT-3, 2 mg/kg; $n = 4$), or an anti- α_4 mAb (TA-2, 4 mg/kg; $n = 4$). (Inset) Administration protocol used for all treatments. In this case, administration of TA-2 at 20 min caused a rapid reduction in leukocyte adhesion. The treatment time points were compared with the initial baseline time points within each animal. The other treatments did not affect leukocyte adhesion. (†) $P < 0.05$ relative to immunized animals not receiving therapy.

$\alpha_4\beta_1$ only used the former to tether and roll in vitro, whereas $\alpha_4\beta_1$ was used primarily for adhesion. Sriramarao et al. (46) demonstrated that human eosinophils rolled in rabbit IL-1-stimulated mesenteric venules. mAbs against the α_4 -integrin and L-selectin each inhibited rolling by $\sim 50\%$, suggesting a contribution of both adhesion molecules to eosinophil rolling. However, in these IL-1-treated venules, adhesion was not observed, making it impossible to establish whether the α_4 -integrin was important in the adhesion of these leukocytes to postcapillary venules. In flow chambers, T cells were observed to roll and adhere to VCAM-1 transfectants, and both processes were inhibited by antibodies to the α_4 - or β_1 -integrins (18, 20). In contrast, IL-1-treated endothelium supported T cell tethering and rolling that was independent of the α_4 -integrin (19). The distinctions observed in these studies may reflect differences in the density or avidity of the α_4 -integrin as well as differences in the expression of ligands for the α_4 -integrins, and make it difficult to extend these data to the role of the α_4 -integrin in chronically inflamed postcapillary venules. In our study, we demonstrate that the α_4 -integrin can support both rolling and subsequent adhesion of PBMCs, in a manner

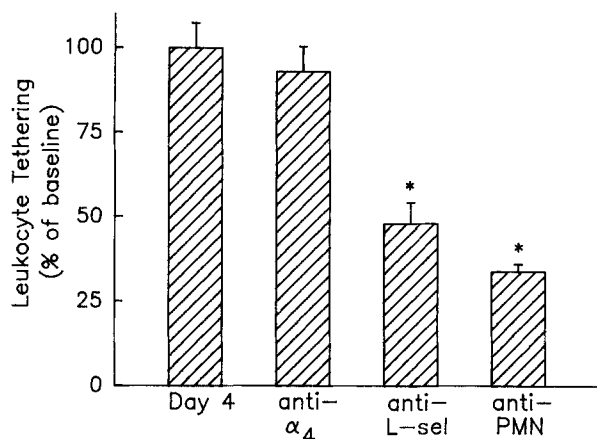


Figure 10. Leukocyte tethering in *M. butyricum*-immunized animals treated with an anti- α_4 mAb (TA-2, 4 mg/kg; $n = 4$), an anti-L-selectin mAb (HRL-3, 1 mg/kg; $n = 4$), or an anti-PMN serum (0.1 ml/rat; $n = 4$). Tethering was measured as the number of leukocyte interactions initiated within a 100- μm venule segment over a 1-min period. (*) $P < 0.05$ relative to immunized animals not receiving treatment.

qualitatively and quantitatively identical to selectin-dependent rolling and CD18-dependent adhesion previously reported for neutrophils. Clearly, the α_4 -integrin could conceivably play the role of both selectins and integrins in the recruitment of PBMCs into inflamed microvessels during the pathogenesis of chronic inflammatory disease.

The anti- α_4 antibody was also able to eliminate PBMC adhesion, whereas the anti-selectin regimens and an anti-CD18 antibody (WT-3) failed to reduce leukocyte adhesion. Clearly, the adhesive mechanisms in *M. butyricum*-treated animals are distinct from the acute inflammatory condition wherein we and others have documented that essentially all of the leukocyte adhesion was CD18 dependent (7, 28, 37, 47). To our knowledge, this is the first documentation that the α_4 -integrin can support both roll-

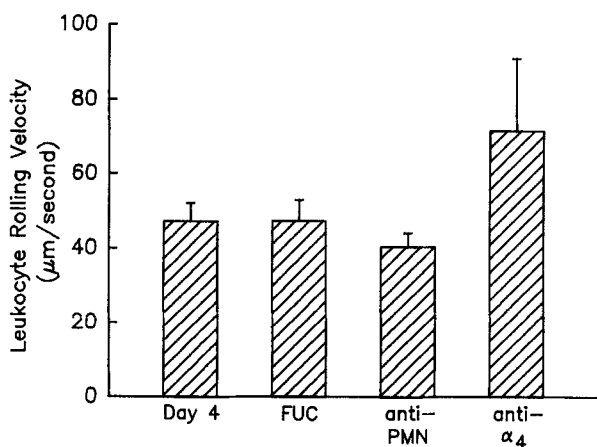


Figure 11. Leukocyte rolling velocity in venules of day 4 *M. butyricum*-immunized animals ($n = 10$), and immunized animals treated with fucoidin (FUC, 25 mg/kg; $n = 8$), an anti-PMN serum (0.1 ml/rat; $n = 7$), or an anti- α_4 mAb (TA-2, 4 mg/kg; $n = 4$). No statistically significant differences were found between different groups.

ing and firm adhesion of endogenous leukocytes at physiologic shear in postcapillary venules in vivo. A provocative finding in this study is that in postcapillary venules 4 d after *M. butyricum* immunization, there was a very selective signal for PBMCs to adhere despite very similar numbers of rolling PMNs and PBMCs. Whether this reflects the particular type of endothelial ligand involved (abundance of VCAM-1 versus lack of ICAM-1), the release of a specific chemotactic agent, or whether it reflects the overproduction of some endogenous antiadhesive molecule (prostacyclin or nitric oxide) specific for neutrophils remains unknown. However this observation clearly demonstrates the exquisite control of the vasculature in recruiting PBMCs versus PMNs in chronic inflammatory processes.

Tethering of leukocytes to the endothelium and rolling of leukocytes along the length of the endothelium have recently been shown to be separable steps that involve distinct ligands. For example, Lawrence et al. (42) demonstrated that L-selectin is required for efficient tethering to purified E-selectin, but is not required for subsequent rolling on this ligand. The α_4 -integrin has also been postulated to be an important tethering molecule; T lymphocytes, as well as a K562 erythroleukemia cell line transfected with $\alpha_4\beta_1$, tethered, rolled, and adhered to recombinant VCAM-1 under flow conditions in vitro (20). Herein, we examined the tethering phenomenon in an in vivo model of inflammation by counting the number of tethering interactions (new attachments) that transpired in 25–50- μm -diameter vessels with shear forces ranging from 4 to 16 dyn/cm^2 . An anti-L-selectin antibody significantly reduced the number of initial contacts that leukocytes made with the endothelium within a specific region under observation, demonstrating for the first time that L-selectin is an important tethering molecule under physiologic shear in postcapillary venules. This observation also suggests that tethering is not due simply to the displacement of leukocytes towards the vessel wall by red blood cells but is mediated specifically by adhesive mechanisms.

It is interesting to note that when an anti- α_4 antibody was administered to *M. butyricum*-immunized animals, the rolling was selectively attenuated whereas the initial brief interactions between cells and endothelium could still be observed. These attachments were manifested as glancing stop-and-go interactions where the obvious impairment was the inability of tethered cells to engage in prolonged rolling interactions. At first glance, these data do not appear to be entirely consistent with the in vitro data. However, it should be noted that tethering to isolated VCAM-1 via $\alpha_4\beta_1$ was observed at 0.73 dyn/cm^2 , but not at higher shear (20). In the same study, when the wall shear stress was increased to 1.8, 3.6, or 7.3 dyn/cm^2 the cells that had tethered at low shear were able to sustain a rolling interaction, but tethering itself did not occur under these physiologic shear conditions. This supports our observation that the α_4 -integrin, expressed at levels that could support PBMC rolling, did not support tethering in vivo. In this respect, it is noteworthy to point out that Jones et al. (19) observed α_4 -dependent tethering and rolling of PBMCs on VCAM-1 transfectants at 1.9 dyn/cm^2 , but could not prevent tethering

and rolling with the same anti- α_4 antibody on cytokine stimulated endothelium. This latter observation raises the possibility that tethering could occur via α_4 -integrin in vessels expressing higher levels of VCAM-1, or in smaller vessels at lower shear.

There are a few other noteworthy characteristics regarding PBMC recruitment in postcapillary venules. Removal of rolling PMNs with the anti-PMN serum permitted us to examine the rolling behavior of PBMCs in postcapillary venules. Although the cells appeared smaller, they rolled with the same velocity as PMNs. This was evident from treatment with the anti- α_4 mAb which likely eliminated the rolling PBMCs; the remaining cells also rolled with the same velocity. These data suggest that for similar rolling ve-

locities to be observed, the on/off rate and perhaps the tensile strength of rolling interactions (48) mediated by the selectins and the α_4 -integrin were of similar duration and magnitude. Finally, the sequence of events for each subpopulation of leukocytes was similar i.e., regardless of treatment, the remaining cells rolled before adhering firmly in postcapillary venules. This observation is somewhat different from the description of leukocytes colliding and adhering within the high endothelial venules of the Peyer's patch without first rolling (49). Clearly in the current study of inflamed postcapillary venules, the cascade of events leading to firm attachment was similar for PMNs and PBMCs despite entirely different molecular mechanisms of adhesion.

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