JLB

Brief Conclusive Report

Human neutrophil integrin α9β1: up-regulation by cell activation and synergy with β2 integrins during adhesion to endothelium under flow

Agnès Mambole,[*,†] Sylvain Bigot,*[*,†] Dominique Baruch,[†,‡] Philippe Lesavre,*[*,†] and Lise Halbwachs-Mecarelli*[*,†,1]*

*INSERM U845, Paris, France; [‡]INSERM U765, Paris, France; and [†]Université Paris Descartes, Paris, France

RECEIVED OCTOBER 30, 2009; REVISED APRIL 8, 2010; ACCEPTED APRIL 9, 2010. DOI: 10.1189/jlb.1009704

ABSTRACT

Neutrophil β1 integrin expression and contribution to cell adhesion were revisited in this study. α9β1 and α5β1 appeared here as the main β1 integrins expressed on the membrane of resting platelet-depleted neutrophils—α6β1 representing <15% and α2β1 undetectable. Neutrophil activation slightly enhanced α5 expression, did not change α6, but resulted in a two- to threefold increase of α9β1, which then became the major β1 integrin of the neutrophil membrane. α9β1 was the only β1 integrin to be up-regulated after transendothelial migration across TNF-α-activated HUVECs. As α9β1 binds VCAM-1, we analyzed its participation to neutrophil adhesion to TNF-α-activated endothelial cells. Blocking anti-α9 mAb had little effect on neutrophil static adhesion, contrasting with the strong inhibition by anti-β2 mAb. Under flow conditions, the anti-α9 mAb had no effect by itself on neutrophil adhesion to activated HUVECs but enhanced the blocking effect of anti-β2 antibodies significantly and further enhanced the velocity of β2–blocked rolling neutrophils. In conclusion, we describe here for the first time a nearly exclusive up-regulation of α9β1 expression among all β1 integrins during neutrophil activation and transendothelial migration and a possibly important synergy between α9β1 and β2 integrins in stabilizing neutrophil adhesion to endothelium under flow conditions. *J. Leukoc. Biol.* 88: 321–327; 2010.

Introduction

Although neutrophils adhere mostly via β2 integrins, they express β1 integrins, as shown by several groups including ours [1–3]. While performing an overall study of β1 integrin functions in neutrophils, we observed peculiar properties of α9β1 and thus, chose to focus on this integrin.

Unlike β2 integrins, which mediate leukocyte cell/cell interactions, the β1 integrin family is mostly devoted to cell adhesion to extracellular matrix, and neutrophils were shown to interact with fibronectin via α5β1 [1, 4, 5], with laminin via α6β1 integrin [2], and with collagen via α2β1 [6]. A major exception to this rule, among β1 integrins, is α4β1, which mediates leukocyte adhesion to endothelial cells by interacting with VCAM-1. Rat and mouse neutrophils express α4β1 and use it to interact with endothelial cells [7–9].

Human neutrophils lack α4β1 [10] but, instead, express the analogous α9β1 integrin [11]. Indeed, α4 and α9 cDNA sequences are 41% identical [12], and α9β1 shares with α4β1 its specificity for endothelial VCAM-1 [13]. Integrin α9β1 is also a receptor for matrix proteins tenascin C and osteopontin and is expressed on cells of various types, muscle cells, hepatocytes, and endothelial or epithelial cells [12].

On leukocytes, high levels of α9β1 are present on PMN and smaller levels on monocytes, and this integrin is absent completely on lymphocytes or eosinophils [11, 13]. Blood circulating neutrophils are decreased dramatically in α9-deficient mice, and a role of α9β1 in granulopoiesis has been proposed, possibly related to its specific interaction with G-CSF and its involvement in G-CSF-induced signaling [14].

The functions of α9β1 integrin on mature neutrophils are not known. Its high level of membrane expression suggested that this integrin could participate in neutrophil recruitment to inflammation sites. However, in vitro experiments resulted in controversial data, showing that anti-α9β1 mAb inhibited [13] or had no effect on transendothelial migration, while the migration through fibroblast barriers was blocked [11]. In transfected COS cell lines, α9β1 inhibits cell spreading and promotes cell migration on tenascin substrates [15, 16], while in neutrophils, it mediates osteopontin-induced chemotaxis [17], thus suggesting a role in cell motility.

Our data here establish for the first time that α9β1 is up-regulated drastically upon neutrophil activation and transmi-

Abbreviations: HBSS^{++}=HBSS with Ca^{++} and Mg^{++}, HBSS^{++}-BSA=HBSS with Ca^{++} and Mg^{++}containing BSA 0.1%, MFI=mean fluorescence intensity, PMN=polymorphonuclear neutrophil(s)

1. Correspondence: INSERM U 845, Batiment Lavoisier, Hopital Necker, 161 rue de Sèvres, 75015 Paris, France. E-mail: lise.halbwachs@inserm.fr

gration and contributes to neutrophil adhesion to activated endothelium.

MATERIALS AND METHODS

Antibodies and reagents

Included were anti-α 9 integrin (clone Y9A2, Chemicon, Hants, UK); anti-CD49f (α6 integrin, GoH3, BD PharMingen, San Diego, CA, USA); anti-CD18 (IB-4, Ancell, Bayport, MN, USA); anti-CD49e (α5 integrin, SAM1), anti-CD62E (E-selectin), and PE-conjugated anti-CD146 (Beckman Coulter, Roissy, France); and anti-VCAM-1 (CD106; R&D Systems, Minneapolis, MN, USA). Four clones of anti-CD49b (α2 integrin) were tested: 12F1-H6 and AK-7 (BD PharMingen); Gi9 (Beckman Coulter); and P1E6 (Chemicon). We used isotype controls from the same firms as mAb. Anti-CD29 (β1 integrin, clone K20) was a generous gift from Alain Bernard (INSERM U576, Nice, France). TNF-α was from PeproTech (Rocky Hill, NJ, USA), and $HBSS^{++}$ and collagenase type I from *Clostridium histolyticum* were from Gibco (Paisley, Scotland). All other reagents were from Sigma-Aldrich (St. Louis, MO, USA).

Neutrophil and endothelial cell isolation

Human neutrophils and endothelial cell HUVECs were isolated as described [18, 19] from platelet-depleted blood and from the umbilical vein, respectively, from healthy volunteers. Neutrophils (2×10^6/ml) were suspended in $HBSS^{++}$-BSA. HUVECs were cultured on gelatin-coated flasks in M199 supplemented with heparin (50 μg/ml), endothelial cell growth supplement (Sigma-Aldrich; 20 μg/ml), and 20% FCS. Third and fourth passage cells were used exclusively.

Flow cytometry

PMN were labeled as described [20], after blocking FcγRs with heat-aggregated goat IgG and analyzed by flow cytometry on FACSCalibur (Becton Dickinson, Mountain View, CA, USA). Results are given as MFI.

Assays for PMN adhesion to HUVEC

Static and flow adhesion of PMN on confluent HUVECs, pretreated for 4 h with 10 ng/ml TNF-α for static adhesion or 0.2 ng/ml TNF-α for flow adhesion, were tested as described [21]. When mentioned, PMN were preincubated for 20 min with blocking anti-α9 clone Y9A2, anti-β2 (clone IB4) mAb or isotype controls, or HUVECs preincubated with anti-VCAM-1 mAb (10 μg/ml) or the isotype control before the adhesion assay.

Flow assay

Cell adhesion was analyzed by real-time videomicroscopy as described [21] in a flow chamber containing confluent HUVECs. Controlled flow rates were applied to generate a wall shear rate of 100 s^{-1} (0.1 Pa), and warmed 37°C buffer and PMN (2×10^6/ml in $HBSS^{++}$-BSA) were perfused sequentially, for 4 min each, over the endothelial cell layer. This was followed by a period of washout with prewarmed buffer. Video recordings were made between 1 and 2 min of washout, as described [22]. The perfusion chamber was set on the stage of an inverted microscope (Axiovert 135, Carl Zeiss, Jena, Germany) with a 10× Hoffman modulation contrast objective and a charged-coupled device camera (Sony, Tokyo, Japan). Histolab, Videomet, and Replay software (Microvision Instruments, Evry, France) allowed quantifying the number of adherent cells visible in at least three fields and measuring instant speeds (μm/s) of each moving cell. The percentage of rolling neutrophils was defined as the percentage of cells in contact with the HUVEC surface and moving by >1 cell diameter in a 1-min time-lapse in a given field.

Statistical analysis

Data were compared using a paired *t*-test analysis and statistical differences in instant velocities of rolling cells evaluated with a two-tailed Mann Whitney U test. Statistical significance was defined as follows: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

RESULTS AND DISCUSSION

Membrane expression of β1 integrins on resting neutrophils

As we pointed out previously [3], the two major pitfalls of β1 or β3 integrin analysis on neutrophils are: i) the presence of neutrophil FcγRs, which may react with IgG aggregates present in anti-integrin antibody preparations. Neutrophil stimuli modulate the FcγR expression and/or activation state [23, 24], which may up- or down-regulate the nonspecific binding of IgGs. ii) The contamination by platelets, which adhere to neutrophils [25] and express high levels of α2β1, α5β1, and α6β1 integrins.

The membrane expression of β1 integrins was thus analyzed on neutrophil preparations devoid of platelets, as shown by the absence of CD41(GPIIb) labeling (data not shown) and after blocking FcγRs with aggregated goat IgGs before labeling [3]. In these conditions, the binding of saturating doses of mAb, specific for each integrin α chain and detected by flow cytometry with the same fluorescent secondary antibody, revealed that resting neutrophils express similar levels of α5 and α9 integrins, each representing about one-half of β1 integrins, and α6 membrane levels only reached 14% of β1 integrins (**Fig. 1**). Three anti-α2 antibodies resulted in negative labeling of native or stimulated neutrophils, while they labeled platelets efficiently (Fig. 1). A fourth anti-α2 mAb, clone 12F1-H6, probably reacted nonspecifically with neutrophils, as it labeled cells stimulated by fMLP exclusively with cytochalasin B (data not shown), a condition known to decrease the repulsive negative charge of the PMN outer membrane [26] and favor the nonspecific binding of antibodies. Although we cannot formally exclude that neutrophils express α2β1, our results thus suggest that this integrin is not present on the membrane of human neutrophils. The discrepancy between our results and in vitro data published previously, describing α2β1 membrane expression on human resting neutrophils [6, 27], could result from the lack of complete platelet depletion before neutrophil isolation or the absence of the FcγR blockade in labeling protocols. Although the presence of functional α2β1 integrins on neutrophils had been suggested by a defective neutrophil recruitment in α2-deficient mice [6], Edelson et al. [28] demonstrated elegantly that this resulted from an indirect effect involving α2 null mast cells, unable to support in vivo neutrophil recruitment.

Our results are in agreement with a previous report showing mainly α9β1 and α5β1 and no α2β1 expression on platelet-depleted neutrophil preparations [29] and confirm that α9β1 is one of the major β1 integrins constitutively expressed on neutrophils [11].

Up-regulation of β1 integrin membrane expression upon neutrophil degranulation

We then submitted neutrophils to increasing concentrations of secretagogue. As shown in **Figure 2A**, micromolar concentrations of fMLP enhanced α5 membrane expression slightly (33±36%

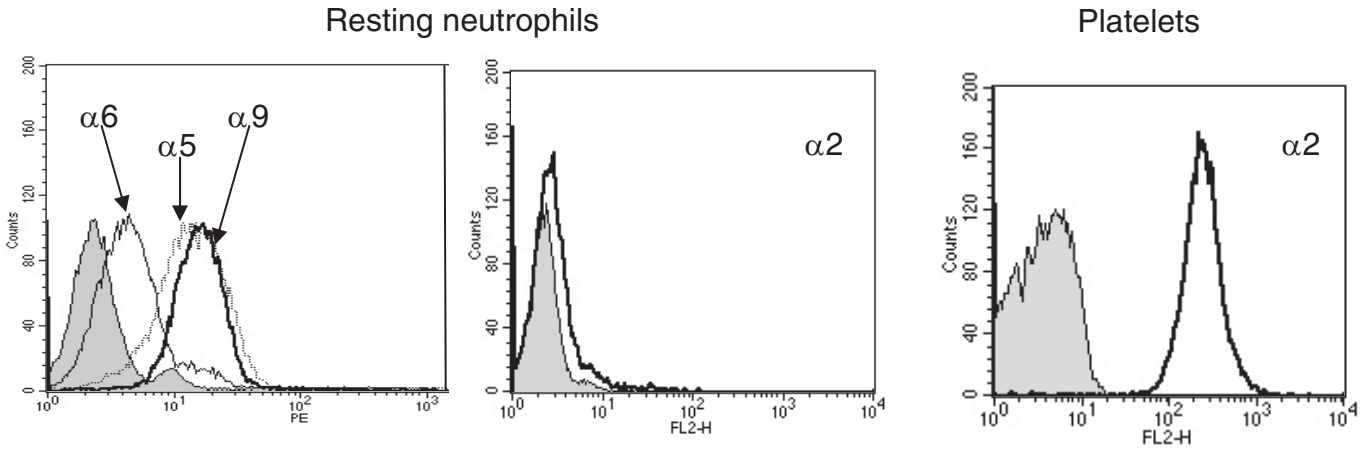

Figure 1. Expression of α5β1, α6β1, and α9β1 and lack of α2 chain expression on resting neutrophils. Resting neutrophils (left and middle panels) were labeled with saturating amounts of mAb directed against the various integrin α chains to measure the respective expression of each α chain by flow cytometry. The shaded peaks represent the isotype control. Platelets (right panel), recovered from the initial blood centrifugation of neutrophil isolation protocol, were labeled with anti-α2 and are used as a positive control. Similar results were obtained with α2 Gi9, AK-7, and P1E6 anti-α2 clones. FL2-H, Fluorescence 2-height.

increase and 75±43% with cytochalasin B priming). fMLP did not modify α6 membrane expression, and the small increase of anti-α6 labeling observed on cytochalasin B-primed neutrophils was nonspecific, as it was similarly observed with the IgG1 isotype control (data not shown). By contrast, a progressive up-regulation of α9 was observed, which was already significant with nanomolar fMLP concentrations (60±40% increase), reached a 165 ± 90% increase with cytochalasin B and fMLP, and paralleled the up-regulation of αMβ2 integrin CD11b/CD18. The membrane expression of the integrin β1 chain increased, as did α9, although nanomolar concentrations of fMLP did not result in significant β1 up-regulation. This could result from a lower efficiency of the anti-β1 mAb, insufficient to detect minute up-regulations, or from the constant amounts of α5β1 and α6β1, which may obscure small increases of β1 levels related to α9β1 expression. α9β1 thus appeared as the major β1 integrin of fMLP-activated PMN (Fig. 2B). A similar α9β1 up-regulation on neutrophils was observed with other agonists, such as TNF-α, IL-8, or PMA (Fig. 2C). Finally, when citrated whole blood was incubated with TNF-α and fMLP, this resulted in increased levels of α9β1 on the neutrophil membrane (Fig. 2D), and we confirmed the absence of α9β1 on lymphocytes or monocytes (data not shown).

Our observation of a parallel increase of α9β1 and CD11b/CD18 membrane expressions upon neutrophil activation suggests the existence of intracellular storage pools of α9β1 similar to those of CD11b/CD18, i.e., in secretory vesicles, mobilized by nanomolar fMLP concentrations, and in specific granules. These results thus confirm the presence of "adhesomes," i.e., intracellular pools of receptors for extracellular matrix, in neutrophil granules [30].

Modulation of β1 integrin membrane expression upon neutrophil adhesion to endothelial cells and transmigration

Up-regulations of α2β1 and α6β1 integrin membrane expression had been reported during neutrophil recruitment to inflammation sites, mostly based on in vivo experiments in rodents [6, 27, 31–34]. We thus investigated modulations of integrin membrane expression resulting from neutrophil migration across a monolayer of confluent HUVECs, cultured on gelatin-coated transwells and preactivated with TNF-α. Neutrophil adhesion to TNF-activated HUVECs did not result in significant modulation of α9β1 integrin expression (**Fig. 3A**). In this case, neutrophils are activated exclusively by stimuli exposed on the membrane of endothelial cells, such as IL-8, platelet-activating factor, or leukotriene B4. The amount of these agonists, in our in vitro setting, may not be sufficient to produce an increase of α9β1 expression detectable by flow cytometry. Alternatively, α9β1 up-regulation could be compensated during PMN adhesion by an endocytic recycling similar to that described previously for αvβ3 and α6β1 integrins on migrating PMN [5, 35]. The observation that neutrophil adhesion to TNF-activated HUVECs or to gelatin-coated plates (data not shown) does not modify α9β1 membrane levels indicates that β2 integrin engagement is not a trigger for α9β1 up-regulation.

Contrasting with cell adhesion, a 30-min transmigration resulted in a significant increase of α9 levels (63±34% increase; $n=7$; Fig. 3A) and of the overall β1 membrane expression (59±57% increase; Fig. 3B) of neutrophils recovered from the lower transwell compartment. Neither α5 nor α6 expression was modified by the transmigration (Fig. 3B), and α2 remained absent (data not shown).

The discrepancy between our results and previous in vivo observations, showing enhanced α2β1 and α6β1 membrane expression on inflammatory neutrophils, could be a result of species differences or the known association of β1-rich platelets with migrating neutrophils [36].

Participation of α9β1 in neutrophil static adhesion to endothelial cells and transendothelial migration

As VCAM-1 is one of α9β1 ligands, we analyzed the role of α9β1 in neutrophil adhesion to TNF-α-activated HUVECs,

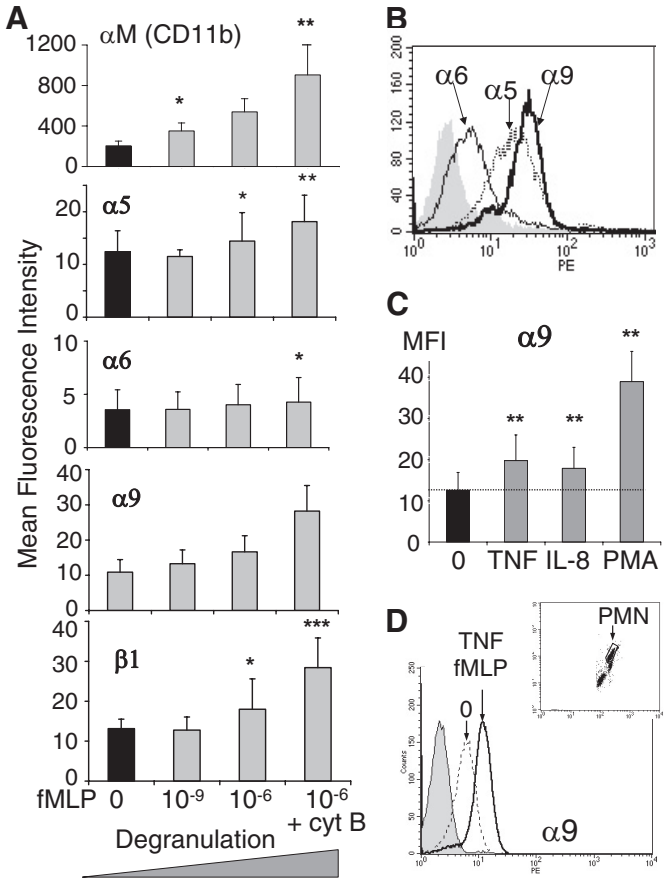

Figure 2. Modulation of β1 integrin expression upon PMN activation. (A) PMN (2×10^6/ml in $HBSS^{++}$-BSA) were gently tumbled every 5 min during their activation at 37°C with increasing concentration of fMLP for 5 min at 37°C. Maximum degranulation was obtained by preincubation of PMN with cytochalasin B (cyt B; 10 μg/ml) for 10 min at 37°C before the addition of 10^{-6} M fMLP. Cells were then labeled with anti-integrin mAb and analyzed by flow cytometry. Results are expressed as mean ± SD of MFI after subtraction of each isotype control MFI (n=7–11). Integrin expressions on activated PMN were compared with those of resting cells (black bars). (B) Quantitative expression of β1 integrins on neutrophils activated with 10^{-6} M fMLP, as measured by flow cytometry, as in Figure 1. (C) Membrane expression of the α9 chain on PMN activated for 30 min at 37°C with 10 ng/ml TNF-α, 25 ng/ml IL-8, or 10 ng/ml PMA and labeled as in A (n=4–10). (D) Neutrophil membrane expression of the α9 chain in citrated whole blood before and after incubation at 37°C with 10 ng/ml TNF for 15 min followed by 10^{-6} M fMLP for 15 min. After labeling with the anti-α9 mAb, erythrocytes were lysed with Becton Dickinson FACS lysing solution before adding the secondary antibody. The flow cytometry histogram describes α9 membrane expression of PMN, selected forward-/side-scatter gating, as shown by the inset. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

which expressed VCAM-1 on their surface (data not shown). **Figure 4A** shows that the anti-α9-blocking mAb resulted in a small, although significant, decrease of PMN adhesion to endothelial cells. As expected, the adhesion was strikingly inhibited by a blocking anti-β2 mAb. Anti-α9 and anti-β2 inhibitory effects were not additive, as no significant difference was observed when PMN were treated with anti-β2 mAb alone or in conjunction with anti-α9 antibodies. This result implies, as reported previously [13], that α9β1 does not promote a β2-independent static adhesion but may modulate β2-mediated effects. Kinetic experiments showed that the effect of anti-α9β1 mAb was detected only when antibody-treated cells were in contact for more than 20 min with endothelial cells (data not shown). One should point out, however, that this inhibition of neutrophil static adhesion by anti-α9β1 mAb, although significant, was limited to 15 ± 10% inhibition, as compared with the 60–80% inhibition by anti-β2 mAb.

The effect of blocking anti-α9 antibodies was then tested on neutrophil migration across HUVEC-coated transwells. Anti-β2 mAb significantly prevented neutrophil transmigration, and anti-α9 antibodies, alone or together with anti-β2 mAb, were without effect (Fig. 4B). The participation of α9β1 in neutrophil transmigration has been a matter of discussion, as transendothelial migration was shown to be inhibited [13] or not modified [11] by the clone Y9A2 anti-α9-blocking mAb. It is worth noting that in the former work of Taooka et al. [13], the inhibition was partial, and we observed a similar inhibition in two out of five experiments. We cannot exclude that some antibody samples, containing aggregated IgGs, result in nonspecific inhibitions of neutrophil functions when compared with control nonaggregated isotypes. On the other hand, we here confirm the data of Shang et al. [11] and observed no inhibitory effect of the same anti-α9-blocking antibody alone or in conjunction with anti-β2-blocking mAb.

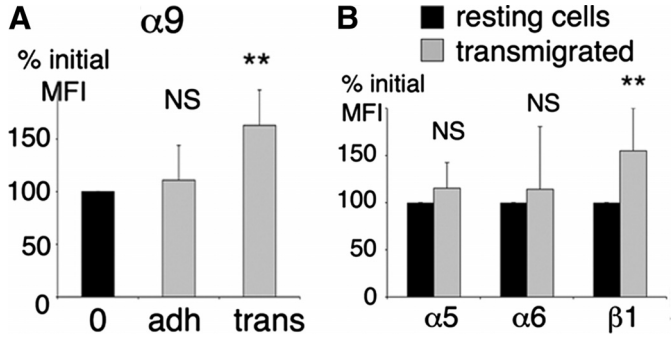

Figure 3. Expression of β1 integrins on adherent and transmigrated neutrophils. PMN (6×10^5; in 300 μl M199 with 0.1% BSA) were allowed to adhere, for 30 min at 37°C, to TNF-activated HUVECs in 48-well plates. Adherent cells (adh) were collected after washing off nonadherent PMN and treatment with 0.5 mg/ml collagenase for 15 min at 37°C. Alternatively, 10^6 PMN in 500 μl M199 with 0.1% BSA were allowed to migrate across TNF-preactivated HUVEC cultured on gelatin-coated, 12 mm diameter transwells with 3 μm pore size (Corning, Corning, NY, USA), with 25 ng/ml IL-8 in the lower compartment. PMN were recovered from this lower compartment (trans) after 30 min migration at 37°C with 5% CO_2. In both cases, cells were labeled with anti-integrin mAb and analyzed by flow cytometry as in Figure 2. PMN and HUVECs were distinguished by forward-/side-scatter gating, confirmed by anti-CD146 labeling (see Fig. 4). (A) Expression of α9β1 on adherent or transmigrated PMN, as compared with resting cells (black bar; n=7). (B) α5, α6, and β1 expression on transmigrated cells, as compared with resting cells (black bars; n=6–20 experiments). NS, Not significant; **, $P < 0.01$.

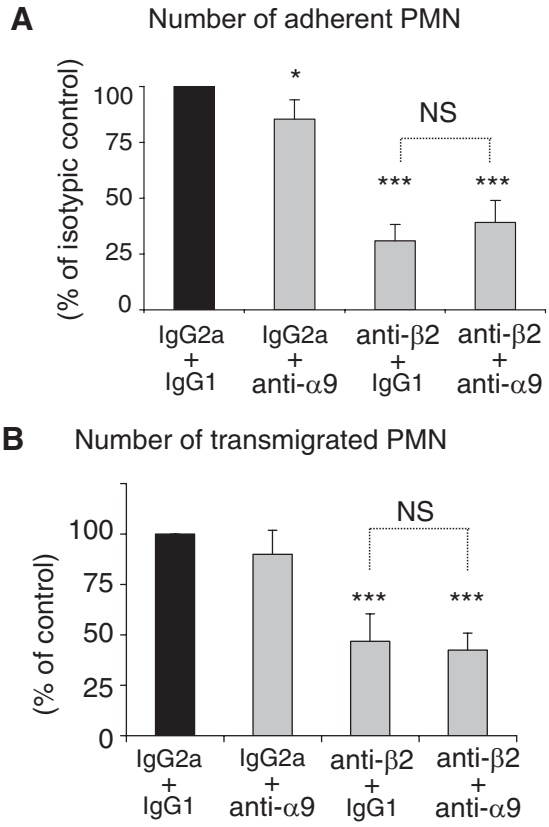

Figure 4. Effects of anti-$\alpha 9$ and anti-$\beta 2$-blocking mAb on neutrophil static adhesion to endothelial cells and transmigration. (A) Static adhesion of PMN to TNF-activated HUVECs was performed as in Figure 3A, except that PMN were preincubated with the 10 μg/ml IgG1 anti-$\alpha 9$ or the IgG2a anti-$\beta 2$-blocking mAb, with corresponding isotype controls, or with a mixture of both anti-integrin clones for 20 min at room temperature before the adhesion assay. After washing off nonadherent cells, adherent PMN were recovered with HUVECs and labeled for CD146. CD146-negative PMN were then counted by flow cytometry with a constant time setting. (B) PMN were allowed to migrate across TNF-activated HUVECs on transwell as in Figure 3B, except that PMN had been preincubated with the anti-$\alpha 9$ and anti-$\beta 2$ as in A. The number of transmigrated PMN, recovered from the transwell lower part, was counted by flow cytometry with a constant time setting. Results are expressed as the number of adherent or transmigrated neutrophils treated with anti-integrin mAb, normalized with the number obtained with isotype controls, considered as 100% (black bar). Results are the mean ± SD of five similar experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Participation of $\alpha 9\beta 1$ in neutrophil adhesion to endothelial cells under flow conditions

We then tested the effect of blocking anti-$\alpha 9$ antibodies on neutrophil adhesion to TNF-activated HUVECs under flow conditions. As shown in **Figure 5A**, the anti-$\alpha 9$ mAb alone had no effect, and anti-$\beta 2$ mAb decreased the overall number of adherent PMN, as compared with control cells treated with the IgG isotype control, in a dose-related manner (left panel, 2 μg/ml anti-$\beta 2$; right panel, 10 μg/ml anti-$\beta 2$). Importantly, anti-$\alpha 9$ mAb further potentiated the inhibition promoted by those limiting anti-$\beta 2$ concentrations. In similar conditions, the anti-$\alpha 6$-blocking mAb GoH3 had no effect on neutrophil adhesion, with or without limiting amounts of anti-$\beta 2$ mAb (data not shown).

One should point out that concentrations of anti-$\beta 2$ mAb higher than 10 μg /ml resulted in complete inhibition of neutrophil adhesion (data not shown), further suggesting that under flow as under static conditions, $\alpha 9\beta 1$ does not promote adhesion by itself. This is reminiscent of a previous report showing that neutrophils with blocked $\beta 2$ integrins were unable to adhere to $\alpha 9\beta 1$-specific substrates in spite of conditions known to activate $\beta 1$ integrins, such as the presence of Mn^{++}, PMA, or anti-$\beta 1$-activating mAb [13]. The inhibitory effect of anti-$\alpha 9\beta 1$ mAb on neutrophils with limiting amounts of functional $\beta 2$ integrins could then be related to a cross-

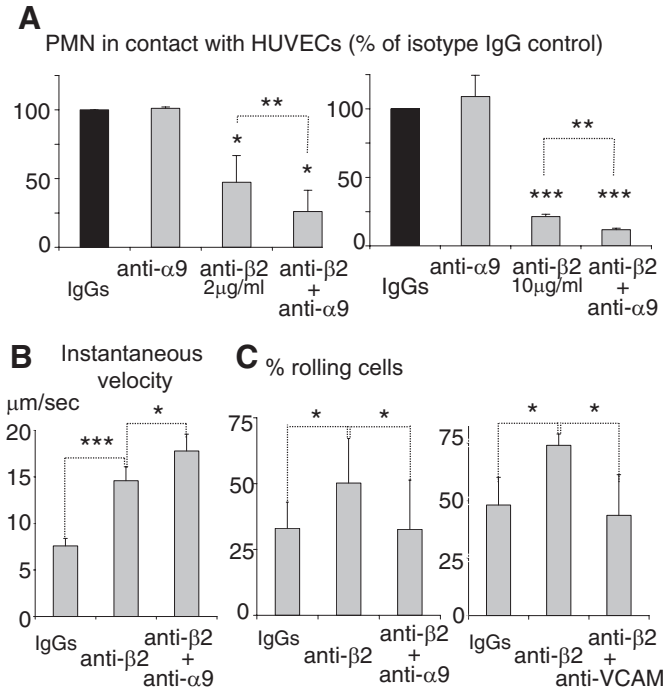

Figure 5. Effects of anti-$\alpha 9$ and anti-$\beta 2$-blocking mAb on neutrophil adhesion to endothelial cells under flow conditions. PMN adhesion to TNF-activated HUVECs under shear flow conditions was analyzed as described in Materials and Methods, after preincubation with anti-$\alpha 9$ and anti-$\beta 2$-blocking mAb or isotype controls as in Figure 4A. (A) Number of adherent PMN treated with IgG1 and IgG2a (anti-$\alpha 9$ and anti-$\beta 2$ isotype controls), 10 μg/ml anti-$\alpha 9$ and IgG2a, 2 μg/ml anti-$\beta 2$ (left panel) or 10 μg/ml anti-$\beta 2$ (right panel), with 10 μg/ml IgG1 or anti-$\alpha 9$ in both panels. Data give the mean ± SD ($n=3$ in each panel) of the mean number of adherent neutrophils per field, normalized with the results obtained with control isotypes (black bars). (B and C) Experiments were performed as in A after PMN preincubation with anti-$\alpha 9$ and anti-$\beta 2$-blocking mAb ($n=5$ experiments). (B) Instantaneous velocities of rolling cells, i.e., distances in μm/s, of each moving cell followed during 30 s–1 min on one to three fields. At least 20 cells per condition were followed. (C) Number of rolling cells, defined as the percentage of PMN in contact with HUVECs, which moved in a lapse-time of 1 min in a given field. Similar experiments were performed with untreated PMN perfused on HUVECs pretreated with anti-VCAM-1 before perfusion (right panel, $n=5$ experiments).*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

talk between $\alpha 9\beta 1$ and $\beta 2$ integrins, similar to that described previously for $\alpha 4\beta 1$ and $\beta 2$ integrins on myeloid cell lines [37].

When analyzing the rolling cells, as defined in Materials and Methods, we observed that the blocking anti-$\beta 2$ mAb led to a two-fold increase of their instantaneous velocities (Fig. 5B), confirming the role of $\beta 2$ integrins in slowing down the rolling movement by promoting a firm adhesion. Importantly, those instantaneous velocities were enhanced further when $\beta 2$ and $\alpha 9\beta 1$ integrins were blocked (Fig. 5B), suggesting a participation of $\alpha 9\beta 1$ in stabilizing PMN adhesion.

The percentage of rolling PMN, among cells in contact with HUVECs, was not affected by the anti-$\alpha 9$ mAb alone (data not shown) but was enhanced by the anti-$\beta 2$ mAb (Fig. 5C). One would indeed expect that preventing the $\beta 2$ integrin-mediated firm adhesion would prolong the rolling duration and thereby, increase the percentage of rolling cells. Interestingly, when neutrophils were incubated with anti-$\alpha 9$ and anti-$\beta 2$ integrin antibodies, the anti-$\alpha 9$ mAb reversed the effect of the anti-$\beta 2$ mAb, i.e., decreased the percentage of these rolling PMN with blocked $\beta 2$ integrins (Fig. 5C). We propose that the inhibition of $\beta 2$ and $\alpha 9\beta 1$ integrins would result in such an unstable adhesion, as suggested by the high rolling velocity, that some PMN detach from the endothelial substrate and are no more counted as rolling or adherent cells.

In summary, the inhibition of $\beta 2$ integrin-induced adhesion resulted in a smaller number of neutrophils in contact with the endothelium. Those were mainly rolling cells with a higher velocity than untreated neutrophils. Anti-$\alpha 9$-blocking antibodies further enhanced the velocity of these rolling PMN with blocked $\beta 2$ integrins, probably leading to cell detachment and decreasing the total number of adherent neutrophils and the percentage of mobile cells.

As VCAM-1 is one of the $\alpha 9\beta 1$ ligands, we looked if pretreating endothelial cells with a blocking anti-VCAM-1 mAb would have an effect similar to neutrophil treatment with the anti-$\alpha 9$ mAb. Anti-VCAM-1 did not significantly modify the number of adherent PMN in the presence or not of anti-$\beta 2$ mAb (data not shown). This could be related to a lower blocking efficiency of anti-VCAM-1 antibodies or alternatively, would suggest the presence of other $\alpha 9\beta 1$ ligands on endothelial cells. However, like anti-$\alpha 9$ mAb-treated PMN, HUVEC pretreatment with anti-VCAM-1 mAb clearly decreased the percentage of rolling PMN with blocked $\beta 2$ integrins (Fig. 5C).

These results suggest that the binding of $\alpha 9\beta 1$ to VCAM-1 stabilizes interactions between rolling neutrophils and the endothelium when the adhesion efficiency of PMN $\beta 2$ integrins is decreased. This proposed role of $\alpha 9\beta 1$ is reminiscent of the reported ability of T cell $\alpha 4\beta 1$ to support the tethering and rolling of leukocytes under shear flow and their firm adhesion under static or flow conditions [38, 39]. Similarities in the signaling used by $\alpha 9\beta 1$ and $\alpha 4\beta 1$ to promote cell migration have indeed been described recently [17]. Selectins/mucins are the major partners of leukocyte rolling, and we confirmed previously that anti-E-selectin-blocking antibodies prevent neutrophil rolling completely on activated endothelial cells in spite of the presence of $\alpha 9\beta 1$ on the former and of VCAM-1 on the latter [21]. One could speculate that although selectins mediate the initial tethering and rolling of leukocytes on endothelial cells, $\alpha 9\beta 1$/VCAM-1 interactions would allow a more prolonged rolling if the following step of $\beta 2$ integrin-mediated stable adhesion is delayed or insufficient. It is possible that shear forces stabilize the $\alpha 9\beta 1$ active conformation, as shown for $\alpha v\beta 3$ [40], so that $\alpha 9\beta 1$ would not participate immediately to neutrophil adhesion but require some time in the shear flow. This would explain the absence of $\alpha 9\beta 1$ participation to neutrophil adhesion to endothelial cells in static conditions, even when $\beta 2$ integrins are blocked. These hypotheses certainly deserve further investigations.

In conclusion, as a result of its rather recent description about neutrophils, as compared with $\alpha 5\beta 1$ and $\alpha 6\beta 1$, the participation of $\alpha 9\beta 1$ in neutrophil functions has been scarcely studied. We confirm here some of the data reported previously and add two original facts: an up-regulation of $\alpha 9\beta 1$ membrane expression by inflammation neutrophil agonists and by transmigration and a new role of $\alpha 9\beta 1$ in stabilizing neutrophil stable adhesion to endothelial cells under shear flow.

AUTHORSHIP

A.M. performed experiments and analyzed data; S.B. performed experiments; D.B. contributed by her expertise in adhesion under flow conditions and with helpful discussions; P.L. analyzed and discussed data and corrected the manuscript; and L.H-M. designed experiments, analyzed data, and wrote the manuscript.

ACKNOWLEDGMENTS

A.M. was supported successively by a grant from the Association pour La Recherche sur la Polyarthrite (Arthritis Foundation) and a grant from the Société Française d'Hématologie. We acknowledge support from the Association pour l'Utilization du Rein Artificiel AURA, from Baxter, and from Amgen. The authors thank Alain Bernard (INSERM U576, Nice, France) for providing the anti-CD29 mAb.

REFERENCES

1. Bohnsack, J. F., Zhou, X. N. (1992) Divalent cation substitution reveals CD18- and very late antigen-dependent pathways that mediate human neutrophil adherence to fibronectin. *J. Immunol.* **149,** 1340–1347.
2. Bohnsack, J. F. (1992) CD11/CD18-independent neutrophil adherence to laminin is mediated by the integrin VLA-6. *Blood* **79,** 1545–1552.
3. Rieu, P., Lesavre, P., Halbwachs-Mecarelli, L. (1993) Evidence for integrins other than β 2 on polymorphonuclear neutrophils: expression of α 6 β 1 heterodimer. *J. Leukoc. Biol.* **53,** 576–582.
4. Everitt, E. A., Malik, A. B., Hendey, B. (1996) Fibronectin enhances the migration rate of human neutrophils in vitro. *J. Leukoc. Biol.* **60,** 199–206.
5. Pierini, L. M., Lawson, M. A., Eddy, R. J., Hendey, B., Maxfield, F. R. (2000) Oriented endocytic recycling of $\alpha 5\beta 1$ in motile neutrophils. *Blood* **95,** 2471–2480.
6. Werr, J., Johansson, J., Eriksson, E. E., Hedqvist, P., Ruoslahti, E., Lindbom, L. (2000) Integrin $\alpha(2)\beta(1)$ (VLA-2) is a principal receptor used by neutrophils for locomotion in extravascular tissue. *Blood* **95,** 1804–1809.
7. Davenpeck, K. L., Sterbinsky, S. A., Bochner, B. S. (1998) Rat neutrophils express $\alpha 4$ and $\beta 1$ integrins and bind to vascular cell adhesion molecule-1 (VCAM-1) and mucosal addressin cell adhesion molecule-1 (MAdCAM-1). *Blood* **91,** 2341–2346.
8. Issekutz, T. B., Miyasaka, M., Issekutz, A. C. (1996) Rat blood neutrophils express very late antigen 4 and it mediates migration to arthritic joint and dermal inflammation. *J. Exp. Med.* **183,** 2175–2184.
9. Henderson, R. B., Lim, L. H., Tessier, P. A., Gavins, F. N., Mathies, M., Perretti, M., Hogg, N. (2001) The use of lymphocyte function-associated antigen (LFA)-1-deficient mice to determine the role of LFA-1, Mac-1, and $\alpha 4$ integrin in the inflammatory response of neutrophils. *J. Exp. Med.* **194,** 219–226.
10. Kirveskari, J., Bono, P., Granfors, K., Leirisalo-Repo, M., Jalkanen, S., Salmi, M. (2000) Expression of $\alpha 4$-integrins on human neutrophils. *J. Leukoc. Biol.* **68,** 243–250.

11. Shang, T., Yednock, T., Issekutz, A. C. (1999) $\alpha 9\beta 1$ integrin is expressed on human neutrophils and contributes to neutrophil migration through human lung and synovial fibroblast barriers. *J. Leukoc. Biol.* **66,** 809–816.
12. Palmer, E. L., Ruegg, C., Ferrando, R., Pytela, R., Sheppard, D. (1993) Sequence and tissue distribution of the integrin α 9 subunit, a novel partner of β 1 that is widely distributed in epithelia and muscle. *J. Cell Biol.* **123,** 1289–1297.
13. Taooka, Y., Chen, J., Yednock, T., Sheppard, D. (1999) The integrin $\alpha 9\beta 1$ mediates adhesion to activated endothelial cells and transendothelial neutrophil migration through interaction with vascular cell adhesion molecule-1. *J. Cell Biol.* **145,** 413–420.
14. Chen, C., Huang, X., Atakilit, A., Zhu, Q. S., Corey, S. J., Sheppard, D. (2006) The integrin $\alpha 9\beta 1$ contributes to granulopoiesis by enhancing granulocyte colony-stimulating factor receptor signaling. *Immunity* **25,** 895–906.
15. Liu, S., Slepak, M., Ginsberg, M. H. (2001) Binding of paxillin to the α 9 integrin cytoplasmic domain inhibits cell spreading. *J. Biol. Chem.* **276,** 37086–37092.
16. Young, B. A., Taooka, Y., Liu, S., Askins, K. J., Yokosaki, Y., Thomas, S. M., Sheppard, D. (2001) The cytoplasmic domain of the integrin α9 subunit requires the adaptor protein paxillin to inhibit cell spreading but promotes cell migration in a paxillin-independent manner. *Mol. Biol. Cell* **12,** 3214–3225.
17. Gupta, S. K., Vlahakis, N. E. (2009) Integrin $\alpha 9\beta 1$ mediates enhanced cell migration through nitric oxide synthase activity regulated by Src tyrosine kinase. *J. Cell Sci.* **122,** 2043–2054.
18. Seveau, S., Lopez, S., Lesavre, P., Guichard, J., Cramer, E. M., Halbwachs-Mecarelli, L. (1997) Leukosialin (CD43, sialophorin) redistribution in uropods of polarized neutrophils is induced by CD43 cross-linking by antibodies, by colchicine or by chemotactic peptides. *J. Cell Sci.* **110,** 1465–1475.
19. Jaffe, E. A., Nachman, R. L., Becker, C. G., Minick, C. R. (1973) Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J. Clin. Invest.* **52,** 2745–2756.
20. Nusbaum, P., Laine, C., Bouaouina, M., Seveau, S., Cramer, E. M., Masse, J. M., Lesavre, P., Halbwachs-Mecarelli, L. (2005) Distinct signaling pathways are involved in leukosialin (CD43) down-regulation, membrane blebbing, and phospholipid scrambling during neutrophil apoptosis. *J. Biol. Chem.* **280,** 5843–5853.
21. Mambole, A., Baruch, D., Nusbaum, P., Suzuki, M., Lesavre, P., Fukuda, M., Halbwachs-Mecarelli, L. (2008) The cleavage of neutrophil leukosialin (CD43) by cathepsin G releases its extracellular domain and triggers its intramembrane proteolysis by presenilin/γ-secretase. *J. Biol. Chem.* **283,** 23627–23635.
22. Radford, D. J., Luu, N. T., Hewins, P., Nash, G. B., Savage, C. O. (2001) Antineutrophil cytoplasmic antibodies stabilize adhesion and promote migration of flowing neutrophils on endothelial cells. *Arthritis Rheum.* **44,** 2851–2861.
23. Huizinga, T. W., van der Schoot, C. E., Jost, C., Klaassen, R., Kleijer, M., von dem Borne, A. E., Roos, D., Tetteroo, P. A. (1988) The PI-linked receptor FcRIII is released on stimulation of neutrophils. *Nature* **333,** 667–669.
24. Nagarajan, S., Fifadara, N. H., Selvaraj, P. (2005) Signal-specific activation and regulation of human neutrophil Fc γ receptors. *J. Immunol.* **174,** 5423–5432.
25. Zarbock, A., Polanowska-Grabowska, R. K., Ley, K. (2007) Platelet-neutrophil-interactions: linking hemostasis and inflammation. *Blood Rev.* **21,** 99–111.
26. Gallin, J. I. (1980) Degranulating stimuli decrease the negative surface charge and increase the adhesiveness of human neutrophils. *J. Clin. Invest.* **65,** 298–306.
27. Roussel, E., Gingras, M. C. (1997) Transendothelial migration induces rapid expression on neutrophils of granule-release VLA6 used for tissue infiltration. *J. Leukoc. Biol.* **62,** 356–362.
28. Edelson, B. T., Li, Z., Pappan, L. K., Zutter, M. M. (2004) Mast cell-mediated inflammatory responses require the α 2 β 1 integrin. *Blood* **103,** 2214–2220.
29. Van den Berg, J. M., Mul, F. P., Schippers, E., Weening, J. J., Roos, D., Kuijpers, T. W. (2001) β1 integrin activation on human neutrophils promotes β2 integrin-mediated adhesion to fibronectin. *Eur. J. Immunol.* **31,** 276–284.
30. Singer, I. I., Scott, S., Kawka, D. W., Kazazis, D. M. (1989) Adhesomes: specific granules containing receptors for laminin, C3bi/fibrinogen, fibronectin, and vitronectin in human polymorphonuclear leukocytes and monocytes. *J. Cell Biol.* **109,** 3169–3182.
31. Dangerfield, J., Larbi, K. Y., Huang, M. T., Dewar, A., Nourshargh, S. (2002) PECAM-1 (CD31) homophilic interaction up-regulates $\alpha 6\beta 1$ on transmigrated neutrophils in vivo and plays a functional role in the ability of α6 integrins to mediate leukocyte migration through the perivascular basement membrane. *J. Exp. Med.* **196,** 1201–1211.
32. Dangerfield, J. P., Wang, S., Nourshargh, S. (2005) Blockade of α6 integrin inhibits IL-1β- but not TNF-α-induced neutrophil transmigration in vivo. *J. Leukoc. Biol.* **77,** 159–165.
33. Kubes, P., Niu, X. F., Smith, C. W., Kehrli Jr., M. E., Reinhardt, P. H., Woodman, R. C. (1995) A novel β 1-dependent adhesion pathway on neutrophils: a mechanism invoked by dihydrocytochalasin B or endothelial transmigration. *FASEB J.* **9,** 1103–1111.
34. Werr, J., Xie, X., Hedqvist, P., Ruoslahti, E., Lindbom, L. (1998) β1 integrins are critically involved in neutrophil locomotion in extravascular tissue in vivo. *J. Exp. Med.* **187,** 2091–2096.
35. Lawson, M. A., Maxfield, F. R. (1995) Ca(2+)- and calcineurin-dependent recycling of an integrin to the front of migrating neutrophils. *Nature* **377,** 75–79.
36. Weissmuller, T., Campbell, E. L., Rosenberger, P., Scully, M., Beck, P. L., Furuta, G. T., Colgan, S. P. (2008) PMNs facilitate translocation of platelets across human and mouse epithelium and together alter fluid homeostasis via epithelial cell-expressed ecto-NTPDases. *J. Clin. Invest.* **118,** 3682–3692.
37. May, A. E., Neumann, F. J., Schomig, A., Preissner, K. T. (2000) VLA-4 ($\alpha(4)\beta(1)$) engagement defines a novel activation pathway for $\beta(2)$ integrin-dependent leukocyte adhesion involving the urokinase receptor. *Blood* **96,** 506–513.
38. Alon, R., Kassner, P. D., Carr, M. W., Finger, E. B., Hemler, M. E., Springer, T. A. (1995) The integrin VLA-4 supports tethering and rolling in flow on VCAM-1. *J. Cell Biol.* **128,** 1243–1253.
39. Berlin, C., Bargatze, R. F., Campbell, J. J., von Andrian, U. H., Szabo, M. C., Hasslen, S. R., Nelson, R. D., Berg, E. L., Erlandsen, S. L., Butcher, E. C. (1995) α 4 Integrins mediate lymphocyte attachment and rolling under physiologic flow. *Cell* **80,** 413–422.
40. Puklin-Faucher, E., Gao, M., Schulten, K., Vogel, V. (2006) How the headpiece hinge angle is opened: new insights into the dynamics of integrin activation. *J. Cell Biol.* **175,** 349–360.

KEY WORDS:
VCAM-1 · endothelial cells · rolling