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Regulation of leukocyte–endothelium interaction and leukocyte transendothelial migration by intercellular adhesion molecule 1–fibrinogen recognition

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ABSTRACT **Although primarily recognized for its role in hemostasis, fibrinogen is also required for competent inflammatory reactions *in vivo*. It is now shown that fibrinogen promotes adhesion to and migration across an endothelial monolayer of terminally differentiated myelomonocytic cells. This process does not require chemotactic/haptotactic gradients or cytokine stimulation of the endothelium and is specific for the association of fibrinogen with intercellular adhesion molecule 1 (ICAM-1) on endothelium. Among other adhesive plasma proteins, fibronectin fails to increase the binding of leukocytes to endothelium, or transendothelial migration, whereas vitronectin promotes the binding but not the migration. The fibrinogen-mediated leukocyte adhesion and transendothelial migration could be inhibited by a peptide from the fibrinogen γ-chain sequence N^{117}NQKIVNLKEKVAQLEA^{133}, which blocks the binding of fibrinogen to ICAM-1. This interaction could also be inhibited by new anti-ICAM-1 monoclonal antibodies that did not affect the ICAM-1–CD11a/CD18 recognition, thus suggesting that the fibrinogen binding site on ICAM-1 may be structurally distinct from regions previously implicated in leukocyte–endothelium interaction. Therefore, binding of fibrinogen to vascular cell receptors is sufficient to initiate (*i*) increased leukocyte adhesion to endothelium and (*ii*) leukocyte transendothelial migration. These two processes are the earliest events of immune inflammatory responses and may also contribute to atherosclerosis.**

Fibrinogen plays a crucial role in primary hemostasis and platelet function, as highlighted by the bleeding disorders of afibrinogenemic individuals (1). However, fibrinogen is also required for efficient inflammatory responses *in vivo*, as originally recognized during delayed-type hypersensitivity (2). More recent studies have demonstrated that defibrinated animals fail to accumulate an inflammatory cell infiltrate after intraabdominal purulent challenges (3), or at the site of biomaterial implant (4), and that such animals are protected from glomerular lesions during the acute phase of immune-complex nephrotoxic nephritis (5). The molecular mechanisms underlying fibrinogen-dependent inflammatory responses are poorly understood. Although fibrinogen fragments generated upon blood activation of coagulation exert chemotactic (6) and immunosuppressive effects (7), a role for the intact fibrinogen molecule in vascular injury and inflammation has been postulated as well.

General inflammatory responses and tissue-specific immune effector functions begin with a regulated "cascade" of adhesive interactions between leukocytes and vascular endothelium, followed by leukocyte extravascular migration across the endothelial cell monolayer (8, 9). In a previous study, we have shown that fibrinogen and normal human plasma enhance the attachment of leukocytes to endothelium by acting as a molecular bridge between the two cell types (10). The endothelial cell fibrinogen receptor mediating this bridging pathway with leukocytes was identified as intercellular adhesion molecule 1 (ICAM-1) (10).

In this study, we show that the fibrinogen–ICAM-1 interaction can also mediate transendothelial migration of leukocytes and identify the sequence in fibrinogen that serves as a binding site for ICAM-1. We also show that fibrinogen binds to a site on ICAM-1 that is spatially distinct from that implicated in the recognition of CD11a/CD18 (9).

MATERIALS AND METHODS

Cells and Cell Cultures. Peripheral blood mononuclear cells (PBMCs) and polymorphonuclear leukocytes (PMNs) were isolated from heparinized blood drawn after informed consent from normal healthy volunteers by differential centrifugation on Ficoll/Hypaque gradient and dextran sedimentation as described (11). The promyelocytic cell line HL-60 was terminally differentiated to a monocyte-like phenotype by a 4- to 5-day culture in the presence of 0.1 μM 1,25-dihydroxyvitamin D_3 (Biomol, Plymouth Meeting, PA) and indomethacin (17.8 μg/ml) (Calbiochem) according to published protocols (12). Previous studies have demonstrated that vitamin D_3-differentiated HL-60 cells constitute a reliable model for *in vitro* monocyte function (13). Human umbilical vein endothelial cells (HUVECs) were prepared by collagenase treatment (14), or purchased from Clonetics (San Diego), and maintained in culture according to the supplier's specifications. For most experiments, HUVECs were used between passages 2 and 5. The integrity of the endothelial cell monolayer was confirmed before each experiment of leukocyte adhesion and transendothelial migration by phase-contrast microscopy and crystal violet staining.

Proteins and Synthetic Peptides. Human fibronectin and fibronectin-depleted fibrinogen were purified according to published protocols (11, 15). Human vitronectin was purchased from GIBCO or kindly donated by Helena Hessle (Telios Pharmaceuticals, San Diego). Control proteins transferrin and bovine serum albumin were purchased from Sigma and dissolved in phosphate-buffered saline (pH 7.2). Partially overlapping synthetic peptides derived from the fibrinogen γ chain, and characterized in previous studies (11), were screened for their ability to inhibit

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Abbreviations: ICAM-1, intercellular adhesion molecule 1; HUVEC, human umbilical vein endothelial cell; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cell; PMN, polymorphonuclear leukocyte; TNF-α, tumor necrosis factor type α.

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binding of ^{125}I-labeled fibrinogen to resting HUVEC monolayers. In these experiments, a synthetic peptide designated γ3 (N^{117}NQKIVNLKEKVAQLEA^{133}) completely abolished in a dose-dependent manner the interaction of ^{125}I-fibrinogen with ICAM-1-expressing cells (IC_{50}, ≈60 μM) and physically associated with genetically engineered ICAM-1 transfectants in direct ^{125}I-labeled peptide binding studies. In contrast, none of the other fibrinogen γ-chain-derived peptides (11), or a control peptide containing a scrambled γ3 sequence (ALENAEVQNLVKKIQKN), was effective under the same experimental conditions (unpublished observations).

Monoclonal Antibodies (mAbs). A panel of murine mAbs was generated against the B-lymphoblastoid cell line Daudi. Hybridoma culture supernatants were initially screened by flow cytometry for their reactivity with Daudi lymphoblasts or with resting or 4-h type α tumor necrosis factor (TNF-α)-activated HUVECs. Three mAbs (2D5, 1G12, and 6E6) specifically bound to genetically engineered ICAM-1 transfectants by flow cytometry and immunoprecipitated a single ≈95-kDa molecule from ^{125}I-surface-labeled detergent-solubilized Daudi lymphocyte extracts.

Leukocyte–Endothelium Interaction and Transendothelial Migration. The experimental procedures for leukocyte adhesion to HUVEC monolayers in the presence of plasma proteins has been described in detail (10). The effect of adhesive proteins on leukocyte transendothelial migration was investigated as follows. HUVEC monolayers were grown to confluency onto gelatin-coated porous membranes (diameter, 3–8 μm; Transwell; Costar) for 1–3 days at 37°C and incubated in duplicate with aliquots (1×10^5 cells per well) of vitamin D_3-differentiated HL-60 cells, PMNs, or PBMCs in the presence or absence of fibrinogen (300–600 μg/ml), vitronectin (40 μg/ml), autologous normal human plasma (1:5 dilution), or serum (1:2 dilution) for 2 h at 37°C. Vitamin D_3-differentiated HL-60 cells, PMNs, or PBMCs were stimulated with 10 μM fMet-Leu-Phe before incubation with HUVECs. After three washes in serum-free RPMI 1640 medium, migrated leukocytes were recovered from the bottom of the well, washed, stained with 0.2% trypan blue, and counted microscopically. Similar experiments were also repeated with HUVEC monolayers stimulated with TNF-α (100 units/ml) for 4 h at 37°C. No difference in leukocyte transendothelial migration was observed when comparable concentrations of fMet-Leu-Phe were simultaneously added to both the lower and the upper Transwell chambers under these experimental conditions. In synthetic peptide or mAb inhibition experiments, vitamin D_3-differentiated HL-60 cell or PMN suspensions were separately preincubated with fibrinogen γ-chain-derived synthetic peptide γ3 or DIII (K^{162}QSGLYFIKPLKAN^{175}) (100–400 μg/ml) or with anti-ICAM-1 mAbs LB-2 (10), 1G12, 2D5, and 6E6, or control mAb 6A11 (25 μg/ml) before addition of fibrinogen (300 μg/ml) or autologous plasma and quantitation of leukocyte–endothelium interaction and transendothelial migration. In another series of experiments, aliquots of ^{51}Cr-labeled HL-60 cells were added in the presence or absence of anti-ICAM-1 mAbs 2D5 and 6E6, anti-CD18 mAb 60.3, or control mAb 6A11 (25 μg/ml) to confluent monolayers of ICAM-1 transfectants or wild-type CHO cells and measurement of specific cell adhesion in the absence of fibrinogen after a 30-min incubation at 22°C was as described (10).

RESULTS

Regulation of Leukocyte–Endothelium Interaction by Plasma Adhesive Proteins. Previous studies have demonstrated that fibrinogen enhances leukocyte adhesion to endothelium by acting as a molecular bridge between the two cell types (10). The possible participation in this mechanism of additional plasma proteins with cell adhesion-promoting properties—i.e., vitronectin and fibronectin—was investigated. While no specific adhesion of undifferentiated HL-60 promyelocytes to resting HUVEC monolayers was observed in the presence of soluble adhesive proteins (data not shown), fibrinogen or vitronectin enhanced by 3- to 4-fold the adhesion of vitamin D_3-differentiated monocyte-like HL-60 cells (12, 13) or PBMCs to resting endothelium (Fig. 1; data not shown). In contrast, comparable concentrations of fibronectin were consistently ineffective under the same experimental condi-

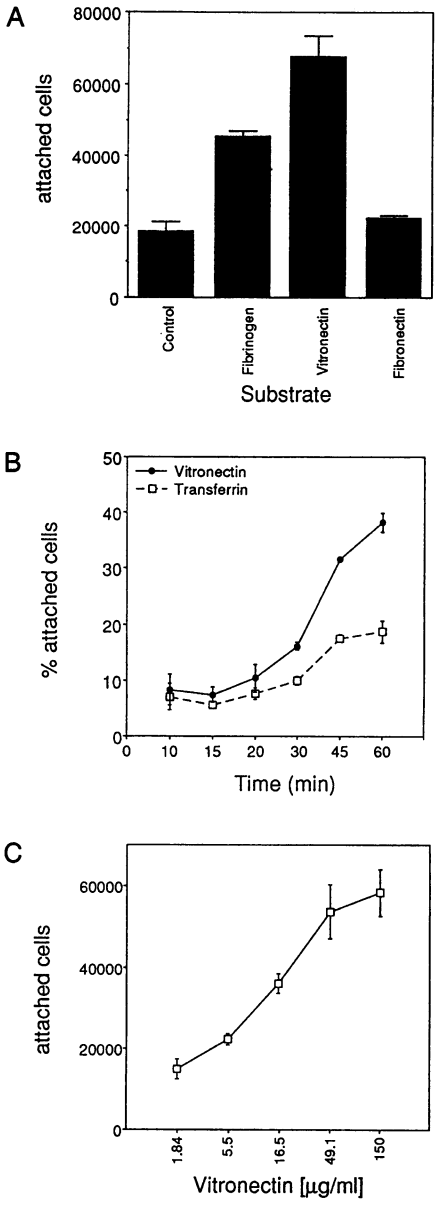

FIG. 1. Leukocyte–endothelium interaction mediated by plasma adhesive proteins. (*A*) Suspensions of vitamin D_3-differentiated ^{51}Cr-labeled HL-60 cells in serum-free RPMI 1640 medium at 1.5×10^6 cells per ml were stimulated with 10 μM fMet-Leu-Phe and equilibrated with fibrinogen, vitronectin, or fibronectin (150 μg/ml) in the presence of 1 mM $CaCl_2$ for 20 min at 22°C. The cell suspension was added to resting HUVEC monolayers for an additional 30-min incubation at 22°C, washed, and solubilized in 10–20% SDS, and radioactivity associated under the various conditions was determined in a scintillation β-counter. (*B*) Experimental conditions are the same as in *A* except that vitamin D_3-differentiated ^{51}Cr-labeled HL-60 cells were equilibrated with vitronectin or control protein transferrin (40 μg/ml) and added to resting HUVECs for increasing time intervals at 22°C before measurement of leukocyte adhesion. (*C*) Vitamin D_3-differentiated ^{51}Cr-labeled HL-60 cells were incubated with the indicated increasing concentrations of vitronectin before addition to HUVEC monolayers for 30 min at 22°C. Data for all panels are means ± SD of triplicate determinations from a representative experiment.

tions, despite the ability of differentiated HL-60 cells to strongly adhere to fibronectin substrate (Fig. 1; data not shown). As shown in Fig. 1, vitronectin enhanced monocytic HL-60 cell adhesion to HUVECs in a time- and dose-dependent reaction (Fig. 1 *B* and *C*), approaching saturation at ≈50 μg/ml, in the range of the physiologic plasma concentrations of vitronectin (Fig. 1*C*). These results are in agreement with a recent study showing that vitronectin specifically binds to the luminal side of endothelial cells (16). Vitronectin did not further increase adhesion of differentiated HL-60 cells to TNF-α-stimulated HUVECs, as compared with control incubation reactions in the presence of transferrin (data not shown).

Effect of Plasma Adhesive Proteins on Leukocyte Transendothelial Migration. Tight adhesion of leukocytes to endothelium is followed by leukocyte transendothelial migration and tissue-specific homing (9, 17). The possibility that plasma adhesive proteins mediating leukocyte–endothelium bridging might also direct leukocyte migration across the endothelium monolayer was investigated. As shown in Fig. 2, in the absence of adhesive proteins or chemotactic gradients, PMNs or vitamin D_3-differentiated HL-60 cells did not migrate across resting HUVEC monolayers, in agreement with previous observations (18) (Fig. 2). In contrast, physiologic concentrations of fibrinogen or normal human plasma, but not vitronectin or serum, stimulated transendothelial migration of both cell types under the same experimental conditions (Fig. 2). Similar results were obtained with PBMCs (data not shown). The effect of cytokine activation of HUVECs on leukocyte transmigration mediated by adhesive proteins was investigated. In agreement with previous observations (18, 19), TNF-α-stimulated HUVECs supported transmigration of 3000 ± 1000 PMNs in the absence of adhesive proteins, as compared with the undetectable values observed for resting endothelium. Under these experimental conditions, fibrinogen promoted migration of 20,500 ± 500 PMNs through TNF-α-stimulated HUVECs, while, consistent with the data presented above, 4000 ± 1000 PMNs migrated through cytokine-activated HUVEC monolayers in the presence of vitronectin.

Structural Requirements of Fibrinogen-Dependent Leukocyte Transendothelial Migration. The potential participation of ICAM-1 and of its recognition for fibrinogen (10) in leukocyte transendothelial migration was investigated in synthetic peptide and mAb inhibition studies. Survey of a large number of fibrinogen γ-chain-derived synthetic peptides identified a peptide designated γ3 (N^{117}NQKIVNLKEKVAQLEA^{133}) that was capable of inhibiting leukocyte–endothelium interaction mediated by fibrinogen. The γ3 peptide completely inhibited binding of ^{125}I-fibrinogen to ICAM-1-expressing cells and specifically bound to ICAM-1 transfectants. Other fibrinogen γ-chain-derived peptides, including peptides shown to bind to the $\alpha_M\beta_2$ integrin (11), were not active under the same experimental conditions (unpublished observations). As shown in Fig. 3*A*, increasing concentrations of γ3 completely inhibited in a concentration-dependent

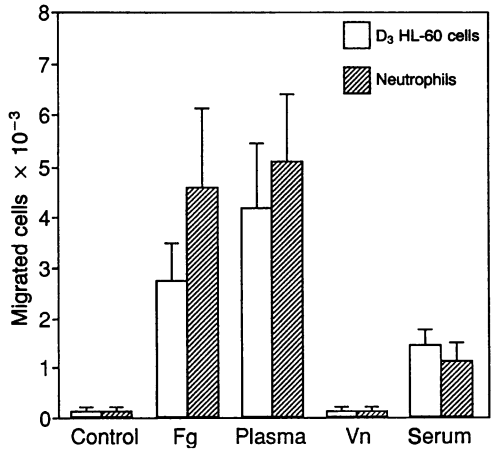

FIG. 2. Effect of plasma adhesive proteins on leukocyte transendothelial migration. Resting HUVEC monolayers were grown to confluency on gelatin-coated porous membranes (Transwell; Costar; diameter, 3–8 μm) and separately incubated with serum-free suspensions of PMNs or vitamin D_3-differentiated HL-60 cells in the presence of 1 mM $CaCl_2$ and 1 mM $MgCl_2$. After separate addition of fibrinogen (Fg) (300 μg/ml), vitronectin (Vn) (40 μg/ml), autologous human plasma (1:5 dilution), or serum, the various incubations were continued for 2 h at 37°C. Cells were washed with three rapid changes of serum-free RPMI 1640 medium, and transmigrated leukocytes at the bottom of the well were recovered and counted in a hemocytometer. Data are expressed as means ± SD of three independent experiments.

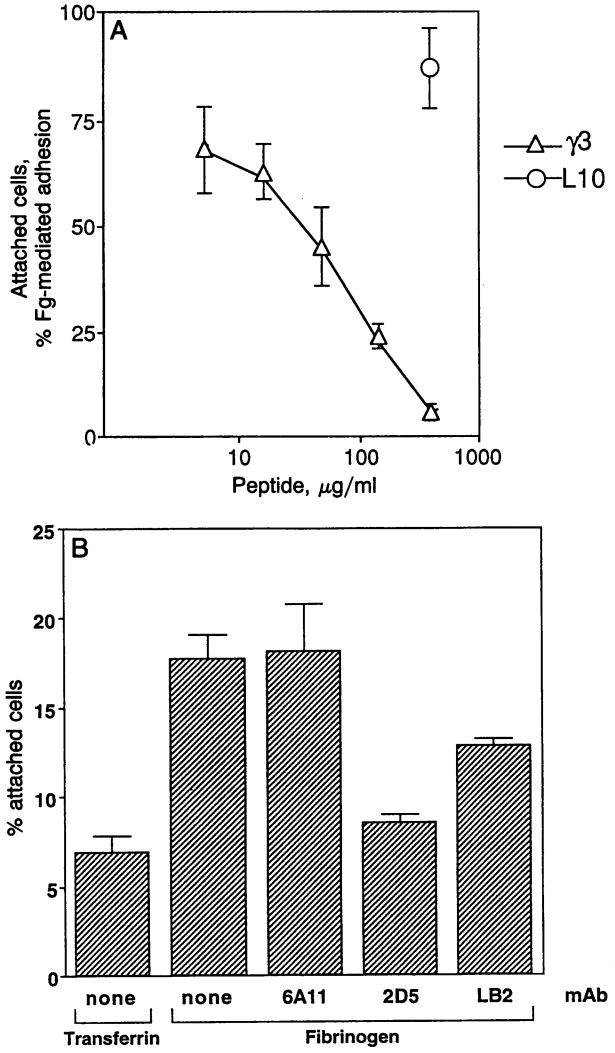

FIG. 3. Effect of antagonists of ICAM-1–fibrinogen interaction on fibrinogen-dependent leukocyte adhesion to HUVECs. (*A*) Serum-free suspensions of ^{51}Cr-labeled vitamin D_3-differentiated HL-60 cells were preincubated with the indicated increasing concentrations of fibrinogen-derived peptide γ3-(117–133) or a single concentration (400 μg/ml) of control peptide L10-(402–411) for 10 min at 22°C before addition to resting HUVEC monolayers in the presence of fibrinogen (Fg) (300 μg/ml). Specifically attached cells under the various conditions tested were calculated after a 30-min incubation at 22°C as described in Fig. 1. (*B*) Experimental conditions are the same as in *A*, except that serum-free suspensions of ^{51}Cr-labeled PBMCs were incubated with saturating concentrations (25 μg/ml) of anti-ICAM-1 mAb 2D5 or LB-2 or control mAb 6A11 for 15 min at 22°C before addition to resting HUVEC monolayers in the presence of fibrinogen (300 μg/ml) or control protein transferrin and measurement of cell adhesion. Data for both panels are expressed as means ± SD of at least two independent experiments.

fashion the adhesion of vitamin D_3-differentiated HL-60 cells to resting HUVEC monolayers mediated by fibrinogen. Similar results were obtained with PBMCs (data not shown). Control peptide L10 (Fig. 3*A*) or a scrambled control peptide based on the γ3 sequence (data not shown) were not inhibitory.

In parallel experiments, an anti-ICAM-1 mAb panel was generated by selecting for inhibition of binding of ^{125}I-fibrinogen to ICAM-1-expressing cells. One of these anti-ICAM-1 mAbs, 2D5, inhibited fibrinogen-mediated adhesion of PBMCs and vitamin D_3-differentiated HL-60 cells to resting HUVEC monolayers to the background values observed in the presence of control protein transferrin (Fig. 3*B*; data not shown). Consistent with previous observations (10), saturating concentrations of anti-ICAM-1 mAb LB-2 inhibited by ≈50% the leukocyte–endothelium interaction, while control mAb 6A11 was ineffective (Fig. 3*B*). In another series of experiments, saturating concentrations of mAb 2D5 failed to diminish HL-60 cell adhesion to monolayers of ICAM-1 transfectants in the absence of fibrinogen (Table 1). In contrast, anti-CD18 mAb 60.3, or the anti-ICAM-1 mAb 6E6, which does not affect fibrinogen recognition, completely inhibited HL-60 cell adhesion to ICAM-1 transfectants (Table 1).

The effect of these two classes of antagonists on fibrinogen-dependent leukocyte transendothelial migration was investigated. As shown in Fig. 4*A*, inhibitory concentrations of γ3 blocked transendothelial migration of vitamin D_3-differentiated HL-60 cells mediated by fibrinogen or by autologous human plasma by 60–70%, respectively, while control peptide DIII (K^{162}QSGLYFIKPLKAN^{175}) was ineffective (Fig. 4*A*). In parallel experiments, anti-ICAM-1 mAb 2D5 also inhibited by 50–75% fibrinogen- or plasma-dependent migration of vitamin D_3-differentiated HL-60 cells across resting HUVEC monolayers (Fig. 4*B*). In contrast, leukocyte preincubation with control mAb 6A11, or with anti-ICAM-1 mAb 6E6 (Table 1), did not diminish leukocyte transendothelial migration mediated by fibrinogen or plasma (Fig. 4*B*).

DISCUSSION

Soluble Adhesive Ligands Regulate Leukocyte–Endothelium Interaction and Leukocyte Transendothelial Migration. In this study, we have shown that the association of fibrinogen with vascular cells is sufficient to promote two among the earliest events of immune inflammatory responses: (*i*) increased adhesion of leukocytes to endothelium, and (*ii*) migration of leukocytes across the endothelial cell monolayer. This adhesion pathway does not require chemotactic/haptotactic gradients, or endothelial cell stimulation by inflammatory cytokines, and is ligand specific for fibrinogen since fibronectin was ineffective in mediating leukocyte–endothelium interaction and both fibronectin and vitronectin failed to mediate transendothelial migration.

Table 1. Effect of anti-ICAM-1 mAbs on HL-60 cell adhesion to ICAM-1 transfectants

mAb	% attached cells	% inhibition
None	28.1 ± 1.7	0
6A11	24.5 ± 1.9	13
2D5	38.3 ± 0.3	—
6E6	8.1 ± 5.5	72
60.3	0	100

Aliquots of ^{51}Cr-labeled HL-60 cells were added in the presence or absence of anti-ICAM-1 mAb 2D5 or 6E6, anti-CD18 mAb 60.3, or control mAb 6A11 (25 μg/ml) to confluent monolayers of ICAM-1 transfectants or wild-type CHO cells. Specific HL-60 cell attachment under the various conditions tested was calculated after a 30-min incubation at 22°C by subtracting the background adhesion observed with wild-type CHO cells. Data are means ± SE of three independent experiments.

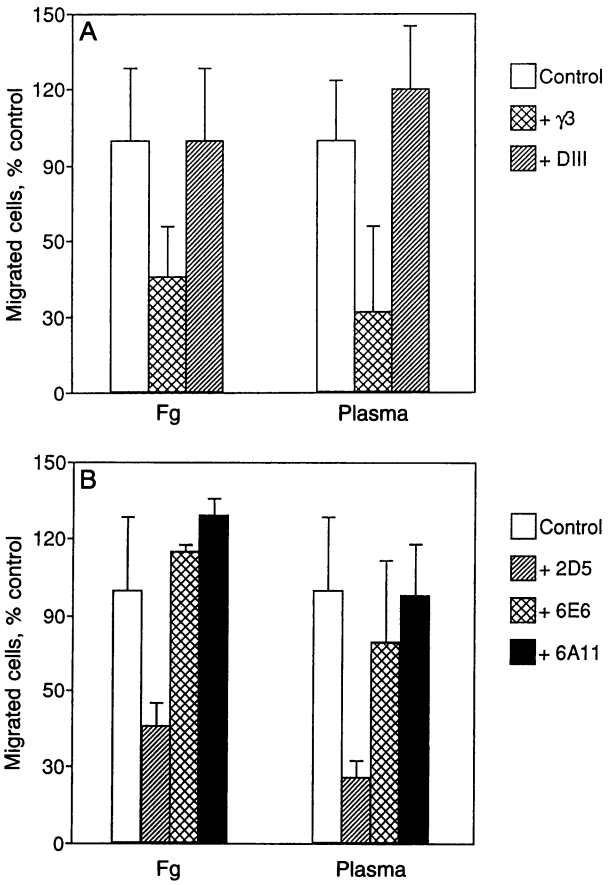

FIG. 4. Effect of antagonists of ICAM-1–fibrinogen interaction on fibrinogen-dependent leukocyte transendothelial migration. (*A*) Serum-free suspensions of vitamin D_3-differentiated HL-60 cells (1×10^5 cells per well) were preincubated with the fibrinogen γ-chain-derived synthetic peptide γ3-(117–133) (400 μg/ml) or control peptide DIII-(162–175) for 10 min at 22°C before addition of fibrinogen (Fg) (300 μg/ml) or plasma (1:5 dilution) and measurement of leukocyte transendothelial migration. (*B*) Experimental conditions are the same as in *A*, except that vitamin D_3-differentiated HL-60 cells were preincubated with anti-ICAM-1 mAb 2D5 or 6E6 or control mAb 6A11 (25 μg/ml) for 15 min at 22°C before addition of fibrinogen (300 μg/ml) or plasma (1:5 dilution) and measurement of leukocyte transendothelial migration. Transmigrated leukocytes in the absence of antagonists (100% of control) were 2715 ± 777 (n = 3) in the presence of fibrinogen and 9037 ± 2190 in the presence of autologous human plasma. Data for both panels are expressed as means ± SD of three independent experiments.

Recent evidence accumulated with systemically defibrinated animals indicates that fibrinogen is absolutely required for inflammatory responses *in vivo* (3–5). The ability of fibrinogen to act as a bridging molecule to enhance leukocyte attachment to endothelium (10, 20), and to direct leukocyte transendothelial migration (this study), might provide a molecular basis for fibrinogen dependency of inflammatory responses *in vivo*. These observations have profound pathogenetic implications in the onset and progression of the atherosclerotic lesion, which is invariably characterized by increased attachment of leukocytes to endothelium followed by intraintimal leukocyte infiltration (21, 22). This possibility is further highlighted by the large deposition of fibrin(ogen) on injured and atherosclerotic endothelium (23) and by the overexpression of ICAM-1 and other leukocyte adhesion-promoting molecules in all types of atherosclerotic lesions (24, 25). Stimulation of ICAM-1 expression by locally generated thrombin (26) or by cytokines released by *in situ* macrophages (27) would further amplify this leukocyte adhesion cascade initiated by fibrinogen at the site of vascular injury.

Structure–Function Relationship of ICAM-1–Fibrinogen Interaction. Based on synthetic peptidyl mimicry of the fibrinogen γ chain, a peptide sequence designated γ3 (N^{117}NQKIVNLKEKVAQLEA^{133}) was identified as responsible for leukocyte–endothelium bridging and transendothelial migration. This structural motif is partially contained within the epitope of the anti-fibrinogen mAb 9F9, which reacts with the conformationally rearranged molecule immobilized on plastic or bound to its platelet receptor (28). Therefore, fibrinogen-mediated leukocyte–endothelium bridging may occur through a sequential docking cascade, in which initial fibrinogen binding to leukocyte CD11b/CD18 (11) may result in spatial conformational changes (28) that render the γ3 sequence more accessible for a secondary interaction with ICAM-1 (10). This model would also be consistent with the ability of this pathway to mediate intercellular bridging in a physiologic plasma milieu (10), containing large concentrations of soluble, competing fibrinogen. The topography of ICAM-1 recognition of fibrinogen was further dissected by generating a panel of anti-ICAM-1 mAbs. Functional characterization of these mAbs has demonstrated that inhibitors of fibrinogen–ICAM-1 interaction—i.e., mAb 2D5—did not affect ICAM-1 association with its counterreceptor CD11a/CD18, as demonstrated by HL-60 cell adhesion to monolayers of ICAM-1 transfectants. Conversely, the anti-ICAM-1 mAb 6E6, which completely inhibited ICAM-1–CD11a/CD18 interaction had no effect on recognition of fibrinogen. Based on these differential and mutually exclusive functional blocking properties, it seems plausible to hypothesize that the fibrinogen binding site on ICAM-1 may be spatially distinct from the complementary CD11a/CD18 recognition site, which also mediates leukocyte adhesion and transendothelial migration (8, 9, 17). This suggests that the fibrinogen-dependent pathway of intercellular bridging may provide an additive and complementary mechanism to the general process of leukocyte–endothelium interaction (9).

In summary, we have delineated a two-step pathway of cell adhesion and leukocyte locomotion mediated by fibrinogen and recapitulated by a physiologic plasma milieu that may contribute to various inflammatory responses *in vivo*. Current experiments performed under flow conditions and analysis of ICAM-1–fibrinogen antagonists—i.e., γ3 and mAb 2D5—in animal models of inflammation should help elucidate the contribution of this pathway to the early molecular mechanisms of vascular injury *in vivo*.

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