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*J Immunol* 2002; 169:3940-3946; doi: 10.4049/jimmunol.169.7.3940
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Polymorphonuclear Leukocytes from Individuals Carrying the G329A Mutation in the \( \alpha_1,3 \)-Fucosyltransferase VII Gene (\( FUT7 \)) Roll on E- and P-Selectins

Per Bengtson,* Arne Lundblad,* Göran Larson,† and Peter Pålsson²*

We recently identified several individuals carrying a missense mutation (G329A; Arg\(^{110}\)-Gln) in the \( FUT7 \) gene encoding fucosyltransferase VII. This enzyme is involved in the biosynthesis of the sialyl Lewis \( \alpha \) (Le\(^\alpha\)) epitope on human leukocytes, which has been identified as an important component of leukocyte ligands for E- and P-selectin. No enzyme activity was measurable in expression studies in COS-7 cells using the mutated \( FUT7 \) construct. One of the identified individuals carried this mutation homozygously. Flow cytometry analysis of polymorphonuclear leukocytes (PMN) from this individual showed a nearly complete absence of staining with mAbs directed against sialyl Le\(^\alpha\) and a diminished staining with an E-selectin IgG chimera. However, staining with P-selectin IgG chimera and Abs directed against P-selectin glycoprotein ligand-1 was not affected by the mutation. PMN from the homozygously mutated individual was further analyzed in an in vitro flow chamber assay. The number of rolling PMN and the rolling velocities on both E- and P-selectin were in the range of PMN from nonmutated individuals. \( FUT4 \) and \( FUT7 \) mRNA was quantified in PMN isolated from individuals carrying the \( FUT7 \) mutation. It was found that PMN from both \( FUT7 \) homozygously and heterozygously mutated individuals exhibited an elevated expression of \( FUT4 \) mRNA compared with PMN from \( FUT7 \) nonmutated individuals. The elevated expression of fucosyltransferase IV was reflected as an increased expression of the Le\(^\alpha\) and CD65s Ags on PMN from these individuals. The significance of the mutation was supported by transfection of BJAB cells. The Journal of Immunology, 2002, 169: 3940–3946.

\( \text{E} \)-selectin and P-selectin are cell adhesion molecules expressed by endothelial cells following activation by several inflammatory cytokines (1, 2). They are both involved in the initial step of leukocyte extravasation into inflamed tissues by mediating tethering and rolling of leukocytes along the vessel wall (2). Both \( \text{E-}\) and \( \text{P-selectin} \) contain C-type lectin domains and bind to glycoprotein counterreceptors expressing carbohydrates such as sialyl Lewis \( \alpha \) (SLex)\(^3\) and related epitopes on the surface of leukocytes (3–5). One glycoprotein, P-selectin glycoprotein ligand-1 (PSGL-1), has been identified as the main counterreceptor for P-selectin (6). However, the exact nature of the glycoproteins or glycolipids involved in E-selectin binding in humans is not clear. Nor are the exact carbohydrate requirements for E- and P-selectin binding fully determined (7).

Several studies have shown that \( \alpha_1,3 \)-fucosylation is a necessary step in biosynthesis of selectin ligands. There are two \( \alpha_1,3 \)-fucosyltransferases (Fuc-T) expressed to a significant degree in human leukocytes and of relevance for the biosynthesis of selectin ligands in these cells, Fuc-TIV and Fuc-TVII (8–10). The importance of these enzymes in the biosynthesis of selectin ligands has been demonstrated in several ways. Mice deficient in Fuc-TVII showed blood leukocytosis and impaired leukocyte extravasation into inflamed tissues, indicating a major role for Fuc-TVII in the generation of selectin ligands in mice (11). Mice deficient in both Fuc-TVII and Fuc-TIV indicated a role for Fuc-TIV as well (12, 13). Cells can be induced to adhere to E- and P-selectins after transfection with cDNA encoding Fuc-TVII (14, 15). In contrast, transfection with Fuc-TIV alone generates different results depending on the type of mammalian cells used (16–19).

Fuc-TIV and Fuc-TVII exhibit distinct acceptor specificities. Fuc-TVII adds fucose residues to \( \alpha_2,3 \)-sialylated lactosamine acceptors to form the SLex epitope. Fuc-TIV has a preference for nonsialylated lactosamine units where it adds fucose \( \alpha_1,3 \)-to Lewis x (Le\(^\alpha\)) epitopes. Fuc-TIV can also act on a sialylated polylactosamine acceptor but then preferentially fucosylates the “inner” lactosamine units to form the CD65s epitope (20, 21).

A third \( \alpha_1,3 \)-fucosyltransferase, Fuc-TIX, is also present in human leukocytes, but expressed at a very low level. Fuc-TIX is mainly involved in Le\(^\alpha\) biosynthesis and has not been shown to contribute to selectin ligand biosynthesis (22–24). Due to its significant role in initiating inflammatory response, Fuc-TIV and Fuc-TVII have been considered to be monomorphic. However, recently we identified an individual homozygously carrying a missense mutation, G329A, in the gene encoding Fuc-TVII, \( FUT7 \) (25). The mutation gives an amino acid shift of arginine to glutamine at position 110 of the enzyme. Enzymatic and immunochromatographic studies of transfected cell lines and isolated polymorphonuclear leukocytes (PMN) from this individual indicated that this mutation essentially inactivated the Fuc-TVII enzyme. The individual carrying the mutation in homozygous form had no history of recurrent infections and the white blood cell
count was repeatedly within the reference range (25). Thus surprisingly, this individual did not exhibit a phenotype similar to the Fuc-TVII−/− mice.

The aim of this study was to further characterize the surface expression of putative selectin ligands on PMN isolated from individuals carrying the FUT7 G329A mutation hetero- or homozygously. Furthermore, the ability of PMN from these individuals to roll on E- and P-selectins was studied in an in vitro flow chamber model.

Materials and Methods

Patients and controls

The individual carrying the FUT7 G329A mutation homozygously (S.J.) is diagnosed with the ulcer disease, noninsulin-dependent diabetes, osteoporosis, spondyloarthrosis, and Sjögren’s syndrome. The latter diagnosis was confirmed by signs of keratoconjunctivitis sicca, sialoadenitis, and a positive titer for antinuclear Abs. However, there was no history of recurrent infections and the white blood cell count was repeatedly within the reference range. Three individuals, one male (R.J.) and two females (M.L. and M.N.) carrying the same mutation heterozygously were included in the study. M.L. and R.J. are both children to the homozygous individual (S.J.) (25). Wild-type individuals will be referred to as R/R, heterozygous individuals as R/Q, and the homozygous individual as Q/Q. Eleven nonmutated individuals, six males and five females, were used as a control group. Local ethical committees in Göteborg and Linköping, Sweden approved the study.

Isolation of PMN

Human PMN were isolated from 9 ml of freshly (1-2 h) drawn acid-citrate dextrose anticoagulated blood using density gradient centrifugation, (Poly- morphprep; Nycoderm Pharma, Oslo, Norway). Isolated PMN were washed once with 4 ml of 0.9% NaCl and centrifuged at 400 × g for 10 min. Remaining erythrocytes were then lysed on ice in hypotonic buffer (0.15 M NH4Cl, 10 mM KHCO3) for 20 min and the suspension was centrifuged at 400 × g for 10 min. The pellet was washed twice in 4 ml HBSS without Ca2+ and Mg2+ (Sigma-Aldrich, St. Louis, MO). The isolated cells were resuspended in HBSS/10 mM HEPES, pH 7.4 (Life Technologies, Sweden), and 2 mM CaCl2 at a concentration of 5 × 106 cells/ml. The purity of PMN was >95% as determined by analysis in an automatic cell counter.

Neuraminidase treatment of PMN

After isolation of PMN, cells were resuspended in 0.1 M NH4Ac buffer, pH 5.5, containing 0.5 U/ml protease-free neuraminidase (Clostridium perfrigens; Calbiochem, La Jolla, CA). The suspension was incubated at 37°C for 2 h. After incubation, the cells were pelleted and resuspended in HBSS/10 mM HEPES, pH 7.4, and 2 mM CaCl2 for flow chamber analysis.

Preparation of adhesion surface

Recombinant E- and P-selectin (R&D Systems Europe, Abingdon, U.K.) were dissolved in TBS containing sodium azide (0.025 M Tris-HCl, 0.15 M NaCl, 0.02% NaN3, pH 8.0, TBS). Polystyrene Petri dishes were coated with 20-μl spots, ~5 mm in diameter, of E-selectin (in concentrations of 0.1, 0.2, 0.4, or 0.8 μg/ml) or P-selectin (in concentrations of 0.2, 0.4, 0.8, or 1.6 μg/ml) for 2 h at room temperature (26). Unspecific binding was blocked by incubating remaining surfaces of the Petri dish with 2% human serum albumin (HSA; Behringwerke, Marburg, Germany) in TBS for 30 min at room temperature. Control spots were coated for 30 min at room temperature with 2% HSA in TBS.

A relative estimate of the number of available selectin binding sites on the coated surface was obtained by ELISA. Polystyrene microtiter plates were coated with different concentrations of E- and P-selectins and blocked with 2% HSA as described above. The subsequent steps in the ELISA were performed as previously described (27). Anti E- and P-selectin Abs (clones BBIG-E1 (1D2) and 9E1, respectively; R&D Systems Europe) directed against the selectin carbohydrate binding domain were used at a concentration of 5 μg/ml and alkaline phosphatase-conjugated secondary Ab (D314; DAKO, Glostrup, Denmark) was used at a concentration of 1.8 μg/ml. Absorbance was measured at 405 nm after the addition of substrate solution (p-nitrophenylphosphate in 1 M diethanolate, pH 9.8).

In vitro flow chamber rolling assay

The flow chamber (Glycotech, Rockville, MD) and the selectin-coated Petri dish were mounted on the slide holder of an inverted phase-contrast microscope. The cell suspension was perfused into the flow chamber using a 10-ml syringe mounted in a syringe pump (TOP, Tokyo, Japan). Silicon tubing was used to connect the syringe pump to the flow chamber. A digital firewire video camera (JTEJ1394; Sony, Tokyo, Japan) mounted on the microscope was used to record rolling cells.

The system was allowed to stabilize at a flow of 1 dyne/cm2 during 2 min. Before recording, the cells were allowed to settle for 5 s before desired flow was applied. Analysis of rolling cells was performed at 1.0 and 2.0 dyne/cm2. Movies were recorded at 30 frames/s directly on a computer. Frame grabbing and editing were done with Snag It 5.1.0 (TechSmith, East Lansing, MI) and QuickEditor 6.0 software (M. Tschopp). The movies were converted into picture stacks, which were analyzed with the public domain program ImageJ (National Institutes of Health, Rockville, MD).

Each setup was recorded at three different areas. Rolling PMN were identified as the visible part passing at <15 μm/s through the plane closest to the Petri dish. As a negative control of selectin-dependent binding, 5 mM glucose was included in the cell suspension.

Rolling velocity was calculated using the cell’s x, y coordinates in the captured area (500 × 500 μm). Rolling cells moved less than a cell length between frames and could be identified from one image to the next. Cells were numbered and their x, y coordinates were measured on the first image and on subsequent images in the image stack. The data for each cell was exported to a calculation spreadsheet and used for the subsequent calculations of velocity. For each setup and area, the velocity of 40 cells was measured. Manual measurement of cell movement during 5- to 6-s intervals confirmed the results obtained from the ImageJ software.

Number of rolling cells

Frames collected in the in vitro flow chamber rolling assay were also used to determine the number of rolling cells. Using the ImageJ software to analyze the frames, the number of rolling cells was determined. Stuck, nonrolling cells were subtracted from the counted cells.

Flow cytometry

Flow cytometry analyses were performed on a FACScan instrument (BD Biosciences, Franklin Lakes, NJ) operating with CellQuest software and calibrated with 6 μm beads of Coulter Science (Coulter, Hialeah, FL) (BD Biosciences). One milliliter of EDTA-anticoagulated peripheral blood was diluted into 50 ml lysis buffer (150 mM NH4Cl, 10 mM KHCO3, 90 mM Titrilplex III (Merck, Stockholm, Sweden), pH 7.3, allowed to stand in room temperature for 7 min, centrifuged, and washed once with 50 ml PBS (150 mM NaCl, 8 mM Na2HPO4, 1.9 mM NaH2PO4), pH 7.2. leukocytes were resuspended in PBS with 0.1% BSA (Sigma-Aldrich) to a final concentration of 5 × 109 cells/ml. Fifty microtiter of cell suspensions were incubated with 5 μl of primary Ab (Leu-M1, directed against Lea (Leu-M1; BD Biosciences)) diluted 1/5; VIM-2 (a gift from Prof. W. Knapp, Institute of Immunology, University of Vienna, Vienna, Austria) diluted 1/100; KM93 (Serotec, Oxford, U.K.) diluted 1/40; CSLEX-1 (BD Biosciences) diluted 1/50; or KPL-1 (BD Biosciences) diluted 1/50, and incubated for 15 min at room temperature. Cells were then washed with 2 ml of PBS, resuspended in 55 μl of FITC-conjugated Fab′(a), rabbit anti-mouse IgG diluted 1/10 in PBS and incubated for another 15 min at room temperature. The cells were washed in PBS and fixed in 200 μl of 1% paraformaldehyde. Mouse FITC-conjugated IgG1 Abs were used as negative controls.

To cell pellets of 2 × 106 cells, washed in SM (DMEM, 2 mM CaCl2, Life Technologies) with 0.1% BSA and 0.1% sodium azide, were added 50 μl of mouse E- and P-selectin human IgG fusion proteins diluted 1/5 and 1/1, respectively. Fusion proteins were kindly provided by Prof. J. B. Lowe (University of Michigan, Ann Arbor, MI). The cells were incubated for 30 min at 4°C, washed four times in SM, centrifuged, incubated with 50 μl of goat anti-human IgG FITC (F0132; Sigma-Aldrich) diluted 1/10 for another 30 min at 4°C, centrifuged, and finally washed twice in SM. SM containing 5 mM EDTA was used as a negative control for all experiments using selectin chimeras. Of 5000 cells counted, only data on the gated granulocyte population is presented.

Quantification of Fuc-TIV and Fuc-TVII mRNA

Total RNA was isolated from fresh (≤1 h) peripheral white blood cells using the SV Total RNA Isolation System (Promega, Madison, WI). The isolated RNA was treated with RQI RNase-free DNase (Promega). From transfected BJAB cells, total RNA was isolated using RNA protect mini-prep kit (Qiagen, Valencia, CA). Including treatment with DNase, reverse transcription system 3500 (Promega) and oligo(dT)15 primers were used according to the manufacturer’s instructions to transcribe 1 μg of total RNA to cDNA.
Quantitative PCR (QPCR) analysis was performed using the TaqMan PCR Core Reagents Kit (Applied Biosystems, Foster City, CA). Reactions for FUT7 and FUT4 quantification were performed in 30 μl with 0.2 μg of cDNA, 3 μl of 10 X TaqMan buffer A (500 mM of KCl, 100 mM Tris-HCl, pH 8.3), 5 mM of MgCl₂, 200 μM each of dATP, dCTP, dGTP, 400 μM of dUTP, 0.3 U of uracil-N-glycosidase, 0.75 U of AmpliTaq Gold DNA polymerase, 50 nM of probe, and 100 nM of sense and antisense primers. The following FUT4 primers were used: FUT4 sense primer, r3s, 5'-AAATGGGCTCCCTGACACC-3'; FUT4 antisense primer, r4s, 5'-CAGCTGGCGAGCCT-3'; and FUT4 probe, 5'-TGGCCGCCCTAAAGTTCACATTG-3'. The following FUT7 primers were used: FUT7 sense primer, r3s, 5'-TTGGTGGCTGACTGACCTGG-3'; FUT7 antisense primer, r2as, 5'-CCCTGGAGCGCTCCG-3'; and FUT7 probe, 5'-CCCTGGGCAAGATTTACTCAGA-3'. The FUT7 probe was designed to cover the sequence over the splice site in FUT7. To further exclude possible contamination of genomic DNA in the mRNA preparations, experiments were performed where incubation with reverse transcriptase was excluded. The PCR program used an initial temperature of 50°C for 2 min and then 95°C for 10 min, followed by 40 amplification cycles run for 15 s at 95°C and 1 min at 60°C. The amplifications were performed on an ABI Prism 7700 sequence detector equipped with a 96-well thermal cycler. Data were collected and analyzed with Sequence Detector v1.6.3 software (Applied Biosystems). Relative quantification of FUT7 and FUT4 mRNA was normalized to the housekeeping gene for β-actin. Reactions for quantifying β-actin were performed exactly as described above except for using 3.5 mM MgCl₂ and 300 nM sense primer, 300 nM antisense primer, and 200 nM probe (Applied Biosystems). The primers and probes for β-actin are commercially available from Applied Biosystems. All analyses were performed in triplicate and with probes labeled with FAM and TAMRA.

Relative quantification of FUT4 and FUT7 mRNA was calculated using the comparative Cₜ method (28). Cₜ is defined as the PCR cycle in which the gain in fluorescence generated by the accumulating specific product exceeds 10 times the SD of the mean baseline fluorescence between cycles 3 and 15. QPCR efficiencies were calculated from the given slopes in 10-fold dilutions plots where Cₜ was plotted against cDNA input. The corresponding QPCR efficiency (E) of one cycle in the exponential phase was calculated according to the equation: E = 10 – (1/slope). Investigated transcripts showed similar QPCR efficiency rates for FUT4 (E = 2.11), FUT7 (E = 2.06), and β-actin (E = 1.99) in the investigated range from 12 pg to 12 ng of cDNA input (means of triplicate determination were used for the calculations).

Plasmid construction

Plasmids containing full-length wild-type FUT7 and FUT7 G329A cDNA have been described earlier (25). The two cDNAs were excised with Xhol and BglII and ligated to the corresponding sites of pcDNA 3.1 plus hygro-mycin (Invitrogen, San Diego, CA). The two different plasmids containing wild-type FUT7 and FUT7 G329A were denoted pcDNA-wt and pcDNA-329. Plasmids were amplified and isolated from Escherichia coli DH5α using a column purification kit (SNAP; Invitrogen). The plasmids were sequenced to confirm the location of the insert (25). The pcDNA 3.1 plasmid containing lacZ gene, denoted pcDNA-lacZ, was obtained from Invitrogen. pcDNA 3.1 without insert was used for mock transfections.

Transfection of BJAB cells

BJAB cells (a kind gift from Prof. A. Rosén, Linköping University, Linköping, Sweden) were cultured at 37°C in complete RPMI 1640 medium, RPMI 1640 Glutamax (Invitrogen) supplemented with 10% heat-inactivated FCS and 1% penicillin/streptomycin (Invitrogen). Before transfection ~10⁵ cells were washed once with RPMI 1640 glutamax and once with ice-cold PBS. The cells were resuspended in 300 μl of ice-cold PBS and 20 μg of pcDNA 3.1 constructs in 20 μl of 10 μM Tris-EDTA buffer, pH 8.0, were added. The mixture was incubated for 5 min on ice before electroporation in a Gene-Pulser II (Bio-Rad, Hercules, CA), using one pulse of 250 V, 975 μF, for 40–50 ms. After transfection the cells were cultured in 20 ml of complete medium. The medium was renewed 24-h posttransfection. Transfected cells were harvested 48-h posttransfection. Cells transfected with pcDNA-lacZ were rinsed in PBS and fixed for 15 min in PBS with 0.2% glutaraldehyde/2% formaldehyde. Samples were then rinsed in PBS and incubated at 37°C for 2 h in 5-bromo-4-chloro-3-indolyl-n-glucosidase PBS solution (1 mg/ml) containing 4 mM KFe(CN)₆, 4 mM K₃Fe(CN)₆, 3H₂O, and 2 mM MgCl₂. All cells that stained blue were considered to express β-galactosidase. Cells transfected with pcDNA-wt or pcDNA-329 were washed twice in HBSS without Ca²⁺ and Mg²⁺ (Sigma-Aldrich) and resuspended in HBSS containing 10 mM HEPES, pH 7.4, and 2 mM CaCl₂ at a concentration of 5 × 10⁵ cells/ml before rolling experiments. Immuno-fluorescence analysis of cells transfected with pcDNA-wt or pcDNA-329 were performed using Abs CSLEX-1 and KM93 directed against SLeα, as described earlier (25).

**Results**

**Effect of the FUT7 G329A mutation on selectin ligand expression on PMN**

PMN were isolated from individuals carrying the FUT7 G329A mutation homozygously (Q/Q), heterozygously (R/Q), or being wild-type (R/R). PMN expression of E- and P-selectin ligands was analyzed by flow cytometry. Expression of SLeα was analyzed using KM-93 Ab. PMN from the Q/Q individual had a much-reduced expression of SLeα compared with PMN from R/R individuals, consistent with a severe reduction in Fuc-TVII activity (Fig. 1, A and C, respectively). However, there was no decrease in expression of SLeβ on PMN from R/Q individuals compared with PMN from R/R individuals (Fig. 1B). Analysis of SLeα expression using the CSLEX-1 Ab showed the same result (25). In concordance with the SLeα expression, there was a decrease in E-selectin ligand expression on PMN isolated from the Q/Q individual compared with PMN from R/R individuals when analyzed using an E-selectin IgG chimera (Fig. 1, D and F). PMN from R/Q individuals showed only a minor decrease in expression of E-selectin ligand compared with PMN from R/R individuals (Fig. 1E). In contrast, there was no reduction of binding of P-selectin IgG chimera to PMN isolated from the Q/Q individual or R/Q individuals compared with PMN from R/R individuals (Fig. 1, G–I). The mutation did not affect PSGL-1 expression as assayed using Ab KPL 1 (Fig. 1, J–L).

**FIGURE 1.** Flow cytometry analysis of selectin ligand epitopes on PMN. Isolated PMN were stained with KM93 (SLeα), KPL1 (PSGL-1), P-selectin-IgG chimera or E-selectin-IgG chimera. The Q/Q individual had lowered expression of SLeα (C) and E-selectin binding epitopes (F) on her PMN but showed no difference in PSGL-1 (L) and P-selectin epitope (I) expression compared with R/R controls (A, D, G, and J). PMN isolated from R/Q individuals showed no differences in epitope expression (B, E, H, and K) compared with R/R controls. Staining by control mAb is indicated in white. Data are presented as histograms of fluorescence intensity for 5000 light scatter-gated events. Median values of fluorescence intensity are indicated in the figures.
PMN isolated from 11 *FUT7* nonmutated individuals showed a wide range in the number of cells interacting with E- or P-selectin at 2.0 dyne/cm² (Fig. 2). This is within the range of the wall shear forces that exist in postcapillary venules (1–10 dyne/cm²; Ref. 29). The number of rolling PMN from R/Q and Q/Q individuals was within the range of rolling PMN from R/R individuals on both E- and P-selectin (Fig. 2). The number of rolling cells was dependent on the site density of selectin as was previously shown for both E- and P-selectins (29, 30). When the selectin coating concentration was decreased a critical coating concentration was reached where no PMN interacted with the selectin-coated surface. This critical coating concentration varied between individuals (Fig. 2), but the critical coating concentration did not differ for the analyzed FucTVII Q/Q and R/Q individuals compared with R/R individuals. To test that these selectin concentrations produced the corresponding increase in selectin binding sites, an ELISA using Abs directed against the carbohydrate binding domains of E- and P-selectin was performed. There was an increase in absorbance corresponding to the increase in selectin concentration indicating that the used selectin coating concentrations did not reach saturation level (data not shown). The rolling of PMN was calcium-dependent and was completely abolished by including 5 mM EGTA in the cell suspension buffer. Furthermore, pretreatment of PMN with neuraminidase abolished adhesion to both E- and P-selectin. Preincubation of PMN with mAb (KPL1) directed against PSGL-1 completely inhibited adhesion of PMN to P-selectin (data not shown). No nonspecific binding was observed on the surface coated only with HSA.

**FIGURE 2.** Number of rolling PMN on E-selectin- (A) or P-selectin- (B) coated surfaces. The number of rolling cells was determined as a function of selectin coating concentration in an in vitro flow chamber assay using a shear rate of 2.0 dyne/cm². The heavy black line represents the results from the Q/Q individual and the thin lines represents results from eleven R/R controls. Data represent mean number of cells bound per recorded surface in three fields of view.

**FIGURE 3.** Mean rolling velocity of PMN on E- or P-selectin determined in an in vitro flow chamber assay. The mean rolling velocity of PMN from R/R (●), R/Q (▲), and Q/Q (●) on recombinant E-selectin (A and B) or P-selectin (C and D), with coating concentrations as indicated, was determined using a constant linear shear force of 1 dyne/cm² (A and C) or 2 dyne/cm² (B and D). Data are presented as mean velocity of two experiments ± SD.

Effect of *FUT7* G329A mutation on rolling velocities of PMN in flow chamber on E- or P-selectin

Mean rolling velocities were obtained for PMN from Q/Q, R/Q, and R/R individuals at two different shear levels. No significant differences in mean rolling velocities on E-selectin were detected between PMN from *FUT7* wild-type controls (R/R) and individuals carrying the *FUT7* G329A mutation (R/Q and Q/Q) (Fig. 3, A and B). The mean rolling velocities of PMN from R/R and R/Q individuals on P-selectin were also similar. However, there was a slightly slower rolling velocity of PMN isolated from the Q/Q individual (Fig. 3, C and D). This difference became more pronounced when the P-selectin coating concentration was lowered. At P-selectin coating concentrations of 0.8 and 0.4 µg/ml, the observed mean rolling velocities of PMN from the FucT-VII Q/Q individual were in the lower quartiles compared with R/R and R/Q individuals. However, these differences were not statistically significant. The decrease in rolling velocity for PMN from the Q/Q
individual on P-selectin was observed at shear stresses of both 1.0 and 2.0 dyne/cm².

The range of PMN rolling velocities observed on both selectins was 1–13 μm/s, which is consistent with rolling velocities observed in other in vitro systems (31). When the distribution of PMN rolling velocities on P-selectin was analyzed, a relatively slower rolling of PMN from the Q/Q individual compared with R/R and R/Q individuals was obvious already at a P-selectin coating concentration of 1.6 μg/ml (Fig. 4B). There were no such differences seen when analyzing the distribution of PMN rolling velocities on E-selectin (Fig. 4A).

Taken together, all analyses of PMN from the Q/Q individual showed that the capacity of these cells to roll on both E- and P-selectin was in the normal range despite inactivation of Fuc-TVII and a near lack of SLex expression on cell surface glycoproteins.

Expression levels of FUT4 and FUT7 mRNA
Expression of FUT4 and FUT7 mRNA in PMN from individuals with or without the FUT7 G329A mutation was examined using QPCR analysis. To avoid amplification of contaminating genomic FUT4 and FUT7 DNA, DNase-treated total RNA preparations were used as a template for cDNA amplification. These experiments showed no PCR product, indicating that there was no contaminating genomic DNA. There were no differences in expression of FUT7 mRNA between R/R, R/Q and Q/Q individuals (Table I). However, expression of FUT4 mRNA was found to be elevated in PMN from both R/Q (18 times compared with R/R) and Q/Q individuals (126 times compared with R/R; Table I).

**Expression of Leα and CD65s is elevated on PMN from the individual carrying the FUT7 G329A mutation**

To analyze the effect of an elevated expression of FUT4 mRNA PMN, surface expression of the carbohydrate epitopes Leα and CD65s, which are both synthesized by the action of Fuc-TIV (21, 32), was examined by flow cytometry analysis. There was a moderate increase in the surface expression of both Leα (Leu-M1) and CD65s (VIM-2) on PMN from the Q/Q individual. However, there was no difference in Leα or CD65s expression on PMN from R/Q and R/R individuals (Fig. 5).

**BJAB cells transfected with FUT7 G329A do not roll on E-selectin**

BJAB cells were transiently transfected with plasmids containing either the mutated or the wild-type FUT7 cDNA sequence (pcDNA-329 and pcDNA-wt, respectively). Transfection efficiency was determined by QPCR. The mRNA levels of FUT7 and FUT7 G329A were comparable in the two transfected cell lines (Table II). BJAB cells transfected with pcDNA-wt interacted with Abs directed against the SLex epitope (CSLEX-1 and KM-93). Both Abs showed intense staining of 6–7% of the cells (Table II). BJAB cells transfected with pcDNA-wt interacted with E-selectin in the flow chamber assay, whereas mock-transfected BJAB cells or BJAB cells transfected with pcDNA-329 failed to roll on E-selectin (Table II).

**Discussion**

We recently identified individuals carrying a missense mutation in the FUT7 gene encoding Fuc-TVII. One of these individuals was...
carrying the mutation homozygously. This mutation, which gives an amino acid substitution of Arg to Gln at position 110, was found to severely reduce the enzyme activity (25). In this study, we have analyzed the functional significance of this mutation for the interaction of PMN from these individuals with E- and P-selectin in vitro.

The individual carrying this mutation homozygously (Q/Q) was found to express only very low amounts of SLe^a on her PMN as determined by flow cytometry. In accordance with several previous studies showing a relationship in SLe^a expression and E-selectin binding (3–5), binding of an E-selectin IgG chimera was decreased on PMN from this individual compared with PMN from R/Q and R/R individuals. In contrast, there was no decrease in P-selectin IgG chimera binding or the expression of PSGL-1 on the PMN surface from the Q/Q individual compared with R/Q and R/R individuals.

Interaction of PMN with selectins was also studied in an in vitro flow chamber assay. The number of rolling PMN interacting with E- and P-selectin was in the same range both for PMN isolated from the Q/Q individuals and PMN isolated from the R/Q and R/R individuals. Furthermore, there was no significant difference (p ≥ 0.1) in rolling velocities between PMN isolated from Q/Q, Q/R and R/R individuals. However, there was a tendency of PMN from the Q/Q individual to roll slower on P-selectin compared with R/Q and R/R individuals.

The fact that PMN from the Q/Q individual did roll on both E- and P-selectin in vitro. However, there was a tendency of PMN isolated from Q/Q, Q/R and R/R individuals. Furthermore, there was no significant difference (p ≥ 0.1) in rolling velocities between PMN isolated from Q/Q, Q/R and R/R individuals. However, there was a tendency of PMN from the Q/Q individual to roll slower on P-selectin compared with R/Q and R/R individuals.

Interaction of PMN with selectins was also studied in an in vitro flow chamber assay. The number of rolling PMN interacting with E- and P-selectin was in the same range both for PMN isolated from the Q/Q individuals and PMN isolated from the R/Q and R/R individuals. Furthermore, there was no significant difference (p ≥ 0.1) in rolling velocities between PMN isolated from Q/Q, Q/R and R/R individuals. However, there was a tendency of PMN from the Q/Q individual to roll slower on P-selectin compared with PMN from R/R individuals.

The fact that the decrease in E-selectin IgG chimera staining of PMN from the Q/Q individual was not reflected in the number or velocity of rolling cells was surprising but may be explained by the differences in experimental systems. In the flow chamber assay, interactions between selectin and ligands must form quickly to generate rolling under linear shear stress. In the flow cytometry assay, in contrast, the E-selectin chimera is allowed to interact with its ligands for 30 min at a lower temperature which would allow detection of ligands both with rapid- and slow-binding kinetics.

The role for Fuc-TVII in selectin ligand biosynthesis has been firmly established in mouse knockout models (11, 12). The fraction of rolling leukocytes measured in vivo by intravital microscopy in Fuc-TVII^−/− mice was clearly diminished compared with wild-type mice. Also, the rolling velocity of leukocytes was somewhat increased in the Fuc-TVII^−/− mice compared with Fuc-TVII^+/− mice (11). Studies using cell lines transfected with anti-sense FUT7 cDNA have shown a clear relationship between Fuc-TVII expression and adhesion of leukocytes to selectins (10). A spontaneous SLe^a-negative variant of HL-60 cells deficient in Fuc-TVII also showed decreased binding to activated endothelial cells consistent with a significant role for Fuc-TVII in selectin ligand biosynthesis (33).

The fact that PMN from the Q/Q individual did roll on both E- and P-selectin despite markedly impaired Fuc-TVII activity may suggest a role for other Fuc-Ts in generation of selectin ligands. There are several lines of evidence that suggest that Fuc-TV may contribute to selectin ligand synthesis. Although reduced, rolling and recruitment of PMN was not completely abolished in Fuc-TVII^−/− mice (11), suggesting that Fuc-TV may synthesize active selectin ligands in the absence of Fuc-TVII. Some strains of Fuc-TV-transfected Chinese hamster ovary cells show E-selectin binding activity (16). Overexpression of Fuc-TV in Jurkat, K562, and BJAB cells also generated active selectin ligands (15, 34). Recent studies on mice deficient in Fuc-TV or both Fuc-TV and Fuc-TVII (double knockouts) further support a role for Fuc-TV in leukocyte rolling on E- and P-selectin (12, 13). These observations suggest that the contribution of Fuc-TV to selectin ligand synthesis is dependent on cell lineage and on the level of Fuc-TV expression.

When PMN from the Q/Q individual was analyzed for expression of FUT4 mRNA there was >100-fold more FUT4 mRNA compared with R/R individuals. This increase was also reflected in increased expression of SLe^a and CD65s structures at the PMN cell surface. The R/Q individuals showed an intermediate increase in FUT4 mRNA compared with R/R individuals. The expression of FUT7 mRNA was similar in the Q/Q individual compared with nonmutated individuals. Studies on human myeloid cell lines have shown that there is a reciprocal expression of Fuc-TIV and Fuc-TVII during cell differentiation (8, 35–37). When the promyelocytic cell line HL60 was allowed to differentiate in the presence of DMSO, there was an increase in cell surface expression of SLe^a and a concomitant fall in the expression of the CD65s Ag. This change correlated with a down-regulation of FUT4 mRNA and an up-regulation of FUT7 mRNA (8, 35). Spontaneous differentiation, in contrast, leads to an increase in FUT4 mRNA and a decrease in FUT7 mRNA levels (36). It is possible that the inactivation of Fuc-TVII in PMN from the Q/Q individual leads to a compensatory increase in Fuc-TV expression that may rescue the rolling capacity of PMN. Another possibility is that the Q/Q individual has a chronic overexpression of Fuc-TIV.

To address whether low residual activity of the mutated Fuc-TVII is sufficient to sustain E-selectin rolling in the absence of Fuc-TV activity, BJAB cells transfected with wild type and mutated FUT7 cDNA was analyzed. BJAB cells have previously been shown not to express detectable amounts of FUT4 or FUT7 mRNA. In addition, these cells do not roll on E-selectin in an in vitro rolling assay, but could be induced to interact with E-selectin after transfection with FUT4 or FUT7 mRNA (15). BJAB cells do not express PSGL-1 and therefore do not bind to P-selectin. Our assay could detect a very low expression of FUT7 mRNA in untransfected cells. After transfection with wild-type FUT7 mRNA rolling on E-selectin was observed. In contrast BJAB cells transfected with FUT7 G329A did not interact with E-selectin in our
system. Transfection of BJAB cells with wild-type FUT7 induced SLe\(^\alpha\) expression, whereas SLe\(^\alpha\) could not be detected in mock-transfected BJAB cells or BJAB cells transfected with the FUT7 G329A construct.

These results show that in this cell system, in the absence of Fuc-TIV activity, the G329A mutation does reduce the Fuc-TIV activity enough to produce a phenotype lacking a functional E-selectin ligand. Although further studies are needed to confirm that FUT7 G329A is a true null mutation, the current report shows that rolling of human PMN on both P- and E-selectin is still functional despite a severe decrease in Fuc-TIV activity. A compensatory increase in Fuc-TIV may be important to support rolling in individuals carrying this mutation.

Due to the major role of Fuc-TVII in the generation of selectin ligands in mouse models, intervention of Fuc-TIV activity has been suggested as a target for suppressing rolling in inflamed tissue. The current study suggests that it may be necessary to intervene not only with the expression of Fuc-TIV, but also with Fuc-TIV to have an active effect on PMN in humans.

Acknowledgments
We thank Gunilla Wallin for excellent technical help with the flow cytometry analyses and Dr. Gösta Bendix for clinical expertise.

References