Regulation and Lectin Activity of the Human Neutrophil Peripheral Lymph Node Homing Receptor

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We characterize the nature and regulation of a human neutrophil cell surface antigen recognized by monoclonal antibodies (the DREG series) against a human lymphocyte peripheral lymph node homing receptor. Human neutrophils express high levels of the DREG antigen, whose expression is downregulated after treatment with phorbol myristate acetate, or the chemotactic factors C5a and FMLP. Interestingly, C5a treatment also downregulated the monocyte DREG antigen, but had no effect on expression of the lymphocyte molecule. Within 3 minutes after treatment with C5a, greater than 80% of neutrophil DREG antigen expression is lost, and essentially the molecule is completely removed from the cell surface by 5 minutes. The human neutrophil DREG antigen is 10 Kd larger than the lymphocyte molecule. These features are similar to those of the mouse neutrophil MEL-14 antigen (murine peripheral lymph node homing receptor). The mannose-6-phosphate rich phosphomannan (PPME) binds human lymphocytes via the DREG antigen. PPME also binds neutrophils, but little difference in binding is seen between unactivated and activated cells. We show that PPME binding to unactivated neutrophils is mediated primarily by a cation- and DREG antigen-dependent mechanism, whereas activated neutrophil-PPME binding is DREG antigen- and cation-independent, and may be due to the translocation of lysosomal mannose-6-phosphate receptors to the cell surface. The DREG antibodies offer powerful tools for analyzing the role of homing receptors in human neutrophil-endothelial cell interactions, and also may prove valuable in the clinical assessment of neutrophil activation.

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EXTRAVASATION of neutrophils from the blood into an inflammatory site requires neutrophil adherence to the vascular endothelium.1 In the murine system, we have recently shown that the MEL-14 antigen, originally described as a "peripheral lymph node-homing receptor" involved in lymphocyte adhesion to high endothelial venules (HEV),2 participates in neutrophil-endothelial cell interaction in vitro and in vivo.3,4 Interestingly, the neutrophil MEL-14 antigen is exquisitely regulated by chemotactic factors released at sites of inflammation that cause a rapid shedding of intact molecule from the cell surface within 1 to 5 minutes.4,5 Our findings have lead us to the hypothesis that the MEL-14 antigen participates in the initial binding of neutrophils to the vascular endothelium before their activation by chemotactic factors released at the inflammatory site. The loss of the peripheral lymph node homing receptor may be a signal for migration through the endothelial cell barrier and into the underlying tissue.

The murine MEL-14 antigen appears to be a mammalian lectin and may bind carbohydrate determinants expressed on endothelial cells.5-10 Mannose-6-phosphate and the mannose-6-phosphate rich phosphomannan (PPME) specifically block neutrophil as well as lymphocyte adhesion to peripheral lymph node HEV.3,6-8 The binding of PPME to lymphocytes is inhibited by MEL-14, suggesting that PPME binds the lymphocyte MEL-14 antigen. Direct binding of PPME to neutrophils has not been shown, nor has the interaction of PPME with the neutrophil peripheral lymph node homing receptor been characterized. It will be important to determine if the neutrophil and lymphocyte molecules interact with distinct carbohydrate determinants expressed by endothelial cells.

The human neutrophil equivalent of the murine MEL-14 antigen has not been characterized previously. This is an important goal, both for understanding the physiology of neutrophil extravasation and for potential clinical diagnostic and therapeutic value. We have recently characterized a panel of new monoclonal antibodies (MoAbs) (DREG series) that react with a human lymphocyte peripheral lymph node homing receptor, which is homologous at the cDNA level to the murine MEL-14 antigen11 and the human MEL-14 equivalent reported by Tedder et al.12 Two antibodies (DREG 56 and DREG 55), which block greater than 90% of lymphocyte adhesion to peripheral lymph node HEV, inhibit lymphocyte binding of PPME.11

We have characterized the nature and regulation of the human neutrophil DREG antigen. In addition, we have performed quantitative flow cytometry to characterize and compare the in vivo expression of peripheral lymph node homing receptors on circulating human neutrophils, monocytes, and lymphocytes, and to evaluate interaction of the neutrophil molecule with PPME.

MATERIALS AND METHODS

Cells. Human peripheral blood was drawn by venipuncture from healthy adult donors and collected into heparin-containing tubes. The blood was diluted 1:2 with Hanks balanced salt solution (HBSS), underlaid with Ficoll-Hypaque-1119 (Histopaque-1119, Sigma, St Louis, MO), and centrifuged at 300g for 30 minutes. After centrifugation the cell layer at the Ficoll-HBSS interface was collected. This layer included lymphocytes, monocytes, and neutrophils. In other experiments, the buffy coat was collected after 1g sedimentation through 1% dextran T500 (Pharmacia, Piscataway,
leukocytes were extensively washed in Ca2+, Mg2+-free HBSS. Spleen cells were removed 4 days later and fused with the Sp2/0 myeloma cells. Hybridoma supernatants were screened for the ability to differentially stain activated and unactivated peripheral blood leukocytes in an immunofluorescence (IF) assay. MoAbs against downregulated antigens were designated as the DREG series. Five wells that gave identical staining patterns, a dramatic loss of staining on activated cells, were subcloned by limiting dilution analysis. The five subclones, all of the immunoglobulin GI (IgG1) subclass, were designated DREG-55, -56, -110, -152, and -200. The DREG antibodies recognize a 70- to 75-Kd glycoprotein, expressed by peripheral blood lymphocytes, which is involved in adhesion to peripheral lymph node HEV. In the experiments presented here, the DREG-56 antibody, which blocks greater than 95% of lymphocyte-HEV adhesion and virtually 100% of the binding of PPME to lymphocytes, was used.

The murine Hermes-3 MoAb, directed against human H-CAM (CD44), has been described previously and was used as a positive control in the fluorescence-activated cell sorter (FACS) analysis described below.

**Immunofluorescence staining and FACS analysis.** IF staining of cells was performed in microtiter plates or 4-mL tubes. Briefly, 1 x 106 peripheral blood leukocytes were initially incubated in 5% rabbit serum (RS) for 10 minutes on ice to saturate Fc receptors. The cells were washed and then incubated with primary antibody at 50 to 100 μg/mL for 20 minutes on ice. After washing, bound antibodies were revealed by incubation with phycoerythrin (PE)-conjugated F(ab′) goat anti-rat IgG(Tago, Burlingame, CA) at a 1:50 dilution in 5% RS in Dulbecco modified Eagle’s medium. Cells were then fixed with 1% paraformaldehyde and stained with the appropriate secondary antibody and fluorescein isothiocyanate (FITC), rhodamine isothiocyanate (RITC), or PE-conjugated F(ab′) goat anti-rat IgG conjugates. The cells were also stained by anti-HCAM conjugated F(ab′) goat anti-rat Ig (Becton Dickinson, Mountain View, CA) as previously described. Neutrophils, monocytes, and lymphocytes were identified by distinctive forward and side light scatter profiles. Data were collected from 10,000 to 50,000 cells, and are presented as either percent positive cells, mean fluorescence, or as histograms.

**Western blot analysis.** Freshly isolated peripheral blood mononuclear cells and polymorphonuclear leukocytes (PMNs) (6 to 8 x 106) were lysed in 2% Nonidet-P40 (Sigma), 150 mmol/L NaCl, 50 mmol/L Tris-HCL (pH 8.0), 0.02% NaN3, and 1 mmol/L phenylmethylsulfonyl fluoride (lysis buffer) for 30 minutes at 4°C. Nuclei were removed by centrifugation at 12,000 rpm for 10 minutes. Lysates were run under nonreducing conditions by sodium dodecyl sulfate (SDS)-9% polyacrylamide gel electrophoresis (PAGE), and transferred to nitrocellulose using a transblot apparatus (BioRad, Richmond, CA). Filters were incubated with horse serum for 30 minutes with gentle agitation, and then subjected to primary MoAb (100 μg/mL) and secondary alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma) using a 25-lane mini blot apparatus (Immunetics, Cambridge, MA), as previously described. After extensive washing with TBST (10 mmol/L Tris-HCl [pH 7.4]), 150 mmol/L NaCl, 0.05% Tween-20, the blots were developed by addition of substrate solution (335 μg/mL nitro blue tetrazolium [Sigma], 165 μg/mL 5-bromo-4-chloro-3-indolyl-phosphate [Sigma] in 100 mmol/L Tris-HCl [pH 9.5], 100 mmol/L NaCl, 5 mmol/L MgCl2).

**PPME binding assay.** Leukocyte binding of PPME, the polyphosphomonoester core from *Hansenula hostil* phosphomannan, has been described previously. Here human peripheral blood PMNs were pretreated with either Hermes-3 or DREG-56 for 30 minutes at 4°C, washed, and then exposed to a 1:100 dilution of fluorescein-conjugated PPME (generous gift of Dr Steve Rosen, University of California at San Francisco) for 30 minutes at 4°C. The cells were washed and then analyzed by flow cytometry for the ability of the MoAbs to inhibit binding of the fluorescein isothiocyanate (FITC)-PPME. To evaluate the effect of EDTA on the binding of PPME, activated and unactivated neutrophils were incubated with 10 mmol/L EDTA for 20 minutes before staining with FITC-PPME.

**RESULTS**

**Expression of the human leukocyte DREG antigen.** Peripheral blood neutrophils, monocytes, and lymphocytes all express high levels of the DREG antigen (Fig 1). Analysis by Western blot PAGE showed that the lymphocyte molecule was approximately 70 to 75 Kd when run under nonreducing conditions (Fig 2, lane 1 and reference 11). In contrast, when analyzed under similar conditions the neutrophil molecule was approximately 80 to 90 Kd (Fig 2, lane 4). The 10 to 15 Kd molecular weight difference between the human neutrophil and lymphocyte peripheral lymph node homing receptor is similar to the difference between the murine neutrophil and lymphocyte MEL-14 antigen. Regulation of the human neutrophil DREG antigen. The effect of recombinant human C5a (1 x 10-8 mol/L) and PMA (100 ng/mL) on the downregulation of the neutrophil DREG antigen was tested. Treatment in vitro for 20 minutes with PMA resulted in marked reduction of neutrophil, monocyte, and lymphocyte staining (Table 1). C5a also caused downregulation of the neutrophil and monocyte molecule. In contrast, C5a had no effect on the lymphocyte molecule even if lymphocytes were cocultured in the presence of activated neutrophils at high cell concentrations (Table 1). Similar results were observed with FMLP (data not shown). Thus, chemotactic factors can downregulate homing receptors in a leukocyte-specific fashion, and the mechanism controlling the release of the antigen appears confined to the cell surface and does not affect bystander cells.

The kinetics of neutrophil DREG antigen downregulation were studied. Neutrophils were treated with 1 x 10-8 mol/L C5a for different periods of time, rapidly fixed by the addition of 1% paraformaldehyde, and stained with the DREG antibody. The cells were also stained by anti-HCAM (CD44) antibody Hermes-3 as a positive control, and with second stage alone. As shown in Table 2, neutrophil staining with DREG-56 is reduced within 1 minute after treatment with C5a, and staining was not apparent after 5 minutes. No significant changes in the expression of H-CAM (the Hermes antigen, CD44) were noted over the time points studied (Table 2), nor was there a significant change in staining with second stage alone.

**PPME binding to unactivated neutrophils is inhibited by DREG-56.** Human neutrophils bind FITC-PPME (Fig 3). Pretreatment of unactivated neutrophils with DREG-56 blocked PPME binding by 90%. In contrast, Hermes-3 MoAb had no effect on FITC-PPME binding to unactivated neutrophils (Fig 3). Thus, PPME binds unactivated neutrophils through a DREG-antigen-dependent mechanism.

**Upregulation of a DREG antigen-independent PPME binding activity on activated neutrophils.** The DREG-56...
antibody, which completely blocks PPME binding to human lymphocytes, did not completely block PPME binding to neutrophils (10% of FITC-PPME staining still remains after treatment with DREG-56). Therefore, it was possible that PPME interacted with other distinct neutrophil cell surface molecule(s). To address this, neutrophils were activated with C5a to remove the DREG antigen from their cell surface, treated with medium, DREG-56, or Hermes-3, and then stained with FITC-PPME. Interestingly, even though the activated neutrophils expressed less than 5% of the level of the DREG antigen, they bound FITC-PPME at high levels and this binding was not inhibited by DREG-56 (Fig 3).

It was possible that the DREG antigen-independent PPME binding to activated neutrophils was mediated by lysosomal mannose-6-phosphate receptors translocated to the cell surface. The predominant lysosomal receptor is cation-independent, whereas PPME binding to the peripheral lymph node homing receptor is cation-dependent. Thus, we tested the effect of 10 mmol/L EDTA on the binding of FITC-PPME to activated and unactivated neutrophils. EDTA blocked approximately 72% of FITC-PPME binding to unactivated neutrophils (mode fluorescence of FITC-PPME staining of unactivated neutrophils = 217, mode fluorescence after EDTA treatment = 61). In contrast, EDTA had no effect on PPME binding to activated neutrophils (mode fluorescence of FITC-PPME staining of activated neutrophils = 149, mode fluorescence after EDTA = 167).

Table 1. Effect of C5a and PMA on the Expression of the DREG Antigen on Human Neutrophils, Monocytes, and Lymphocytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Neutrophils</th>
<th>Monocytes</th>
<th>Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>PMA</td>
<td>4</td>
<td>8</td>
<td>11</td>
</tr>
<tr>
<td>C5a</td>
<td>3</td>
<td>10</td>
<td>96</td>
</tr>
</tbody>
</table>

*Values represent the mean fluorescence of DREG-56–stained C5a (1 x 10^{-8} mol/L) or PMA (100 ng/ml)-treated cells divided by the mean fluorescence of DREG-56–stained untreated control cells x 100. The second-stage reagent was PE-conjugated goat F(ab)2 anti-mouse Ig. Results are data pooled from two separate experiments.
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Table 2. Kinetics of the Downregulation of the Neutrophil DREG Antigen After Treatment With C5a

<table>
<thead>
<tr>
<th>Time After C5a (min)</th>
<th>Percentage of Control Mean Fluorescence*</th>
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<tbody>
<tr>
<td></td>
<td>DREG AG</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>89</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
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<td>5</td>
<td>3</td>
</tr>
<tr>
<td>15</td>
<td>0</td>
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</table>

*Values represent the mean fluorescence of DREG-56- or Hermes-3 (anti-HCAM)-stained C5a-treated neutrophils divided by the mean fluorescence of DREG-56- or Hermes-3-stained untreated control neutrophils x 100. The second stage reagent was PE-conjugated goat F(ab')2 anti-mouse Ig. Results are data pooled from two separate experiments.

Therefore, neutrophils express two PPME receptors that are inversely regulated by activation: the cation-dependent DREG antigen and a cation-independent molecule that may be related or identical to the lysosomal mannose-6-phosphate transport receptor.

DISCUSSION

The peripheral lymph node homing receptor is thought to be important in lymphocyte adhesion to peripheral lymph node HEV and in murine neutrophil extravasation to sites of inflammation. Here we characterize the regulation and nature of a peripheral lymph node homing receptor (DREG antigen) expressed by human neutrophils. This is important if the homing receptor class of adhesion molecules is to be exploited in the development of novel treatments or diagnostic procedures. The neutrophil molecule has a molecular weight of 80 to 90 Kd, 10 to 15 Kd larger than the molecule expressed by lymphocytes. This difference may be due to distinct glycosylation patterns or other posttranslational modifications. Interestingly, activation of human neutrophils by the chemotactic factors C5a and FMLP causes a rapid (within minutes) downregulation of the peripheral lymph node homing receptor. Importantly, we demonstrate for the first time that mannose-6-phosphate-rich sugars (PPME) bind unactivated neutrophils via a mechanism involving homing receptors. The molecular weight and regulation of the human neutrophil peripheral lymph node homing receptor are similar to the murine neutrophil antigen recognized by the MEL-14 antibody.

A number of leukocyte-specific adhesion molecules have been shown to be involved in human neutrophil-endothelial cell adhesion. These include predominantly the CD11a-c/CD18 (LFA-1, Mac-1, p150/95) family of cell surface glycoproteins. The importance of the CD 11/18 complex is dramatically illustrated in human patients lacking expression of these molecules on the surface of their leukocytes (leukocyte adhesion deficiency [LAD]). These patients have recurrent bacterial infections and exhibit inflammatory lesions that are devoid of neutrophils. However, CD11/18-independent human neutrophil-endothelial cell adhesive interactions exist. For example, LAD neutrophils bind well to endothelial cell monolayers activated by inflammatory cytokines. Activated endothelial cells have been shown to express a neutrophil adhesion molecule (ELAM-1) that mediates binding independent of the CD11/18 complex. The neutrophil cell surface adhesion molecules mediating these CD11/18-independent interactions have yet to be characterized. The DREG antibodies offer powerful tools to analyze the role of the DREG antigen in CD11/18-independent human neutrophil-endothelial cell interactions. In preliminary experiments, DREG antibodies block a significant percentage of the CD11/18-independent neutrophil adhesion to cytokine-stimulated endothelial cells (Hallman R, Jutila MA, Smith CW, Anderson DC, Kishimoto TK, Butcher EC: in preparation).

The lectin activity of the peripheral lymph node homing receptor is thought to be important in the function of this molecule. Mannose-6-phosphate and the polysaccharide PPME block both lymphocyte and neutrophil adhesion to lymph node HEV. PPME specifically binds the mouse lymphocyte peripheral lymph node homing receptor. PPME binding to lymphocytes is cation-dependent and inhibitable by anti-homing receptor antibodies (MEL-14 and the DREG series). We demonstrate here that PPME interacts with the neutrophil DREG antigen in a manner similar to the lymphocyte molecule. However, PPME also interacts with neutrophils through a cation- and DREG antigen-independent mechanism that is upregulated on the surface of the cell.

Fig 3. Binding of FITC-PPME to unactivated neutrophils via a DREG antigen-dependent mechanism (left) and to activated neutrophils via a DREG antigen-independent mechanism (right). Unactivated and C5a-activated neutrophils were treated with medium alone, DREG-56, or Hermes-3 (H-3) for 20 minutes and then stained with FITC-PPME. Histograms of the log fluorescence of FITC-PPME staining after each treatment are presented.
on activation. The cation-independent lysosomal mannose-6-phosphate receptor has been shown previously to be expressed on the surface of a variety of cells. It is interesting to speculate that the surface form of the lysosomal mannose-6-phosphate receptor could be functionally important in the interaction of activated neutrophils with carbohydrate determinants expressed on other cells.

Importantly, the regulation of human neutrophil peripheral lymph node homing receptor is leukocyte-specific. For example, C5a and FMLP, which are potent chemotactic factors for neutrophils, cause downregulation of the neutrophil DREG antigen, but have no effect on the regulation of the lymphocyte molecule. Because the lymphocyte molecule can be downregulated by phorbol esters, this observation suggests that downregulation of homing receptors will only occur if the cell is directly activated. This feature offers an elegant pathway for rapid and leukocyte-specific regulation of homing mechanisms.

The rapid downregulation of peripheral lymph node homing receptors on human neutrophils has potential to provide a highly sensitive marker of leukocyte activation in vivo. Monitoring the loss of the DREG antigen on circulating leukocytes in vivo may provide an important diagnostic tool in evaluating the progression of inflammatory diseases in humans. As one example, the loss of the DREG antigen on circulating leukocytes may be an early marker of inflammation associated with allograft rejection. If true, this might offer a relatively noninvasive means of evaluating the host response to grafted tissues.

In summary, we have identified and characterized the regulation of the human neutrophil peripheral lymph node homing receptor (DREG antigen) on neutrophils. Like its lymphocyte counterpart, the neutrophil homing receptor appears to be able to function as a lectin, binding PPME in experimental settings. It may interact with inflammation-induced endothelial cell carbohydrate determinants in vivo. Further analysis of the role of the peripheral lymph node homing receptor in neutrophil–endothelial cell interactions is now possible using the DREG antibodies. Because only hematolymphoid cells express the antigen and its primary role is most likely restricted to adhesion to endothelial cells, the DREG antigen is an attractive target for therapeutic intervention designed to block leukocyte extravasation and local inflammatory responses in vivo.

REFERENCES

phenotypes of heritable Mac-1, LFA-1 deficiency: Their quantitative definition and relation to leukocyte dysfunction and clinical features. J Infect Dis 152:668, 1985


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MA Jutila, TK Kishimoto and EC Butcher