# In Vivo Behavior of Neutrophils from Two Patients with Distinct Inherited Leukocyte Adhesion Deficiency Syndromes

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## Abstract

**The selectins and the β2-integrins (CD11/CD18) mediate distinct adhesive interactions between neutrophils and endothelial cells. Selectins are believed to initiate binding by mediating neutrophil rolling, whereas β2-integrins are required for subsequent activation-induced firm sticking and emigration. In vitro evidence suggests that two endothelial cell selectins, P- and E-selectin, can mediate rolling by binding to the carbohydrate ligand sialyl-Lewis$^x$ (sLe$^x$) on neutrophil surface glycoconjugates. To test the relative contribution of selectins and β2-integrins in vivo we used intravital microscopy to study the behavior of neutrophils from two patients with distinct inherited leukocyte adhesion deficiency syndromes. Neutrophils from a patient suffering from CD18 deficiency showed normal rolling behavior but were incapable of sticking or emigrating upon chemotactic stimulation. Neutrophils from a second patient with a newly described adhesion deficiency had normal CD18 but did not express sLe$^x$. These neutrophils rolled poorly and also failed to stick in venules under shear force. Under static conditions, however, chemoattractant-induced sticking and emigration could be observed. This demonstrates that both selectin-carbohydrate–mediated initiation of adhesion and subsequent activation-induced β2-integrin engagement are essential for the normal function of human neutrophils in vivo. (*J. Clin. Invest.* 1993. 91:2893–2897.) Key words: intravital microscopy • leukocyte adhesion deficiency syndromes • β2-integrins • selectins • sialyl Lewis$^x$**

## Introduction

It has recently been proposed that adhesive interactions between leukocytes and endothelial cells (EC)[1] involve multiple sequential steps (1–4). Venular EC adjacent to inflamed tissue are induced to express cell adhesion molecules on their surface, thus increasing their adhesiveness for circulating leukocytes. The initial interaction of neutrophils capable of recognizing activated EC is relatively weak but allows cells to partly resist the shear force exerted by the flowing blood, resulting in slow rolling along the venular wall. Subsequently, rolling cells become activated when they encounter appropriate activating or chemotactic stimuli generated on the EC surface or in the extravascular compartment. Neutrophil activation leads to functional activation and up-regulation of β2-integrins (CD11/CD18) whose engagement enables rolling cells to stop and stick firmly to venular EC. Recent in vivo experiments suggest that this chain of events is essential for neutrophils to be able to emigrate into the extravascular space and to fulfill their role as effector cells of the immune system (5, 6).

The first adhesive step, the rolling phenomenon, is believed to be mediated by members of the family of selectin adhesion molecules (1, 2, 6–8) which recognize fucosylated carbohydrate ligands, especially structures containing sialyl-Lewis$^x$ (sLe$^x$; [NeuAcα2,3Galβ1,3 (Fucα1,3) GlcNAc]) (9–11). A central role is played by neutrophil L-selectin which may function simultaneously as a constitutively functional lectin recognizing inducible EC surface ligands (12, 13) and as a structure presenting sLe$^x$ to both P- and E-selectin (13, 14) whose expression on EC is induced by inflammatory stimuli (reviewed in reference 15). In contrast to rolling, firm adhesion is mainly mediated by a protein-protein interaction involving CD11/CD18 on the neutrophil surface and, among other structures, the intercellular adhesion molecule-1 (ICAM-1) on EC (2, 5). An inherited defect in the synthesis of CD18, the common β chain of β2-integrins, causes a syndrome termed leukocyte adhesion deficiency (LAD) (16, 17). Affected patients suffer from severe recurrent bacterial infections characteristically without pus formation despite markedly elevated systemic leukocyte counts. More recently, a syndrome has been reported in two unrelated children with similar clinical symptoms (18) and impaired neutrophil adhesion despite intact function and expression of β2-integrins (18). The underlying mechanism for this immune defect, which was proposed to be called LAD type 2 (vs. LAD type 1 for CD18 deficiency), was ascribed to a congenital defect of the endogenous fucose metabolism that causes an inability to synthesize fucosylated carbohydrate molecules, including sLe$^x$ and related structures (19). We speculated that the apparent neutrophil dysfunction in these patients could be explained by the lack of appropriate carbohydrate ligands for selectins which may result in a defect in the initiation of adhesion and may be characterized by impaired rolling behavior. To test this hypothesis we employed a modified

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1. *Abbreviations used in this paper:* EC, endothelial cell(s); LAD, leukocyte adhesion deficiency; $LTB_4$, leukotriene $B_4$; RF, rolling fraction; sLe$^x$, sialyl-Lewis$^x$.

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model of the rabbit mesentery preparation to study the behavior of neutrophils isolated from blood of two healthy donors and from two patients, one with LAD type 1 (LAD-I; patient 1 in reference 17) and one with LAD type 2 (LAD-II; patient 1 in reference 19).

## Methods

*Neutrophil isolation and labeling.* Neutrophils were isolated from whole blood using dextran sedimentation followed by discontinuous density gradient centrifugation over Ficoll-Paque and Percoll (Pharmacia, Inc., Piscataway, NJ) as described (6). After this procedure, it was necessary to remove residual contaminating red cells in the LAD-II cell preparation by centrifugation on 65% Percoll after the cell suspension was made slightly hypotonic to induce red cell swelling but no lysis. Viability and purity of all cell preparations was > 90%. Isolated neutrophils were fluorescently labeled by 30-min incubation with 20 $\mu$g/ml carboxyfluorescein diacetate (Molecular Probes, Inc., Eugene, OR). Cells were washed, resuspended to $1 \times 10^7$ cells/ml in PBS + 0.01% glucose, and kept in the dark until use.

*Immunofluorescence flow cytometry.* Expression of CD18 and L-selectin on isolated neutrophils was determined on a FACScan flow cytometer (Becton Dickinson & Co., Mountain View, CA) using FITC-labeled anti-CD18 mAb IB4 and anti-L-selectin mAb DREG-200 as described (1, 6). Expression of $sLe^x$ was determined in whole blood by indirect immunofluorescence using anti-$sLe^x$ mAb CSLEX-1 and FITC-labeled goat anti-mouse-Ig serum.

*Animal preparation.* Two New Zealand White rabbits (1.2 kg body wt) were sedated by i.m. injection of 0.1 ml of 1% fluanison and 0.02% fentanyl (Hypnorm, Janssen Pharmaceutica, Beerse, Belgium) and 1,000 U recombinant human IL-1$\beta$ (gift from Dr. Witt, Repligen) in 5 ml of PBS were administered i.p. for local stimulation of mesenteric EC. 2 h later, surgical anesthesia was induced (5–10 ml of 20% urethane i.v.) and animals were prepared for in vivo observation of the mesenteric microvasculature on an intravital microscope (E. Leitz, Rockleigh, NJ) as described (1). Subsequently, a side branch of the superior mesentery artery was cannulated with polyethylene tubing (6).

*Intravital microscopy.* Samples of fluorescent neutrophils were consecutively injected through the catheter into the terminal mesentery artery blood stream 4–6 h after i.p. application of IL-1. Cells passing through venules of a mesenteric segment in the downstream circulation were made visible by stroboscopic epi-illumination through an epi-illuminator (Ploemopak, E. Leitz) employing an N2 filter block and a ×10 water immersion objective (Nikon Inc., Melville, NY).

After determination of baseline rolling (see below) of each neutrophil sample, mesentery preparations were superfused with buffer containing $5 \times 10^{-8}$ M leukotriene $B_4$ ($LTB_4$) and fluorescently labeled neutrophils were again injected as described above. After assessment of $LTB_4$-induced neutrophil sticking under conditions of physiologic intravascular shear force, arterial blood flow was transiently stopped in one preparation by inflating a balloon catheter wrapped around the main stem of the mesentery artery upstream of the side branch used for cell injection. Immediately thereafter, LAD-II neutrophils were injected into the unperfused mesenteric microvasculature during simultaneous superfusion with $LTB_4$. Blood flow was restored 10 min after addition of cells by deflation of the balloon catheter and neutrophil behavior in the presence of extravascular $LTB_4$ was monitored for 30 min more without further injection of cells. All scenes were recorded on video tape using a silicon intensified tube camera (Dage MTI Inc., Michigan City, IN) and a SVHS video recorder (JVC).

*Image analysis.* Video recordings of 10 venules were analyzed for assessment of rolling of control cells and patient neutrophils as described previously (6). Briefly, the rolling fraction (RF) was determined as the percentage of the flux of rolling cells ($F_r$) in the total flux ($F_t$) of fluorescent neutrophils passing a venule during one injection: $RF = F_r/F_t \times 100$ [%]. RF has been shown to be a highly sensitive parameter for assessment of the relative ability of neutrophils to initiate interactions with inflamed venular EC (6).

In addition, velocities of individual neutrophils were determined. Control cells, LAD-I cells, and LAD-II cells were analyzed using a computer-based interactive image analysis system designed for use in microcirculatory research (20). The centerline velocity, $V_c$, of the blood stream was assessed from each sample according to Ley and Gaethgens (21): $V_c = 2 \times V_{max}/(2 - \epsilon^2)$, where $V_{max}$ is the highest velocity measured in each cell sample and $\epsilon$ is the ratio of neutrophil diameter (assumed to be 7 $\mu$m) to venular diameter (24 $\mu$m). Individual velocities were expressed relative to $V_c$.

For assessment of neutrophil responsiveness to the chemotactic stimulus supplied by $LTB_4$ superfusion, neutrophil sticking was analyzed in eight venules. Neutrophils were defined as sticking cells when they remained stationary in a venule for at least 30 s. Results are given as percentage of sticking cells in the total flux passing a venule during one injection.

*Statistics.* Rolling and sticking fractions of control and patient neutrophils were compared using the Quade test for nonparametric two-way analysis of variance and multiple comparisons on ranks of several paired samples. $P$ values of $< 0.05$ were considered statistically significant. All results are given as mean±standard deviation (SD).

## Results

Flow cytometric analysis of the surface expression of adhesion receptors on isolated cells (Fig. 1) confirmed a complete absence of CD18 on neutrophils from the LAD-I patient (17) and normal expression on control cells and LAD-II neutrophils (19). All samples stained positively with anti-L-selectin mAbs. However, in agreement with a previous report (22), LAD-I neutrophils expressed ~ 50% less L-selectin than control or LAD-II cells. Neutrophils from controls and the LAD-I patient had equal levels of $sLe^x$. In contrast, as reported recently (19), $sLe^x$ expression was not detectable on LAD-II cells.

To determine the relative ability of patient neutrophils to roll, we studied the in vivo behavior of fluorescently labeled neutrophils during their passage through inflamed venules in rabbit mesenteries. Control neutrophils behaved essentially as described previously for this model (6, 13). 4–6 h after local stimulation of mesenteric EC by intraperitoneal administration of 1,000 U interleukin-1$\beta$, the RF was 28.2±11.7% (mean±SD) ranging from 8.2% to 51.3% (Fig. 2). Analysis of the velocity distribution (Fig. 3) of control neutrophils in a representative venular segment revealed a typical distinction of free cells with velocities close to the mean blood flow velocity (1.1–1.2 mm/s) from rolling cells with much lower velocities (0.17±0.12 mm/s).

Neutrophils rolled only in venules, not arterioles or capillaries, and rolling cells did not stick spontaneously. This is in agreement with previous studies in this model that have demonstrated that the mere contact of a rolling neutrophil with venular EC, even after IL-1 stimulation, is not sufficient to elicit CD18-dependent sticking in vivo (6). Sticking was reproducibly induced, however, by superfusion of the preparations with buffer containing $LTB_4$ as a chemotactic stimulus. On average, 5.5% of the total flux of neutrophils in each venule became stuck in these experiments when $LTB_4$ was applied (Fig. 4).

Rolling of CD18-deficient neutrophils (RF = 25.5±14.4%, range: 5.9–47.4%; rolling velocity: 0.21±0.16 mm/s) was slightly lower (58–90% of average control) in 6 out of 10 venules analyzed. However, RF was equal to controls in three venules and slightly elevated (113% of control) in one venule. Statistical analysis revealed no significant difference in the rolling behavior of LAD-I and control neutrophils ($P > 0.05$). In contrast, $LTB_4$ superfusion completely failed to induce sticking. Instead, LAD-I neutrophils continued to roll along the venular

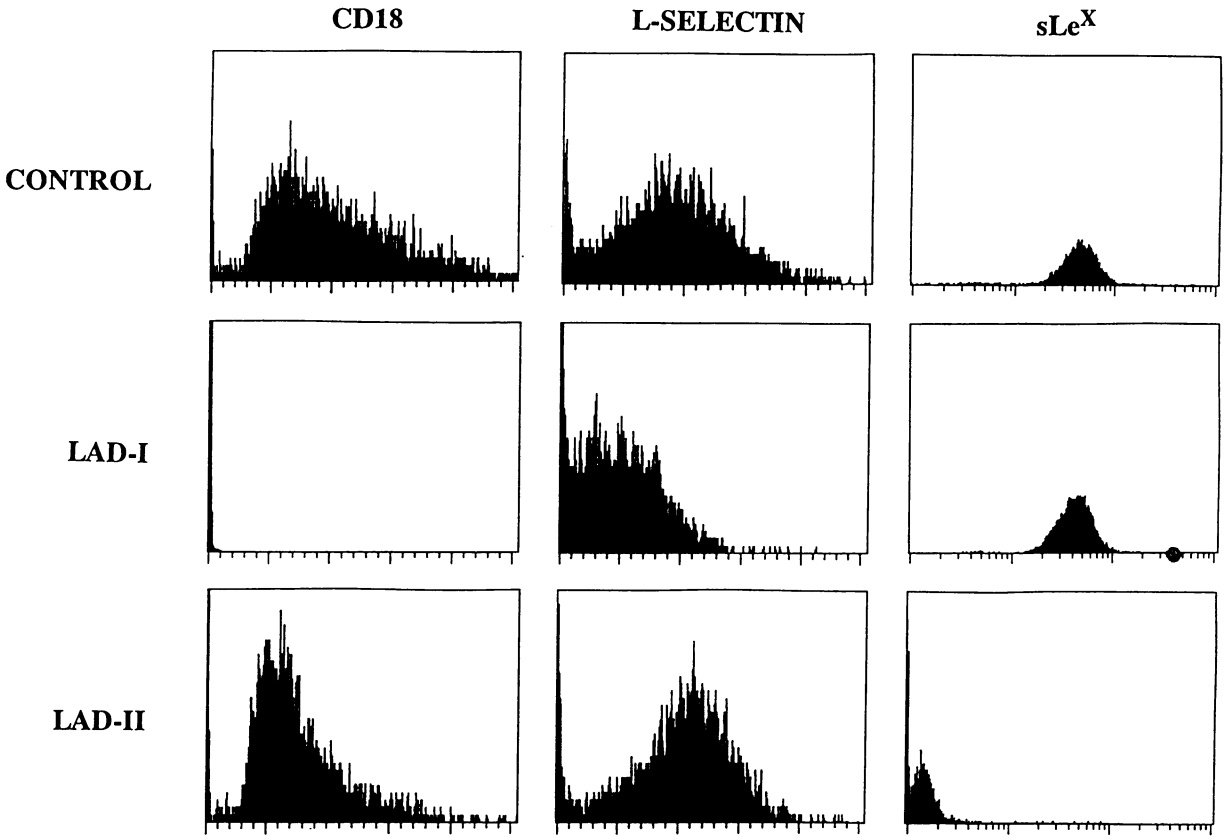


*Figure 1.* Flow cytometric analyses of surface expression of CD18, L-selectin, and sialyl-Lewis$^x$ (sLe$^x$) on neutrophils from a healthy donor (control) and two patients with leukocyte adhesion deficiency type 1 and type 2. CD18 and L-selectin expression was determined on isolated neutrophils on a FACScan flow cytometer (Becton Dickinson & Co.) using FITC-labeled anti-CD18 mAb IB4 and anti-L-selectin mAb DREG-200 as described (1). Expression of sLe$^x$ was measured in whole blood using anti-sLe$^x$ mAb CSLEX-1 and FITC-labeled goat anti-mouse-Ig serum. The signal obtained from LAD-II neutrophils with anti-sLe$^x$ mAb was not different from a nonbinding control mAb.

wall despite a marked $LTB_4$-induced accumulation of sticking rabbit leukocytes. These observations are in agreement with previous in vivo reports showing that the transition from rolling to sticking, but not rolling itself, is CD18 dependent (1, 5, 6).

In contrast to LAD-I cells, LAD-II neutrophils lacking fucosylated carbohydrates rolled poorly; RF was 4.9±4.7% (range: 0–15.2%) and most LAD-II neutrophils that did interact with venular EC had a higher rolling velocity (0.49±0.22 mm/s), rolled only over short distances, and frequently detached from the vessel wall. LAD-II neutrophils reacted poorly to the chemotactic stimulus supplied by $LTB_4$ in the presence of blood flow. Sticking in response to $LTB_4$ was observed in only 0.7% of the total venular flux. This remarkable inability of LAD-II neutrophils to react to an inflammatory stimulus appears to be dependent on the presence of intravascular shear force. In an additional experiment, the mesenteric blood flow was stopped upstream of the cannulated arterial side branch and LAD-II cells were injected into the unperfused microvasculature while $LTB_4$ was superfused. When blood flow was restored 10 min later, numerous LAD-II cells had become stuck and were not detached by the flowing blood. Eventually, several sticking cells emigrated into the extravascular space, indicating that the immune defect in LAD-II patients is in fact due to a shear-dependent inability of neutrophils to roll and slow down in inflamed venules and not due to a dysfunction of shear-independent later steps in the intravascular adhesion process.

## Discussion

Here we have compared the in vivo behavior of neutrophils from healthy normal subjects and from two patients suffering from distinct inherited leukocyte adhesion deficiencies. CD18-deficient neutrophils from a patient with a classic LAD-I syndrome rolled normally, suggesting that they were capable of initiating adhesive interactions with inflamed EC. However, these cells failed to perform activation-dependent, $\beta$2-integrin–mediated adhesion steps and did not stick or emigrate when challenged with a chemotactic stimulus. In contrast, LAD-II neutrophils lacking fucosylated carbohydrate structures such as sLe$^x$ were severely affected in both their ability to roll and to stick.

The rolling phenomenon has been shown to be a prerequisite for $\beta$2-integrin–mediated neutrophil adhesion in the presence of shear force (2, 6). Thus, it appears possible that the inability of LAD-II cells to stick in response to challenge with $LTB_4$ is due to their incompetence to marginate and slow down in the blood stream. This is supported by the observation that LAD-II neutrophils can respond to a chemotactic signal in vivo when no intravascular shear force is present. Furthermore, our findings are in agreement with recent in vitro studies on LAD-II neutrophils; these sLe$^x$-deficient cells fail to adhere to E-selectin expressed on Il-1 $\beta$–treated EC (19) whereas CD18-mediated adhesion events are not affected (J.M.H., unpublished observation). E-selectin can bind normal neutrophils in the

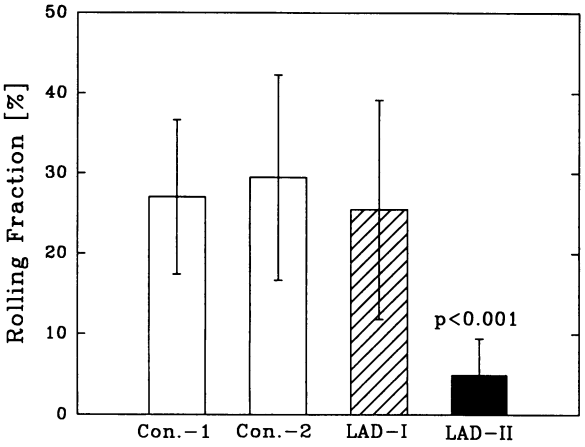


*Figure 2.* Quantitation of neutrophil rolling from control subjects and patients with LAD-I and LAD-II in inflamed mesenteric venules in rabbits. Samples of fluorescent neutrophils were injected into the terminal mesentery artery blood stream 4–6 h after i.p. injection of IL-1. Cells passing through venules in the downstream segment were made visible by stroboscopic epi-illumination and were recorded on video tape. Tapes were analyzed for assessment of neutrophil rolling behavior. The rolling fraction was determined as the percentage of rolling neutrophils in the total flux of fluorescent cells passing a venule during an injection (6). A total of 10 venules was analyzed, five vessels for each neutrophil sample in each rabbit. Mean±SD are shown.

presence of shear force in vitro (8) and is also involved in leukocyte rolling in IL-1-treated rabbit mesenteries (M. Olofsson, U. H. von Andrian, and K.-E. Arfors, manuscript in preparation). In contrast, CD11/CD18 alone readily mediates neu-

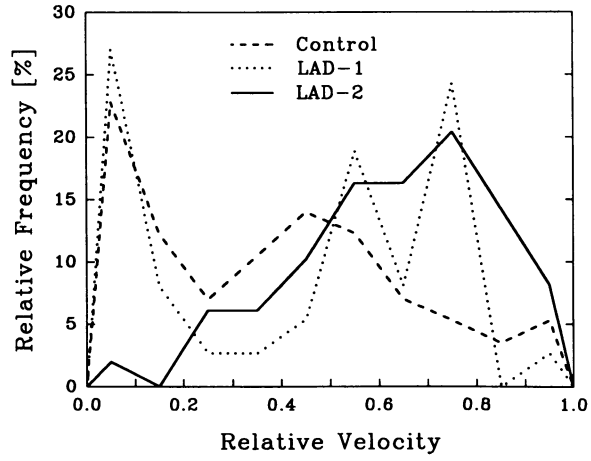


*Figure 3.* Velocity profiles of control, LAD-I, and LAD-II neutrophils passing a mesenteric venule. The individual velocities of neutrophils passing a 250-$\mu$m stretch of a representative venule (24-$\mu$m diam) were determined from a control subject ($n$ = 57 cells), a LAD-I patient ($n$ = 37 cells), and a LAD-II patient ($n$ = 49 cells). The velocity of each neutrophil was expressed relative to the centerline velocity in the venule which was assigned a value of 1. Lines were drawn after assessing the relative frequency of neutrophils in classes from 0 to < 0.1, 0.1 to < 0.2, and so on. The mean blood flow velocity, calculated as $V_c/2$ (assuming a parabolic flow profile), was 1.1 mm/s during injection of control and LAD-I cells and 1.2 mm/s during injection of LAD-II cells. Neutrophils passing this venule with a relative velocity of < 0.25 are likely to be rolling cells which actively interact with the vessel wall as their low velocity cannot be explained by microhemodynamic factors.

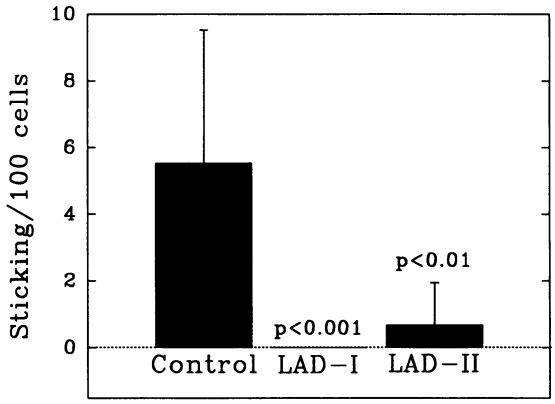


*Figure 4.* Leukotriene $B_4$–induced neutrophil sticking in rabbit mesentery venules. Two mesentery preparations were continuously superfused with buffer containing $5 \times 10^{-8}$ M $LTB_4$ and fluorescently labeled neutrophils were injected locally. Recordings from neutrophils passing through a total of eight venules were analyzed. Neutrophils were defined as sticking cells when they remained stationary in a venule for at least 30 seconds. Neutrophils from both the LAD-I and the LAD-II patient were significantly reduced in their ability to react to the chemotactic stimulus; there was no statistically significant difference between LAD-I and LAD-II. Mean±SD are shown.

trophil adhesion in static assays (2, 6, 22) and potentially under conditions of very low flow. However, it cannot engage in the presence of physiologic shear rates as they are encountered in the rabbit mesentery unless adhesion is initiated through selectins (1, 2, 6). Taken together, these results suggest an important role for $sLe^x$ (and/or other fucosylated structures) as a neutrophil surface determinant that mediates rolling.

It has been shown earlier that treatment of normal neutrophils with chymotrypsin or activation through a variety of inflammatory mediators dramatically decreases neutrophil rolling in this model (6, 13). However, these treatments were shown to have little or no effect on overall $sLe^x$ expression (13, 14, 23). Thus, although a multitude of glycosylated molecules (including CD18) on neutrophils can be decorated with $sLe^x$, it appears that only a small fraction of the total surface $sLe^x$ participates in rolling (13). Both chymotrypsin treatment (6, 13, 14, 23) and activation (1, 6, 24) effectively remove L-selectin from the neutrophil surface and L-selectin has been shown to be critically involved in rolling of rat (7), rabbit (1), and human (6) neutrophils. L-selectin on neutrophils can present $sLe^x$ as a ligand to both vascular selectins in vitro (14) and CD18-independent neutrophil binding to IL-1–treated EC was shown to be mediated by an adhesion pathway that appears to be shared by L- and E-selectin (25). The results presented here are consistent with the hypothesis that presentation of fucosylated carbohydrates may be a major mechanism of L-selectin-mediated rolling in vivo (13).

The residual capacity of LAD-II cells to roll could be mediated by direct interaction of the L-selectin lectin domain with endothelial ligand(s) (12). In support of this possibility is our finding that murine pre-B lymphoma cells, which normally do not express L-selectin or carbohydrate ligands for vascular selectins and do not roll, acquire a weak ability to roll when transfected with human L-selectin but not with the vector alone (13). Furthermore, we tested a subfraction of LAD-II neutrophils which could not be sufficiently purified from red cells using the methods described above. These cells were iso-

lated by hypotonic lysis of contaminating erythrocytes. Neutrophils from this preparation did not bind anti-L-selectin mAb and expressed elevated levels of CD18 probably due to activation induced by the isolation process. When tested in vivo, these neutrophils completely failed to roll (not shown).

The endothelial counterreceptor (other than the vascular selectins) for L-selectin in acutely inflamed extralymphoid venules has not been identified yet. However, it is conceivable that such molecule(s) may be partly carbohydrate in nature and act as ligand(s) for the lectin domain of L-selectin (12). Thus, it cannot be excluded that fucose is required for this interaction, in which case the ability of $sLe^x$-deficient neutrophils to roll and stick in venules of LAD-II patients with a global fucosylation defect may be even more reduced than in our experiments using stimulated rabbit endothelium.

Interestingly, LAD-I neutrophils rolled remarkably well in our experiments despite a decreased surface expression of L-selectin. A comparable activation-induced reduction in L-selectin levels on normal neutrophils was shown to cause a concomitant decrease in rolling (6, 22). Consistent with our findings, the frequency of adhesion of LAD-I neutrophils to IL-1–stimulated cultured EC in the presence of shear stress has been reported to be equal to that of normal neutrophils (although higher rolling velocities were observed [26]). The mechanism(s) by which CD18-deficient neutrophils manage to roll as well as control cells despite reduced L-selectin expression cannot be explained from these experiments. However, the degree of substitution with $sLe^x$ or the lectin affinity of the remaining L-selectin or a cell surface topography and receptor distribution that may favor the presentation of L-selectin and/or other glycoproteins decorated with $sLe^x$ are potential factors that could possibly compensate for decreased L-selectin levels on LAD-I cells. Nevertheless, despite their ability to roll well in inflamed venules, LAD-I neutrophils could not respond adequately to chemotactic stimulation confirming the pivotal role of neutrophil activation and $\beta 2$ integrin function for adhesion and emigration.

In summary, our in vivo observations suggest that $sLe^x$- and CD18-dependent steps in the adhesion cascade are distinct events of crucial importance for neutrophil function in humans. Interaction of $sLe^x$-like structures with vascular selectins is required for adhesion initiation and increases the efficiency of neutrophil recruitment at physiologic shear rates. Subsequent engagement of CD18 is triggered by neutrophil activation and functions in adhesion strengthening. A defect in either of the two steps causes a severe impairment of neutrophil function and becomes clinically manifest as LAD syndrome.

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