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Receptors Involved in Lymphocyte Homing: Relationship between a Carbohydrate-binding Receptor and the MEL-14 Antigen

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Abstract. Blood-borne lymphocytes extravasate in large numbers within peripheral lymph nodes (PN) and other secondary lymphoid organs. It has been proposed that the initiation of extravasation is based upon a family of cell adhesion molecules (homing receptors) that mediate lymphocyte attachment to specialized high endothelial venules (HEV) within the lymphoid tissues. A putative homing receptor has been identified by the monoclonal antibody, MEL-14, which recognizes an 80–90-kD glycoprotein on the surface of mouse lymphocytes and blocks the attachment of lymphocytes to PN HEV. In a companion study we characterize a carbohydrate-binding receptor on the surface of mouse lymphocytes that also appears to be involved in the interaction of lymphocytes with PN HEV. This receptor selectively binds to fluorescent beads derivatized with PPME, a polysaccharide rich in mannose-6-phosphate. In this report we examine the relationship between this carbohydrate-binding receptor and the putative homing receptor identified by the MEL-14 antibody. We found that: (a) MEL-14 completely and selectively blocks the activity of the carbohydrate-binding receptor on mouse lymphocytes; (b) the ability of six lymphoma cell lines to bind PPME beads correlates with cell-surface expression of the MEL-14 antigen, as well as PN HEV-binding activity; (c) selection of lymphoma cell line variants for PPME-bead binding by fluorescence-activated cell sorting (FACS) produces highly correlated (r = 0.974, P < 0.001) and selective changes in MEL-14 antigen expression. These results show that the carbohydrate-binding receptor on lymphocytes and the MEL-14 antigen, which have been independently implicated as receptors involved in PN-specific HEV attachment, are very closely related, if not identical, molecules.

During lymphocyte recirculation, lymphocytes continuously cycle from the blood to lymphoid organs and back to the blood. This process is critical to immune surveillance as it allows the body's repertoire of immunocompetent cells to be brought into contact with sequestered antigens in peripheral lymphoid tissues. Migration of lymphocytes into lymph nodes and other secondary lymphoid organs is initiated by the binding of blood–borne lymphocytes to specialized venules, known as high endothelial venules or HEV (Gowans and Knight, 1964; Marchesi and Gowans, 1964). In vitro and in vivo studies have established that lymphocyte attachment to HEV is a highly specific cell recognition event that depends on the immunologic class and state of differentiation of the lymphocyte as well as the anatomical site of the HEV (Stamper and Woodruff, 1976; Butcher et al., 1980; Stevens et al., 1982; Dailey et al., 1982; Reichert et al., 1983). Such specificity is widely thought to be based upon a family of lymphocyte adhesive receptors (homing receptors) that recognize organ-specific attachment sites on HEV (Gallatin et al., 1986).

Candidates for homing receptors on murine lymphocytes have been identified as cell-surface antigens recognized by adhesion-blocking antibodies (Chin et al., 1982; Gallatin et al., 1983; Rasmussen et al., 1985) or as lymphocyte-derived soluble factors (found in lymph) that react with HEV and block lymphocyte attachment (Chin et al., 1980, 1984). In mouse, an 80–90-kD glycoprotein recognized by the MEL-14 monoclonal antibody has been implicated as a homing receptor as follows (Gallatin et al., 1983): (a) expression of the antigen on lymphocyte and lymphoma populations is closely correlated with the ability of these cells to bind to peripheral lymph node (PN) HEV in vitro (Stamper–Woodruff assay); (b) MEL-14 blocks the attachment of lymphocytes to PN HEV in vitro, but not to HEV in Peyer's patches, a

I. Abbreviations used in this paper: FACS, fluorescence-activated cell sorting; M6P, o-mannose-6-phosphate; HEV, high endothelial venules(s); PN, peripheral lymph node; PPME, phosphomannan monoester core from *Hansenula hostii* Y-2448.
demonstrated directly on the lymphocyte cell surface with a drate-binding receptor on the surface of lymphocytes is in-
Reagents

perhaps identical to the MEL-14 antigen.

and to PN HEV; (f) selection of $49 variants for their ability weaker binding than mature lymphocytes to PPME beads
and the thymic lymphoma cell line, $49, exhibit 10-fold
tions; (d) both interactions require calcium; (e) thymocytes
fucose-rich polysaccharide, PPME; (b) fucoidin, a sulfated fucose-rich polysaccharide is an extremely potent inhibitor in both assays; (c) mild treat-
ment of lymphocytes with trypsin eliminates both interactions; (d) both interactions require calcium; (e) thymocytes and the thymic lymphoma cell line, S49, exhibit Ig-f0 Ig weaker binding than mature lymphocytes to PPME beads
and to PN HEV.

These findings provide strong evidence that the carbohydrate-binding receptor, detected by PPME beads, is involved in lymphocyte attachment to PN HEV. We now report that this carbohydrate-binding receptor is closely related and perhaps identical to the MEL-14 antigen.

Materials and Methods

Reagents

Chondroitin sulfate (C3254), dextran sulfate (D7515), heparin (H3251), kappa carrageenan (C1263), BSA (A4503), cyanogen bromide (C6388), and 2-mercaptoethanol (M6250) were obtained from Sigma Chemical Co. (St.

Louis, MO). Fucoidin was purchased from K & K Laboratories, Inc. (Plain-
view, NY). The yeast mannan, man I, was kindly supplied by Dr. C. E.
Ballou (Department of Biochemistry, University of California, Berkeley, CA) and PPME, the polyphosphomonoester core from Hansenula hostii
phosphomannan (Słodki et al., 1973; Kaplan et al., 1978), was a generous gift of Dr. M. E. Słodki (U.S. Department of Agriculture, Northern Regional Research Center, Peoria, IL). Disodium EDTA and bovine acid had been obtained from Mallinkrodt Inc. (Paris, KY). Reagents for tissue culture came from the Cell Culture Facility at the University of California, San Francisco.

Cell Lines

The following mouse cell lines were obtained as previously described (Gal-
latin et al., 1983): RAW 112 (Abelson virus-induced, Balb/c, pre-B cell lympho-

moma), 38C-13 (cloned C3H/eb, B cell lymphoma), Sp 20 (fusion-partner myeloma), and EL4 (chemical carcinogen-induced, C57Bl, T cell lympho-
moma). BW 5147 (T cell lymphoma from spontaneous AKR/J tumor) was
obtained from the American Type Culture Collection, Rockville, MD (ATCC), No. TIB 47. Each of these cell lines was maintained by suspension culture in RPMI 1640 growth medium (pH 7.4), supplemented with 10% FBS, 2 g/liter NaHCO3, 2 mM L-glutamine, 1 mM Na pyruvate, nonessential amino acids, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% heat-inactivated horse serum. The cells were grown in suspension at 37°C with 5% atmospheric CO2, and were subcultured every 3-4 d by a 1:14 dilution into fresh medium (maximum density of 106 cells/ml).

Preparation of Cell Suspensions

Peripheral lymphocytes were obtained from mesenteric lymph nodes of cerially dislocated mice (Balb/c female, 6-10 wk) or from peripheral lymph nodes of ether-anesthetized rats (Sprague- Dawley female, 180 g) killed by cardiac puncture. Nodes were teased with fine needles and flushed with Dul-
becco's PBS containing 1 mg/ml BSA to release lymphocytes. After cen-
trifugation (5 min, 250 g, 4°C) the cells were resuspended in fresh PBS with 1 mg/ml BSA and adjusted to an appropriate concentration of viable (trypan blue-excluding) cells.

Preparation of Derivatized Beads

Beads were derivatized with polysaccharide as described in the companion study (Yednock et al., 1987). PPME beads contain ~1.4 × 10-9 g of PPME/bead corresponding to 64,000 M6P moieties/bead. Heparin beads contain ~1.6 × 10-9 g heparin/bead. The degree of carbohydrate substitution of carrageenan beads was not determined.

Bead Binding Assay

Cells were preincubated with antibodies diluted in PBS with 1 mg/ml BSA for 30 min at 4°C. The cells were then exposed to a 1:100 dilution of fluorescent carbohydrate-derivatized beads or nonderivatized-beads as described previously (Yednock et al., 1987). Briefly, lymphocytes and beads were mixed in wells of a flat-bottomed microtiter plate and centrifuged at 225 g for 12 min at 4°C. The plate was carefully transferred to ice and left undisturbed for 60 min. Cells were resuspended by pipetting and were washed free of unbound beads by centrifugation through PBS containing 7% BSA and 1 mM sodium azide. In most of the studies with the $49 cell lines, the procedures were essentially the same except that the assay was performed in modified $49 growth medium (containing 10 mM Hepes, rather than NaHCO3, and supplemented with 2% heat-inactivated horse serum) and the cells were separated from unbound beads by centrifugation through heat-inactivated horse serum containing 1 mM sodium azide. The degree of bead binding to single cells was determined by flow cytometry (FACS Ana-
lyzer; Becton-Dickinson & Co., Paramus, N J) and fluorescence values were converted to linear numbers for comparison. In most experiments, the degree of specific PPME bead binding was determined by subtracting the level of binding by nondonrivatized beads. The means and ranges (shown as error bars) are based on analysis of two 5,000-cell samples from independent replicates.

Immunofluorescence Measurement of Antibody-treated Cells

Cells were exposed to antibodies as described above, washed by centrifuga-
tion through PBS containing 7% BSA and 1 mM sodium azide, and resuspended in a 1:10 dilution of fluorescein-conjugated second antibody (goat anti-rat IgG; cat. No. F6258, Sigma Chemical Co.) in PBS with 5% mouse serum and 1 mM sodium azide. After 15 min at 4°C the samples were washed, resuspended in PBS containing 1 mM sodium azide, and ana-
yzed immediately by flow cytometry as described above.

Results

Effects of Antibodies on PPME-bead Binding to Lymphocytes

We found that MEL-14 potently inhibited the attachment of PPME beads to lymphocytes. As shown in Fig. 1, the degree of PPME-bead binding decreased in close reciprocal corre-
centrations with the level of MEL-14 bound to the cell surface. MEL-14 had no effect on the attachment of nondonrivatized beads to lymphocytes.

In contrast to MEL-14, a series of antibodies that recognize
Inhibition of Antibody Binding by Polysaccharides

The fact that MEL-14 blocks PPME-bead binding to lymphocytes suggested that this antibody might recognize the active site of the carbohydrate-binding receptor. We therefore sought to determine whether polysaccharides that inhibit PPME-bead binding to lymphocytes would compete with the binding of the MEL-14 antibody to cells. Competition by a particular carbohydrate would suggest that this substance binds at or near the epitope recognized by the monoclonal antibody. We found that fucoidin at 1 mg/ml substantially inhibited MEL-14 binding to lymphocytes but had no significant activity on the cell-surface binding of a series of other monoclonal antibodies (Fig. 4 a). When several anionic polysaccharides were compared at 1 mg/ml for inhibition of MEL-14 binding to lymphocytes, only fucoidin showed significant activity (Fig. 4 b). Surprisingly, PPME was among the inactive substances when tested at 1 mg/ml (Fig. 4 b) and even at 10 mg/ml in the presence of a minimal concentration of MEL-14 (not shown). A discussion of these diver-
Figure 3. Effect of MEL-14 on the attachment of PPME beads to mouse or rat lymphocytes. Mouse lymphocytes (dark bars) or rat lymphocytes (stippled bars) were preincubated with MEL-14 and then exposed to PPME beads. The level of PPME-bead binding to both cell types (with no MEL-14) was five times that of nonderivatized-bead binding, which was subtracted from the PPME-bead signal to yield the degree of specific PPME-bead binding. The degree of specific PPME-bead binding to each cell type with no MEL-14 was set at 100%.

Figure 4. Competition of antibody binding to mouse lymphocytes by polysaccharides. (a) Lymphocytes were preincubated with 1 mg/ml fucoidin for 30 min at 4°C and then to subsaturating concentrations of the indicated antibodies in the presence of fucoidin. The fluorescence of cells after exposure to the second antibody (fluorescein-conjugated) was determined as described in Materials and Methods. The fluorescence of cells exposed to the second antibody alone was one-tenth that of cells first exposed to MEL-14 (in the absence of polysaccharide). None of the polysaccharides affected cell viability, as determined by trypan blue exclusion.

Table I. Six Mouse Lymphoma Cell Lines vs. Normal Peripheral Lymphocytes: Degree of Divalent Cation-dependent PPME-Bead Binding, Ability to Bind PN HEV, and Expression of the MEL-14 Antigen

| Cell type | Level of EDTA-sensitive PPME-bead binding (% of control) | MEL-14 expression (%) | PN HEV binding (%) |
|-----------|----------------------------------------------------------|-----------------------|--------------------|
| Lymphocytes | 100 ± 11 | 100 | + |
| 38C-13 | 426 ± 30 | 569 | + |
| S49 | 23 ± 3 | 11 | ± |
| RAW 112 | 0 | 0 | − |
| EL4 | 0 | 0 | − |
| BW 5147 | 0 | 0 | − |
| Sp20 | 0 | 0 | − |

The lymphoma cell lines (described in Materials and Methods) were exposed to PPME beads in the presence of control buffer (PBS with 1 mg/ml BSA) or buffer with 2 mM EDTA. The level of bead binding to each cell line in the presence of EDTA was subtracted from the level of binding in the absence of EDTA to yield the level of divalent cation-dependent binding (see text). This number is expressed as a percentage of the value determined for mouse peripheral lymphocytes. The level of the MEL-14 antigen expressed on each cell type was determined by exposing the cells to 0.5 μg MEL-14/ml, followed by detection with a second fluorescent antibody as described in Materials and Methods. Higher concentrations of MEL-14 did not increase binding to any of the cell types (i.e., MEL-14 was at saturation). The mean level of MEL-14 antigen expressed per cell by each population is indicated as a percentage of the amount on peripheral lymphocytes. The ability of the cell lines to bind to mouse PN HEV was determined using a modified version of the Stammer-Woodruff (Stamper and Woodruff, 1976) in vitro assay (described in detail in Stoolman and Rosen, 1983). In brief, each cell type (at a concentration of 2 x 10⁷ cells/ml PBS with 1 mg/ml BSA) were exposed (with gyration at 80 rpm) to frozen, paraformaldehyde-fixed sections of mouse peripheral lymph nodes for 30 min at 7°C. The cell suspensions were decanted and the slides were post-fixed with glutaraldehyde and stained for viewing. Cell attachment to HEV was scored on a ± basis. At least 20 segments of HEV were evaluated on each of five independent replicate sections.

Discussion. Gent effects of PPME and fucoidin is presented below (see Discussion).

Properties of Lymphoma Cell Lines

Six lymphoma cell lines were compared with normal mouse peripheral lymphocytes (isolated from mesenteric lymph nodes) for their ability to bind to PPME beads, their expression of the MEL-14 antigen, and their ability to bind to PN HEV. The attachment of normal lymphocytes to PPME beads as well as their binding to HEV requires calcium (Yednock et al., 1987). We found that some of the lymphoma lines exhibited variable and substantial binding to PPME beads, as well as to nonderivatized beads. To restrict consideration to those receptors that require divalent cations, we examined EDTA-inhibitable PPME-bead binding. As shown in Table I, two cell lines, 38C-13 and S49, demonstrated EDTA-sensitive PPME-bead binding. 38C-13 bound to PPME beads 4- to sixfold better than peripheral lymphocytes. This cell line expressed five- to sixfold higher levels of the MEL-14 antigen than peripheral lymphocytes and strongly interacted with PN HEV. S49 exhibited a low but measurable degree of EDTA-sensitive PPME-bead binding. S49 cells expressed a tenfold lower level of the MEL-14 antigen than peripheral lymphocytes and only weakly bound to PN HEV. The other cell lines tested failed to exhibit EDTA-inhibitable PPME-bead binding. These cells did not express detectable MEL-14 antigen, nor were they able to bind to PN HEV. These data demonstrate a correlation among the presence of the carbohydrate-binding receptor on lymphoma cells, the expression of the MEL-14 antigen, and the ability of the cells to bind PN HEV.
binding and MEL-14 antigen expression. In each case, the parental line was also examined for use as a standard reference. Cells were incubated in the presence or absence of 0.5 μg MEL-14/ml (a saturating concentration) for 30 min on ice. Half of each sample was then exposed to beads and the other half to a fluorescent second antibody and the samples were analyzed by flow cytometry (see Materials and Methods). The low level of nonderivatized-bead binding was subtracted from the level of PPME-bead binding to yield an index of specific PPME-bead binding. MEL-14 always inhibited PPME-bead binding to the level of nonderivatized-bead binding, which was not affected by MEL-14 and which did not change during the selection procedures. For immunofluorescence measurements, the degree of fluorescence exhibited by cells exposed to second antibody alone was subtracted from the level of fluorescence of cells exposed to MEL-14 before second antibody. The degree of specific PPME bead attachment (x-axis) and the level of specific MEL-14 binding (y-axis) are expressed relative to the values obtained for the S49 parental cell line, which were normalized to unity. The strength of the relationship between normalized PPME-bead binding and normalized MEL-14 expression was determined by computing the Pearson product-moment correlation coefficient (r). The significance of the r value was calculated by the t test (see Glantz, 1981).

To investigate this correlation further, we examined S49 cells that had been subjected to a serial selection procedure for PPME-bead binding with FACS (see companion study, Yednock et al., 1987). During the course of this selection protocol, 14 cell-line isolates were evaluated for PPME-bead binding and MEL-14 expression. A highly significant correlation (r = 0.974, P < 0.001) was found between these two parameters (Fig. 5). Fig. 6 shows a fluorescence histogram comparing MEL-14 expression on the S49 parental cell line and the S49 variant obtained after nine rounds of selection with PPME beads. This isolate bound to PPME beads 17-fold better than the parental line and expressed a 20-fold higher level of the MEL-14 antigen.

In the companion study (Yednock et al., 1987), we describe an earlier isolate, referred to as S49-PBIII, that was obtained after seven rounds of selection. S49-PBIII has been well characterized as a stable cell line that binds seven- to eightfold higher levels of PPME beads than the parental S49 line and shows a parallel increase in its ability to bind PN HEV. As shown in Fig. 7, S49-PBIII cells expressed a fivefold higher level of the MEL-14 antigen at the cell surface than the parental cells. As expected, the attachment of PPME beads to S49-PBIII cells was completely inhibited by MEL-14, whereas the binding of beads derivatized with heparin or...
Table II. Effect of MEL-14 on the Attachment of S49-PBm Cells to Beads Derivatized with PPME, Heparin, or Sulfated Galactan

| Bead type | Level of control binding | Level of binding to S49-PBm treated with MEL-14 |
|-----------|-------------------------|-----------------------------------------------|
| PPME      | 100 ± 2                 | 1 ± 1                                         |
| Heparin   | 332 ± 5                 | 334 ± 9                                       |
| Sulfated galactan | 84 ± 6   | 74 ± 7                                       |

S49-PBm cells, untreated or pretreated with a saturating concentration of MEL-14 (0.5 μg/ml) for 30 min on ice, were exposed to the indicated type of bead and the level of bead-binding was quantified as described in Materials and Methods. PPME-bead binding (in the absence of MEL-14) was 10 times that of nonderivatized beads. The level of nonderivatized-bead binding was subtracted from all values to yield the index of specific bead binding. These values are expressed as a percentage of specific PPME-bead binding to cells in the absence of MEL-14.

The MEL-14 antibody, which recognizes a single 80–90-kD glycoprotein on the surface of mouse lymphocytes, blocks lymphocyte binding to PN HEV both in vivo and in vitro. Here we have shown that this monoclonal antibody interferes with the activity of a lymphocyte surface carbohydrate-binding receptor that has been independently implicated as a receptor involved in lymphocyte attachment to PN HEV. The inhibitory effect of MEL-14 on the interaction of lymphocytes with PPME beads is highly selective since saturating levels of other antibodies are without effect. Moreover, the MEL-14 antibody does not inhibit lymphocyte binding to nonderivatized beads, nor does it inhibit the attachment of beads derivatized with heparin or sulfated galactan to the S49-PBm cell line. In reciprocal inhibition studies, we found that fucoidin, the most potent carbohydrate inhibitor of PPME-bead binding, prevented MEL-14 binding to the cell surface of lymphocytes.

The analysis of lymphoma cell lines greatly strengthens the case for a close relationship between the MEL-14 antigen and the carbohydrate-binding receptor. Only the two lymphoma lines that exhibited EDTA-sensitive PPME-bead binding expressed the MEL-14 antigen and could bind to PN HEV, whereas the four cell lines that failed to bind PPME beads in an EDTA-sensitive manner were negative for MEL-14 antigen expression and PN HEV attachment. Moreover, when we selected variants of the S49 cell line for PPME-bead binding, there was a highly correlated change ($r = 0.974$) in the level of the MEL-14 antigen at the cell surface during both positive and negative selections. The ability of these cells to bind PN HEV also changed in parallel (Yednock et al., 1987). The specificity of the changes in MEL-14 antigen expression was striking in that several other cell surface antigens did not change during the selections.

Taken together, these results strongly argue that the MEL-14 antigen and the receptor for PPME beads are closely related if not identical molecules. To establish biochemical identity conclusively, it will be necessary to demonstrate that the isolated antigen can bind to PPME with the same characteristics found for the cell surface receptor. The PN HEV-binding factor isolated from lymph (Chin et al., 1980, 1982) and presumed to be a shed lymphocyte homing receptor should also be tested for PPME binding.

It is likely that M6P interacts at an active site of a receptor involved in lymphocyte homing because: (a) M6P and the M6P-rich polysaccharide, PPME, potently inhibit lymphocyte attachment to PN HEV and (b) the binding of PPME beads to cells closely mimics the attachment of lymphocytes to PN HEV (Yednock et al., 1987). Soluble PPME, even at great excess with a minimally detectable level of MEL-14, does not affect MEL-14 binding to the cell surface. Thus, the presumptive active site where PPME binds is probably distinct from the antigen epitope for MEL-14. Recently it has been shown that the glycoprotein recognized by MEL-14 is covalently modified with ubiquitin, an 8-kD peptide associated with a number of cytoplasmic and cell-surface proteins (Siegelman et al., 1986). Ubiquitin, apparently in a rare conformation, forms part of the epitope recognized by MEL-14. It is plausible that MEL-14 binding to this determinant either sterically hinders or induces conformational changes in the physically separate active site of the receptor. Fucoidin, which inhibits MEL-14 binding to the cell surface, may interact directly with the ubiquitin-dependent determinant and block receptor activity by an indirect mechanism analogous to that of the MEL-14 antibody. Consistent with the proposal that fucoidin interacts at sites different from or additional to PPME are the recent findings that the binding of fucoidin to lymphocytes is not selectively inhibited by M6P or FIP, nor does it require calcium (Brandley et al., 1985, and personal communication). It is noteworthy that fucoidin has been reported to interact with several known and putative adhesive proteins (Roberts et al., 1985, 1986; Bolwell et al., 1980; Grabel et al., 1983; Glabe et al., 1982, 1983; Huang and Yanagimachi, 1984; Ahuja, 1982; Töpfer-Petersen et al., 1985) in a variety of systems ranging from sea urchin fertilization to aggregation of teratocarcinoma stem cells. Perhaps the adhesion molecules involved in this diverse collection of systems are ubiquitinated, or share a similar site for ubiquitination with which fucoidin can interact. Clearly, other explanations of fucoidin's activity in the lymphocyte recirculation system can be suggested and further analysis is therefore required.

The MEL-14 antigen and the lectin-like receptor identified by PPME beads were previously implicated as candidates for homing receptors by two independent approaches. The results reported here establish a close relationship, if not identity, between these two molecules. The convergence of these two disparate lines of evidence adds considerable weight to the proposed cell adhesion function of these molecules. Furthermore, the knowledge that carbohydrate recognition is in-

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togonal to the function of a homing receptor should greatly expedite further analysis of the undoubtedly complex adhesive system underling lymphocyte attachment to HEV.

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