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The peripheral lymph node homing receptor, LECAM-1, is involved in CD18-independent adhesion of human neutrophils to endothelium

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The binding of polymorphonuclear granulocytes (PMN) to activated vascular endothelium is a crucial step in the recruitment of PMN to an inflammatory site. Studies employing cytokine-activated endothelium in culture have shown that PMN binding involves the CD18 family of leukocyte integrins, but also CD18-independent adhesion mechanism(s) on PMN that have not been defined. We unify here two previously disparate approaches to study cell adhesion events between endothelial cells and leukocytes. We show that antibodies to human LECAM-1, the peripheral lymph node homing receptor that is also expressed on PMN, partially inhibit the adhesion of human PMN not only to HEV in frozen sections of lymph node tissue, but also to cytokine-activated human umbilical vein endothelium in vitro. Inhibition with anti-LECAM-1 antibodies and anti-CD18 antibodies is additive. Furthermore, the anti-LECAM-1 antibodies inhibit the adhesion of CD18-deficient PMN to cytokine activated human endothelial cells. These findings indicate that LECAM-1 and CD18-mediated binding mechanisms are independent, and act coordinately or sequentially to mediate PMN attachment to cytokine activated endothelium. © 1991 Academic Press, Inc.

Accumulation of PMN at a site of inflammation is thought to require local activation of the vascular endothelium allowing the blood-borne neutrophil to adhere to and migrate through the endothelium into the surrounding tissue. In vitro activation of cultured human umbilical vein endothelial cells with the inflammatory stimuli tumor necrosis factor (TNFα) or Interleukin 1 (IL-1) dramatically increases the binding of unactivated PMN (1,2,3). Early studies demonstrated an essential role for the leukocyte integrins in neutrophil binding to IL-1 or TNF stimulated HUVEC (1,4,5,6). This finding was strongly supported by studies of the clinical syndrome 'leukocyte adhesion deficiency' (LAD)(7), characterized by

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defective neutrophil extravasation in patients with a genetic defect in the common leukocyte integrin β-chain (CD18).

More recently attention has turned to additional adhesion mechanisms, independent of the leukocyte integrins, that participate in neutrophil-endothelial cell interactions (4,5,6,8,9). Two endothelial cell surface molecules involved have been identified. Interestingly these molecules, ELAM-1 and GMP-140, are both members of the LECAM/ selectin family of lectin adhesion molecules (2,10,11).

Neutrophil surface components involved in CD18 - independent adhesion to activated endothelial cells in culture have not been identified previously. However, our earlier studies in the mouse implicated the neutrophil LECAM-1, defined by the monoclonal antibody (mAb) MEL-14 to the lymphocyte peripheral lymph node homing receptor (12) in neutrophil interactions with lymph node high endothelial venules (8). Furthermore, MEL-14 dramatically inhibited the extravasation of neutrophils from the blood into dermal (8) or peritoneal sites (13) of inflammation in the mouse. These results raised the possibility that LECAM-1 is capable of mediating leukocyte interactions not only with HEV in lymph nodes, but also with activated endothelial cells in extralymphoid tissues.

We have recently produced a series of monoclonal antibodies to human LECAM-1 (14,15). In contrast to other reported mAbs to the human peripheral node homing receptor (Leu8, TQ1 (16), anti-LAM-1 (17)) several of our monoclonal antibodies, and in particular DREG 56 and DREG 55, very effectively block lymphocyte binding to HEV in fresh frozen sections of peripheral lymph nodes (14). These antibodies offered us an opportunity to examine the role of neutrophil LECAM-1 in neutrophil binding to cultured endothelial cells in the now standard cytokine-activated HUVEC model.

We show here in three different adhesion assays that anti LECAM-1 antibodies inhibit binding of human neutrophils not only to high endothelial venules, but also to cytokine activated HUVEC.

Materials and Methods

Endothelial cell culture. HUVEC were cultured by standard methods (18,19) in complete RPMI, 20% FCS (cRPMI20) and Endogro (Vectec Inc., NY). HUVEC of passages 2 to four were used in the adhesion assays.

Antibodies and cytokines.

The antibodies used were all of the mouse IgG1-isotype and were partially purified except for TS1/18 which was used as a 1:100 diluted ascites. DREG55, DREG56 and DREG200 monoclonal antibodies have been described (14,15). TS1/18 is an anti-CD18 antibody (20,21). H1-53 is an anti-HCAM mAb (Picker, unpublished). Rb4 is an anti-endothelium control antibody, and PJ32 is a mouse control antibody directed against endothelium and PMN (Hallmann and Picker, unpublished). L3B12 is an anti human leukocyte common antigen (CD45) monoclonal antibody (22).

PMN isolation.

Human peripheral blood was freshly collected into heparin-containing tubes. The blood was diluted 1:2 with Ca^{2+}, Mg^{2+}-free Hanks buffered salt solution (HBSS) and centrifuged at 2500rpm(Sorvall tabletop) through Ficoll-Hypaque 1077 (Sigma) for 30min at RT to separate the peripheral blood mononuclear cells. The pellet, containing erythrocytes and PMN, was diluted with HBSS to the original blood volume and 20% (v/v) of a 6% Dextran T500 solution (Pharmacia) were added. Human PMN were isolated by 1g-sedimentation over 20 min and washed twice in HBSS. Remaining red blood cells were disrupted by hypotonic lysis in water.

Adhesion assays.

Assay I. The adhesion assay of leukocytes binding to frozen tissue sections has been described extensively(8,23). Non-pathological human peripheral lymph node sections (10μm) were freshly cut and incubated with $3x10^6$ PMN for 20min at 7°C on a horizontal shaker (85 RpM). The PMN were incubated for 20min with 100μg/ml monoclonal antibodies in HBSS on ice as indicated in Results. The assays were fixed in 1.5% glutaraldehyde in PBS. Blood vessels on the section were visualized using FITC-labelled antibodies to factorVIIIrAg (Atlantic Antibodies, Charles River). The number of bound PMN per venule was determined and calculated as percent of binding of medium-treated PMN. The data are presented as mean ± SEM.

Assay II. HUVEC of passage no. 2 or 3 were trypsinized and seeded into 8-chamber labtek slides (Labtek) coated with 0.5% gelatin (Sigma). The HUVEC were grown for 24-48h to subconfluency before being treated with 50 U/ml rhuTNFα in cRPMI20 for 4h at 37°C. All consecutive steps were performed in cRPMI without serum. Cytokine-treated HUVEC were washed three times with cRPMI and the slides were transferred to a horizontal shaker (100RpM) at 7°C. PMN ($6x10^6$/ml) were treated for 20min with 20-100μg/ml antibody in HBSS on ice. The sample volume was adjusted with cRPMI and the PMN were immediately added to the endothelium. The PMN, 10^6cells/well in 0.4ml cRPMI, were incubated with the endothelium under shear (100Rpm) for 20min. The disassembled labtek-slides were washed three times in cRPMI before fixation in 1.5% glutaraldehyde in cRPMI. For statistical analysis, adherent PMN in three randomly chosen microscope fields (100X) were counted using an ocular grid, and averaged as one data point. This value was expressed in percent of adhesion of medium-treated PMN. Data, presented as mean ± SEM from 12 assays, are derived from four independent experiments. Significances were calculated using a two-tailed Student's T-test.

Assay III. The assay III has been described in detail (3). Briefly, passage two HUVEC, cultured on gelatin-coated coverslips, were activated with 3U/ml IL-1 (Genzyme; cell-derived) for 4h at 37°C and washed with PBS. The coverslips were inserted into a modified Sykes-Moore chamber and PMN plus the antibodies were added into the chamber. In some experiments the PMN were activated with 0.1μM fMLP (Sigma) for 15min at 37°C prior to injection. The number of bound PMN (contact cells) after incubation for 500s at RT was determined by counting 5-10 microscope fields (400x). The chambers were inverted for another 500s and the percentage of PMN remaining in contact with the HUVEC was determined. The data presented as mean ± SEM were derived from experiments with normal PMN of four healthy donors and with CD18-deficient PMN of three patients with severe LAD deficiency.

Results and Discussion

We studied the effect of the anti-LECAM-1 antibodies DREG55, DREG56 and DREG200 on the adhesion of human neutrophils to endothelium in three different assay systems. The first one, a modification of the ex vivo assay of Stamper and Woodruff (24), examined the binding of peripheral blood PMN to the high endothelial venules in human peripheral lymph nodes at 7°C and under shear (designated here assay I). Under these conditions, binding of leukocytes to venules closely mimicks leukocyte - endothelial cell interactions in vivo(9,25). Fig.1 shows that preincubation of PMN with DREG56 inhibited the adhesion of unactivated human PMN by 55±3%. Control antibodies, H1-53 against H-CAM (CD44) and L3B12 against the lymphocyte common antigen (CD45), had no significant effect. These results indicate that LECAM-1 is involved in human neutrophil binding to the specialized endothelium of HEV, as previously shown in the mouse.

We next wished to define the role of the LECAM-1 in PMN binding to activated endothelial cells. Because the conditions of the ex vivo frozen section assay have allowed demonstration of several leukocyte -endothelial cell interactions (9,25), we first employed

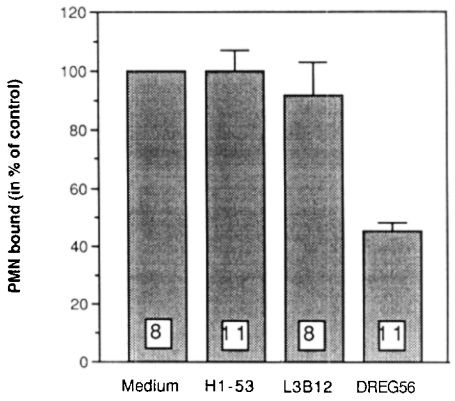

Fig.1. The adhesion of human PMN to the endothelium in frozen human lymph node sections at 7°C under shear is inhibited by DREG56 (assay I).The column inserts give the number of assays performed. Results are given as mean ± SEM. The number of experiments performed is imprinted into the columns.

conditions similar to those of the HEV system -- ie, binding was carried out at 7°C under shear stress (designated assay II). The adhesion of unactivated PMN to unactivated HUVEC was negligible in this assay (Fig.2A), but activation of HUVEC with TNFα dramatically increased the binding (Fig.2B). Adhesion was significantly reduced by preincubation of the PMN with DREG56 (Fig2C), whereas preincubation with the anti-CD45-antibody had no effect (Fig.2D). Preincubation of the unactivated PMN with the different DREG-antibodies inhibited the binding to HUVEC by 50-80% in different experiments (mean values are given in Fig.3). Similar inhibition was obtained when the PMN were precoated with mAbs and washed extensively prior to the assay, but preincubation of HUVEC with the antibodies had no effect on the binding (data not shown). Thus, inhibition is due to an effect on neutrophil surface components. The anti-CD18 antibody TS1/18 inhibited the PMN adhesion by 65±11% (Fig.3). Four isotype-matched control antibodies against abundant leukocyte antigens had no effect on the adhesion (Fig.3).

The adhesion of PMN to activated HUVEC was also studied at RT without shear stress (assay III) as described previously (6)(Fig.4). Under these conditions DREG 56 reduced the adhesion of normal unactivated PMN to IL-1 activated HUVEC by 30±2% ($p < 0.05$ vs control). TS1/18 inhibited the adhesion by 57±4%. The combination of both antibodies resulted in an additive effect, inhibiting PMN binding by 76±2% (Fig.4a), which suggests that the LECAM-1 and CD11/CD18 adhesion mechanisms are distinct.

To explore further the relationship between leukocyte integrin and LECAM-1 mediated adhesion, we studied the binding of PMN from patients with severe Leukocyte Adhesion Defiency (LAD), a genetic deficiency in CD18 expression(26). Preincubation with DREG 56 blocked the adhesion of LAD PMN to IL-1 stimulated HUVEC by 34±4% ($p < 0.001$), whereas the anti-CD45-antibody L3B12 had no inhibitory effect (Fig.4b). Similar results were obtained using TNF-activated HUVEC in assay III. DREG 56 inhibited the adhesion of LAD-PMN in assay II as well (data not shown). We conclude that the LECAM-1-mediated binding of PMN to endothelium is largely CD18-independent.

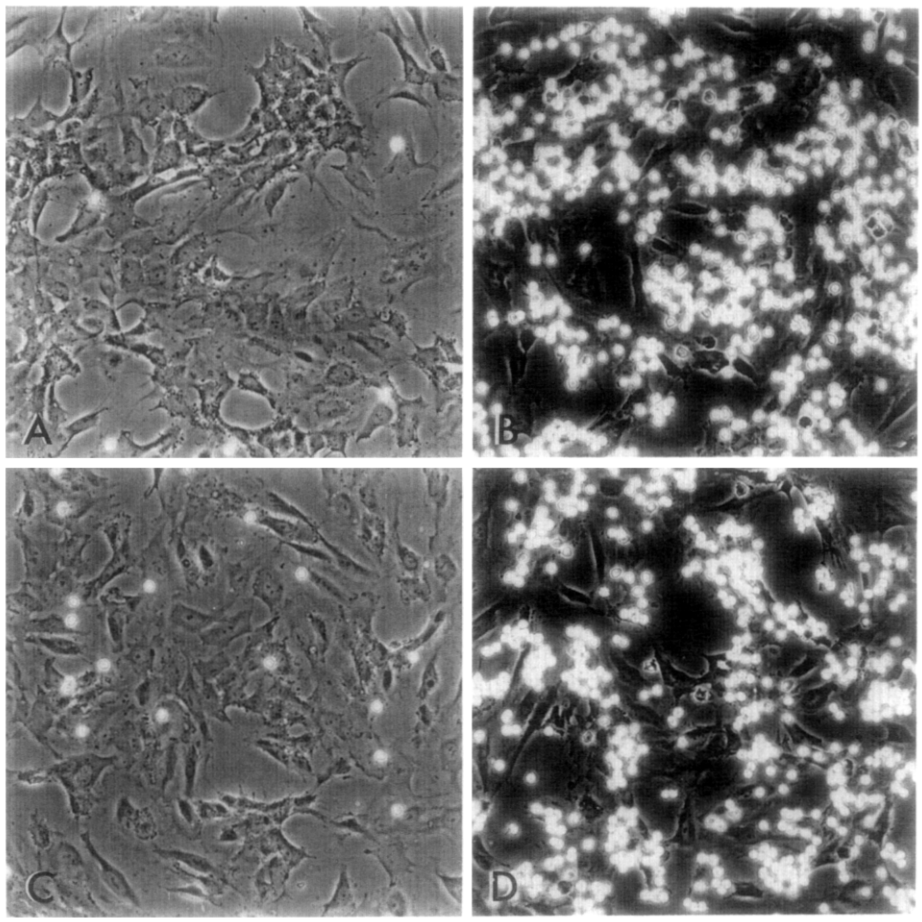

Fig.2. The adhesion of unactivated human PMN to HUVEC at 7°C under shear (assay II) is greatly increased by activation of HUVEC with TNF, and this binding is inhibited by DREG56. Human PMN were incubated with unstimulated (**A**) or TNF-activated (**B-D**) HUVE cells. PMN rarely bound (8 PMN/unit area) to unactivated HUVEC(**A**), while they bound in high numbers (2120 PMN/unit area) to TNF-activated HUVEC (**B**). PMN preferentially adhered to the endothelium and not to the gelatin-coated surface. Binding could be significantly reduced by preincubation of the PMN with DREG56 antibody (**C**). Preincubation of the PMN with the isotype-matched control-antibody L3B12 (anti-CD45) did not affect the binding to the HUVEC (**D**). Bar equals 150μm.

fMLP-activation of PMN results in a rapid release of the DREG antigen from PMN and upregulates expression of the leukocyte integrin CD11b/CD18 within minutes(14). FACS analysis of the fMLP-activated human PMN studied here showed an 80% loss of LECAM-1 on both normal and LAD PMN and a 4 fold increase of CD18 only on normal PMN (data not shown). Interestingly, fMLP activation only slightly effected the binding of normal PMN in assayIII, and this binding was inhibited by TS1/18 by 87±2% (Fig.4a). This is consistent with the finding that activation shifts the adhesion of normal PMN nearly completely to a CD18-dependent mechanism(6). In contrast, fMLP activation of LAD-PMN had a more pronounced effect on their binding, causing a reduction in adhesion of these CD18-deficient PMN by 65±4% (Fig.4b). Neither anti-CD18 antibodies (data not shown) nor DREG56 (Fig.4b) had any additional effect. The residual binding of activated

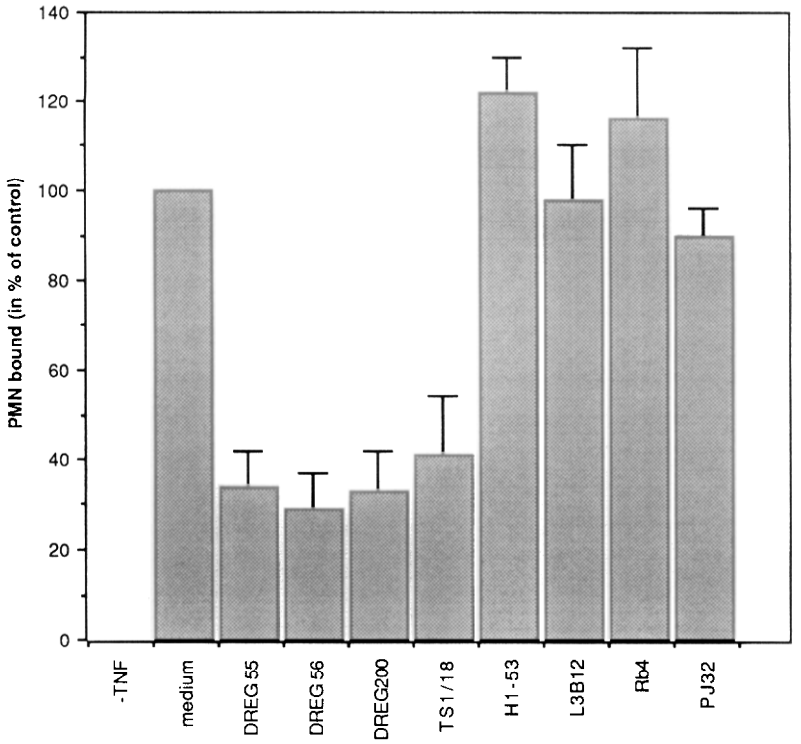

Fig.3. Adhesion of unactivated PMN to TNF-activated HUVEC at 7°C under shear (assay II) is inhibited by DREG antibodies and TS1/18 (antiCD18) , while four isotype matched controls showed no inhibitory effect. Results are mean values ± SEM from 12 assays, derived from four independent experiments.

neutrophils (18 ± 2% of contact cells), even in the presence of DREG-56 and TS1/18, raises the possibility that additional cell adhesion molecules participate in neutrophil binding.

The results presented here are consistent with a important role for LECAM-1 in CD18-independent adhesion, and with the loss of this LECAM-1-based adhesion mechanism in association with shedding of LECAM-1 during neutrophil activation. Recent experiments suggest that the activated endothelium per se could signal the attached PMN (27,28), triggering the shedding of LECAM-1(29) and arresting the PMN through activation of the leukocyte integrins. The leukocyte integrins probably play an important role in subsequent diapedesis as well, since in vitro studies of neutrophil interactions with cytokine-activated endothelium demonstrate the importance of CD18-dependent mechanisms both in adhesion and subsequent transmural migration (6,21).

Furthermore, the findings demonstrate a common molecular basis underlying two previously uncompared models for studying leukocyte - endothelial cell interactions, the Stamper-Woodruff frozen section assay (24) of binding to venules in frozen sections, and the cultured HUVEC model. This common basis strongly supports the physiologic relevance of both systems.

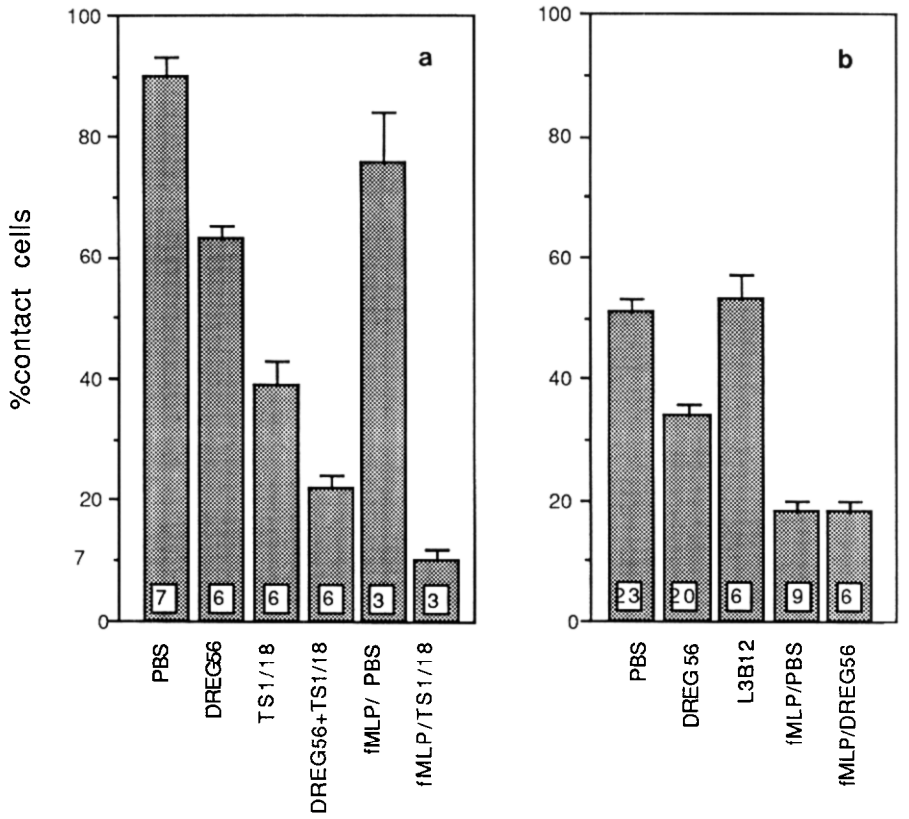

Fig.4. The DREG antibodies inhibit adhesion of normal (a) and LAD (CD18-deficient) (b) PMN to IL-1-activated HUVEC at RT without shear (assay III). Preincubation of LAD-PMN with the control-antibody L3B12 (anti-CD45) had no effect. Activation of LAD-PMN with fMLP resulted in a low level adhesion independent from CD18 and LECAM-1. Results are mean values ± SEM. The number of experiments performed is imprinted into the columns.

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