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The β_1 integrin, very late activation antigen-4 on human neutrophils can contribute to neutrophil migration through connective tissue fibroblast barriers

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SUMMARY

Polymorphonuclear leucocyte (PMNL) accumulation in extravascular tissues and inflammatory exudates is dependent on their migration through blood vessel endothelium and then through connective tissue. Previously we utilized a barrier of human synovial and dermal fibroblasts (HSF or HDF) grown on microporous filters, as a model of PMNL migration through connective tissue. Those studies showed that β_2 (CD18) and the β_1 integrins, very late activation antigen-5 (VLA-5) and VLA-6, in part mediate this PMNL migration. Here we report that VLA-4, which can also be expressed at low levels on activated PMNL, is also involved in PMNL migration induced by C5a through fibroblast (HSF and HDF) barriers, because monoclonal antibody (mAb) to VLA-4 significantly inhibited (by 20–30%) PMNL migration. Blocking the function of CD18, VLA-5 or VLA-6 was not required for detection of the VLA-4-mediated migration. Combination treatment with mAb to VLA-4 and with mAb to VLA-5 or to VLA-6 further inhibited PMNL migration, irrespective of whether CD11/CD18 mechanisms were blocked with anti-CD18 mAb or not. Treatment of PMNL with a peptide based on the VLA-4-binding domain in the CS-1 fragment of fibronectin, but not a control peptide, inhibited PMNL migration to a comparable extent to treatment with mAb to VLA-4. A low level of VLA-4 was expressed on C5a-activated PMNL, detected by immunofluorescence flow cytometry. These results suggest that VLA-4 can be mobilized by human peripheral blood PMNL and can, in addition to VLA-5, VLA-6 and CD11/CD18 integrins, mediate PMNL migration through connective tissue. This is in marked contrast to PMNL transendothelial migration, where β_1 integrins appear to play no significant role.

INTRODUCTION

Leucocyte, including polymorphonuclear leucocyte (PMNL) accumulation in connective tissue, is a prominent feature of inflammatory diseases. In some conditions, such as for example arthritis, meningitis, peritonitis, or pneumonitis, the leucocytes and PMNL also accumulate in large numbers in the fluid component of an inflammatory exudate, e.g. in synovial, cerebrospinal, or peritoneal fluids. These PMNL may contribute to local tissue injury.[1] The PMNL accumulation in extravascular connective tissues and cavities requires PMNL migration first through vascular endothelium and subsequently through connective tissue. The interaction between PMNL and endothelium has been extensively investigated. It is recognized that a number of adhesion molecule families expressed on PMNL and endothelium, e.g. selectins, β_2 (CD11/CD18) integrins and the immunoglobulin supergene cell adhesion molecules are involved.[2–4] However, the mechanisms of PMNL migration through connective tissue barriers in any tissue, once the PMNL are in the extravascular space, is much less understood. It is likely that in order to migrate in this space, adhesion molecules on PMNL may bind to ligands on stromal cells such as fibroblasts and extracellular matrix (ECM) proteins produced by such cells, e.g. fibronectin, vitronectin, collagen etc.[5] The β_1 (CD29) and β_2 (CD18) integrins on PMNL have been shown to mediate PMNL adhesion to ECM protein such as laminin, fibronectin, collagen, as well as to denatured proteins.[6–12] Recently, we reported that PMNL migration through a barrier of synovial or dermal fibroblasts is in part mediated by β_2 (CD11/CD18) and by β_1 (CD29) integrins.[13,14]

The β_1 integrins consist of heterodimers of individual α chains and a common β_1 chain (CD29). There are at least nine known heterodimers.[15–17] Most of the members on leucocytes and connective tissue cells are receptors for ECM proteins such as fibronectin, laminin and collagen. The very late

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antigen-5 (VLA-5, $\alpha_5\beta_1$) and VLA-6 ($\alpha_6\beta_1$) are PMNL receptors for fibronectin and laminin, respectively and can mediate PMNL adhesion.[6,11] Recently we also found that PMNL migration through synovial and dermal fibroblast monolayers in response to a chemotactic gradient is dependent on the function of VLA-5 and VLA-6, as well as the β_2 (CD18) integrins.[13,14] Another member of the β_1 integrins, VLA-4 ($\alpha_4\beta_1$) can bind to fibronectin, as well as to vascular cell adhesion molecule-1 (VCAM-1) and it is known to mediate monocyte, lymphocyte and eosinophil migration.[3,18–23] VLA-4 is expressed by PMNL precursors in the bone marrow, but VLA-4 expression by blood PMNL has been difficult to demonstrate.[16,24–26] Recently, low level expression of VLA-4 on degranulated or activated PMNL has been observed.[27] In a previous study, we found that inhibition of the functions of VLA-5 and VLA-6 by monoclonal antibodies (mAb) did not completely block CD18-independent PMNL migration in response to C5a through fibroblast barriers.[13,14] Therefore, we investigated whether VLA-4 may also contribute to PMNL migration across such connective tissue barriers. Here we report that mAb to α_4 of VLA-4 or a soluble peptide derived from the CS-1 fibronectin-binding region for VLA-4, partially inhibit PMNL migration through synovial or dermal fibroblast barriers. Thus, VLA-4 may play a role in PMNL migration through connective tissue in conjunction with VLA-5, VLA-6 and β_2 (CD18) integrins.

MATERIALS AND METHODS

Monoclonal antibodies

The following murine mAb against human antigens were used as purified IgG: 60.3 (anti-CD18, IgG_{2a}, provided by Bristol-Myers Squibb, Seattle, WA),[28] R15.7 (anti-CD18, IgG_1, provided by Dr R. Rothlein, Boehringer Ingelheim, Ridgefield, CT),[29] HP1/2 (anti-α_4 chain of VLA-4 integrin, IgG_1 from Dr R. Lobb, Biogen Inc., Cambridge, MA),[30] rat mAb 16 (anti-α_5 chain of VLA-5; provided by Dr K. Yamada, National Institutes of Health, Bethesda, MD)[31] and mAb 3S3 (anti-β_1 integrin; gift from Dr J. Wilkins, University of Manitoba, Winnipeg, MA). The following mAb were used as ascites: 450-30A1 (anti-α_6 chain of VLA-6, IgG_1 from Dr S. J. Kennel, Oak Ridge National Laboratory, TN),[32] 3H11B9 (anti-pertussis toxin, IgG_1, from Dr S. Halperin, Halifax, NS), mAb 543 (anti-CR1, IgG_1) and 3C10 (anti-CD14, IgG_1) were from the American Type Culture Collection (Bethesda, MD).

Reagents

Recombinant human tumour necrosis factor-α (TNF-α) (specific activity of 5×10^7 U/mg), a kind gift from Genentech Inc. (South San Francisco, CA) was diluted immediately before use in 0·1% lipopolysaccharide (LPS)-free human serum albumin (HSA) (Connaught Laboratories, Don Mills, Ontario, Canada) in PBS. Recombinant human C5a was a gift from CIBA-Geigy Pharmaceuticals (Summit, NJ). The CS-1 fibronectin peptide (sequence-EILDVPST) was a gift from Dr R. Lobb (Biogen, Cambridge, MA) and also purchased along with the control peptide (EILEVPST), from Peninsula Laboratories (Belmont, CA).

Isolation and growth of human synovial and dermal fibroblasts

Human synovial fibroblasts (HSF) were aseptically isolated from synovium obtained at surgery or arthroscopy of knee or hip joints of patients with rheumatoid arthritis (RA) (provided by Dr J. Hanly, Division of Rheumatology, Victoria General Hospital, Halifax, NS, Canada) and human dermal fibroblasts (HDF) were isolated from skin resected during minor plastic surgery (kindly provided by Dr K. Wilson, Izaak Walton Killam Children's Hospital, Halifax, NS, Canada), as reported previously.[14] Briefly, the minced tissue was digested with 2 mg/ml collagenase type IV (512 units/mg; Sigma Chemical Co., St. Louis, MO) in α-minimal essential medium (αMEM; Sigma) containing 10% heat-inactivated fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT) and penicillin G/streptomycin (Gibco, Grand Island, NY), by incubation in a shaker (250 r.p.m.) at 37° for 4 hr. Single cells were recovered by centrifugation, washed and cultured in αMEM–10% FBS, 50 μM 2-mercaptoethanol (2-ME) and penicillin G/streptomycin until cells grew confluent. The cultures became homogeneous by the second passage and the third to the twelfth passage were used. Cells were harvested with 0·05% trypsin/0·02% ethylenediamine tetraacetic acid (EDTA) (Flow Laboratories, Mississauga, Ont., Canada) and seeded onto polycarbonate filters bearing 5 μm pore in Transwell culture plate inserts (6·5-mm diameter, Transwell 3421; Costar, Cambridge, MA), which were precoated with 0·01% gelatin overnight. Seeding density was 2×10^4 of HDF or 3×10^4 HSF in 0·1 ml–10% FBS, 2-ME and antibiotics above the filter and 0·6 ml of this medium was added to the lower compartment beneath the filter. After 6–7 days culture, confluent monolayers had formed on the filters, which allowed the diffusion of $<5\%$ of ^{125}I-labelled human serum albumin (HSA) in 45 min compared to diffusion of 25–30% across bare filters.

Isolation of human PMNL

Blood PMNL were isolated as described previously.[33] Briefly, peripheral venous blood from healthy donors was collected into heparin (5 U/ml blood) and acid citrate dextrose (1·6 ml/10 ml blood; ACD formula A, Travenol, Malton, Ont., Canada) anticoagulant. The red blood cells were sedimented by 6% dextran saline (1 part to 5 parts blood) (Travenol, Malton, Ont., Canada). The leucocyte-rich plasma (LRP) was harvested and centrifuged at 150 *g* for 10 min. The pellet containing leucocytes and residual red blood cells was resuspended in Ca^{2+}, Mg^{2+}-free Tyrode's solution with 5% autologous platelet-poor plasma (PPP) and labelled with ^{51}Cr sodium chromate (40 μCi/ml; Amersham Corp. Oakville, Ont., Canada) by incubation for 30 min at 37°. The labelled PMNL were separated on discontinuous 10% PPP–Percoll (Pharmacia Fine Chemicals, Dorval, Quebec, Canada) gradients (58%/73%) by centrifugation (30 min at 300 *g*). The PMNL were washed and suspended in RPMI-1640 medium containing 0·5% HSA and 10 mM HEPES (RHSA), pH 7·4 to a final concentration of 2×10^6/ml. This method yielded $>95\%$ pure PMNL with $>98\%$ viability with essentially no red blood cell contamination.

Assay of PMNL migration across fibroblast

Migration assay was performed as previously.[33,34] Briefly, HSF or HDF monolayers on the filters and the lower compartment were washed with RPMI-1640 and incubated for 4 hr in fresh RPMI-1640 with 10% FBS. After incubation, the filters were washed on the upper and lower surfaces with RPMI-1640 and transferred to a new, clean well (lower compartment). To

this well, 0·6 ml RPMI-1640 medium with 0·5% HSA (RHSA) was added with or without the chemotactic stimulus, C5a. Before immersion of the filter unit, 0·1 ml of medium containing 2×10^5 ^{51}Cr-labelled PMNL was added into the upper compartment above the monolayer filter. After incubation, migration was stopped by washing of the upper compartment twice with 150 μl RPMI-1640 to remove non-adherent PMNL. The undersurface of the filter was then swabbed with a cotton swab soaked in ice-cold PBS/0·2% EDTA and this was combined with the contents of the lower compartment and considered to contain the migrated PMNL. The monolayer/filter unit was then transferred to another well containing 0·7 ml of 0·5 M NaOH to release all ^{51}Cr from PMNL remaining adherent to the monolayer. The cells which migrated into the lower compartment were lysed by addition of 0·5% Triton-X-100 and the medium in this compartment, including the swab, were analysed for ^{51}Cr and were considered 'migrated PMNL'. To measure PMNL adherence, the NaOH solution bathing the monolayer/filter unit was also analysed for ^{51}Cr content. The results are expressed as per cent of the total ^{51}Cr-labelled PMNL added above the HSF or HDF monolayers, which migrated across the HSF, or HDF/filter unit or remained adherent to the monolayers. All the treatment conditions were performed in triplicate.

Antibody treatment and immunofluorescence flow cytometry

In some experiments, ^{51}Cr-labelled PMNL were treated for 20 min at room temperature with saturating mAb (30–50 μg/ml) before being added for migration. The antibodies were present throughout the migration assay unless otherwise indicated. Immunofluorescence staining of mixed leucocytes or purified PMNL employed a standard protocol using a primary mAb followed by reaction with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (BioCan Scientific, Mississauga, Ont., Canada) and fluorescence detection in a Becton-Dickinson FACScan flow cytometer using forward and side scatter parameters to gate on granulocytes. Data were analysed using PC-LYSIS software.

Statistical analysis

One-way analysis of variance (ANOVA) or Student's t-test were used for analysis of the data. Values of $P < 0.05$ were considered to be significant.

RESULTS

Effect of antibody to VLA-4 on the migration of PMNL through synovial fibroblast monolayers

The migration of PMNL across a monolayer of human synovial fibroblasts (HSF) occurs spontaneously to only a small extent, about 6–8% of added PMNL in 90 min (Fig. 2). However, migration was dramatically increased by adding the chemotactic factor, C5a into the compartment beneath the monolayer, reaching upto 70% of added PMNL, as shown in Fig. 1 and reported previously in greater detail.[13] The role of specific β_1 integrins, in particular of VLA-4, in this PMNL transfibroblast migration was evaluated. Previously, we found that β_1 integrin mediated migration of PMNL across HSF monolayers involved the VLA-5 and VLA-6 members of the β_1 integrin family.[13] VLA-4 has not been identified on unstimulated human blood PMNL, although it is expressed on PMNL

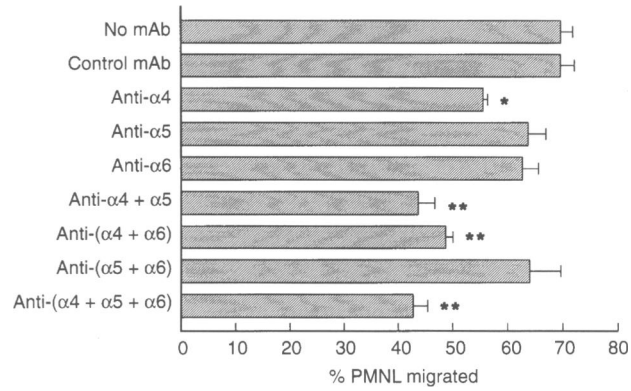

Figure 1. The effect of monoclonal antibody to α_4 chain of VLA-4 on PMNL migration across HSF monolayers. The ^{51}Cr-labelled PMNL were treated (20 min, 22°) with mAb to α_4 of VLA-4 (HP1/2, 40 μg/ml), to α_5 chain of VLA-5 (ab16, 50 μg/ml), or to α_6 chain of VLA-6 (450-30A1, 1 : 100 ascites) alone or in combination as indicated. Monoclonal antibodies to CD14 or to CR1 were used as control mAb. All mAb were present during 90 min migration induced by C5a (3×10^{-9} M). Values are mean + SEM of three experiments performed in triplicates. *$P < 0.05$; **$P < 0.01$ as compared with control mAb treatment analysed by paired t-test.

precursors in bone marrow and on blood PMNL of rodents.[24,26,35] Since VLA-4 is known to mediate migration of other leucocyte types *in vivo* and *in vitro*, we examined whether a function blocking mAb to VLA-4 (HP1/2) might reveal a role for any VLA-4 that might be expressed on human PMNL during migration. As shown in Fig. 1, treatment of PMNL with mAb to VLA-4 (anti-α_4) (HP1/2) alone slightly, but significantly inhibited (by 20%) PMNL migration across HSF monolayer in response to C5a, as compared with control mAb treatment. In contrast, treatment with mAb to VLA-5 or VLA-6 alone or in combination did not significantly inhibit PMNL migration. Treatment with mAb to VLA-4 plus mAb to VLA-5 (anti-α_5) or mAb to VLA-6 (anti-α_6) further and additively inhibited PMNL migration and either of these combinations was more effective than combining anti-VLA-5 plus anti-VLA-6 mAb. None of the mAb treatments, in the absence of C5a, stimulated PMNL transmigration.

Effect of blocking the function of CD18 on VLA-4-mediated PMNL migration

We reported that detection of C5a-induced, VLA-5 and VLA-6 integrin-mediated migration of PMNL across HSF monolayers required simultaneous inhibition of the function of the β_2 (CD18) integrins.[13] Although blocking the function of β_2 integrin for detection of VLA-4-mediated PMNL migration was not necessary, as shown in Fig. 1, it was of interest to determine the effect of blocking CD18 on VLA-4 mediated migration. The findings were more complicated than expected. As shown in Fig. 2 when: (a) mAb to CD18 was combined with either anti-α_4, anti-α_5, or anti-α_6, no further (additive) inhibitory effect on migration, compared with treatment with mAb to CD18 alone was observed; (b) treatment with anti-CD18 plus *both* anti-α_4 and anti-α_5 further inhibited PMNL migration, which was comparable to treatment with anti-CD18 plus anti-α_5 and anti-α_6; (c) combined treatment with mAb to α_4, α_5 and α_6 plus anti-CD18 did not further inhibit migration

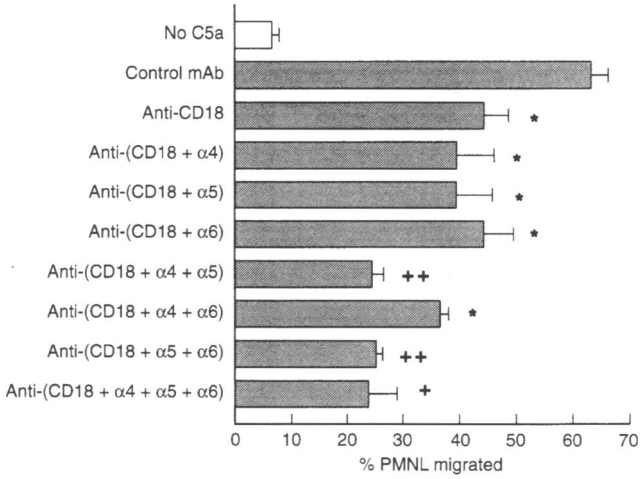

Figure 2. The effect of blocking CD18 on VLA-4-mediated PMNL migration across HSF monolayers. The ^{51}Cr-labelled PMNL were treated (20 min, 22°) with mAb to CD18 (60·3; 30 μg/ml) alone or in combinations with mAb to α_4 (HP1/2), to α_5 (ab16) or to α_6 (450-30A1) (40 μg/ml). PMNL migration was induced by C5a for 90 min. Values are mean ± SEM of three or four experiments performed in triplicate. $*P<0.05$, as compared with control mAb treatment; $+P<0.05$, and $++P<0.01$ as compared with treatment with anti-CD18 mAb.

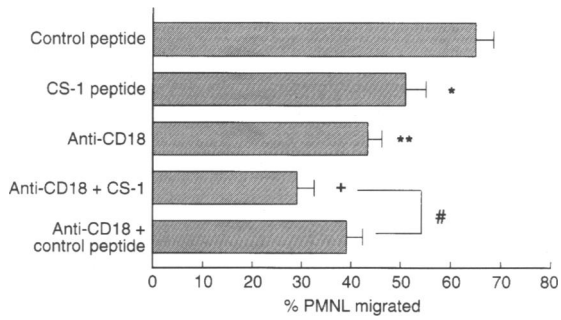

Figure 3. The effect of CS-1 peptide on PMNL migration through HSF monolayers. The ^{51}Cr-labelled PMNL were treated (20 min, 22°) with CS-1 peptide (0·5 mg/ml), a control sequence peptide (0·5 mg/ml), or mAb to CD18 (60·3; 30 μg/ml) alone or in combination, as indicated. Migration (90 min) was induced by C5a (3×10^{-9} M). Values are mean ± SEM of seven experiments performed with triplicates. $*P<0.05$ and $**P<0.01$ as compared with control mAb treatment; $+P<0.01$, as compared with treatment with anti-CD18 mAb; $\#P<0.05$; analysis by paired t-test.

compared with (b); and (d) combination treatment with anti-CD18 plus anti-α_4 and anti-α_6 did not inhibit relative to anti-CD18 ± anti-α_4. The inhibitory effects of mAb to α_4, α_5, or α_6 appeared to be due to their binding to PMNL, because no inhibition was observed when only the HSF were treated with the mAb and then washed, before addition of untreated PMNL (not shown but reported in part previously).[13,14] Furthermore, no increase in the low level of PMNL adhesion (4–8%) to the HSF monolayer, was observed with any of the mAb treatments (not shown). The results in Figs 1 and 2 suggest that some functional α_4, probably VLA-4 integrin, may be present on PMNL, and can contribute, especially in conjunction with VLA-5 and CD18 integrins to PMNL migration.

Inhibitory effect of CS-1 peptide on PMNL migration

Fibroblasts produce ECM proteins such as fibronectin. It is known that the VLA-4 integrin can bind to the CS-1 region of alternatively spliced fibronectin and soluble peptides encompassing or mimicking the CS-1 fibronectin region can bind to VLA-4 and block VLA-4-mediated adhesion of lymphocytes to fibronectin or adhesion and transendothelial migration of monocytes.[30,36–38] Therefore, to explore further the possible role of VLA-4 on PMNL, we examined whether a CS-1 peptide encompassing the VLA-4 recognition region may effect PMNL transfibroblast migration. Treatment of PMNL with CS-1 peptide (500 μg/ml) alone slightly, but significantly inhibited PMNL migration, an effect comparable in magnitude to mAb to VLA-4 (Fig. 3, compared to Fig. 1). The inhibitory effect of CS-1 peptide was additive with mAb to CD18 (60·3). In contrast, treatment of PMNL with a control peptide, with an amino acid substitution (LEV) in the LDV recognition sequence, either alone or in combination with mAb to CD18 (60·3), had no significant effect on PMNL migration (Fig. 3).

The effect of mAb to VLA-4 on PMNL migration through dermal fibroblast monolayer

We also investigated whether VLA-4 may play a role in PMNL migration across human dermal fibroblasts (HDF) for comparison with migration across HSF monolayers. As shown in Fig. 4(a), mAb to α_4, but not to α_5 or α_6, inhibited migration partially but significantly and combination with mAb to α_5 or α_6 had no greater inhibitory effect. The effect of mAb to VLA-4 was similar to that on PMNL migration through HSF (Fig. 1), except that with HSF, addition of anti-α_5 or anti-α_6 mAb enhanced the inhibitory effect of anti-α_4 mAb (Fig. 1). When the function of CD18 was blocked, as shown in Fig. 4(b), treatment with mAb to α_4, α_5, or α_6 alone or in combination as indicated, further inhibited PMNL migration, as compared with treatment with mAb to CD18 alone. These results suggest that VLA-4 may contribute to migration of PMNL across HDF as well as HSF barriers.

Expression of VLA-4, VLA-5 and VLA-6 on PMNL

The presence of VLA-5 and VLA-6 on human PMNL has been described,[6,9,11] but VLA-4 expression on human blood PMNL has been difficult to detect,[24–26] although a recent report indicates that low level expression can be observed.[27] The above functional effects of CS-1 peptide and of mAb to α_4 of VLA-4 suggested that some VLA-4 may be mobilized by PMNL. We analysed expression of VLA-4, VLA-5 and VLA-6 on purified PMNL activated with C5a (3×10^{-9} M), by flow cytometry using mAb to α_4 of VLA-4 (HP1/2), α_5 of VLA-5 (ab16) and α_6 of VLA-6 (450-30A1). The histograms of staining with mAb to α_4, α_6 and β_1 integrins are shown in Fig. 5. Staining with mAb to α_5 was virtually superimposable with the profile for anti-α_4, so for the sake of clarity, it was not included in Fig. 5. All three of these β_1 integrins were expressed on the PMNL, although the expression was quite low. An identical profile for α_4 (VLA-4) expression was observed when a different control and anti-α_4 mAb (HP2/1) were used as primary antibodies. Thus, with both α_4 mAb, there was essentially a unimodal profile with a shift of the

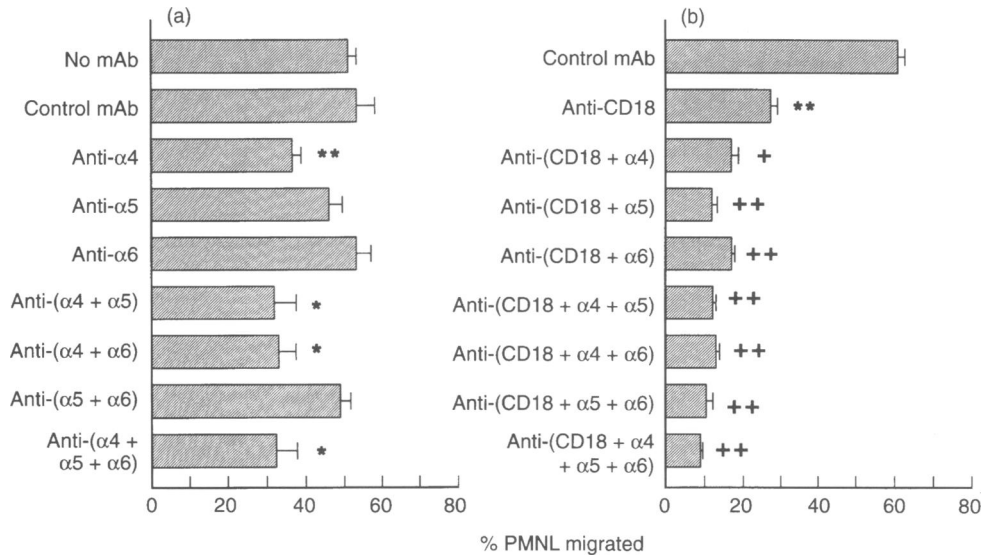

Figure 4. The effect of mAb to α_4, α_5 and α_6 integrin chain on migration of PMNL across human dermal fibroblast monolayers. The ^{51}Cr-labelled PMNL were treated (20 min, 22°) with mAb to CD18 (60·3; 30 μg/ml), α_4 chain of VLA-4, α_5 chain of VLA-5, α_6 chain of VLA-6 alone or in combination as indicated. The mAb anti-CR1 (543) or anti-CD14 (3C10) were used as control mAb. Spontaneous, unstimulated PMNL migration was 3·1 ± 0·3% of added cells. Migration was induced by C5a (2×10^{-9} M) for 90 min. Values are mean ± SEM of three or four experiments performed with triplicates. $*P<0.05$ and $**P<0.01$ as compared with control mAb treatment; $+P<0.05$; $++P<0.01$ compared with treatment with mAb CD18.

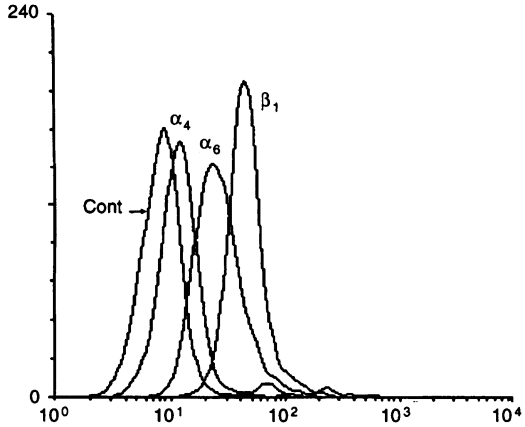

Figure 5. Immunofluorescence flow cytometry analysis of VLA-4, VLA-5, VLA-6 and β_1 integrin expression on neutrophils. Human blood neutrophils were activated at 37° (15 min) with 3×10^{-9} C5a and then stained with mAb to α_4 of VLA-4 (HP1/2), α_5 of VLA-5 (ab 16), α_6 of VLA-6 (450-30A), the common β_1 integrin (mAb 3S3) or irrelevant isotype-matched control mAb (3H11B9, anti-Pertussis). The profile for α_5 (VLA-5) expression was essentially superimposable with the α_4 profile and therefore, for the clarity of the α_4 profile, was not plotted on the figure.

population, e.g. as in Fig. 5, from mean fluorescence intensity of 8·7 for the control to 14·1 with mAb to α_4.

DISCUSSION

Our previous investigations have shown that the major component of PMNL migration through fibroblast (HSF, HDF) monolayers is CD18 independent.[13,14] The β_1 integrins, VLA-5 and VLA-6, were identified as mediating a part of this CD18-independent migration. In this study, we extend these observations to show that another β_1 integrin, VLA-4 also contributes to PMNL migration through HSF and HDF barriers, since treatment of PMNL with mAb to α_4 chain of VLA-4-inhibited migration by 20–30% (Figs 1 and 4). This conclusion from the anti-α_4 mAb treatment results is supported by the inhibition of PMNL migration by soluble CS-1 peptide, containing the CS-1 region of fibronectin to which VLA-4 is known to bind.[23,37,38] This peptide inhibited migration to a comparable degree to anti-α_4 mAb treatment (Fig. 3). No significant inhibition was observed with a control sequence peptide lacking the LDV sequence.

Whether VLA-4 is expressed on mature PMNL is controversial. Generally, no VLA-4 has been detected on peripheral blood PMNL.[24–26] However, a recent report has implicated VLA-4 in PMNL adhesion and these authors have shown that VLA-4 can be mobilized from intracellular granules to the plasma membrane in human blood PMNL upon appropriate cell activation or during transendothelial migration.[27] Our results in Fig. 5 are in agreement with this observation in that C5a-activated PMNL also express low but detectable levels of VLA-4, comparable to the level of VLA-5, but less than VLA-6. These findings, combined with those reported here in which PMNL were activated with C5a to migrate through a novel connective tissue-like barrier, may explain why the role of VLA-4 on PMNL has heretofore not been recognized. Since the PMNL may mobilize VLA-4 during activation, previous approaches employing immunofluorescence flow cytometry analysis or surface iodination and immunoprecipitation of unstimulated PMNL would not have detected the VLA-4. Interestingly, rat blood PMNL, which have also been considered to lack VLA-4, have recently been shown to utilize VLA-4 in addition to the CD11/CD18 (β_2 integrins) to migrate to arthritis and to be capable of expressing VLA-4.[35] It is known that neutrophil precursors in bone marrow express

VLA-4.[24,26] It now appears that although VLA-4 synthesis during terminal neutrophil differentiation may be very low, VLA-4 may become incorporated during myeloid maturation into granules from where it can be made functionally available to contribute to adhesion[27,39] and PMNL migration through connective tissue barriers, as shown here.

Recently, we reported that the β_1 integrins VLA-5 and VLA-6 mediate PMNL migration through HSF and HDF monolayers in response to C5a.[13,14] However, detection of this mechanism required blocking the function of CD11/CD18 integrins (Figs 2 and 4b), because treatment of PMNL with mAb to α_5 or α_6 alone or in combination did not significantly inhibit PMNL migration[13,14] (Figs 1 and 4a). In contrast, blocking the function of CD18 was not required to observe inhibition of PMNL migration by mAb to α_4 (VLA-4) through HSF and HDF barriers (Figs 1 and 4a). The reason for this difference is not clear. However, the fact that VLA-4 can bind to multiple ligands including at least two sequences in fibronectin (the CS-1 domain and an RGD region), as well as to VCAM-1, in which it also recognizes two domains (1 and 4), and since VLA-4 also displays preferential binding to these dependent on its activation state, may have a bearing on the more apparent role of VLA-4,[23,30,37,38,40–42] even when the CD11/CD18 integrins are not blocked.

It is possible that at least two VLA-4 pathways (VLA-4/CS-1 fibronectin and VLA-4/VCAM-1) are involved in the observed PMNL migration, as has been shown for VLA-4-mediated monocyte transendothelial migration.[36,43] The finding that soluble CS-1 peptide inhibited PMNL migration (Fig. 3) does not exclude that VLA-4 may also engage VCAM-1, since this peptide can block VLA-4 binding to VCAM-1 as well.[37] VCAM-1 is constitutively expressed on HSF,[44,45] but not on HDF.[44,46] However, this could not be reliably investigated with blocking mAb to VCAM-1 because anti-VCAM-1 mAb treatment of HSF appeared to weaken the HSF barrier, causing an increase in permeability to ^{125}I-labelled albumin and variable PMNL migration results (not shown). The migration of PMNL across HDF monolayers, which in our studies also did not express VCAM-1, was not inhibited by treatment with mAb to VCAM-1 (not shown). These observations, especially with HDF, suggest that the VLA-4 on PMNL can efficiently utilize ligands other than VCAM-1, likely alternatively spliced fibronectin, to migrate through connective tissue. This is in contrast to PMNL migration through human umbilical vein endothelium (HUVE), where virtually all transendothelial migration is mediated by CD11/CD18 integrins.[47–50] In fact, employing a migration assay analogous to that used here but using HUVE monolayers, even when these were cytokine activated, we did not observe a significant role for VLA-4 or other β_1 integrins in PMNL transendothelial migration.[50] Thus, PMNL and perhaps other leucocytes can utilize distinct and novel mechanisms for migration in various tissue compartments during inflammation. Further definition of these mechanisms may contribute to strategies for controlling extravascular leucocyte migration, accumulation and retention in connective tissues, events which may contribute to inflammatory tissue injury.

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