Rat Blood Neutrophils Express Very Late Antigen 4 and it Mediates Migration to Arthritic Joint and Dermal Inflammation

By Thomas B. Issekutz,* Masayuki Miyasaka,‡ and Andrew C. Issekutz§

*From the *Departments of Medicine, Pediatrics and Immunology, University of Toronto, Toronto, Ontario, M5G 2C4 Canada; the ‡Department of Bioregulation, Osaka University Medical School, Yamadaoka, Suita, 565 Japan; and the §Departments of Pediatrics and Microbiology-Immunology, Dalhousie University, Halifax, Nova Scotia, B3J 3G9 Canada*

Summary

Blood neutrophils contribute to joint injury in human and experimental models of arthritis. Neutrophil migration out of the blood in joint inflammation involves both the CD18 (β_2) integrins and a CD18 integrin-independent pathway. To investigate this migration, radiolabeled rat blood neutrophils were used to measure neutrophil accumulation in the inflamed joints of rats with adjuvant arthritis and the role of leukocyte integrins in migration to these joints and to dermal inflammation was determined. Neutrophils migrated rapidly (<2 h) to the inflamed joints 14–18 d after immunization with adjuvant. Blocking monoclonal antibodies (mAbs) to both LFA-1 and Mac-1 together, as well as a mAb to CD18, inhibited neutrophil accumulation in the inflamed joints by 50–75%. However, migration to dermal inflammation induced by $C5a_{des\ Arg}$, tumor necrosis factor α, lipopolysaccharide, and poly-inosine:cytosine was inhibited by ~90%. Flow cytometry revealed the expression of low levels of very late antigen 4 (VLA-4) on nearly all rat blood neutrophils. Treatment with anti-VLA-4 plus anti-LFA-1 but neither mAb alone, strongly (60–75%) inhibited neutrophil accumulation in arthritic joints. This mAb combination also inhibited neutrophil migration to dermal inflammatory reactions by 30–70%. Blocking VLA-4 together with the CD18 integrins inhibited neutrophil accumulation by 95–99%, virtually abolishing neutrophil accumulation in cutaneous inflammation. A similar blockade of VLA-4 and CD18 decreased neutrophil accumulation in the inflamed joints by 70–83%, but a significant portion of the neutrophil accumulation to these joints still remained. In conclusion, rat blood neutrophils express functional VLA-4 that can mediate neutrophil migration to both inflamed joints and dermal inflammatory sites. VLA-4 appears to be able to substitute for LFA-1 in this migration and is particularly important for accumulation in inflamed joints. However, there exists an additional CD18- and VLA-4–independent pathway of neutrophil migration to arthritic joints that is not involved in acute dermal inflammation.

Migration of leukocytes out of the blood into inflammatory tissues is governed by a complex series of adhesive events involving selectins, sialomucin glycoproteins, and integrins on the leukocyte that control leukocyte attachment to the vascular endothelium (1, 2). The integrins are a family of cell surface molecules that mediate cell–cell and cell–extracellular matrix adhesion (3). Each of the integrins consists of an α/β heterodimer that binds to one or more ligands. Lymphocytes express CD11a/CD18 (LFA-1) and several β_1 integrins, including $\alpha_4\beta_1$ (CD49d/CD29, very late antigen 4 [VLA-4][1]). These receptors mediate lymphocyte adhesion to vascular endothelial cells and the migration of lymphocytes into cutaneous inflammatory reactions (4–7). Monocytes express three integrins sharing in common the β_2 (CD18) chain, $\alpha_L\beta_2$ (LFA-1), $\alpha_M\beta_2$ (CD11b/CD18, Mac-1), and $\alpha_X\beta_2$ (CD11c/CD18, p150,95), as well as the β_1 integrin, VLA-4. The CD18 integrins and VLA-4 have been shown to mediate monocyte–endothelial cell adhesion and transendothelial migration in vitro, and in vivo monocyte migration to inflammation in the skin, peritoneum, and inflamed joints (8–10). Blood neutrophils express high levels of the CD18 integrins, LFA-1, Mac-1, and p150,95, and these mediate neutrophil–endothelial adhesion and migration to inflammation in the skin, in the lung, and in ischemic reperfusion injury (11–15). There is also, however, evidence that neutrophils can infiltrate lungs,

[1]*Abbreviations used in this paper:* i.d., intradermal; poly I:C, poly-inosine: cytosine; VLA-4, very late antigen 4; ZAS, zymosan-activated serum.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/96/05/2175/10 $2.00
Volume 183 May 1996 2175–2184

joints, and the peritoneal cavity via a CD18-independent mechanism (10, 14, 16).

Recent studies have also suggested that human neutrophils may also express some β_1 integrins. Neutrophil adherence to laminin is mediated by $\alpha_6\beta_1$ (VLA-6), and these cells can bind to fibronectin via $\alpha_5\beta_1$ (VLA-5; 17, 18). During myelopoesis, neutrophils also express VLA-4, although human blood neutrophils are thought to express little or no VLA-4 (19).

Previously, our laboratory demonstrated that neutrophil migration to inflammation induced with a variety of agents (C5a, TNF-α, IL-1, and LPS) was strongly inhibited by blocking the CD18 integrins, LFA-1 and Mac-1 (13). On the other hand, the migration of blood neutrophils to inflamed joints was only partially (50–70%) CD18 dependent (16). This suggested that an alternate adhesion pathway may be responsible for part of the migration of neutrophils from the blood into these tissues. Since virtually all of the migration of monocytes into inflamed joints could be inhibited by combined blockade of the CD18 integrins and VLA-4 (9), we reasoned that neutrophils may also use VLA-4 for migrating to inflamed joints.

Our results demonstrate that rat blood neutrophils express low levels of VLA-4, and that this VLA-4 is functional. VLA-4 can mediate a part of the neutrophil migration to inflammatory reactions in the skin, and it plays an important role in the accumulation of neutrophils in inflamed joints. Furthermore, there exists an additional component of neutrophil accumulation in arthritis that is both CD18 and VLA-4 independent.

Materials and Methods

Animals. Adjuvant arthritis was induced in 6–8-wk-old inbred male Lewis strain rats by immunization on the lower back with 1.0 mg *Mycobacterium butyricum* (Difco Laboratories, Inc., Detroit, MI) in 0.1 ml mineral oil subcutaneously at the base of the tail. These animals developed polyarticular arthritis 10–11 d after immunization, and were studied between days 14 and 16.

PMN Isolation and Labeling. Rat blood neutrophils (PMNs) for migration studies were obtained using the hydroxyethyl-starch exchange transfusion technique of Williams et al. (20) as modified by us previously (13). Briefly, the total blood volume of a donor rat was exchanged using 50 ml 6% hydroxyethyl-starch-saline (Dupont Chemical Co., Dorval, Quebec, Canada). After the collection of 40–50 ml blood hydroxyethyl-starch perfusate, erythrocytes were allowed to sediment at 1 *g* and the leukocyte-rich plasma was collected. The leukocytes were recovered by centrifugation, resuspended in calcium magnesium–free Tyrode's solution plus 10% platelet-poor plasma (Tyr-10% PPP), and layered onto 63% isotonic Percoll (Pharmacia Fine Chemicals, Dorval, Quebec, Canada) layered above 74% Percoll. After centrifugation (400 *g* for 30 min), the mononuclear layer on the top of the 63% Percoll was removed, and the purified neutrophils at the 63/74% interface were recovered. This layer consisted of >98% granulocytes with minimal erythrocyte contamination and was >98% viable by Trypan blue dye exclusion. The neutrophils were washed and incubated for 10 min with 1 μCi ^{111}In oxine (Amersham, Inc., Oakville, ON, Canada) per 10^7 cells. These cells were washed twice and resuspended for i.v. injection. Each rat received 10^7 labeled PMNs carrying 2–5 × 10^5 cpm.

The functional integrity of these PMNs was demonstrated by very active accumulation of labeled cells in dermal inflammatory reactions and the retention of >95% of the label on the cells in the circulation after i.v. injection, as reported previously (21). The purified neutrophils also showed no significant (<6%) increase in Mac-1 expression as measured by immunofluorescence flow cytometry compared with PMNs in blood.

mAbs and Ab Treatment. The mAb MRC OX-42 was a kind gift from Dr. D.W. Mason (University of Oxford, Oxford, UK). It is a mouse IgG2a mAb that reacts with CD11b of rat Mac-1 and blocks its adhesive functions, as previously shown (13). It was used as the $F(ab)_2$ fragment generated by pepsin digestion. The TA-3 mAb is an IgG1 mAb that reacts with the α chain of rat LFA-1 and blocks its function (6). The TA-2 mAb is an IgG1 mAb that reacts with α_4 and blocks in vitro adhesion and in vivo lymphocyte and monocyte migration mediated by VLA-4 (8, 9, 22, 23). The WT.3 mAb is an IgG1 mAb that reacts with the β chain (CD18) of the rat β_2 integrins and blocks its function (24).

Control mAbs included 2CB4E1 (IgG2a) which was generated in our laboratory and reacts with rat neutrophils to approximately the same intensity as a saturating concentration of OX-42 and TA-3, as determined by flow cytometry. It immunoprecipitates a 65-kD polypeptide from rat leukocytes. mAb B9 (IgG1), which reacts with pertussis toxin, was also used as a nonbinding control mAb (25). Since the results with these control mAbs were comparable, these measurements were pooled. All of the mAbs were grown in ascites in mice, and ammonium sulphate–precipitated IgG was used.

The effect of the mAbs on neutrophil migration in vivo was determined by giving animals an i.v. injection of 1–2 mg TA-2, TA-3, or WT.3 IgG, or the $F(ab)_2$ fragment of OX-42, immediately before i.v. injection of labeled neutrophils. None of the mAb treatments caused neutropenia or clearance of the ^{111}In-labeled neutrophils from the circulation when compared with animals not receiving any Abs.

Dermal Inflammatory Reactions. Inflammatory reactions, which we have previously shown to induce neutrophil accumulation, were induced in rats by intradermal (i.d.) injection (13, 26). Recombinant mouse TNF-α was kindly provided by Genentech, Inc. (South San Francisco, CA). *Escherichia coli* LPS was from List Biologicals (Campbell, CA). Zymosan-activated serum (ZAS), a source of $C5a_{des\ Arg}$ chemotactic factor, was generated by activating C′ in normal rat serum with 5 mg/ml of Zymosan A (Sigma Chemical Co., St. Louis, MO) for 60 min at 37°C and removing the zymosan as described previously (27). Poly-inosine:cytosine (poly I:C) was obtained from Sigma Chemical Co.

Measurement of Neutrophil Migration. Rats previously immunized with *M. butyricum* to induce adjuvant arthritis were anesthetized and injected intravenously with ^{111}In-labeled neutrophils. Immediately afterward, the skin on the back of the animals was shaved and inflammatory stimuli in a volume of 0.05 ml were injected intradermally into several sites along with diluent controls. 2 h later, the animals were euthanized and blood was collected in acid citrate dextrose anticoagulant. The dorsal skin, including the area of dermal inflammatory reaction, was removed and frozen; the injected skin sites were punched out with a 12-mm punch and counted in a gamma spectrometer. The forelimbs were sectioned, leaving the forepaws (containing the metacarpal and phalangeal joints) and the carpal joints as separate samples for gamma counting. Similarly, the hind limbs were sectioned just above and below the tibiotalar joint, providing hindpaws (containing metatar-

sophalangeal and phalangeal joints) and talar joint samples. The red blood cells in 1 ml of blood were lysed with 0.84% NH_4Cl, the leukocytes pelleted by centrifugation, and the number of neutrophils and ^{111}In content of the cells determined. The accumulation of ^{111}In-neutrophils is expressed as the number of neutrophils $\times 10^6$ per tissue calculated from the blood neutrophil-specific activity.

Statistical Methods. Differences between groups were tested using a one-way analysis of variance and posthoc testing with the Tukey-Kramer Multiple Comparisons Test.

Results

Effect of mAbs to LFA-1, Mac-1, and CD18 on Neutrophil Accumulation in Arthritic Joints. To evaluate the role of the CD18 integrins in neutrophil accumulation in arthritic joints, animals were studied 14–18 d after immunization, when the joints were markedly inflamed. Animals received an i.v. injection of mAb OX-42 to Mac-1, mAb TA-3 to LFA-1, the combination of both of these mAbs, or mAb WT.3 to CD18, or a control mAb, and immediately afterward, an injection of ^{111}In-labeled neutrophils. Animals were killed 2 h later. As shown in Fig. 1, there was considerable neutrophil accumulation in the joints of the animals receiving the control mAb treatment with ~16 million neutrophils accumulating in the talar joint, which was the most severely affected, and 2–4 million in the other joints. Treatment with mAb to Mac-1 or to LFA-1 had no significant effect on neutrophil accumulation in any of the joints, although there was some decrease in neutrophil accumulation in the talar joints of animals treated with anti-LFA-1. The combination of mAbs to Mac-1 and LFA-1 strongly inhibited neutrophil accumulation to the large talar joints and also significantly reduced accumulation in the hindpaw, carpal, and forepaw joints. Treatment of rats with mAb to CD18 had a similar inhibitory effect. Both combined LFA-1 and Mac-1 blockade, or CD18 blockade, inhibited neutrophil accumulation in the talar joints by ~75%, and in the hindpaw and carpal joints by ~50%.

Effect of mAb to LFA-1, Mac-1, and CD18 on Neutrophil Migration to Cutaneous Inflammation. To compare the role of the CD18 integrins in neutrophil migration to inflamed joints with inflammation in the skin, the same animals injected with mAb and labeled neutrophils for the experiments in Fig. 1 were injected intradermally with stimuli to induce a dermal inflammatory reaction. As shown in Fig. 2, rats were injected with ZAS, a source of $C5a_{des\ Arg}$, TNF-α, LPS, and poly I:C, a potent cytokine inducer. Anti-Mac-1 had no effect on neutrophil accumulation to any of the inflammatory reactions. Anti-LFA-1 alone caused a small inhibition of neutrophil accumulation to poly I:C, but much more dramatic inhibition was induced by blocking the combination of both Mac-1 and LFA-1. The combination of anti-Mac-1 and anti-LFA-1 inhibited 80–90% of the neutrophil accumulation, and treatment with anti-CD18 inhibited >90% of the migration to the four inflammatory stimuli. Doubling the dose of the mAb treatment did not further suppress neutrophil accumulation. Thus, neutrophil accumulation to dermal inflammation was strongly suppressed (>90%) by blocking CD18 integrins, whereas accumulation in inflamed joints was inhibited only by 50–75%. Therefore, the contribution of VLA-4 to this latter migration was investigated.

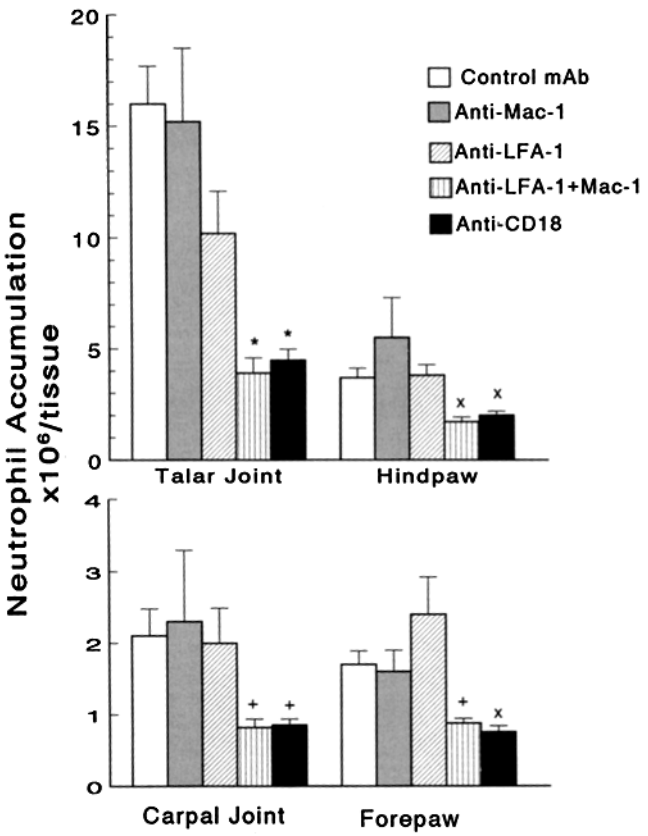

Figure 1. The effect of treatment with mAb to LFA-1, Mac-1, and CD18 on neutrophil migration to arthritic joints. Rats with polyarticular arthritis between days 14 and 16 after immunization were injected intravenously with control mAb (2CB4E1 or B9), anti-Mac-1 (mAb MRC OX-42), anti-LFA-1 (mAb TA-3), a combination of anti-LFA-1 plus anti-Mac-1, or anti-CD18 (mAb WT-3) as indicated. Immediately afterward, the animals were injected with ^{111}In-labeled blood neutrophils. 2 h later, the rats were euthanized and ^{111}In-neutrophil accumulation was determined. Values are mean ± SEM of 5–17 animals in each group. Values for background neutrophil accumulation in corresponding joints of normal nonarthritic animals were 0.34×10^6 PMN/talar joint, 0.26×10^6 PMN/hindpaw, 0.14×10^6 PMN/carpal joint, and 0.07×10^6 PMN/forepaw. $^+P < 0.05$, $^xP < 0.01$, $^*P < 0.001$.

Immunofluorescence Analysis of Neutrophil Staining with Anti-VLA-4. Fig. 3 shows that the TA-2 mAb to VLA-4 stained nearly all blood neutrophils. Although the intensity of the staining was relatively low, it was clearly greater than that of the isotype-matched negative control mAb.

Effect of mAb to VLA-4, LFA-1, and Mac-1 on Neutrophil Accumulation in Arthritic Joints. Rats were treated with anti-VLA-4, anti-LFA-1, both of these mAbs, or control mAb, and neutrophil migration to the talar, hindpaw, and carpal joints was determined (Fig. 4). Anti-α_4 by itself had no effect on neutrophil accumulation, but when combined with anti-LFA-1, it strongly inhibited neutrophil accumulation

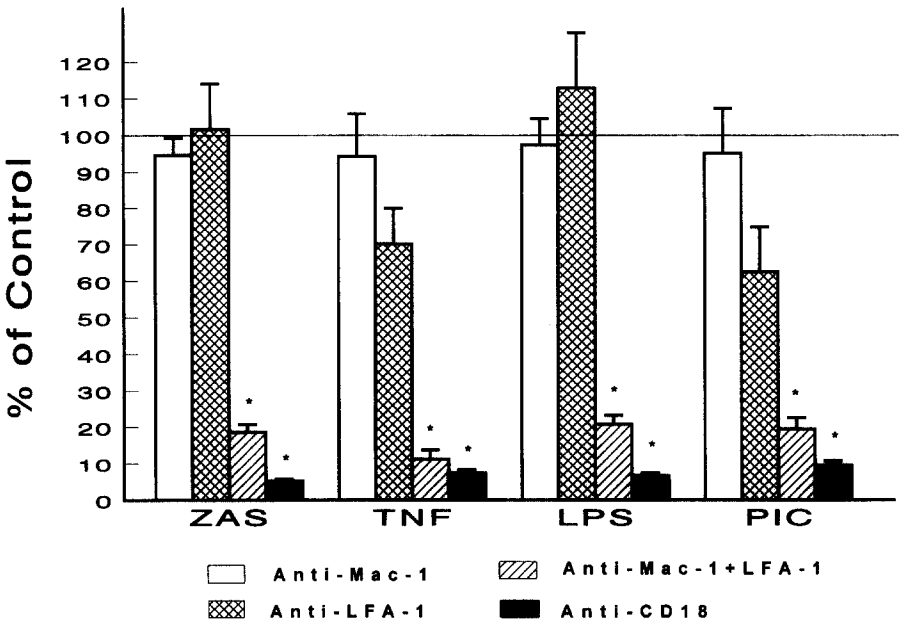

Figure 2. The effect of treatment with mAb to Mac-1, LFA-1, and CD18 on neutrophil migration to dermal inflammation. In the animals in Fig. 1, immediately after i.v. injection of the mAbs and ^{111}In-labeled neutrophils, cutaneous inflammatory reactions were induced by i.d. injection of 50% ZAS, 1 ng of endotoxin (*LPS*), 10 ng mouse TNF-α, and 200 ng poly I:C (*PIC*) per site. The labeled cells were allowed to accumulate in the skin sites for 2 h, while at the same time they were also migrating to the inflamed joints. Each bar represents the mean percentage of the control antibody-treated animals ± SEM with 5–17 animals per group. The neutrophil accumulation in the control animals was 3.65×10^6 PMN/site for ZAS, 3.08×10^6 PMN/site for TNF-α, 3.24×10^6 PMN/site for LPS, and 1.14×10^6 PMN/site for poly I:C. Values of control diluent injected sites were 0.08–0.13×10^6 PMN/site. $^*P < 0.001$.

in all three joints. The talar and carpal joints were inhibited by 75%, and the hindpaw by 60%. In all cases, blocking α_4 was more inhibitory than anti-LFA-1 alone.

Fig. 5 shows that blocking Mac-1 in combination with anti-VLA-4, unlike blocking LFA-1, did not significantly inhibit neutrophil accumulation in the inflamed joints.

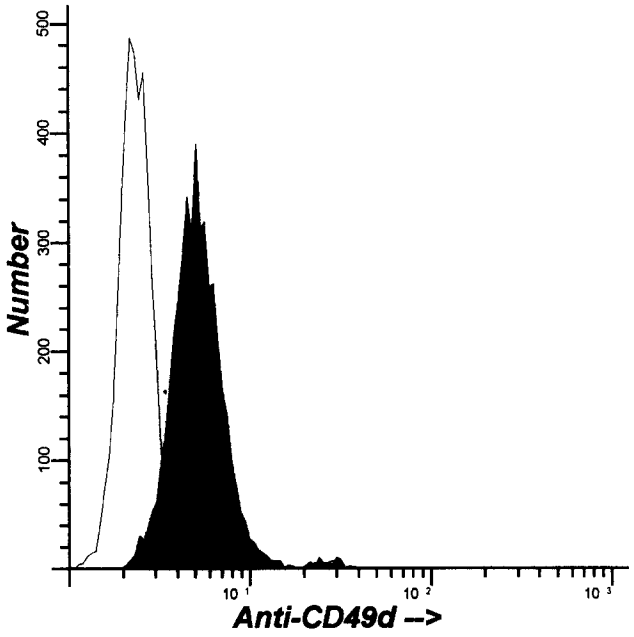

Figure 3. Flow cytometry analysis of immunofluorescence-stained rat blood neutrophils were stained with the anti-VLA-4 (*CD49d*) mAb TA-2 (*solid histogram*), or an isotyped-matched control mAb (B9), washed, and stained with streptavidin-conjugated phycoerythrin. Cells were analyzed by flow cytometry and one representative result of five similar assays is shown. The neutrophils were found to be >90% positive for staining with anti-CD49d.

There was a tendency for less neutrophil accumulation in the presence of dual Mac-1 VLA-4 blockade, but this effect was not significant.

Effect of mAbs to VLA-4 and LFA-1 on Neutrophil Accumulation in Dermal Inflammatory Reactions. The effect of blocking VLA-4 and LFA-1 on PMN migration to inflamed skin was also determined. As shown in Fig. 6, treatment with anti-α_4 had no effect on neutrophil accumulation to ZAS, LPS, and poly I:C, and produced a small decrease in migration to TNF-α. Anti-LFA-1 alone also caused only a small decrease in neutrophil accumulation to some of the lesions, whereas the combination of blocking both VLA-4 and LFA-1 inhibited neutrophil accumulation by 35–70%, with the greatest effect observed in response to TNF-α. The combination of blocking both VLA-4 and Mac-1 was no more effective than VLA-4 alone (data not shown). Although blocking both VLA-4 and LFA-1 was not as effective as inhibiting LFA-1 and Mac-1 in migration to cutaneous inflammation (Fig. 2), these results showed that VLA-4 on neutrophils could mediate part of the accumulation in cutaneous inflammation, especially when LFA-1 was blocked.

Effect of VLA-4 and Combined CD18 Blockade on Neutrophil Accumulation in Arthritic Joints. Blockade of the CD18 integrins only partially inhibited neutrophil migration to inflamed joints, therefore the effect of blocking VLA-4, as well, was also examined (Fig. 7). Blocking VLA-4, LFA-1, and Mac-1 was not different from blocking the combination of LFA-1 and Mac-1. However, the addition of anti-VLA-4 to anti-CD18 treatment further decreased neutrophil accumulation to the talar, hindpaw, and carpal joints, and this decrease was significantly greater in the case of the larger joints of the hind limbs. This combined VLA-4 and CD18 blockade inhibited neutrophil accumulation into these joints by 70–83%, and suggested that VLA-4 contributed to a component of the CD18-independent neutrophil

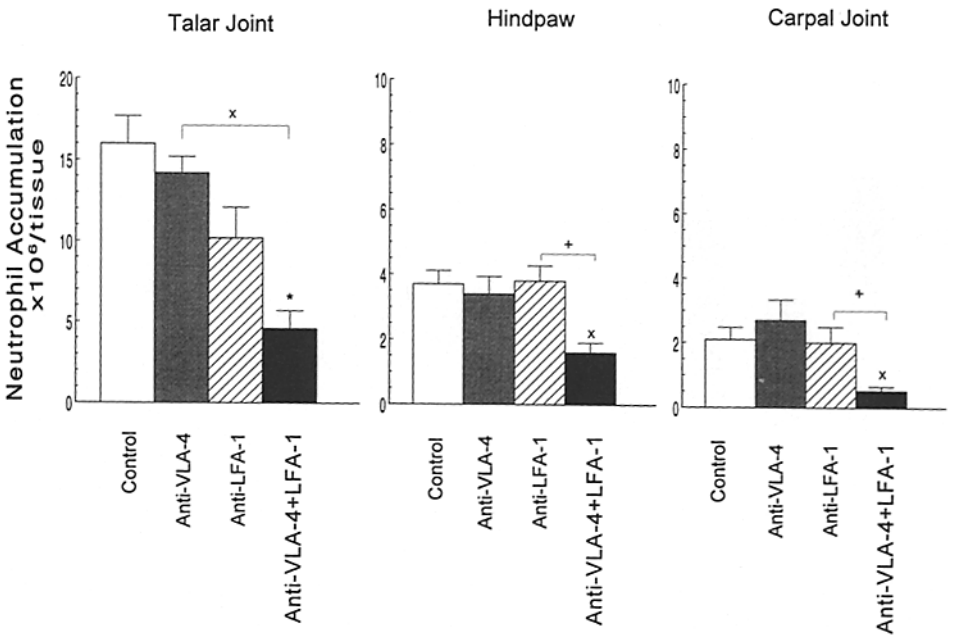

Figure 4. The effect of mAb to VLA-4 and LFA-1 on neutrophil migration to arthritic joints. Animals with arthritis as in Fig. 1 were injected intravenously with anti-VLA-4 (mAb TA-2), anti-LFA-1 (mAb TA-3), a combination of anti-VLA-4 plus anti-LFA-1, or control mAb as indicated. Immediately afterward, labeled neutrophils were given and their accumulation in the indicated joints was measured after 2 h and is expressed as in Fig. 1. Values are mean ± SEM of 5–17 animals in each group. $^{+}P < 0.05$, $^{x}P < 0.01$, $^{*}P < 0.001$.

migration into arthritic joints. Furthermore, there remained a component of ~25% that might be independent of these four integrins.

Effect of VLA-4 and CD18 Blockade on Neutrophil Accumulation in Dermal Inflammation. The effect of VLA-4 and CD18 integrin blockade on neutrophil migration to dermal inflammation is shown in Fig. 8. Treatment with anti-VLA-4 plus anti-Mac-1 and anti-LFA-1 was significantly more inhibitory than blockade of the combination of LFA-1 and Mac-1. Similarly, treatment with anti-VLA-4 and anti-CD18 was significantly more effective than blocking CD18 alone. Anti-VLA-4 plus anti-CD18 inhibited 95–98.5% of the neutrophil accumulation in the cutaneous inflammatory sites, suggesting that virtually all of the neutrophil migration was mediated by these integrins.

It should also be pointed out that in spite of this profound suppression of neutrophil accumulation in the dermal inflammatory sites, migration in these same animals to arthritic joints was only partially suppressed, and the level of circulating labeled blood neutrophils was actually slightly (~13%) increased.

Discussion

Neutrophil migration out of blood vessels is thought to involve an initial tethering and rolling adhesion to the vascular endothelium, a G–protein mediated activation of the neutrophil, and firm adhesion mediated by neutrophil integrins (1, 2). Several studies, including our own, have shown that the CD18 integrins, notably LFA-1 and Mac-1,

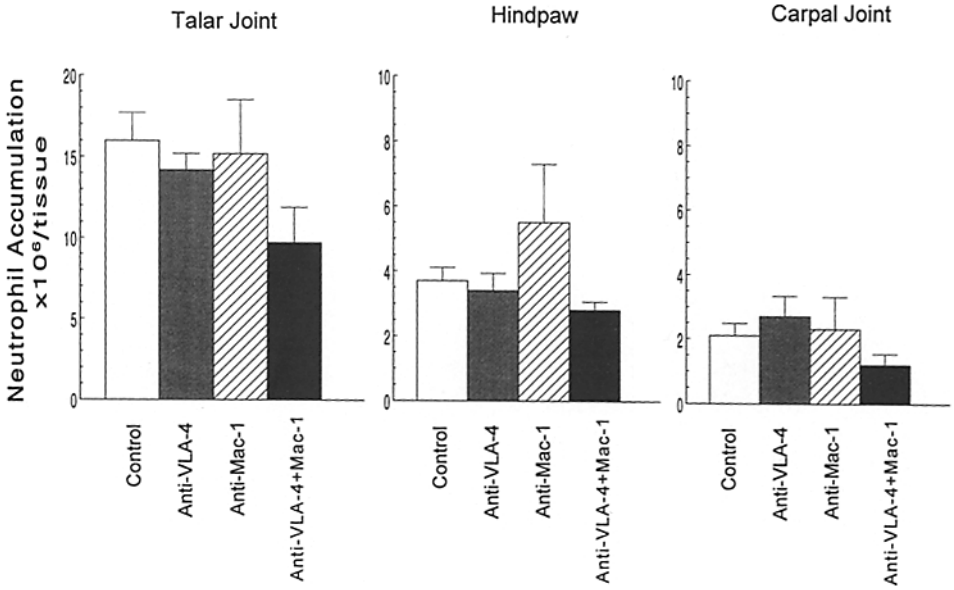

Figure 5. The effect of treatment with antibody to VLA-4 and to Mac-1 on neutrophil migration to arthritic joints. Animals with arthritis as in Figs. 1 and 4 were injected intravenously with anti-VLA-4 (mAb TA-2), anti-Mac-1 (OX-42), anti-VLA-4 plus anti-Mac-1, or control mAb. Labeled neutrophils were injected intravenously and neutrophil accumulation was measured after 2 h and is expressed as in Figs. 1 and 4. Values are mean ± SEM of 5–17 animals in each group.

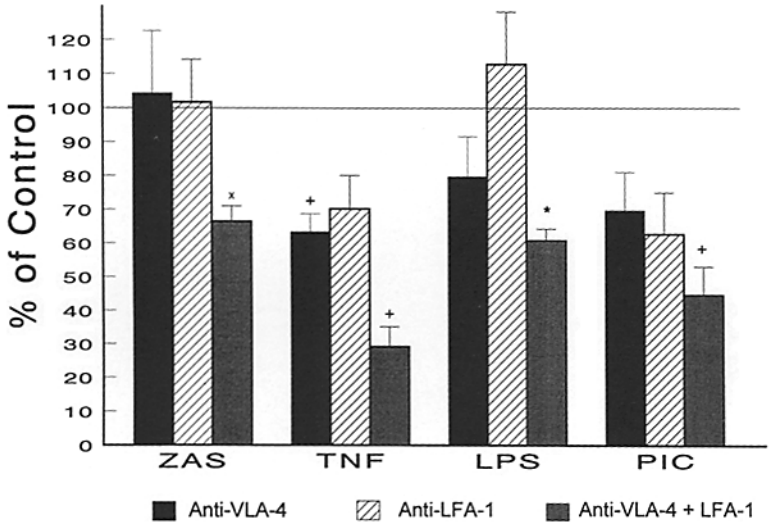

Figure 6. The effect of anti-VLA-4 and anti-LFA-1 on neutrophil migration to dermal inflammation. Arthritic rats were given an i.v. injection of anti-VLA-4, anti-LFA-1, anti-VLA-4 plus anti-LFA-1, or control mAb, and immediately thereafter labeled neutrophils were injected intravenously and cutaneous inflammatory reactions were induced by i.d. injection of 50% ZAS, 10 ng TNF-α, 1 ng LPS, or 200 ng poly I:C per site as outlined in Fig. 2. The labeled cells were allowed to accumulate in the skin sites for 2 h. Values are mean ± SEM of 5–17 animals in each group. $^{+}P < 0.05$, $^{x}P < 0.01$, and $^{*}P < 0.001$.

can mediate neutrophil adhesion and in vivo neutrophil migration to inflammatory sites (11–14). As reported here, 80–90% of the neutrophil infiltration in dermal inflammatory lesions could be inhibited by blocking LFA-1 and Mac-1 or all three members of the CD18 family. In contrast, a few investigations have also shown that neutrophils can utilize CD18-independent mechanisms for migration to the lung (14) and peritoneum (10), and to the inflamed joint (16). These studies show that 25–50% of the neutrophil accumulation in adjuvant arthritis was not inhibitable by anti-CD18 mAb treatment, even though this treatment inhibited >90% of the accumulation in dermal inflammation in the same animals (Figs. 1 and 2). Our previous studies also showed that neutrophil migration to an intra-articular delayed-type hypersensitivity reaction was also CD18 independent whereas migration to intra-articular IL-1 injection was CD18 dependent (26). These findings suggested that neutrophils utilized additional adhesion pathways for infiltration in adjuvant arthritis. Since monocyte migration to inflamed joints was highly dependent upon VLA-4, we wished to determine whether VLA-4 on neutrophils might contribute to the CD18-independent neutrophil accumulation in arthritis.

The findings reported here are novel in that they are the first to demonstrate the expression of α_4 integrins on rat blood neutrophils, the first to demonstrate that the VLA-4 on neutrophils is functional and mediates, together with the CD18 (β_2) integrins, part of the migration of neutrophils to inflamed joints and dermal inflammation, and that in addition to the CD18 and α_4 integrin pathways, neutrophil accumulation in arthritis involves at least one additional CD18- and VLA-4–independent pathway.

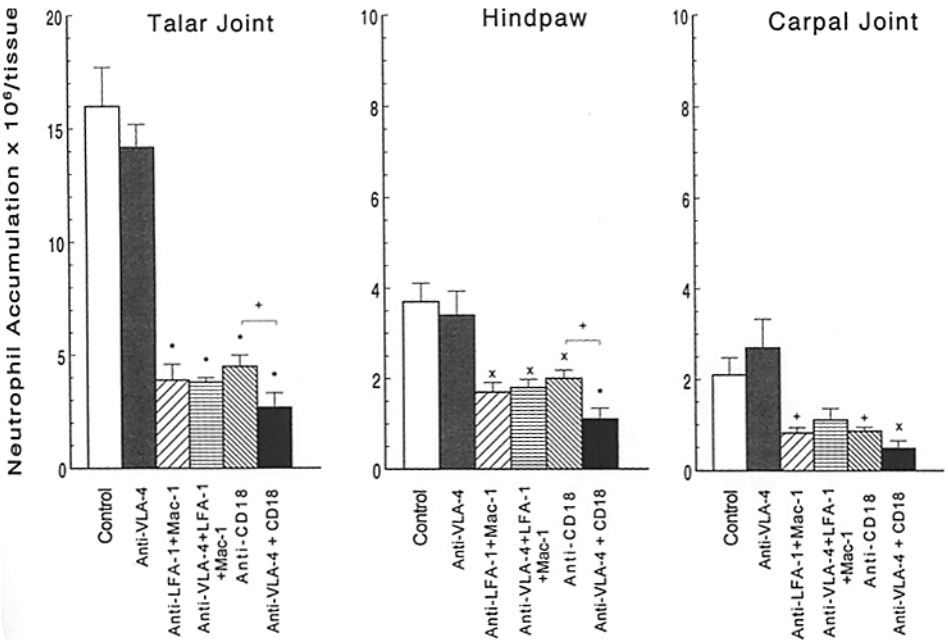

Figure 7. The effect of Ab to VLA-4 and CD18 integrins on neutrophil migration to arthritic joints. Animals with arthritis as in Figs. 1 and 4 were injected intravenously with anti-VLA-4, anti-CD18, anti-Mac-1 plus anti-LFA-1, or combinations of anti-VLA-4 with anti-CD18 and anti-LFA-1 plus anti-Mac-1 or control mAbs. ^{111}In-labeled blood neutrophils were injected intravenously and neutrophil accumulation in the joints was measured after 2 h and is expressed as in Fig. 1. Values are mean ± SEM of 5–17 animals in each group. $^{+}P < 0.05$, $^{x}P < 0.01$, and $^{*}P < 0.001$.

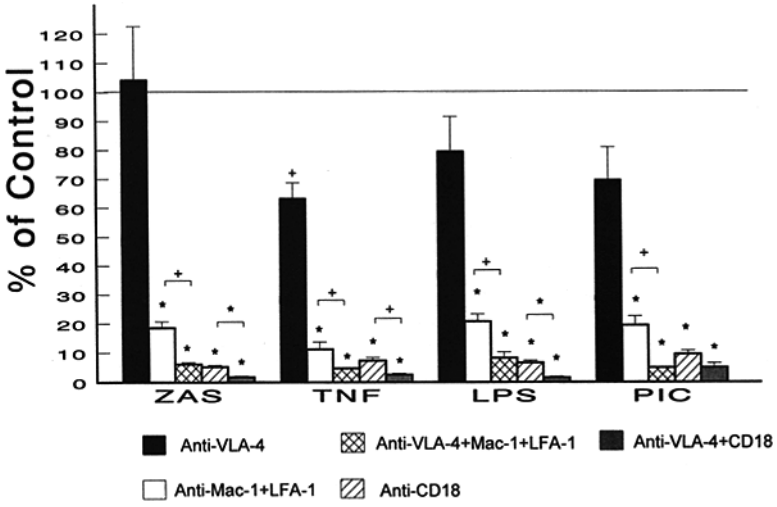

Figure 8. The effect of blocking VLA-4 and CD18 integrins on neutrophil migration to dermal inflammation. In the animals in Fig. 7, immediately after i.v. injection of the mAbs and labeled blood neutrophils, cutaneous inflammatory reactions were induced by the i.d. injection of 50% ZAS, 10 ng TNF-α, 1 ng LPS, and 200 ng poly I:C per site. The labeled cells were allowed to accumulate in the skin sites for 2 h. Values are mean ± SEM of 5–17 animals in each group. $^{+}P < 0.05$, $^{x}P < 0.01$, $^{*}P < 0.001$.

Our studies showed that virtually all of the neutrophils in the blood react with an anti-α_4 mAb, TA-2 (Fig. 3). Although α_4 can associate with β_1 and β_7 to form VLA-4 and $\alpha_4\beta_7$, LPAM-1, the latter has only been found on lymphocytes and is absent from both neutrophils and monocytes (28). Therefore, the α_4 integrin on the neutrophil in our studies is most likely VLA-4.

Eosinophils, monocytes, and lymphocytes all express VLA-4, which can mediate adhesion to cytokine-stimulated endothelium by binding to the vascular cell adhesion molecule 1 (VCAM-1) on endothelial cells (8, 22, 29–34). The TA-2 mAb reacts with VLA-4, blocks its adhesive function, and inhibits the migration of T lymphocytes and monocytes to inflammatory reactions (8, 22, 23). Blocking α_4 with TA-2 alone produced only a small decrease in neutrophil migration to i.d. TNF-α and had no effect on accumulation in inflamed joints. This suggests that, by itself, VLA-4 plays a minor role in neutrophil accumulation in most of the inflammatory sites examined. However, the functional importance of VLA-4 could be seen when LFA-1 was also blocked with an α_L (LFA-1)-specific mAb. Blocking VLA-4 significantly decreased neutrophil migration to inflamed joints by 60–75% if LFA-1 was also blocked (Fig. 4). Similarly, neutrophil migration to the dermal inflammatory sites was also inhibited by blocking VLA-4 together with LFA-1, although the magnitude of this effect depended on the inflammatory stimulus in the skin (Fig. 6). This suggests that VLA-4 on neutrophils is functional. In addition, VLA-4 can act as an alternate adhesion pathway to LFA-1, since blocking both VLA-4 and LFA-1 was required for significant inhibition of neutrophil accumulation in most of the inflammatory tissues. The effect of dual VLA-4 and LFA-1 blockade appeared to be greater on neutrophil accumulation in inflamed joints and TNF-α–injected skin than in the other dermal inflammatory sites. Since previous studies have shown that TNF-α is a key mediator of neutrophil migration to joints in adjuvant arthritis (35), the marked effect of dual blockade of VLA-4 and LFA-1 in adjuvant arthritis and cutaneous TNF-α reactions and anti-VLA-4 alone on i.d. TNF-α might be the result of more efficient utilization by neutrophils of VLA-4 to bind to VCAM-1, which is strongly induced on endothelium by TNF-α (36, 37).

These findings regarding neutrophil migration in adjuvant arthritis are similar to those observed with monocyte recruitment to arthritic joints. Both VLA-4 and the CD18 integrins, particularly LFA-1, mediate monocyte accumulation in inflamed joints (9). In addition, monocyte migration to dermal inflammation is much more strongly inhibited by blocking CD18 integrins than migration to arthritic joints, where VLA-4 appears to be more important.

Blocking Mac-1 alone, or in combination with VLA-4, did not significantly inhibit neutrophil accumulation to either inflamed joints or skin (Figs. 1 and 2), suggesting that LFA-1 is sufficient to allow near normal neutrophil migration. However, blocking both LFA-1 and Mac-1, or all three CD18 integrins (with anti-β_2 mAb) inhibited >90% of the neutrophil migration to dermal inflammation, and 50–75% of the migration to arthritic joints. Anti-VLA-4 treatment further inhibited neutrophil migration to dermal inflammation, such that neutrophil accumulation in cutaneous sites was nearly abolished (Fig. 8). Blocking VLA-4 also enhanced the effect of anti-CD18 treatment on neutrophil accumulation in arthritic joints (Fig. 7). This effect, however, was only partial. The increased inhibition of neutrophil accumulation with combined CD18/VLA-4 blockade further supports the contribution of VLA-4 to neutrophil accumulation in arthritic joints and dermal inflammation. The contribution of the CD18 integrins and VLA-4 in neutrophil migration to skin again parallels their roles in monocyte migration, although a larger proportion of monocyte migration to dermal inflammation is VLA-4 dependent (9).

Interestingly, anti-VLA-4 in combination with anti-LFA-1 and anti-Mac-1 was no more effective at inhibiting neutrophil accumulation in joints than blockade of the latter two integrins, whereas the addition of anti-VLA-4 to anti-CD18 treatment was more effective than anti-CD18 alone (Fig. 7). Although differences in Ab affinity and

blocking activity of the anti-LFA-1, anti-Mac-1, and anti-CD18 mAbs may explain these results, one possible explanation for the enhanced effectiveness of anti-CD18 plus anti-VLA-4 treatment may be that CD11c/CD18 may have an additional role in neutrophil migration in adjuvant arthritis.

Finally, one important result from these studies is the fact that neutrophil accumulation in joints in adjuvant arthritis is not entirely abolished by blocking CD18 and VLA-4 integrins, even though this treatment can virtually abolish neutrophil accumulation in dermal inflammation. Our previous studies demonstrated that there is a CD18-independent neutrophil migration to arthritic joints, and CD18-independent migration was observed to delayed-type hypersensitivity reactions in joints (16, 26). The current report demonstrates that VLA-4 contributes to a portion of the CD18-independent migration, but that another pathway is probably also involved. The adhesion pathway mediating this migration is not clear. The selectins, L-, E-, and P-selectin, which can mediate initial tethering of leukocytes to the vascular endothelium, are not thought to be able to mediate the firm leukocyte adhesion required for transendothelial migration (1, 2). VLA-5 and VLA-6 have recently been reported to be also present on neutrophils, and to mediate binding to fibronectin and laminin and migration across synovial fibroblast barriers (17, 18, 38). Neutrophil adhesion via these integrins to extracellular matrix proteins might contribute to part of the neutrophil infiltration in a chronically inflamed tissue such as the joint in adjuvant arthritis, in contrast to an acute dermal inflammatory reaction. However, there is no evidence that these integrins mediate cell adhesion before transendothelial migration.

A recent in vitro study has suggested that human blood neutrophils, stimulated with chemotactic factors in the presence of dihydrocytochalasin B, can express VLA-4 (39). Similarly, transendothelial migration by neutrophils could also result in increased VLA-4 expression. Our studies suggest that in vivo, even in the absence of a microfilament disrupting agent such as cytochalasin, rat neutrophils have functional VLA-4, and that this VLA-4 may be particularly important in migration to selected inflammatory sites.

Other investigations have also demonstrated CD18-independent neutrophil migration to some types of inflammation in the lung and the peritoneal cavity (10, 14). In the lung, the role of VLA-4 in neutrophil accumulation has not been examined, whereas in peritoneal inflammation in rabbits, blocking both VLA-4 and CD18 inhibited neutrophil accumulation, although the basis of this effect is unknown (10). Recent studies by A. Issekutz (40) have demonstrated that human blood neutrophils in vitro can migrate across unstimulated endothelium in response to C5a in a CD18-dependent manner, but that migration across cytokine-stimulated endothelium in the presence of C5a was partially CD11/CD18 independent and was not inhibitable by mAb to VLA-4. This suggests that, similar to the migration of rat neutrophils to inflamed joints, human neutrophils also demonstrate a CD18- and VLA-4–independent migration under the appropriate conditions. The mechanism operative in this migration, with human or rat neutrophils, awaits further characterization.

In conclusion, our studies have demonstrated that rat blood neutrophils express α_4 integrins and that these are functional and mediate, together with β_2 integrins, virtually all of the migration to cutaneous inflammation, but migration to inflamed joints involves not only β_2 and α_4 integrins, but also another pathway. The identification of this additional pathway and the full range of receptors mediating the migration of neutrophils in different inflammatory situations is yet to be fully clarified.

The authors gratefully acknowledge the excellent technical assistance of Ms. N. MacPhee, C. Jordan, and J. Stoltz. We also thank Ms. S. Gruman for her expert secretarial help in manuscript preparation.

This work was supported by grant 89036 from The Arthritis Society and grant MA8575 from the Medical Research Council of Canada. T.B. Issekutz is a recipient of a Research Chair supported by the MRC/PMAC Health Program and Sandoz Canada, Inc.

Address correspondence to Dr. Thomas B. Issekutz, Conacher Research Wing, 2-855, The Toronto Hospital, 101 College Street, Toronto, Ontario M5G 2C4, Canada.

Received for publication 31 October 1995 and in revised form 19 February 1996.

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