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Research Article

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Leukocyte Adhesion Deficiency Type 1 (LAD-1)/Variant

A Novel Immunodeficiency Syndrome Characterized by Dysfunctional β_2 Integrins

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Abstract

Leukocyte adhesion deficiency (LAD) is characterized by the inability of leukocytes, in particular neutrophilic granulocytes, to emigrate from the bloodstream towards sites of inflammation. Infectious foci are nonpurulent and may eventually become necrotic because of abnormal wound healing. LAD-1 is characterized by the absence of the β_2 integrins (CD11/CD18) on leukocytes. When expression is completely absent, patients often die within the first year. However, low levels of β_2 expression may result in a milder clinical picture of recurrent infection, which offers a better prognosis.

In this paper, we describe the in vivo and in vitro findings on a patient with clinical features of a mild LAD-1 disorder, i.e., suffering from bacterial infections without apparent pus formation in the presence of a striking granulocytosis, showing no delayed-type hypersensitivity reaction upon skin testing, no specific antibody generation, but normal in vitro T cell proliferation responses after immunization. Expression levels of CD11/CD18 proteins were completely normal, but leukocyte activation did not result in CD11/CD18 activation and high-avidity ligand-binding. In vitro chemotaxis and endothelial transmigration of the neutrophils as well as leukocyte aggregation responses were almost absent. On the other hand, β_1 and β_3 integrin–mediated adhesion functions were completely normal. During follow-up, a bleeding tendency related to decreased β_3 activation became clinically apparent, different from previously described cellular adhesion molecule variants. Therefore, this is the first well-documented case of a clinical combined immunodeficiency syndrome that results from nonfunctional CD11/CD18 molecules, and thus designated LAD-1/variant. (*J. Clin. Invest.* 1997. 100:1725–1733.) Key words: leukocyte adhesion deficiency • bleeding tendency • integrins • aggregation • chemotaxis

Introduction

Inflammation involves a complex series of events, including vasodilatation, increased vascular permeability, and exudation of fluids and plasma proteins. These processes are followed by or coincide with an influx of inflammatory cells. Several inflammatory mediators are generated at or near the site of the lesion, e.g., complement fragments, lipid mediators, and chemokines such as IL-8, monocyte chemoattractant protein 1, macrophage inflammatory protein 1α/β, or regulated upon activation, normal T cell expressed and secreted (1). These mediators possess chemotactic activity and can attract leukocytes to the inflamed tissues. Leukocytes in the bloodstream go through a series of well-regulated adhesive steps before these cells actually leave the circulation. These steps are mediated by families of adhesion molecules, including integrins, members of the Ig supergene family, and selectins (2). The initial adhesive step consists of leukocyte rolling along the endothelial cell lining, as was shown first in studies with neutrophils (2–5). Rolling of leukocytes (step 1) occurs via low-avidity interactions between sialylated and fucosylated oligosaccharides such as sialylated Lewis-X antigen (SLe^x or CD15S)[1] and adhesion molecules of the CD62 selectin family. Rolling decreases the velocity of the leukocytes, and the cells will finally come to a stop. This last event depends on cell stimulation (step 2), which results in the activation of the leukocytic β_1 (CD29) and β_2 (CD18) integrin proteins from a low- to a high-avidity ligand-binding state through intramolecular changes in conformation (step 3) (2). Thereafter, integrin-dependent transmigration may follow along a gradient of chemotactic factors diffusing from extravascular inflammatory sites.

Leukocyte adhesion deficiency type 1 (LAD-1) was first described as a separate entity among immunodeficiencies in 1979 by Hayward et al. (6). LAD-1 is a rare autosomal recessive disease characterized by recurrent and/or chronic bacterial and sometimes fungal infections without pus formation, despite persistent leukocytosis (i.e., granulocytosis) in the circulation. A remarkable delay in the natural detachment of the umbilical cord is often the first symptom of this disorder and may result in serious omphalitis caused by manipulations. The

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1. *Abbreviations used in this paper:* CLB, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service; DTH, delayed-type hypersensitivity; FN, fibronectin; HE, hydroethidine; HUVEC, human umbilical vein endothelial cells; ICAM, intracellular adhesion molecule; KLH, keyhole limpet hemocyanin; LAD, leukocyte adhesion deficiency; NK, natural killer cell; SFDA, sulfofluorescein diacetate; SLe^x, sialylated Lewis-X antigen; STZ, serum-treated zymosan; VLA, very late antigen.

defect in LAD-1 is caused by mutations in the β_2 integrin (CD18) gene at chromosome 22q21. The aberrant β_2 integrin precursor protein is either undetectable or synthesized in a mutant form unable to associate with the respective α subunits. Hence, the expression of all three heterodimeric structures $\alpha_L\beta_2$ (CD11a/CD18 or LFA-1), $\alpha_M\beta_2$ (CD11b/CD18 or CR3), and $\alpha_X\beta_2$ (CD11c/CD18 or p150,95) will be deficient, and may range from complete absence to 20–30% of normal (7, 8). Severity of the disease correlates directly to expression levels (i.e., to the nature of the genetic defect).

In vitro neutrophil functions that depend on CR3, such as adhesive reactions, chemotaxis, and binding and ingestion of C3bi-coated microorganisms or particles, are dramatically deficient in LAD-1 (9). LAD-1 neutrophils show initial rolling along the endothelium but are unable to switch to a high-avidity state of ligand-binding for further emigration. The endothelial ligands for β_2 integrins that have been identified thus far are intracellular adhesion molecule (ICAM)-1 and -2, while the leukocytes themselves express ICAM-1 and another homologous cellular ligand, ICAM-3. All three ICAMs are members of the large Ig-like supergene family (2, and references therein).

In LAD-1 patients, lymphocytic functions such as proliferation, cytotoxic T-lymphycote responses, and natural killer cell (NK) activity (9) are also affected upon in vitro measurement, but to a lesser extent than neutrophil function in the same patients. Moreover, most LAD-1 patients have no serious problems with severe viral infections, and show normal delayed-type hypersensitivity (DTH) reactions in vivo. Antibody generation in vivo is generally, although not always, present to some extent (9). The relatively normal lymphocyte functions in LAD patients have been explained by the expression of β_1 integrins on these cells, in particular very late antigen (VLA)-4 ($\alpha_4\beta_1$, CD49d/CD29), which may rescue lymphocyte adhesive functions through binding to the (endothelial) cellular ligand vascular cell adhesion molecule 1 or to a splice-variant of the extracellular matrix protein fibronectin (FN) that contains the so-called CS-1 region (2, 10–15).

In this paper, we describe a patient with clinical features of congenital LAD-1, but with normal expression levels of CD11/CD18 members on leukocytes. However, cellular activation did not result in a conformational change in CD11/CD18 integrin receptors from a low-avidity to a high-avidity ligand-binding state. On the other hand, β_1 and β_3 integrin–mediated functions were completely normal initially. However, during follow-up, the patient developed at the age of 2 yr a clinically overt bleeding tendency unrelated to a plasma coagulation or fibrinolytic defect, but instead caused by a β_3 integrin dysfunction.

Methods

Patient. The propositus is a boy born in July, 1992, after an uneventful pregnancy and delivery, as the first child of healthy consanguineous Turkish parents. After 5 wk his umbilical cord detached spontaneously. From birth onward, his history is characterized by a persistent leukocytosis (i.e., granulocytosis) and bouts of nonpussing inflammatory lesions. Plasma levels of Igs were low, although the Ig spectrum did not reveal any bias towards a particular isotype or subclass. Upon standard immunizations at 3, 4, and 5 mo of age, specific anti–tetanus toxoid antibodies were not detected in his serum. In May, 1993, maturation of the immune system was still not apparent, and immune prophylaxis was begun (cotrimoxazole and monthly gammaglobulin infusions). Until now, serious disease or complications of bacterial or viral infections have not occurred. The boy is regularly admitted with bacterial infections for which intravenous treatment with high-dose antibiotics is needed. Wound healing takes place, but very slowly. Since the beginning of 1994, he has shown signs of hemorrhagic diathesis (mucosal lesions) with a prolonged bleeding time (15–16 min; normal value $<$ 6 min) in the presence of normal platelet counts. Plasma coagulation parameters have always been within normal limits, with normal concentrations of all known coagulation factors, FXIII, plasminogen and tissue plasminogen activator (tPA) antigen, and plasminogen activator inhibitor (PAI)-1 antigen, and normal activity. A 1-desamino-8-*D*-arginine vasopressin (DDAVP) test was performed for the determination of endothelial function, with normal increase of von Willebrand factor; however, the bleeding time was unaffected by DDAVP. Platelet aggregation was initially unaffected (see Results). In vitro tissue factor activity on LPS-stimulated monocytes was normal. Immunization with keyhole limpet hemocyanin (KLH, 0.5 mg subcutaneously; reference 16) was performed while patient was on regular Ig infusions to measure specific antibody generation and antigen-specific T cell responsiveness.

Reagents. PMA and fMLP were purchased from Sigma Chemical Co. (St. Louis, MO). These agents were dissolved in DMSO at 1,000 times the final concentration for cell stimulation, and were stored at −20°C. Indo-1/AM, NBD-Phallacidin, and sulfofluorescein diacetate (SFDA) were from Molecular Probes, Inc. (Eugene City, OR), and hydroethidine (HE) was from Polysciences Inc. (Warington, PA). SFDA and HE were dissolved as described (17).

mAbs. The following mAbs were used (produced in the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB) or obtained from the IIIrd, IVth, or Vth Leukocyte Antigen Typing Workshops, unless stated otherwise): CD2 (CLB-T11.1/1 and CLB-T11.2/1), CD3 (CLB-T3/4.1), CD4 (CLB-T4/1), CD8 (CLB-T8/1), CD11a (CLB-LFA1/2; activating CD11a NKI-L16, a kind gift of C. Figdor, Radboud Hospital, Nijmegen, The Netherlands), CD11b [CLB-B2.12, 60.1, Bear-1, OKM1 (Becton Dickinson, San Jose, CA) and the conformation-dependent mAb CBRM1/5, a kind gift of Dr. T.A. Springer, Harvard Medical School and the Center for Blood Research, Boston, MA], CD11c (Leu-M5, Becton Dickinson), CD14 (CLB-mon/1), CD16 (CLB-FcR-gran.1), CD18 (CLB-LFA1/1), CD19 (CLB-B4/1), CD27 (CLB-CD27/1), CD28 (CLB-CD28.1), CD29 (8A2, a kind gift of Dr. N. Kovach, University of Washington, Seattle, WA), CD31 (CLB-HEC/75), CD32 (IV.3, Medarex, Inc., W. Lebanon, NH), CD41 (CLB-tromb/7), CD42b (CLB-MB45), CD44 (F10-44), CD45 (CLB-3D3), CD45RO (UCHL-1), CD49d (HP1/2, a kind gift of F. Sanchez-Madrid, Hopital de la Princesa, Madrid, Spain; and P3E3, Cytel Corp., San Diego, CA), CD49e (CLB-SAM1), CD54 (RR1/1), CD61 (CLB-C17) and CD41/CD61 conformation–dependent mAb PAC-1 against CD41/CD61 (a kind gift of S.J. Shattil, Scripps Clinics, Los Angeles, CA), CD62L (Leu-8, Becton Dickinson), CD62P (CLB-C2), CD62E (ENA-2, a kind gift of W. Buurman, University of Maastricht, The Netherlands), CD63 (CLB-gran/12), CD64 (32), CD65 (VIM-2), CD66b (CLB-B13.9), anti-HLA class-I mAb W6/32, and anti-HLA class-II (CLB-E/1.1).

Cell isolation. Leukocytes were purified from human blood anticoagulated with 0.4% (wt/vol) trisodium citrate (pH 7.4) by density gradient centrifugation over isotonic Percoll with a specific gravity of 1.076 g/ml (18). The interphase, containing the mononuclear cells, was removed for further purification of monocytes and lymphocytes by negative selection with magnetic beads precoated with specific mAbs. The pellet fraction, containing erythrocytes and granulocytes, was treated for 10 min with ice-cold isotonic NH_4Cl solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.4) to lyse the erythrocytes. Granulocytes were resuspended in incubation medium containing 132 mM NaCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 6 mM KCl, 1.2 mM KH_2PO_4, 20 mM Hepes, 5.5 mM glucose, and 0.5% (wt/vol) HSA, pH 7.4.

Determination of surface antigen expression. Indirect immunofluorescence and flow cytometric analysis on a FACScan® (Becton Dickinson, Mountain View, CA) were performed to determine the

antigen expression on purified neutrophils, monocytes or lymphocytes. In some experiments, the neutrophils were activated before the addition of the primary antibody. After addition of the stimulating agent (e.g., FMLP, 1 μM) to prewarmed cells, activation was stopped after 15 min by addition of an excess of ice-cold PBS (19). The neutrophils were washed twice, incubated with primary antibody for 30 min at 4°C, and washed again twice in excess PBS. The procedure was repeated with FITC-labeled goat anti–mouse Ig for another 30 min at 4°C. Neutrophils, monocytes, and lymphocytes were distinguished by forward–side scatter pattern. Data were collected from 10,000 cells, and are represented as mean fluorescence intensity. Subpopulations were not observed.

Double-color FACS® assay. The double-color FACS® analysis has been described extensively elsewhere (17). In short, neutrophils (2×10^6 cells/ml) were divided into two portions and stained either with SFDA (100 μM) or HE (40 μg/ml) for 60 min at 37°C. After extensive washes, the cells (2.5×10^6 cells/ml in incubation buffer) were mixed in a ratio of 1:1 in siliconized cuvettes and activated after 10 min of preincubation. Samples were taken from the cuvette at various times and were immediately fixed in 1% paraformaldehyde (wt/vol). The number of double-colored cell clusters was determined in a FACScan® as percentage of the total number of colored cells counted.

Chromium-51 labeling. Purified neutrophils (or clonal T cells) were radiolabeled with chromium-51 according to Gallin et al. (20). Briefly, 10^7 cells/ml were incubated with 1 μCi chromium-51 per 10^6 cells (sodium chromate, 200–500 Ci/g; New England Nuclear, Boston, MA) in incubation medium (containing 0.1% HSA instead of 0.5% HSA) at 37°C for 1 h under gentle shaking and subsequently washed. Viability remained > 95%.

Endothelial cell culture, adherence, and transmigration. Endothelial cells were isolated from human umbilical cord veins (HUVEC) according to Jaffe et al. (21), with minor modifications (22). Adhesion and transmigration assays were performed essentially as described previously (23). Only second or third passages of HUVEC were used. Tissue culture medium–treated polycarbonate membranes (8.0-μm pore size, 24-mm-diameter) of transwell cell culture chamber inserts (Costar Corp., Cambridge, MA) were used for migration studies; filters were precoated with FN. In some experiments, HUVEC monolayers were pretreated with optimal concentrations of human recombinant IL-1β (10 U/ml; a kind gift of Dr. P. Lomedico, Hoffmann-La Roche, Nutley, NJ) or TNF-α (20 ng/ml; a kind gift of Dr. A. Creasy, Cetus Corp., Oakland, CA) for the indicated time before subsequent use. In other experiments, prewarmed medium with an optimal amount of the chemoattractant FMLP (10 nM) was added to the lower chambers. Prewarmed ^{51}Cr-labeled neutrophils (10^6 cells/ml) were added to the upper compartments, and the chamber plates were incubated at 37°C in a 5% CO_2 incubator for 30 min. Neutrophil fractions were collected from the upper and lower compartments and diluted with a fixed amount of incubation medium that had been used to wash the compartments. Radioactivity was determined in three cell fractions: luminal (upper compartment), abluminal (lower compartment), and in the membrane cut out of its container. Recovery was always > 92%. Results were expressed as percentage of radioactivity added to the chambers.

Neutrophil functional assays. Cytosolic free Ca^{2+}, $[Ca^{2+}]_i$, was measured with a spectrofluorometer (model RF540; Shimadzu Corp., Kyoto, Japan) at excitation and emission wavelengths of 340 and 390 nm, respectively, in neutrophils preloaded with indo-1/AM (Molecular Probes, Inc.). Oxygen consumption was measured with an oxygen electrode. Actin polymerization in neutrophils stimulated with FMLP (1 μM) was measured by FACS® analysis of NBD-Phallacidin–stained cells (Molecular Probes, Inc.). We refer to previous studies for a more detailed description of these assays (24).

Specific antibody measurement. KLH-specific Ab generation in vivo was measured in serum by an ELISA, as described (25). The Ab response is expressed in 1,3-di(2-tolyl)guanidine (DTG) units per milliliter; one unit is defined as the amount of KLH-specific IgG present in 1 ml of a 1,000,000 dilution of a reference serum provided by Dr. A. Fauci (National Institutes of Health, Bethesda, MD).

Cell proliferation assay. Lymphocyte proliferation was measured with standard [^{3}H]thymidine ([^{3}H]dThd) incorporation assays (26). PBMC were cultured in 96-well flat-bottomed tissue culture plates (Costar Corp.) at 10^4 cells per well in 200 μl of RPMI 1640 with Hepes and 10% FCS, with either 10 μg/ml CD3 mAb or a combination of CD2 mAbs in the absence or presence of a costimulatory CD28 mAb, or with KLH at 0.1 mg/ml (Calbiochem-Behring, Hoechst Holland BV, Amsterdam, The Netherlands). At day 4 of stimulation, 0.2 μCi (7.4 kBq) [^{3}H]dThd (2 Ci/mM, Amersham International, Buckingshire, UK) was added to each well, and the incorporated radioactivity was counted 4 h later.

Lymphocyte aggregation. Aggregation of clonal T cells (2×10^5/ml) was induced by phorbol ester (PMA, 100 ng/ml), a combination of CD2 mAbs (CLB-T11.1/1 and CLB-T11.1/2), or by CD3 mAb (CLB-T3/4.1) at a final concentration of 5 μg/ml each in flat-bottomed tissue culture plates at 37°C. Epstein Barr virus (EBV)-transformed B cell lines (2×10^5/ml) were induced by phorbol ester (PMA, 100 ng/ml). The activating CD11a mAb NKI-L16 as well as the blocking CD18 mAb CLB-LFA1/1 were used at 5 μg/ml final concentration. The blocking CD18 mAb was added to the cells 10 min before the stimulus. After 45 min, the cells were fixed. Aggregates were scored microscopically in a semiquantitative fashion (27).

T cell clones and adhesion to FN. Influenza virus–specific T cell clones, either $CD4^+$ or $CD8^+$, were used for binding to FN-precoated 24-well plates (Costar Corp.). The patient's T cell clones were compared with clones from healthy individuals, as previously described (26). T cell clones were radiolabeled with chromium-51 (see above), washed, and resuspended at 2×10^6 cells/ml. Cells were allowed to bind to FN-coated plates for 60 min at 37°C in the presence or absence of PMA (100 ng/ml) or the activating CD29 mAb 8A2 at an optimal concentration (2 μg/ml). Thereafter, the nonadhering cells were removed, the plates were washed three times with prewarmed incubation medium, and the adhering cells were lysed. Nonadherent cells and adherent cell fractions were counted separately. Recovery was > 95%. Blocking antibodies against VLA-4 (HP1/2 or P3E3) and VLA-5 (CLB-SAM1) were used in some experiments at a final concentration of 10 μg/ml; the cells were preincubated with these mAbs for 15 min before addition to the plates and further activation. A JY cell line ($\alpha_4\beta_1^-$, $\alpha_5\beta_1^-$, $\alpha_4\beta_7^+$) was used as CD29-negative control. Binding of JY cells to FN could be induced by PMA but not by 8A2, and was blocked by HP1/2 (not shown). Cellular viability was tested by lactate dehydrogenase LDH release (< 5%).

Platelet studies. Platelet aggregation studies in 400 μl platelet-rich plasma were performed in a two-channel aggregometer (Chrono-Log Corp., Havertown, PA). Under standard conditions, final concentrations of stimuli were as follows: collagen 0.4 μg/ml (Organon Teknika, Durham, NC), AA 0.25–0.50 mg/ml (Chrono-Log Corp.), ADP 1.0 μg/ml (Boehringer Mannheim, Mannheim, Germany), the endoperoxide analogue U46619 (Sigma Chemical Co.), and ristocetin 1.2 mg/ml (Paesel-Lorei, Hanau, Germany). TxB2, a stable metabolite of TxA2, was measured by RIA (NEN DuPont, Wilmington, DE), according to the procedures of the manufacturer, in the supernatant of platelets after activation with AA (0.50 mg/ml for 3 min) in triplicate samples prepared on two different occasions.

Sequence analysis of the CD18 subunit. RNA was isolated by dissolving mononuclear cells in 4 M guanidine thiocyanate and by centrifugation through 5.7 M cesium chloride. cDNA was synthesized with 200 U MoMuLV reverse transcriptase (GIBCO-BRL, Gaithersburg, MD), in a volume of 25 μl containing 7.5 μl of stretched RNA template, 30 OD pd$(N)_6$ (Pharmacia Diagnostics AB, Uppsala, Sweden), 0.5 mM dNTP mix (dATP, dCTP, dGTP, dTTP), 10 mM DTT, 30 U RNAsin (Promega Corp., Madison, WI) and the 5× reaction buffer at 42°C according to the instructions of GIBCO-BRL. 50 μl water was added to the cDNA mix. Suitable oligonucleotide primers were used in the PCR to amplify the coding regions of CD18 cDNA in six overlapping fragments (8). The positions of the oligonucleotide

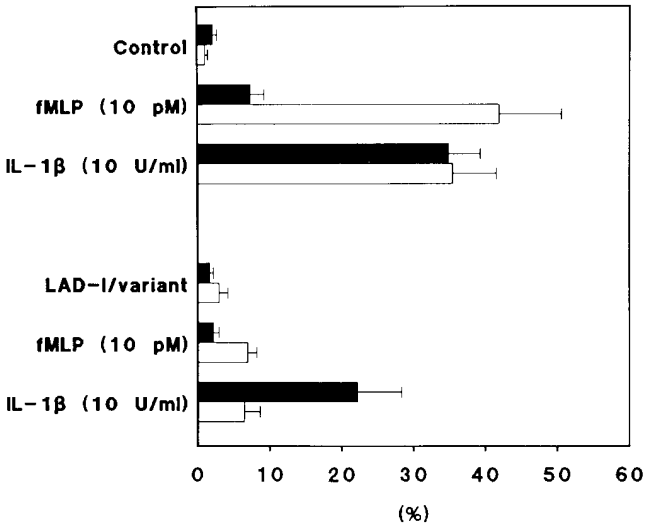

Figure 1. Neutrophil adhesion to and migration across HUVEC monolayers in a two-compartment transwell system (*filled* and *open bars*, respectively). Neutrophils were allowed to migrate in response to a chemotactic dose of FMLP (10^{-8} M) in the lower compartment across resting HUVEC, or across HUVEC preactivated for 4 h with rIL-1β (10 U/ml) or TNF (50 ng/ml; data not shown). Migration of patient neutrophils was significantly reduced compared to control neutrophils ($P < 0.05$).

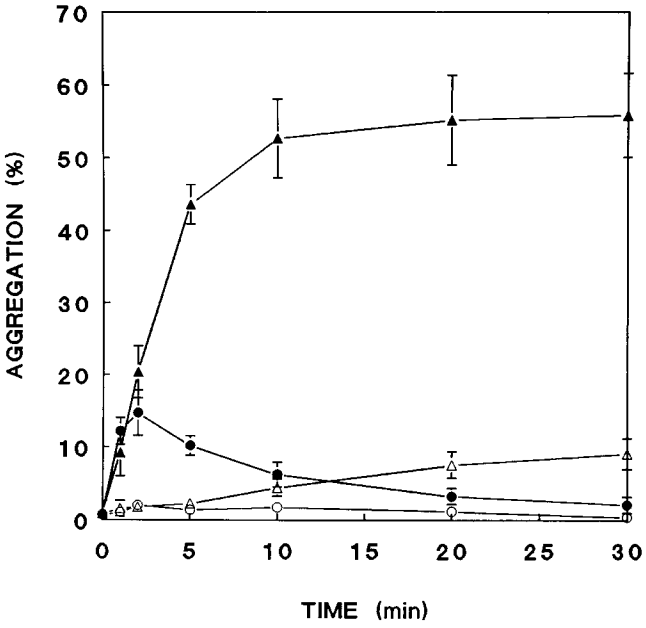

Figure 2. Homotypic aggregation of purified neutrophils in response to FMLP (1 μM, *circles*) or PMA (100 ng/ml, *triangles*). Whereas control neutrophils (*filled symbols*) show normal aggregation, patient neutrophils show almost none (*open symbols*). Control values, i.e., without addition of any stimulus, were < 3% throughout the 30-min period of testing.

primers complementary to the CD18 cDNA sequence were as follows: L1 sense (−70 to −51), 5′-GGGCAGACTGGTAGCAAAGC-3′; L2 sense (305 to 327), 5′-GTGACGCTTTACCTGCGACCAGG-3′; L3 antisense (432 to 413), 5′-CCTGAGGTCATCAAGCATGG-3′; L4 sense (712 to 731), 5′-GACGCCATGATGCAGGTCGC-3′; L5 antisense (873 to 854), 5′-GTTGTCCTCCAGGTGACAGC-3′; L6 sense (1132 to 1151), 5′-CTGAAAGTCACCTACGACTC-3′; L7 antisense (1239 to 1221), 5′-TCACCTGGAAGGTGATCGGG-3′; L8 sense (1540 to 1557), 5′-TGCGGGCAGTGCCTGTGC-3′; L9 antisense (1647 to 1628), 5′-GCAGACCTGGCCGTTGTAGC-3′; L10 sense (1949 to 1968), 5′-CTGCAGCTGTCGAACAACCC-3′; L11 antisense (2031 to 2012), 5′-CTCCAGCGTGTAGGCCACCC-3′; and L12 antisense (2396 to 2377), 5′-TGTGGCCAAGCCATGTCTCG-3′. PCR was performed with cDNA as a template, picomoles of each primer, and 2 U Taq DNA polymerase (GIBCO-BRL) in a buffer recommended by the manufacturer, in a total volume of 50 μl. The first cycle of PCR was performed at 95°C for 5 min, followed by 34 cycles of 1 min at 95°C, 1.5 min at 52°C, and 2.5 min at 72°C, and finally 1 cycle of 1 min at 95°C, 1.5 min at 52°C, and 9 min at 72°C. Nucleotide sequences of the PCR products were determined directly by the dideoxy-chain–termination method using cycle sequencing (GIBCO-BRL).

Statistical analysis. Significance was defined by Student's *t* tests. *P* values > 0.05 were considered not significant.

Table I. Expression of Surface Antigens on Purified Neutrophils Before and After Cell Activation

	Control		Patient	
	Resting	Activated	Resting	Activated
Control (murine IgG_1/2a)	10±3	16±6	17±4	22±5
CD11b (CLB-B2.12)	363±132	575±141	333±128	603±136
CD18(CLB-LFA1/1)	235±89	398±125	216±65	409±102
CD16b(CLB-gran/1)	798±194	286±97	817±110	375±129
CD32 (IV.3)	228±34	212±47	187±65	194±33
CD45 (UCHL-1)	122±25	235±41	114±37	190±13
CD62L (Leu8)	207±89	38±18	121±30	32±24
SLe^x (CSLEX-1)	2029±327	1696±431	1490±235	1204±372
Le^x (CLB-B4.3)	2472±198	2545±402	3102±222	3682±586
CD66B (CLB-B13.9)	124±39	362±73	120±27	238±44

Mean of three experiments ±SEM performed on different occasions. Neutrophils were incubated while shaking in a water bath at 37°C for 15 min in the presence or absence of 1 μM of the chemoattractant FMLP. Activation was stopped by the addition of ice-cold PBS. Thereafter, incubation steps with antibodies for flow cytometric analysis were performed as described in Methods.

Results

Abnormal β_2 integrin activation upon neutrophil activation. Neutrophil studies indicated a clear defect in adhesive properties of the patient's cells: chemotaxis and transmigration across resting and cytokine-preactivated HUVEC were significantly reduced (Fig. 1; $P < 0.05$), binding to FN coatings was strongly deficient (not shown), and homotypic aggregation was almost absent (Fig. 2; $P < 0.005$). Because of the clinical features of late separation of the umbilical cord and nonpurulent bacterial infections in the presence of a persistent neutrophilia ($> 30 \times 10^6$/ml), a diagnosis of LAD-1 seemed possible. However, normal expression levels of CD11/CD18 proteins on neutrophils as well as on mononuclear cells excluded this diagnosis (Tables I and II). The expression of these proteins was increased by cellular activation, concomitant with a normal fusion of specific granules to the plasma membrane, as evidenced by upregulation of CD66b (Table I) and release of vitamin B_{12}-binding protein from these granules (not shown).

Further studies excluded a defect in selectin-mediated interactions. First, neutrophil adhesion to cytokine-preactivated HUVEC was completely blocked by $F(Ab'_2)$ fragments of CD62E mAb (24). Second, neutrophil binding to a CD62E-expressing murine B cell line pMRB107 (a gift of M. Robinson, Celltech, Berkshire, UK) was not different from that of control neutrophils (18.5 vs. 17.6%; no binding occurred to the sham-transfected B cell line pMRB101). Third, in a CD62P-dependent rosetting assay of activated platelets around neutrophils (28), adhesion was normal (in autologous and heterologous fashion: patient platelets to patient neutrophils, patient platelets to control neutrophils, and control platelets to patient neutrophils) (data not shown). Fourth, L-selectin expression was normal for age and was shed as readily upon activation as observed with control neutrophils (Table I). Fifth, SLe^x and Le^x expression were normal, thus excluding LAD-2 (Table I).

Table II. Surface Antigen Expression on Leukocyte Subsets As Determined By Flow Cytometry

	Lymphocytes		Monocytes	
	Control	Patient	Control	Patient
Control (murine IgG_1/2a)	11	11	18	22
CD11a (CLB-LFA1/1)	268	138	293	307
CD11b (CLB-B2.12)	89	111	166	251
CD11c (LeuM5)	15	16	84	198
CD18 (CLB-LFA1/2)	86	45	118	155
Sle^x (CSLEX1)	92	51	1180	618
Le^x (CLB-B4.3)	18	20	2025	1211
CD65 (VIM-2)	11	14	95	197
CD62L (Leu8)	178	257	298	108
VLA-4α (HP1/2)	47	54	51	79
FcγR-I (32.2)	11	11	44	49
FcγR-II (IV.3)	22	22	90	65
FcγR-III (CLB-gran/1)	50	23	25	26
CD14 (CLB-CD14)	15	12	179	109
CD3 (CLB-T3/4.1)	241	243	25	27

Antigen expression expressed as mean of two different experiments. CD11/CD18 levels were checked more often, with results always comparable to those obtained with normal control leukocytes.

To test whether the CR3 integrin was unable to function at all, we measured its function by oxygen consumption after addition of serum-treated zymosan (STZ), a response that is completely dependent on CD11b/CD18 (29, 30). Both the number of FITC-labeled STZ particles (30) that bound to patient neutrophils (not shown) and, as a consequence, NADPH oxidase activity (6.5 nmol $O_2/10^6$ neutrophils/min) induced upon binding were not significantly different with control neutrophils. Integrin function in adhesion is related to cellular activation in two ways: inside-out activation of integrins for increased binding of ligands, and outside-in activation of neutrophil functions by this ligand-binding (31). Because STZ-binding and functional responses were normal, outside-in signaling seemed to be intact, in contrast to inside-out activation of ligand-binding to integrins. This assumption was further substantiated. In contrast to normal neutrophils, the activation epitope on CD11b/CD18 as recognized by CBRM1/5 (32) remained absent in the patient's cells upon neutrophil activation (Fig. 3), showing that neutrophil activation did not result in the intramolecular conformational changes that normally coincide with changes from a low- to a high-avidity ligand-binding state required for aggregation, adhesion, and migration (Figs. 1 and 2). Other functional assays yielded normal responses: normal $[Ca^{2+}]_i$ and actin polymerization responses were measured upon stimulation of neutrophils with the chemotactic stimuli FMLP, C5a, PAF, or IL-8 used at both low or high concentrations (i.e., 10^{-8} M and 10^{-6} M). NADPH oxidase activity was induced normally by FMLP (10^{-6} M) or phorbol ester (100 ng/ml) (data not shown). From these findings, we conclude that the lack of neutrophil adhesion cannot be explained by the lack or dysfunction of chemotaxin receptors, inherently disturbed $[Ca^{2+}]_i$, actin metabolism, or deficient protein kinase activity.

Abnormal in vitro and in vivo lymphocyte functions related to β_2 integrins. Antigen expression levels of CD11a/CD18 (LFA-1) on peripheral blood lymphocytes and monocytes were also found to be normal (Table II). Homotypic aggregation of clonal T cells and EBV-transformed B lymphoblasts was dramatically reduced upon activation with CD3 or CD2 mAbs, or with PMA, respectively (Table III), as we had observed previously with true CD18-deficient cells (33). On the other hand, normal induction of large aggregates was observed

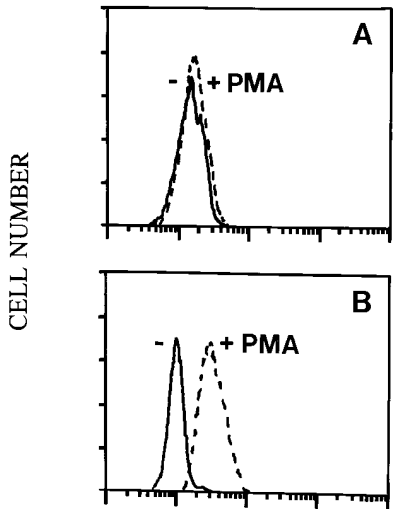

Figure 3. Expression of the activation epitope CBRM1/5 on neutrophils in the absence (−) or presence (+) of the stimulus PMA. Neutrophils were obtained from the patient (*A*) or from a healthy control (*B*). These experiments were performed on three different occasions with similar results.

Table III. Homotypic Aggregation of EBV-transformed B Cells and Clonal T Cells

	EBV-transformed B cell line		T cell clone	
	Patient	Control	Patient	Control
No addition	0	1	0	0
CD3 mAb (CLB-T3/4.1)	0	1	0	3
CD2 mAb (CLB-T11.1/1)	NT		0	0
CD2 mAbs combination (CLB-T11.1/1 + (CLB-T11.2/1)	NT		1	4
PMA	1	4	1	4
CD18 (CLB-LFA1/1)	0	0	0	0
CD18 + PMA	0	0	0	0
CD11a (NKI-L16)	4	4	4	4
CD18 + CD11a NKI-L16	0	0	0	0

NT, not tested. Data are representative of two different experiments on two different occasions. Results are scored 0 (no cells in aggregates), 1 (< 10% of the cells in aggregates), 2 (10–50% of the cells in aggregates), 3 (50–80% of the cells in aggregates), and 4 (> 80% of the cells in aggregates).

when the activating CD11a mAb NKI-L16 (33) was used (Table III). This last finding shows that the β_2 integrin receptors were able to recognize their ligand(s) and hence made it less likely that the adhesion deficiency was due to a defect located in the extracellular β_2 domain.

T cell proliferation was normal except for the proliferative response induced by CD2 in the absence of costimulatory signals, as we often observe in cases of immunodeficiency, among them LAD-1 patients (Table IV and our unpublished observations).

A further distinction was made between inside-out and outside-in signaling with respect to ligand-binding of CD11/CD18 integrins: the inability to activate CD11a/CD18-mediated aggregation upon addition of exogenous stimuli such as CD2 and CD3 mAbs or the phorbol ester PMA contrasted with a normal positive and negative modulation of CD3-induced T cell proliferation upon addition of CD11a or CD18 mAbs (26, 34), respectively (Fig. 4). This modulatory effect of CD11a and CD18 mAbs is absent in LAD-1 PBL (34).

Primary humoral and cellular immune responses in vivo were investigated upon immunization with KLH, an immunogenic T cell–dependent neoantigen (16) that can be safely used to determine immune responses irrespective of the gammaglobulin infusions the patient was receiving regularly. Blood was drawn at days 0, 7, and 14 to test in vitro T cell proliferation and in vivo antibody generation. In vitro T cell proliferation to KLH was detected normally, whereas in vivo anti-KLH antibody generation was absent. A disturbed T–B cell communication and the lack of an adequate antigen-specific B cell stimulation in LAD-1 have been observed previously (8, 9). Further experiments showed that the patient's T cells were unable to induce antibody production in autologous B cells. On the other hand, addition of normal T cells drove the patient's B cells to synthesize and secrete normal amounts of Ig. As we will expand upon elsewhere (van Lier, R.A.W., D. Hamann, and T.W. Kuijpers, manuscript in preparation), normal affinity regulation of CD11a/CD18 (LFA-1) integrin receptors is required for proper costimulatory signaling in lymphocytes in order to produce and secrete Ig in vitro. The migratory properties of T cells were also measured in vivo by means of DTH reactions. 1 mo after the immunization, skin tests with intradermal KLH injections (at 1, 10, or even 100 μg) were negative at 24, 48, and 72 h, which suggests that T cell infiltration was inadequate even though KLH-specific T cells were detected by in vitro proliferation at normal frequency in the peripheral blood after KLH immunization. Immunohistochemistry of biopsies of noninduced versus KLH-induced skin showed significant upregulation of vascular CD62E and vascular cell adhesion molecule 1 (although less than in controls) in the absence of a T cell influx in the KLH-induced skin biopsies (when hemorrhagic diathesis was not yet apparent; data not shown).

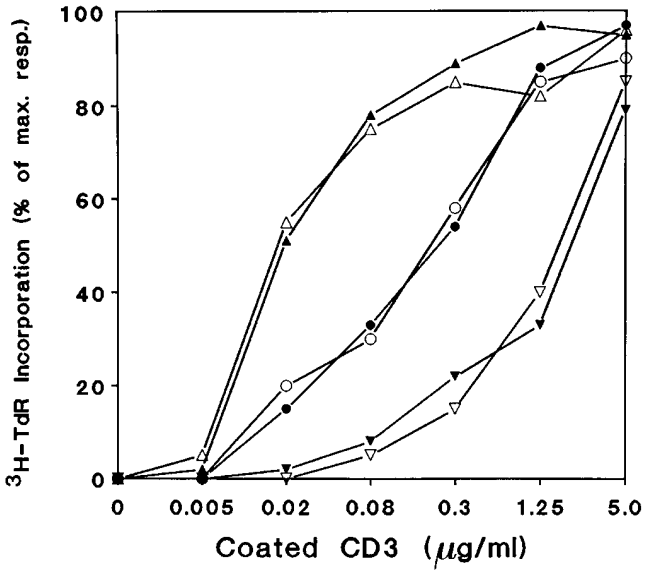

Figure 4. Proliferation of purified PBLs in response to graded amounts of coated CD3 mAb (CLB-T3/4.1, *circles*) in the presence of CD11a (CLB-LFA1/2, *upward triangles*) or CD18 (CLB-LFA1/1, *inverted triangles*) mAb (1/1,000 final dilution of ascites). Patient PBLs (*open symbols*) were compared with control PBLs (*closed symbols*). Results are expressed as mean of triplicate cultures. Maximal responses for PBLs from patient or control were 22,540 cpm and 18,820 cpm, respectively.

Table IV. Proliferation of Lymphocytes

	Control	Patient
CD3	31793	26873
CD2*	35550	449
CD2*/CD28	42981	38333
ConA	31978	41332

*In these proliferation tests, a combination of CD2 mAbs (CLB-T11.1/1 and CLB-T11.1/2) was used; either of these, CD2 mAbs or the CD28 mAb CLB-CD28.1, used as a single antibody, fails to induce any proliferative response by itself. Proliferation is expressed in counts per minute (cpm) of [^{3}H]thymidine incorporation measured after 4 h of uptake at day 5. Data are representative of different experiments on several occasions during follow-up.

PCR analysis of the β_2 integrin subunit. Extensive and repeated analysis of CD18 cDNA was applied in an attempt to

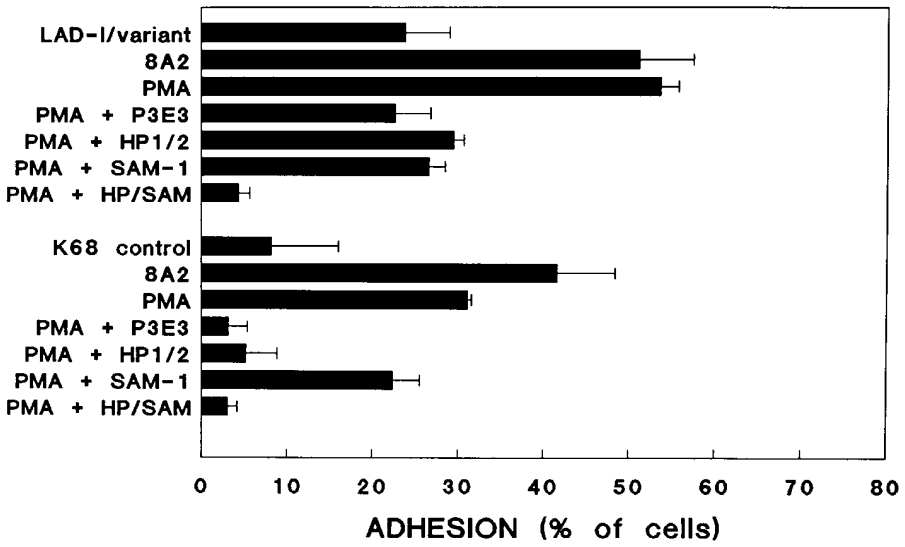

Figure 5. Binding of influenza virus–specific T cell clones of the patient and control (K68) to FN in response to PMA or the activating CD29 mAb 8A2. In some of the experiments, T cells were preincubated for 15 min with inhibitory anti–VLA-4α (HP1/2 or P3E3) or anti–VLA-5α (CLB-SAM1) mAbs (or a combination thereof) to define the contribution of either of these T cell integrin receptors to FN-binding. Results are expressed as mean±SEM for three different experiments.

find a mutation or deletion of the common subunit of the CD11/CD18 adhesion molecules. Six overlapping fragments of the cDNA were amplified, spanning the entire coding region. The following oligonucleotide primer combinations were used: L1/L3, L2/L5, L4/L7, L6/L9, L8/L11, and L10/L12. The PCR products were electrophoresed on agarose gels to control the size of the fragments. Direct nucleotide sequence analysis of the cDNA of the patient was then performed. Although we were successful in characterizing various mutations in the β_2 chain of several patients with deficient levels of CD11/CD18 expression, we did not find any defect in the β_2 subunit of the patient described here.

Normal β_1 integrin–mediated adhesion responses with progressive loss of β_3 integrin function. T cell clones were used to show that T cell adhesion to FN, mediated predominantly through VLA-4 and VLA-5, was intact (Fig. 5). When inhibitory CD49d and CD49e mAbs were added in combination, 95% or more of the cells were blocked in binding to FN as induced by PMA or the activating CD29 mAb 8A2. These findings are compatible with an intact β_1 integrin function that has not deteriorated during follow-up.

Platelet studies were performed to test β_3 integrin function and expression. In January of 1994, there was no defect in platelet function or glycoprotein GPIIbIIIa and GPIb expression. Aggregation responses were normal for all stimuli tested at standard concentrations, i.e., ristocetin, collagen, ADP, and AA. However, the child slowly developed a clinical bleeding tendency during follow-up. In November of 1995, platelet aggregation had become clearly defective even at four- to eight-fold higher doses of these stimuli (Fig. 6), compatible with a dysfunction of β_3 integrins. The patient's platelets again showed normal GPIIbIIIa expression. Upon platelet activation, expression of GPIIbIIIa was normally upregulated (as well as the granular markers CD62P and CD63), but without a clear expression of the conformation-dependent GPIIbIIIa-activation epitope on platelets, as recognized by mAb PAC-1 (35) (data not shown). The GPIb-dependent reactions to ristocetin have remained stable over time.

The possibility of a defect in AA metabolism was excluded by measuring TxB2, a stable metabolite of TxA2, in the supernatant of the platelets after activation with AA (123±36.2 vs. 108±42.7 pmol/10^7 platelets; patient vs. control). The endoperoxide analogue U46619, a stable TxA2 mimetic, was also able to induce a slight aggregation response, albeit at high concentrations, excluding a defect at the TxA2 receptor level (data not shown).

Discussion

The diagnosis of LAD-1, either the severe or milder variant, relies both on clinical and in vitro observations. In the classical form, delayed detachment of the umbilical cord, nonpurulent bacterial infectious foci in the presence of granulocytosis, and impaired wound healing are the clinical symptoms, with in vitro correlates of strongly reduced neutrophil adhesion and migration, deficient CD11/CD18 integrin expression on leukocytes, and, as final proof, a genetic defect of the β_2 integrin subunit. We have identified a patient whose clinical presentation is compatible with the diagnosis of an LAD syndrome: neutrophil functions were disturbed with respect to adhesive phenomena (Figs. 1–3), in the presence, however, of normal CD11/CD18 levels on all leukocyte types tested. Neutrophil actin dysfunction (24, 36), LAD-2 (37), or (combined) selectin deficiencies similar to those found in CD62 knockout mice (38–41) were excluded. Because the neutrophils and lymphocytes behaved normally in the various functional assays (NADPH oxidase activity, changes in $[Ca^{2+}]_i$, actin polymerization, release of proteins from azurophilic and specific granules, and lymphocyte proliferation), the defect seemed limited to the function of adhesion molecules.

Integrin avidity regulation and ligand-binding have received much attention. Intramolecular changes result in high-avidity ligand-binding (2, 31). The patient's leukocytes were found to be dramatically deficient in β_2-mediated adhesion ligand-binding upon stimulation by chemotactic stimuli, antigen-receptor stimulation, or direct activation with phorbol esters. Integrin function is presently understood in terms of inside-out and outside-in signaling events. Cellular activation induces various changes at the cytoplasmic tail of integrin subunits (e.g., phosphorylation, engagement of cytoskeletal ele-

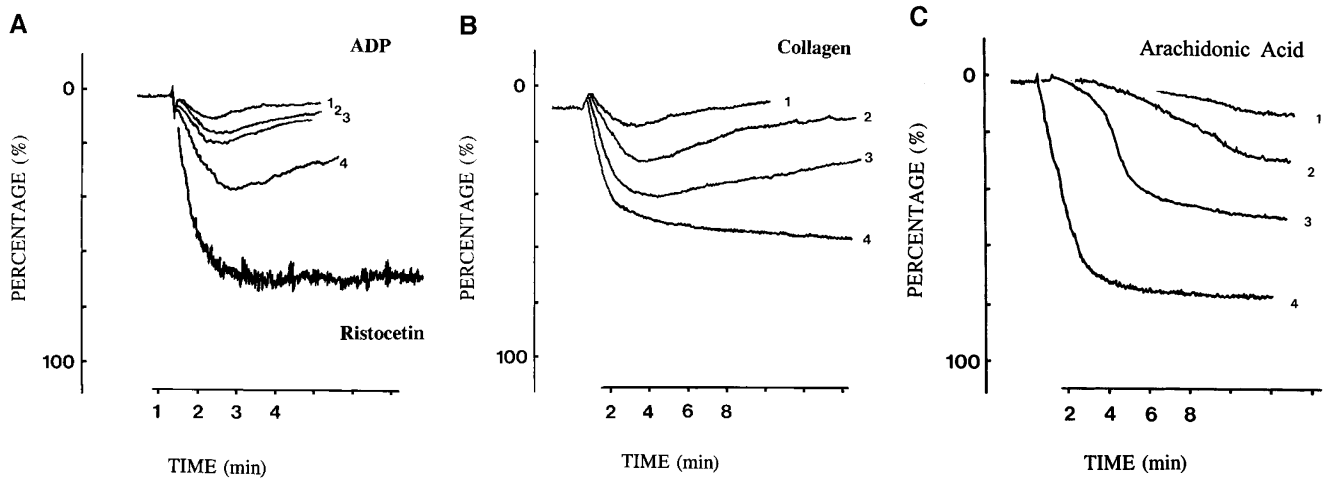

Figure 6. Platelet aggregation responses to various stimuli. (*A*) Patient platelet aggregation was induced with increasing doses of ADP (*1*: 1 μg/ml; *2*: 2 μg/ml; *3*: 8 μg/ml) compared to control platelets (*4*: 1 μg/ml). The patient platelet response to ristocetin (1.2 mg/ml) was identical to that of the control (data not shown). (*B*) Aggregation responses of the patient platelets to increasing doses of collagen (*1*: 0.4 μg/ml; *2*: 0.4 μg/ml; *3*: 1.6 μg/ml) compared to control platelets (*4*: 0.4 μg/ml). (*C*) AA-induced aggregation of patient platelets at high doses (*1*: 1 mg/ml; *2*: 1.5 mg/ml; *3*: 2 mg/ml) compared to control platelets (*4*: 0.5 mg/ml).

ments, as well as kinase activity; references 31, 42–46). These events are part of a coinciding change in affinity of the extracellular part of the integrin receptor for adhesion ligands (inside-out activation). These conformational changes are reflected by mAb binding to newly exposed epitopes, such as those we have measured in the case of CD11b/CD18 on neutrophils with mAb CBRM1/5 (Fig. 3). On the other hand, outside-in signaling, as occurs via (artificial) binding to ligands presented as densely coated substrate on plastic or particles, results in promoting various signaling events in the cells (31, 45). We tested the β_2 integrin function by means of STZ-induced NADPH oxidase activity in neutrophils and CD11a/CD18-modulated T cell proliferation. These β_2-dependent outside-in signaling events were normal (Fig. 4). A structural defect in the extracellular domain of CD11a/CD18 resulting in an inherent molecular inability to recognize ICAM-1 was excluded by the observation that mAb NKI-L16 (33) induced clonal T cells as well as EBV-transformed B lymphoblasts to aggregate in a strictly LFA-1/ICAM-1–dependent way (Table III). A structural defect (deletion or point mutation) in the β_2 subunit (with special attention to the transmembrane and cytoplasmic tail) was excluded by sequencing CD18 cDNA, indicating that this patient did not suffer from classical LAD-1.

Definite defects in expression or function of β_1 and β_3 integrin receptors were not observed initially. Lymphocyte-binding via β_1 integrins VLA-4 and VLA-5 to FN remained unaffected (Fig. 5). However, during follow-up, the patient developed a bleeding tendency compatible with thrombastenic platelets related to a well-expressed but dysfunctional β_3 integrin receptor (Fig. 6).

In conclusion, we have identified a patient with strong in vivo and in vitro immunological resemblance to classical LAD-1. Nonetheless, CD11/CD18 molecules are expressed at normal levels but cannot be activated to a high-affinity ligand-binding state through inside-out signaling. However, these β_2 integrins can transduce signals as soon as they are activated directly by substrates such as antibodies (CD11a/CD18) or STZ (CD11b/CD18). The lack of a genetic defect in the common β_2 subunit further excluded the diagnosis of classical LAD-1. Development of Glanzmann's disease–like thrombasthenia further indicates that a defect in adhesive properties of leukocytes and platelets is most likely not located at the level of β_2 or β_3 integrin subunits per se, but at some integrin-associated level. The remaining β_1 integrin function is obviously a fortunate situation for the child. Most likely, a β_2 and β_3 function–associated molecule involved in inside-out signaling leading to high-avidity ligand-binding is deficient. Elucidation of the primary defect awaits further study; until then, we propose that this syndrome be designated LAD-1/variant or LAD-1b.

Acknowledgments

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