Characterization of an Adhesion Molecule that Mediates Leukocyte Rolling on 24 h Cytokine- or Lipopolysaccharide-stimulated Bovine Endothelial Cells under Flow Conditions

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Summary

Bovine γ/δ T cells and neutrophils roll on 24 h cytokine- or lipopolysaccharide-stimulated bovine fetal umbilical cord endothelial cells in assays done under physiological flow. An antibody directed against E- and L-selectin has minimal blocking effect on this rolling interaction. mAbs were raised against the stimulated bovine endothelial cells and screened for inhibition of γ/δ T cell rolling. One mAb (GR113) was identified that recognizes an antigen (GR antigen) selectively expressed by stimulated bovine endothelial cells isolated from fetal umbilical cord, mesenteric lymph nodes, and aorta. GR113 blocked bovine γ/δ T cell as well as neutrophil rolling on the 24 h-activated endothelial cells. The GR antigen was constitutively expressed at low levels on the cell surface of platelets and its expression was not upregulated after stimulation of these cells with thrombin or phorbol myristate acetate. However, stimulated platelets released a soluble, functionally active form of the molecule that selectively bound in solution to γ/δ T cells in a mixed lymphocyte preparation. GR113 mAb blocked the binding of the soluble platelet molecule to the γ/δ T cells. Soluble GR antigen also bound a subset of human lymphocytes. Cutaneous lymphocyte-associated antigen (CLA) bright human lymphocytes exhibited the greatest capacity to bind the GR antigen, though CLA was not required for binding. Subsets of both human CD4 and CD8 T cells bound the GR antigen. Immunoprecipitation experiments showed the GR antigen to be 110-120 kD M_r. The binding of soluble GR antigen was inhibited by EDTA and *O*-sialoglycoprotease, but not neuraminidase treatment of the target cells.

Leukocyte recruitment into sites of inflammation involves a multi-step process that leads to the accumulation of cells from the blood into the underlying inflamed tissue (1–5). Endothelial cells lining venules in sites of inflammation respond to inflammatory mediators, which leads to expression of adhesion molecules on their cell surface. Leukocytes within the flow of blood recognize the newly expressed adhesion molecules, bind and begin to roll along the vessel wall. Eventually, many of the rolling leukocytes tightly adhere to the endothelial cell lining, and migrate into the underlying tissue (1–5).

Most leukocyte rolling interactions are mediated by a family of adhesion proteins called selectins and their diverse ligands, though important exceptions exist. To date, three selectins have been defined: E-selectin on inflamed endothelial cells; P-selectin on activated platelets and endothelial cells; and L-selectin on circulating leukocytes (2). Antibodies against each of the selectins can block leukocyte rolling under various conditions in vitro and in vivo (6–19). Other molecules that can mediate rolling include the integrins, α4/β1 and α4/β7 on mononuclear cells, and vascular cell adhesion molecule-1 (VCAM-1)[1] and mucosal addressin cell adhesion molecule-1 (MAdCAM-1) on endothelial cells (15, 19, 20–22). Though antibodies against selectins and their ligands and integrins and their ligands block most rolling interactions that have been defined in vitro, a recent report suggests that 24 h cytokine stimulation of cultured human endothelial cells leads to leukocyte rolling that does not involve any of the known adhesion systems (23). The rolling interaction exhibits the characteristics of a selectin-like event, yet it is distinct from E-, P-, and L-selectin (23).

Recently, we have characterized the interaction of bovine γ/δ T cells with acutely inflamed (<6 h) endothelium in vitro and defined a prominent role for selectins in mediating the rolling of these cells under physiological flow (12, 24, 25). Here, we found that 24-h stimulation (LPS or cy-

[1] *Abbreviations used in this paper:* BUVECs, bovine umbilical vein endothelial cells; CLA, cutaneous lymphocyte-associated antigen; MAdCAM-1, mucosal addressin cell adhesion molecule-1; PSGL-1, P-selectin glycoprotein ligand-1; PVDF, polyvinylidene difluoride; VCAM, vascular cell adhesion molecule-1.

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tokine) of bovine umbilical vein endothelial cells leads to a reproducible rolling interaction of γ/δ T cells under flow conditions, which is not blocked by antibodies against E- or L-selectin. A mAb (GR113) was raised against the activated endothelial cells that blocks the rolling event. GR113 recognizes a 110–120-kD M_r surface antigen on cytokine-stimulated endothelial cells. Platelets release a functionally active form of the molecule upon stimulation, which selectively binds γ/δ T cells in mixed bovine lymphocyte cell preparations. The soluble antigen also binds human leukocytes and optimal binding correlates with high expression of the cutaneous lymphocyte-associated (CLA) E-selectin ligand on human T cells, but CLA is not required for binding. EDTA and *O*-sialoglycoprotease treatments block the binding interaction. The antigen is expressed by postcapillary venules in sites associated with inflammatory leukocytes in a chronically inflamed tissue.

Materials and Methods

Animals. BALB/c mice, ranging in age from 6–12 wk, were used for the generation of mAbs. The mice were housed in the small animal facility at Montana State University (Bozeman, MT). 1-wk–3-mo-old calves, which were housed in the MSU large animal facility, were used as sources of peripheral blood and for inflamed tissues.

Cell Preparations. Leukocytes were harvested from the peripheral blood of cattle and humans, as previously described (6, 11, 24, 25). In brief, blood was collected into citrate, EDTA or heparin anticoagulant tubes, diluted 1:2 with warm HBSS, underlayed with Histopaque 1077 (Sigma Chemical Corp., St. Louis, MO), and centrifuged at 800 *g* for 30 min at room temperature. Mononuclear cells were collected from the Histopaque/plasma interface. Neutrophil preparations were made by hypotonic lysis of RBCs. Monocytes were removed from the mononuclear cell preparation by adhesion to plastic and γ/δ T cells were purified by a 30-min incubation on monolayers of E-selectin cDNA-transfected L-cells. After the incubation period, the γ/δ T cells were removed from the monolayer by incubation in 2 mM EDTA. This procedure yielded a population >90% γ/δ T cells (12, 24, 25, 26). In some experiments human lymphocytes were cultured in Ex Vivo 15 medium (BioWhittaker, Waltersville, MD), plus 5 μg/ml Con A (5×10^7 cells in 15 ml; Sigma) for 6 d. In others, bovine lymphocytes were treated with a 3-d PMA-stimulated lymphocyte supernatant fluid for 2 h at 37°C.

Platelets were isolated from Histopaque-separated leukocytes by three successive low speed spins (100 *g*), which resulted in pelleting of the leukocytes while the platelets remained in suspension. The platelets were resuspended in HBSS and kept on ice until used in the capillary tube shear assay described below or they were used as antigen sources. Bovine umbilical vein endothelial cells (BUVECs) or aorta derived endothelial cells were isolated by collagenase (type 2; Worthington Biochemical Corp., Freehold, NJ) digestion of the lumenal surface of cords or aorta, and cultures were established as described previously (12, 25). Primary endothelial cell cultures were established from stroma of mesenteric lymph nodes as well. In brief, mesenteric lymph nodes were minced with a razor blade. The stromal fragments were extensively washed in HBSS to remove nonadherent lymphocytes, dispersed with collagenase (type A1; Sigma), washed multiple times, and then cultured in endothelial cell medium (EGM; Clonetics, San Diego, CA) to select for endothelial cells. Colonies that grew out from the fragments, which exhibited the distinctive cobblestone morphology of endothelial cells, were physically removed from the cultures and passaged into new flasks. Only endothelial cells from passage five or less were used in functional analyses.

The following antibodies were used in this study: GD3.8, a mouse IgG1 that selectively stains bovine γ/δ T cells (26); HECA 452, a rat IgM that stains CLA on human leukocytes (27, 28); EL-246, a mouse IgG1 that stains bovine L- and E-selectin (29); and rabbit polyclonal anti-sera specific for bovine P-selectin and E-selectin (30–33). Preimmune rabbit serum, irrelevant isotype-matched mAbs, or second stage reagents were used as negative controls.

Bovine thrombin (Sigma), PMA (Sigma), Cell Tak (Collaborative Biomedical Products, Bedford, MA), neuraminidase (Calbiochem Novabiochem Inc., San Diego, CA), LPS (from *Serratia marcescens*; Sigma) and *O*-sialoglycoprotease from *Pastuerella haemolytica* (Pierce Chemicals Inc., Rockford, IL) were used. Pig TNF-α, which crossreacts in cattle, was purchased from Endogen, Inc. (Boston, MA). Mouse IFN-γ was a gift of E. Amento while at Genentech, Inc. (South San Francisco, CA).

Capillary Tube Shear-dependent Rolling Assay. We and others have previously described an approach for measuring the interaction of leukocytes with cells expressing adhesion molecules under controlled shear forces (6, 11, 12, 20, 25, 34). For the functional studies described here, mouse IFN-γ (1×10^4 U/ml for 24 h)/TNF-α (500 ng/ml, 5–6 h)-stimulated BUVECs were grown on the internal surface of sterile glass 1.4-mm capillary tubes (Drummond Scientific, Broomall, Penn) (12, 25). Endothelial cells (1×10^5) were seeded into the tubes and cultured for 24 h, which consistently resulted in a confluent monolayer. Tubing (internal diameter of 1.5 mm) was attached to each end of the capillary tube to form a closed system in which fluid and cells could be recirculated using a variable peristaltic pump (Cole Parmer, Barrington, IL) containing 6 rollers to reduce pulsation. The capillary tube was mounted on an inverted microscope (Nikon Diaphot) modified for video microscopy (Sony CCD Camera). Purified γ/δ T cells or neutrophils were injected into the system at 4×10^6 cells/ml in DMEM plus 20 mM Hepes. A reproducible rolling interaction on the cytokine-activated endothelial cells was detected under a shear force of ~2 dynes/cm^2 (20). The number of rolling γ/δ T cells on cytokine-stimulated endothelial cells, and in the assays below, at 60-s intervals was determined by analysis of individual frames of the videotape recording using macro-driven NIH Image software and a Macintosh 660 AV computer (Apple, Cupertino, CA). Data were recorded as the number of rolling cells within the field of view versus time. Aggregates were excluded from the analyses.

For P-selectin assays, platelets isolated from bovine plasma were activated with 1 μg/ml bovine thrombin and coated at 1.5×10^9 cells/ml onto the internal surface of Cell Tak-coated capillary tubes, and allowed to attach for 15–30 min. Shear assays were then performed, as previously described (12). In brief, bovine γ/δ T cells were injected at 4×10^6 cells/ml in DMEM plus 20 mM Hepes. The interaction of the leukocytes with the bound platelets was monitored by video microscopy at 2 dynes/cm^2, as described above.

Generation of mAbs that Selectively Stain Cytokine-stimulated Endothelial Cells. Bovine endothelial cells stimulated for 24 h with IFN-γ (1×10^4/ml) followed by TNF-α (500 ng/ml) or LPS (1 μg/ml) for 4-6 h, which avidly supported γ/δ T cell rolling under flow conditions, were used to immunize mice (5×10^7-1×10^8 cells) until significant titers (>1:1,000) of serum antibodies

were generated that selectively stained the cytokine-stimulated endothelial cells and blocked the rolling of the γ/δ T cells. Fusions were done, as previously described (26, 29, 35), and the supernatant fluids from the hybridoma cells were screened on control versus cytokine-activated endothelial cells using an ELISA-based screening assay. In brief, endothelial cells were grown in the wells of a 96-well plate and some were stimulated with IFN-γ for 24 h followed by a 5-h treatment with TNF-α or LPS, as described above, whereas others were left untreated. Hybridoma supernatant fluids were added to PBS washed wells and allowed to incubate at 37°C for 30 min. The wells were washed in PBS and then treated with 1% paraformaldehyde for 30 min at room temperature. The plates were washed again in PBS and second stage anti–mouse Ig coupled to biotin (Biosource, Inc., Camarillo, CA) was added and incubated for 30 min at room temperature. The plates were washed and streptavidin-peroxidase (Biosource, Inc.) was added, incubated for 20 min at room temperature, and developed by the addition of AEC substrate (Sigma). Hybridomas that produced antibodies, which selectively stained the cytokine-stimulated endothelial cells, were expanded and subcloned by limiting dilution. One mAb, GR113 (mouse IgG1), was characterized in this report.

Soluble Platelet Antigen Binding Assay. Isolated platelets from 150 ml of blood were resuspended in 10 ml of RPMI and stimulated with thrombin (1 μg/ml) or PMA (100 ng/ml) for 2 h at 37°C. The platelets were removed by high-speed centrifugation (800 *g*) and the clarified supernatant fluid collected. Bovine or human leukocytes were incubated with the platelet supernatant fluid with or without various activating agents at 37°C for 1.5 h at 37°C. After the incubation period, the treated leukocytes were washed and then stained for flow cytometric analysis to detect bound GR antigen and to determine the phenotype of the binding population. For two-color analysis after the binding assay, cells were first stained with GR113 followed by anti-mouse Ig-PE in one color. The cells were then washed, incubated in 10% mouse serum to block free sites on the second stage, and then stained with FITC-labeled HECA452 (anti-CLA), anti-CD4 (Becton Dickinson, San Jose, CA), or anti-CD45RO (Becton Dickinson) for human cells, or FITC-labeled GD3.8 (anti-bovine γ/δ T cells) for bovine cells. Specificity controls included treating the platelet supernatant fluid with blocking GR113 and nonblocking isotype matched mAbs and EDTA. Neuraminidase and *O*-sialoglycoprotease treatments of the leukocytes before the binding assays were also done. EDTA was used at a 2 mM concentration. Neuraminidase (0.1 μg/ml) and *O*-sialoglycoprotease (50 μg/ml) were used to treat the leukocyte preparations for 30–45 min at 37°C or room temperature before the binding assay, as described (23–25). Relevant buffers were used as controls for the treatments. The cells were analyzed on a Becton Dickinson FACScan® or FACSCalibur® (San Jose, CA).

SDS-PAGE/Western Blot and Immunoprecipitation Procedures. Isolated platelets were lysed in a 1% Triton X-100 detergent buffer containing 0.005% SDS, 1 mM EDTA, PBS and protease inhibitors at 4°C for 60 min. Cell debris was removed by centrifugation (microfuge) and then the lysate was run on an 8% PAGE gel under reducing or nonreducing conditions. In some experiments, GR113 mAb was covalently attached to cyanogen bromide-activated Sepharose beads, per manufacturer's recommendations (Pharmacia LKB Inc., Piscataway, NJ), and used to affinity purify the GR113 antigen. The purified antigen was then run on an 8% SDS-PAGE gel. The separated platelet proteins were transferred to nitrocellulose overnight and then the nitrocellulose blot was blocked with horse serum for 30 min. The nitrocellulose was then blotted with GR113, a polyclonal anti-serum against bovine P-selectin, or various negative control antibodies using a 24-well miniblotter apparatus (Immunetics Inc., Cambridge, MA). After a 1-h incubation at room temperature, the blots were washed and then second-stage alkaline phosphatase-coupled reagents specific for either mouse Ig (Sigma) or rabbit Ig (Sigma) were added and allowed to incubate with the blots for 1 h at room temperature. The blots were washed extensively and then developed by the addition of nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma) in AP buffer (100 mM Tris, pH 9.5, 100 mM NaCl, and 5 mM $MgCl_2$).

For the immunoprecipitation procedures, endothelial cells were grown in T175 flasks and activated for 6 h with 1 ng/ml LPS to get both GR antigen and E-selectin expressed. The endothelial cell monolayers were surface labeled with biotin (10 ml of 0.55 mg/ml immuno-pure NHS-LC-biotin sulfosuccinimidyl 6-biotinamido heranoate [Pierce] in PBS) for 30 min at room temperature. The monolayers were washed, physically removed from the flasks using a cell scrapper, and washed three additional times in PBS to remove all residual biotin. The endothelial cells were lysed in the Triton X-100 lysis buffer, as described above. The biotin-labeled lysate was divided into two fractions and precleared with either 5% rabbit serum or 5% mouse serum followed by protein–A agarose (Sigma) or protein–G agarose (Boehringer Mannheim, Indianapolis, IN), respectively, for 4–5 h at 4°C. The protein A and protein G beads were removed by centrifugation and the procedure repeated a second time. The precleared lysates were then immunoprecipitated with GR113 (lysate precleared with mouse serum) or rabbit polyclonal antiserum against either E-or P-selectin (lysate precleared with rabbit serum), as previously described (26). The immunoprecipitated samples were resuspended in nonreducing SDS-sample buffer and run on an 8% PAGE-gel. After the electrophoresis step, the gels were transferred to polyvinylidene difluoride (PVDF) membrane overnight and then the PVDF membrane was blocked in a 7% BSA NaCl buffer for 2 h at room temperature. The blot was washed and then incubated for 30 min with streptavidin-horseradish peroxidase conjugate (Amersham, Inc., Arlington Heights, IL) diluted 1:5,000 in PBS, followed by development with ECL chemiluminescent detection reagents from Amersham.

Chronic Inflammation Lesions. To induce a chronic inflammatory lesion, plastic golf ball–sized wiffle balls were inserted under the skin of young calves. The balls were allowed to be encapsulated and then were injected with 1 ml of a 1:20 dilution of a saturated potassium permanganate preparation. The resulting inflammatory response was allowed to continue until the tissue within the ball became solid, as detected by X-irradiation and autoradiography. The wiffle ball was removed and the inflammatory tissue cut and frozen in O.C.T. freezing medium (Tissue-Tek, Inc., Elkhart, IN). To induce acute inflammatory lesions, LPS was injected into the dermis and tissue samples were collected 4–6 h later. For immunohistology, frozen sections were allowed to air dry, fixed in acetone, and then stained for immunohistology, as previously described (24).

Results

GR113 Selectively Stains Cytokine- or LPS-stimulated Endothelial Cells and Blocks Their Capacity to Support γ/δ T Cell and Neutrophil Rolling under Physiological Flow. Endothelial cells stimulated for 24 h with IFN-γ followed by LPS or TNF-α for 4–6 h avidly supported γ/δ T cell rolling under

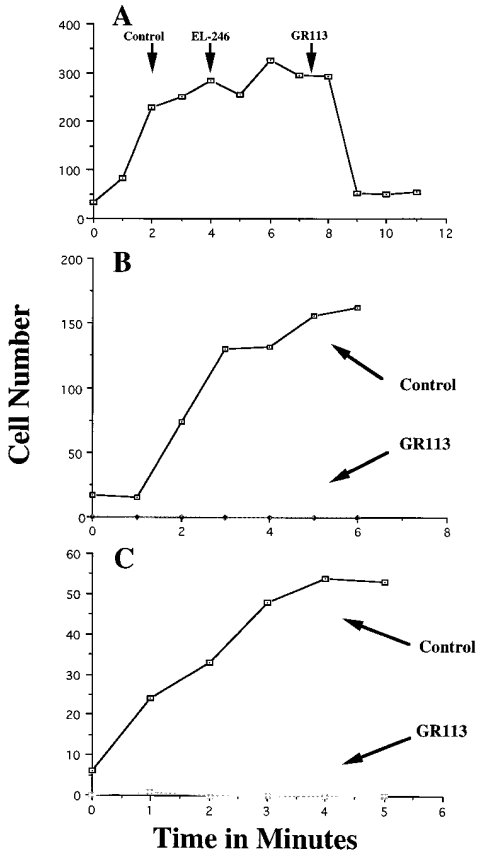

Figure 1. GR113 blocks γ/δ T cell and neutrophil rolling on 24 h cytokine-activated endothelial cells. Endothelial cells were grown on the internal surface of glass capillary tubes, activated with IFN-γ/TNF-α, and integrated into the flow assay, as described in Materials and Methods. In *A*, γ/δ T cell rolling was established first and then an isotype-matched negative control mAb (*control*), EL-246 (anti–L- and E-selectin), and GR113 were sequentially added. In *B*, the endothelial cell monolayer was either pretreated with GR113 or a control mAb for 30 min before the addition of the γ/δ T cells. In *C*, the rolling of neutrophils on endothelial cells pretreated with GR113 was compared with antibody control. These experiments are representative of >3 other experiments showing inhibition of leukocyte rolling on the 24 h cytokine- or LPS-activated endothelial cells.

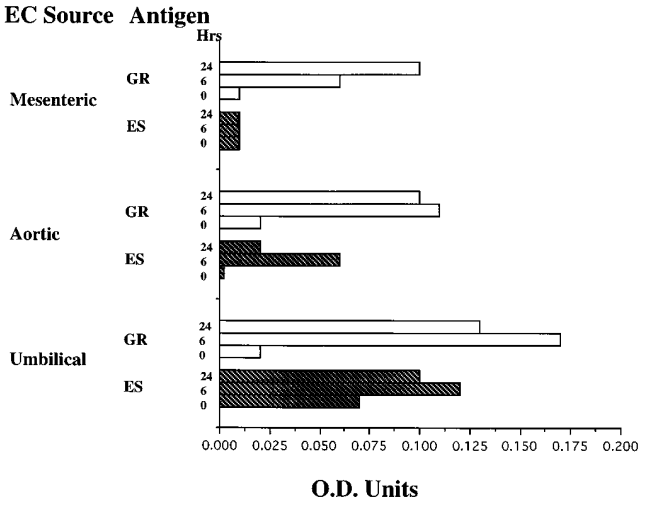

Figure 2. GR113 stains 6 h and 24 h LPS-stimulated endothelial cells isolated from mesenteric lymph nodes, aorta, and umbilical cords. Endothelial cell cultures in 96 well plates were stimulated with LPS for 6 and 24 h and then stained with GR113 (denoted *GR antigen*) or EL-246 (anti–E-selectin, denoted *ES antigen*), as described in Materials and Methods. The data represent a single comparison and are the average of duplicate measurements. OD values reflect OD of positive staining mAb minus OD of negative control antibody staining. The staining of the umbilical cord endothelial cells which were used in all subsequent assays was repeated in >3 different experiments.

flow conditions; however, the rolling interaction was not blocked by an antibody directed against L- and E-selectin (EL-246) (Fig. 1 *A*). Similar rolling interactions occurred on endothelial cells treated with LPS or TNF-α alone for 24 h, showing that the IFN-γ pretreatment was not prerequisite, but the consistency of the induction of the capacity support rolling was enhanced by IFN-γ (data not shown).

mAbs were raised against the 24 h cytokine-stimulated endothelial cells, as described in Materials and Methods. One mAb, GR113, was identified that selectively stained endothelial cells treated for 24 h. Untreated umbilical cord endothelial cells expressed little if any GR antigen on their cell surface or in intracellular pools (data not shown, also see Fig. 2). In one experiment, the expression of the GR antigen was compared with E-selectin after 6 and 24 h LPS treatment of endothelial cells isolated from mesenteric lymph nodes, aorta, and fetal umbilical cords. As shown in Fig. 2, GR113 selectively stained LPS-stimulated endothelial cells isolated from all three sources. 6 h stimulation with LPS (or TNF-α, data not shown) alone led to increased staining of endothelial cells in all three cases and, for the most part, maximal or close to maximal expression was maintained after 24 h (Fig. 2). In contrast, induction of E-selectin expression was highly variable, with endothelial cells from umbilical cords expressing significant antigen before stimulation; whereas little E-selectin was expressed on endothelial cells from mesenteric lymph nodes after either 6 or 24 h incubation in the presence of LPS (Fig. 2). The reason for the variability in E-selectin expression or the consistency of this variability was not determined, but it could have been related to the proliferative state of the cells (33). Flow cytometric analysis showed that virtually all 24 h stimulated umbilical cord endothelial cells expressed the GR antigen, though there was heterogeneity in the level of expression by different cells in the culture (Fig. 3). Overall, we have shown that TNF-α, LPS, or a combination of IFN-γ plus LPS or TNF-α, but not IFN-γ alone, induced significant levels of the GR antigen on bovine endothelial cells (data not shown). Since much of our initial analyses were on umbilical cord endothelial cells pretreated with IFN-γ for 24 h followed by LPS or TNF-α treatment, this treatment regime was used in the functional analyses reported below.

GR113 was tested in the capillary tube shear assay for its ability to block γ/δ T cell rolling on the 24 h stimulated umbilical cord endothelial cells. In the first series of experiments, rolling interactions were established, a negative control an-

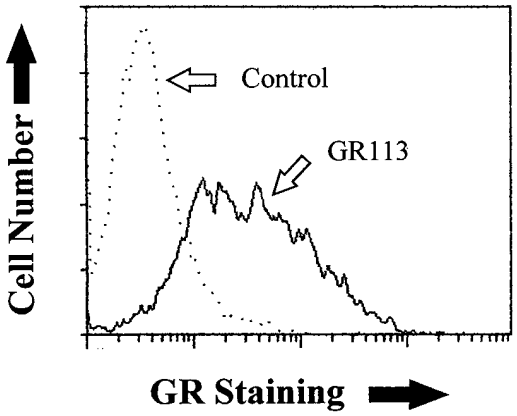

Figure 3. GR113 stains 24 hour LPS-stimulated umbilical cord endothelial cells as measured by flow cytometric analysis. Stimulated endothelial cells were lifted by incubation in EDTA and physical scrapping of the monolayers, dissociated, washed, stained with GR113 and analyzed by flow cytometry. Solid line reflects staining with GR113 and the dotted line represents background staining with a negative control. Expression is in arbitrary fluorescence units on a log scale.

tibody injected, which was then followed sequentially by EL-246 (anti–L- and E-selectin), which had no effect, and GR113, which almost completely blocked the rolling interaction within 1 min (Fig. 1 *A*). As shown in Fig. 1 *B*, pretreatment of endothelial cells completely prevented their ability to support γ/δ T cell rolling. As seen in the studies using human endothelial cells (23), neutrophils rolled on the 24 h stimulated endothelial cells, though not nearly as well as the lymphocytes (compare the number of rolling cells). Pretreatment of the endothelial cells with GR113 completely blocked the neutrophil rolling interaction (Fig. 1 *C*).

Distribution of the GR Antigen. Since GR113 selectively stained cytokine-stimulated endothelial cells in vitro, the staining pattern in chronic inflammatory lesions in vivo was tested. As shown in Fig. 4 *A*, GR113 intensely stained vessels associated with leukocytes in a chronic inflammatory lesion induced by potassium permanganate. Upon closer examination of the GR113 positive vessels it was noted that not only did the antibody stain endothelial cells, scattered granular platelet-like particles (also see below) were also stained (Fig. 4 *B*). Note in Fig. 4 *B* that leukocytes were adhered to the lumenal surface of the large GR113 positive vessel. We detected variable, low level of staining of GR113 on vessels in uninflamed skin. 4 h LPS-inflamed skin exhibited positive vessels, but much of the staining was associated with platelet-like particles. In contrast, E-selectin was expressed on many vessels in the 4 h inflamed tissues, whereas minimal expression was seen in the chronic lesion (data not shown).

Flow cytometric analysis was done to determine if GR113 reacted with any blood cells, particularly platelets. As shown in Fig. 5 *A*, the antibody weakly stained platelets by flow cytometric analysis. In some experiments, reactivity of GR113 on platelets was barely above background (data not shown). As shown in Fig. 5 *A*, thrombin activation did not lead to increased GR113 staining. The thrombin-activated platelets supported avid rolling of bovine γ/δ T cells in the capillary tube flow assay, suggesting that they did express P-selectin; however, GR113 did not block the interaction (Fig. 5 *B*), as seen with γ/δ T cell rolling on 24 h cytokine-stimulated endothelial cells (Fig. 1).

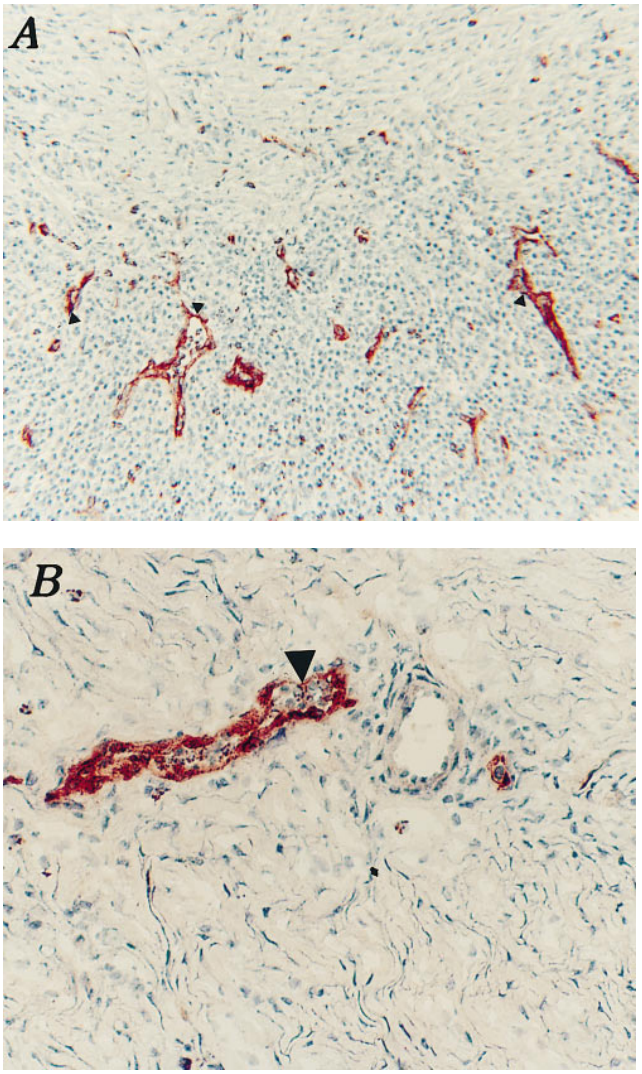

Figure 4. GR113 stains venules in a site of chronic inflammation. A chronic inflammatory lesion was induced by potassium permanganate, as described in Materials and Methods, which was characterized by an infiltration of mononuclear leukocytes with many of the inflammatory cells being lymphocytes (data not shown). Frozen sections were stained with GR113 by standard immunoperoxidase techniques. *A* shows a low power (125× final magnification) field demonstrating many GR113 positive vessels in the site of leukocyte infiltration (*arrowheads*). *B* shows a high power field (250× final magnification) of the same tissue. The arrow head points to a GR113 positive vessel containing leukocytes bound to its lumenal surface.

GR Antigen Is Released in a Functional Form by Activated Platelets. A series of activation experiments were initially done to determine if the GR antigen could be induced on the surface of blood leukocytes. Treatment of a mixed population of mononuclear cells, containing lymphocytes, monocytes, and platelets, with PMA or an activated lymphocyte

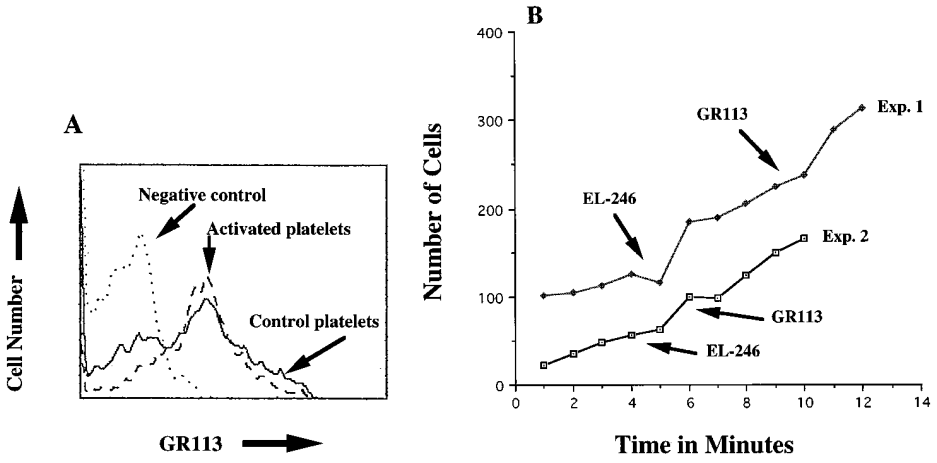

Figure 5. Staining of GR113 on platelets and its effect on leukocyte rolling on thrombin-activated platelets. Platelets, isolated as described in Materials and Methods, were stained with the GR113 mAb and analyzed by flow cytometry or they were used in rolling assays. *A* shows GR113 staining of control and thrombin-activated (15 min) platelets versus background staining (second stage alone). *B* shows the effects of GR113 on the rolling of bovine γ/δ T cells on immobilized, thrombin-activated platelets. Two different experiments are shown in which rolling interactions were established first, and negative control (EL-246) and GR113 mAbs sequentially injected.

supernatant fluid (3 d PMA-treated lymphocyte supernatant fluid shown in another study to be a potent activator of bovine lymphocytes) led to reproducible staining of a large percentage of γ/δ T cells with GR113 within two h (Fig. 6, *A* and *B*). However, if purified γ/δ T cells (purified by panning on E-selectin transfectants, as described in Materials and Methods, and lacking most other leukocytes and platelets) were directly stimulated, they did not express the GR antigen (Fig. 6, *C* and *D*).

Subsequently, it was found that platelets released the antigen upon PMA or thrombin stimulation (data not shown). To determine if the leukocyte staining shown in Fig. 6 *B*, could be due to released GR antigen from contaminating platelets, supernatant fluid from PMA or thrombin-stimulated platelets (see Materials and Methods) was collected (both worked equally well) and used to treat purified bovine γ/δ T cells. After 1.5 h, the treated cells were washed and stained with GR113 for flow cytometric analyses. As shown in Fig. 6 (*E* and *F*), GR113 specifically stained the supernatant fluid-treated γ/δ T cells. In mixed lymphocyte preparations, γ/δ T cells were the only cell that bound the soluble GR antigen (data not shown).

To determine the specificity of the GR antigen binding to γ/δ T cells in suspension, the platelet supernatant fluid was pretreated with the GR113 antibody before the binding assay. As shown in Fig. 7 (*A* and *B*), GR113 pretreatment prevented binding of the GR antigen to the γ/δ T cells. Importantly, antibodies directed against L-selectin or other γ/δ T cell surface antigens had no effect on the binding of the GR antigen (Fig. 7 and data not shown). Since the blocking GR113 mAb could detect bound antigen on the surface of the γ/δ T cells, this suggested that the soluble molecule was likely aggregated, creating multiple binding sites, some of which were unoccupied when bound to the leukocyte. Binding of soluble GR antigen to leukocytes was inhibited by EDTA (2 mM) and O-sialoglycoprotease (50 μg/ml), but not neuraminidase (0.1 U/ml) treatment of the γ/δ T cells (Fig. 7). EDTA and *O*-sialoglycoprotease treatment of γ/δ T cells also blocked their capacity to roll on the 24 h stimulated endothelial cells (data not shown).

Soluble GR Antigen Specifically Binds a Subpopulation of Human Lymphocytes. The GR113 antibody did not cross-react with human cells (data not shown). To determine if human leukocytes express receptors for the GR antigen, the binding of soluble bovine GR antigen to human mononuclear cells was tested. GR antigen containing supernatant fluid from bovine platelets was used to treat human mononuclear cells. The cells were washed and then analyzed by

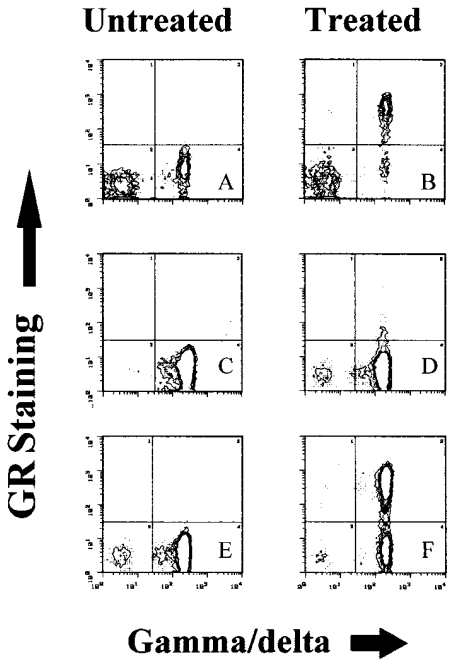

Figure 6. Staining of blood leukocytes with GR113. Two-color flow cytometric analysis was done using GR113 (GR staining) versus a γ/δ T cell specific mAb (GD3.8) on bovine lymphocytes treated by different protocols. *A* and *B* shows GR113 staining of bovine peripheral blood mononuclear cells before (*A*) and after (*B*) treatment (1.5 h) with a PMA-induced lymphocyte supernatant fluid, which we had previously shown in other studies to be a potent activator of bovine lymphocytes. *C* and *D* show GR113 staining of purified γ/δ T cells before (*C*) and after (*D*) treatment with the PMA-induced lymphocyte supernatant fluid. *E* and *F* show GR113 staining of purified γ/δ T cells before and after treatment with a clarified supernatant fluid from thrombin-activated platelets, as described in Materials and Methods. The quadrants reflect the upper end of background staining seen with negative controls. Both the y and x axis are in log units. The results are representative of at least three other experiments with similar results.

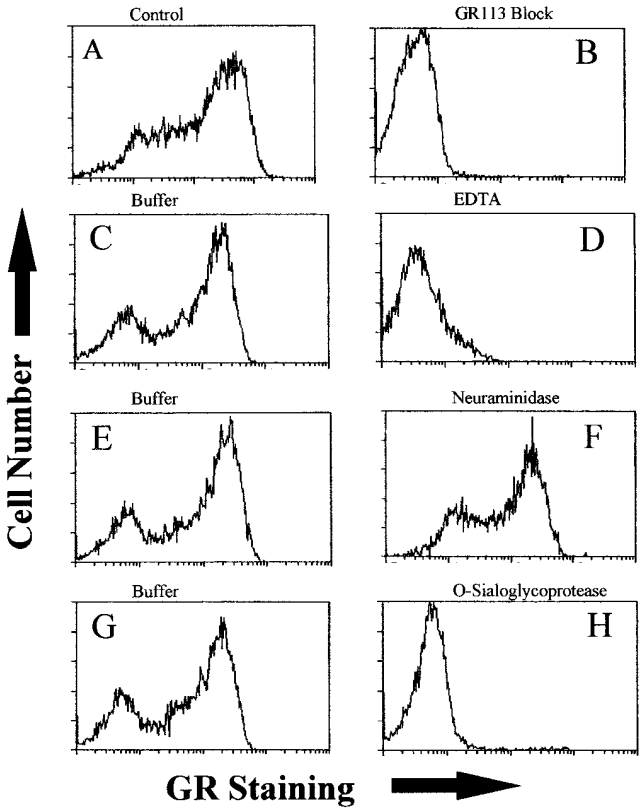

Figure 7. Effect of different inhibitors on GR staining of γ/δ T cells treated with platelet supernatant fluid. Bovine lymphocytes were treated with neuraminidase (0.1 μg/ml for 30 min at room temperature) or *O*-sialoglycoprotease (50 μg/ml for 45 min at room temperature) before the addition of supernatant fluid from thrombin-activated platelets, or EDTA (2 mM), GR113 antibody, or negative control antibody was added to the platelet supernatant fluid before addition to the bovine lymphocytes. After the treatments, the lymphocytes were washed, stained with GR antibody, and flow cytometric analysis performed. The data are presented as histograms. Expression is shown in arbitrary fluorescence units on a log scale. *A* and *B* show the effects of an antibody negative control and GR113, respectively. *C* and *D* show the effects of the buffer control and EDTA, respectively. *E* and *F* show the effects of the buffer control and neuraminidase, respectively. *G* and *H* show the effects of the buffer control and *O*-sialoglycoprotease, respectively. These experiments are representative of at least two others showing the same result.

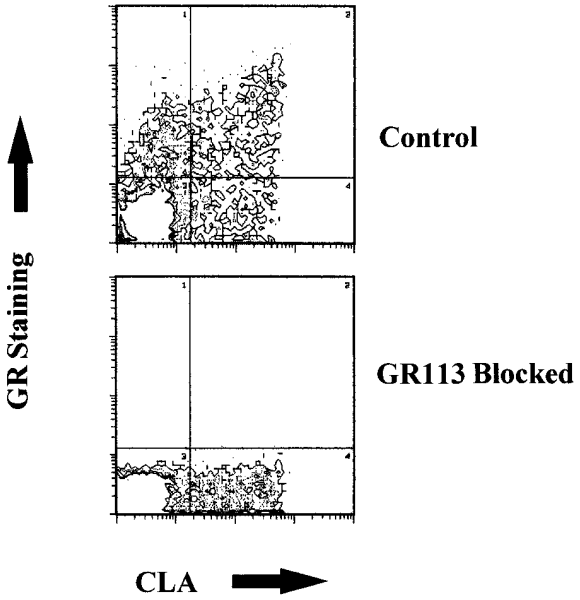

Figure 8. GR113 stains HECA 452 bright human lymphocytes. Human lymphocytes were treated with supernatant fluid from thrombin-activated bovine platelets for 1.5 h, as described in Materials and Methods, washed, and then analyzed by two-color flow cytometry using the GR113 and HECA 452 mAb. *A* and *B* show the staining of the platelet supernatant fluid treated cells in the absence or in the presence of the blocking GR antibody, respectively. The quadrants reflect the upper level of staining with a negative control antibody. These flow cytometric plots are representative of at least three different experiments showing the same result.

two-color analysis using GR113 and HECA 452 to stain the E-selectin binding lymphocyte population (CLA) (27), as a comparison. These analyses showed that most HECA 452 bright cells bound the GR antigen (GR antigen also bound some HECA 452-negative cells, Fig. 8). Importantly, the binding of the soluble GR antigen to human lymphocytes was completely blocked by pretreatment of the platelet supernatant fluid with GR113 (Fig. 8). As with bovine cells, neuraminidase treatment had no effect on GR antigen binding, though HECA 452 reactivity was greatly reduced (data not shown). Human monocytes and neutrophils also bound the GR antigen and rolled on 24 h activated bovine endothelial cells, which was blocked by the GR113 mAb (data not shown).

The percentage of human lymphocytes that bound the GR antigen varied considerably from donor to donor, making extensive phenotypic analysis of the cells difficult. Therefore, the binding of GR antigen to cultured human lymphocytes expanded in the presence of Con A was tested. As shown in Fig. 9, after 6 d culture in Ex Vivo 15 medium plus 5 μg/ml Con A, almost all of the lymphocytes exhibited a memory phenotype as defined by expression of the CD45RO isoform. GR antigen bound a large percentage, but clearly not all, of the Con A–treated, CD45RO-positive lymphocytes and, again, optimal GR antigen binding correlated with CLA expression (Fig. 9). Anti-CD4 staining was also done in this experiment, which showed that a signficant percentage of CD4-positive T cells bound the GR antigen (Fig. 9). Indeed, in this experiment, most of the cells binding the greatest amount of GR antigen were CD4 positive. GR antigen binding was also seen on CD8 positive T cells in other experiments (data not shown).

Comparison of the GR Antigen with P- and E-Selectin by SDS-PAGE/Western Blot and Immunoprecipitation. Though the distribution of the GR antigen is not consistent with either P- (surface of activated platelets) or E-selectin (cytokine-stimulated and/or proliferating endothelium only), most of our understanding of the selectins have come from work with human cells and it was possible that bovine P- or E-selectin were simply different and that the GR antigen represented one or the other selectin. In Western blot analysis of bovine platelet antigens, GR113 recognized a 110–120-kD glycoprotein under nonreducing conditions (Fig. 10 *A*, lane *1*). In contrast, a polyclonal anti-bovine P-selectin an-

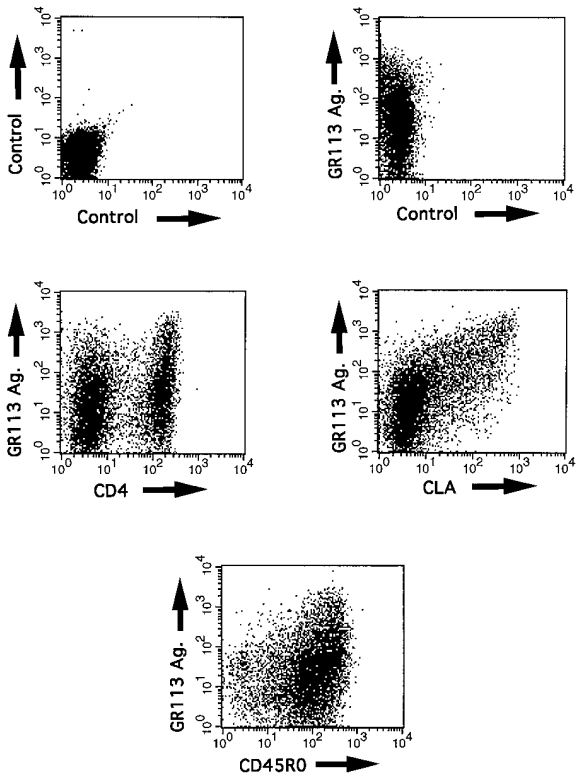

Figure 9. GR113 antigen binds to a subset of Con A expanded human T cells expressing CD45RO, CD4, and CLA. Human lymphocytes were cultured in Ex Vivo 15 medium, plus 5 μg/ml Con A for 6 d and then a soluble GR113 antigen binding assay was done as described for Fig. 8. Two-color analyses was done to compare the GR113 antigen binding population with cells expressing CD4 and CD45RO, in addition to CLA, as described in Materials and Methods. Controls represent background due to second-stage alone and autofluorescence of the cell population.

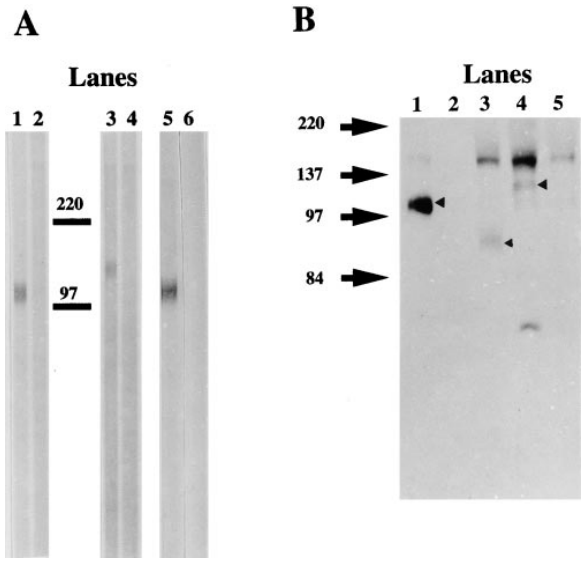

Figure 10. SDS-PAGE/Western blot and immunoprecipitation analysis of the platelet and endothelial cell GR antigen. In one experiment, bovine platelets were lysed in detergent lysis buffer, run on an 8% PAGE gel, transferred to nitrocellulose, and blotted with GR113 and a polyclonal antiserum against bovine P-selectin. *A* shows the results. GR113 and negative control antibody are represented in lanes *1* and *2*, which were run under nonreducing conditions. Lanes *3* and *4* represent polyclonal antiserum against bovine P-selectin and preimmune control, respectively, on samples run under reducing conditions. In another experiment, affinity purified GR antigen was analyzed. Lanes *5* and *6* represent affinity-purified GR antigen run under nonreducing (lane *5*) and reducing (lane *6*) conditions. GR113 was used to probe the nonreduced sample, whereas polyclonal anti–P-selectin was used to probe the reduced sample, as done in lane *3*. The results of the immunoprecipitation analysis of endothelial cells are shown in *B*. Biotin-labeled endothelial cells were lysed in detergent and antigens precipitated with polyclonal antiserum against either bovine E-selectin or P-selectin, or with GR113. GR113, negative control, polyclonal antiserum against E-selectin, polyclonal antiserum against P-selectin, and preimmune rabbit sera are shown in lanes *1–5*, respectively. Arrow heads point to specific bands. The GR113 band has been repeated >3 times and a similar size E-selectin band has been repeated with a mAb against E-selectin. The P-selectin–specific band in the endothelial cell analysis has not been clearly seen in five other experiments.

tiserum recognized a band at 130 kD under reducing conditions (Fig. 10 *A*, lane *3*), exactly as previously reported (30). The polyclonal anti–P-selectin antibody did not work as well on nonreduced samples. In most instances the band was very faint on the nonreduced gel (data not shown). The latter observation may be related to the fact that the polyclonal antiserum was raised against recombinant bovine P-selectin expressed in *E. coli* and not the native protein (30). GR113 did not react on reduced samples (data not shown). To determine if the anti–P-selectin polyclonal antibody reacted with reduced GR antigen, the GR antigen was affinity purified as described (26), run on a SDS-PAGE gel under nonreducing and reducing conditions, and probed with GR113 or the anti–P-selectin antiserum, respectively. As shown in Fig. 10 *A*, lanes *5* and *6*, the anti–P-selectin antiserum did not react with reduced, purified GR antigen.

We were particularly interested in determining the nature of the GR antigen on the surface of activated endothelial cells. To enhance getting E-selectin and the GR antigen expressed on the cell surface at the same time for a comparison, 6 h LPS-stimulated endothelial cells were surface labeled with biotin. The labeled endothelial cells were lysed in detergent and precipitated with polyclonal antiserum against either bovine E- or P-selectin, or with GR113. A variable background band of 150 kD was detected with specific antibody as well as preimmune serum controls (Fig. 10 *B*). GR113 specifically immunoprecipitated a surface molecule from the endothelial cells of 110-120 kD (Fig. 10 *B*, lane *1*, *arrowhead*). The E-selectin polyclonal antiserum weakly immunoprecipitated a 90-kD glycoprotein (Fig. 10 *B*, lane *3*, *arrowhead*), which was also seen in other experiments using EL-246 (data not shown). In the experiment shown in Fig. 10 *B*, the polyclonal anti–P-selectin antiserum immunoprecipitated a 130-kD molecule from the endothelial cell lysates (lane *4*, *arrowhead*); however, this result was not repeated in four other experiments, thus the identity of that band could not be confirmed.

Discussion

Here, we report on the characterization of an adhesion molecule (GR antigen) expressed by chronically stimulated

bovine endothelial cells in vitro and in vivo. The GR antigen on endothelial cells supports myeloid cell rolling, as well as the rolling of a subset of lymphocytes under flow conditions. Platelets also produce the antigen, but very little is expressed on their cell surface; whereas a functional form is released upon activation of the platelet with thrombin or PMA. The bovine antigen also has the capacity to bind human leukocytes. The binding activity of the GR antigen is inhibited by EDTA and *O*-sialoglycoprotease treatment of target leukocytes.

Though characteristics, such as inhibiton by EDTA and support of leukocyte rolling, of the GR antigen are similar to the known vascular selectins, E- and P-selectin, considerable data suggests that the GR antigen is likely unique. First, in precipitation studies the GR antigen is distinct from the major forms of either P- or E-selectin. The GR antigen has a molecular mass of 110–120 kD; whereas, bovine P- and E-selectin have masses of 130 and 90–100 kD, respectively (bovine selectins are slightly smaller than the homologues in humans, because they contain fewer SCR domains [30–32]). The GR antigen also exhibits other characteristics that distinguish it from known adhesion proteins. It is maintained at optimal levels by endothelial cells from three different tissues after 24 h stimulation in vitro, which distinguishes it in most, but not all, instances from E- and P-selectin, but not VCAM-1 (36–38). The GR antigen is also produced by platelets, which is different from E-selectin and VCAM-1 (36, 38). GR113 mAb stains unactivated and activated platelets equally well, a potential difference with P-selectin (37). Inhibition of GR antigen binding to leukocytes by *O*-sialoglycoprotease is another difference with the interaction mediated by E-selectin (12, 13). Thrombin-activated platelets release soluble GR antigen, which retains full functional activity. Indeed, though antibody reactivity with unactivated platelets can clearly be detected by flow cytometric analysis, the expression levels are very low and the majority of the platelet antigen appears to be internal and secreted. P-selectin is a conventional transmembrane glycoprotein on platelets (37), but it certainly could be released by damaged platelets. GR antigen is also distinct from the recently described VAP-1 endothelial cell adhesion molecule, which is glycoprotein of 90 and 170 kD that mediates carbohydrate-dependent binding of lymphocytes (39). Therefore, based on its expression profiles, size, and functional activity, the GR antigen is likely distinct from any other previously defined adhesion molecule involved in leukocyte rolling interactions under physiological flow. However, though the data is quite compelling, we are still limited in the bovine system with respect to available reagents, such as panels of mAbs, to make definitive conclusions. Confirmation that the GR antigen represents a new adhesion molecule (either a previously described molecule with a new function or a new molecule) or an alternate form of a known molecule, such as P-selectin, will only be shown once its molecular structure is determined.

Though the GR antigen has been defined in the bovine system, evidence points to the existence of a similar molecule in humans. GR113 mAb does not crossreact with human cells; however, the GR antigen has the capacity to bind human myeloid cells and a subset of lymphocytes, the same pattern as defined with bovine leukocytes. The binding of the antigen to the human cells is blocked by EDTA and GR113, demonstrating that it is a specific interaction. Interestingly, lymphocytes taken directly from the blood and expressing the highest levels of CLA, an antigen associated with E-selectin binding (13, 27), bind the GR antigen. CLA is expressed by CD45RO positive T cells (memory cells). In preliminary experiments, we found that GR antigen bound a subset of Con A expanded lymphocytes, which homogenously expressed CD45RO, indicting that expression of this suface antigen does not correlate precisely with GR antigen binding. GR antigen is bound by both CD4 and CD8 human T cells.

The intent of this study was not the characterization of the counter-receptor for the GR antigen, but certain insights have been gained. First and foremost, though CLA bright cells bind the GR antigen and cells from some donors show a linear correlation between CLA expression and GR antigen binding, CLA is not a prerequisite nor sufficient for binding. CLA is a carbohydrate moiety associated with E-selectin binding expressed on a variety of different glycoproteins (13, 27, 40, 41). Neuraminidase treatment (0.1 U/ml for 30 min) of bovine γ/δ T cells or human lymphocytes does not block binding of soluble GR antigen, though HECA 452 reactivity is greatly reduced on the human cells. *O*-sialoglycoprotease treatment of leukocytes effectively blocks their ability to bind the GR antigen, suggesting that the ligand for the GR antigen is a mucin. *O*-sialoglycoprotease has no effect on CLA (13). In contrast, at least one of the counter-receptors for the GR antigen likely is similar if not identical to the P-selectin ligand on leukocytes, P-selectin glycoprotein ligand-1 (PSGL-1) (42, 43). *O*-sialoglycoprotease treatment of leukocytes blocks their ability to bind P-selectin, by cleaving PSGL-1 (44, 45).

Our functional studies point to a potential role for the GR antigen in directing leukocytes to sites of chronic inflammation. We have found from in vivo antibody blocking studies that inhibition of L- and E-selectin, though quite effective in blocking acute ($<$6 h) inflammatory events, is not as effective in calves in blocking chronic inflammation, such as that associated with DTH reactions (24 and 48 h) (Jutila, M.A., unpublished observations). The limited chronic inflammatory lesions examined in this report have many vessels that express high levels of the GR antigen. Indeed, there is a striking correlation between the expression of the GR antigen and the site of leukocyte infiltration into a chronic lesion induced by potassium permanganate. A few scattered GR antigen positive venules can be found in uninflamed skin and expression can be increased by treatment with LPS for 4–6 h, but the level of endothelial cell expression of GR antigen at this early timepoint is far less than the level of E-selectin expression. We have been unable to detect E-selectin positive vessels in the potassium permanganate lesion. From these initial studies, we predict that the GR antigen has a minimal role early in inflammation, but may have a major contribution in sites of chronic inflammation,

complementing the role of VCAM-1, E-selectin, VAP-1, or other adhesion proteins. Currently, a greater survey of inflamed tissues is being studied to identify relevant in vivo settings to examine the functional importance of the GR antigen in the animal.

The function of the GR antigen in supporting adhesion appears restricted to the endothelium, even though the antigen is also produced by platelets. The low level expression of the GR antigen on the surface of platelets does not support leukocyte rolling. The platelet antigen is released in a soluble and functional form following stimulation of the cell with thrombin or PMA. The soluble platelet antigen is quite effective in binding to leukocytes, which suggest a possible function in down regulating leukocyte endothelial cell interactions during the course of overt inflammation. It may be that as platelets accumulate at sites of vessel damage, this dampens the leukocyte inflammatory response through release of the GR antigen from intracellular stores. If true, this would represent another mode of regulation of inflammation.

In summary, from functional studies with human and now bovine cells, evidence supports the existence of a potentially new adhesion molecule that mediates leukocyte rolling under flow conditions on chronically stimulated endothelial cells. To date, the identification of this new molecule has not been reported. Here we provide the initial characterization of an endothelial cell transmembrane and soluble platelet molecule that may represent this undefined adhesion protein (a new molecule or a previously characterized molecule with a new function). Molecular characterization of the bovine molecule as well as identification of the human homologue may result in the identification of a new adhesion system that can be exploited in the development of novel antiinflammatory therapies.

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