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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.

**D**URING 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<sup>1</sup> (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

1. *Abbreviations used in this paper:* FACS, fluorescence-activated cell sorting; M6P, D-mannose-6-phosphate; HEV, high endothelial venule(s); PN, peripheral lymph node; PPME, phosphomannan monoester core from *Hansenula hostii* Y-2448.

gut-associated lymphoid organ; and (c) MEL-14 selectively inhibits *in vivo* localization of lymphocytes to peripheral lymph nodes.

Independent studies by us have indicated that a carbohydrate-binding receptor on the surface of lymphocytes is involved in lymphocyte attachment to PN HEV (Stoolman and Rosen, 1983; Stoolman et al., 1984). This receptor has been demonstrated directly on the lymphocyte cell surface with a solid-phase probe consisting of fluorescent beads covalently derivatized with the mannose-6-phosphate (M6P)-rich polysaccharide PPME (Yednock et al., 1987). As shown in the companion study (Yednock et al., 1987), the interaction of lymphocytes with PPME beads (monitored by flow cytometry) closely mimics lymphocyte attachment to PN HEV (measured in the Stamper-Woodruff *in vitro* assay [Stamper and Woodruff, 1976]): (a) both interactions are selectively inhibited by the structurally related monosaccharides M6P and fructose-1-phosphate and by the M6P-rich polysaccharide, PPME; (b) fucoidin, a sulfated fucose-rich polysaccharide is an extremely potent inhibitor in both assays; (c) mild treatment of lymphocytes with trypsin eliminates both interactions; (d) both interactions require calcium; (e) thymocytes and the thymic lymphoma cell line, S49, exhibit 10-fold weaker binding than mature lymphocytes to PPME beads and to PN HEV; (f) selection of S49 variants for their ability to bind PPME beads produces parallel changes in their ability to bind 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 (H3125), 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. (Plainview, NY). The yeast mannan, mnn 1, 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 (Slodki et al., 1973; Kaplan et al., 1978), was a generous gift of Dr. M. E. Slodki (U.S. Department of Agriculture, Northern Regional Research Center, Peoria, IL). Disodium EDTA and boric acid were obtained from Mallinckrodt 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 (Gallatin et al., 1983): RAW 112 (Abelson virus-induced, Balb/c, pre-B cell lymphoma), 38C-13 (cloned C3H/eb, B cell lymphoma), Sp 20 (fusion-partner myeloma), and EL4 (chemical carcinogen-induced, C57BL, T cell lymphoma). 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 NaHCO<sub>3</sub>, 2mM L-glutamine, 1 mM Na pyruvate, nonessential amino acids, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2-mercaptoethanol ( $1 \times 10^{-5}$  M). The cells were grown at 37°C in a humidified incubator with 5% CO<sub>2</sub> and were diluted from 1:5 to 1:20 into fresh medium every 3–4 d. S49 (Balb/c, T cell lymphoma, ATCC No. TIB 28) was obtained from the Cell Culture Facility at the University of California,

San Francisco. This cell line, and the variants selected from it, were grown in DME-H16 medium, containing 3 g/liter glucose, 3.7 g/liter NaHCO<sub>3</sub>, 0.11 g/liter Na pyruvate, 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 CO<sub>2</sub>, and were subcultured every 3–4 d by a 1:14 dilution into fresh medium (maximum density of 10<sup>6</sup> cells/ml).

### Preparation of Cell Suspensions

Peripheral lymphocytes were obtained from mesenteric lymph nodes of cervically 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 Dulbecco's PBS containing 1 mg/ml BSA to release lymphocytes. After centrifugation (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  $\sim 1.4 \times 10^{-16}$  g of PPME/bead corresponding to 64,000 M6P moieties/bead. Heparin beads contain  $\sim 1.6 \times 10^{-16}$  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 S49 cell lines, the procedures were essentially the same except that the assay was performed in modified S49 growth medium (containing 10 mM HEPES, rather than NaHCO<sub>3</sub>, 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 Analyzer; Becton-Dickinson & Co., Paramus, NJ) 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 nonderivatized 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

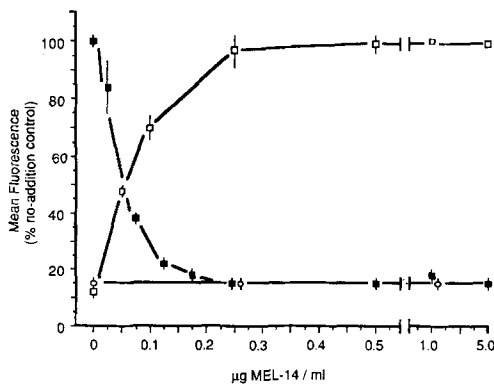
Cells were exposed to antibodies as described above, washed by centrifugation through PBS containing 7% BSA and 1 mM sodium azide, and resuspended in a 1:10 dilution of fluorescein-conjugated second antibody (goat anti-rat IgG; cat. No. F6258; Sigma Chemical Co.) in PBS with 5% mouse serum and 1 mM sodium azide. After 15 min at 4°C the samples were washed, resuspended in PBS containing 1 mM sodium azide, and analyzed 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 correspondence with the level of MEL-14 bound to the cell surface. MEL-14 had no effect on the attachment of nonderivatized beads to lymphocytes.

In contrast to MEL-14, a series of antibodies that recognize



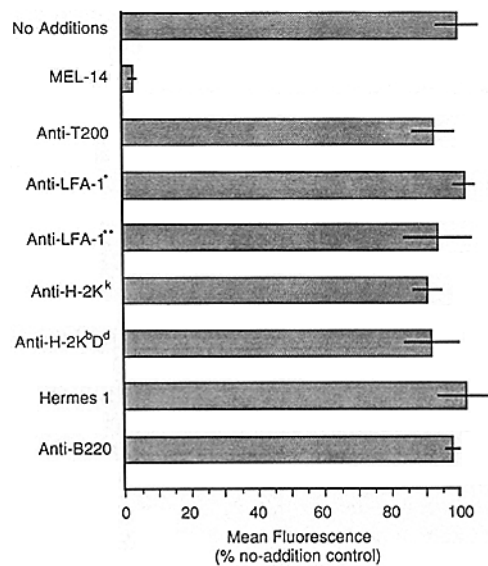
**Figure 1.** Effect of MEL-14 on the attachment of mouse lymphocytes to PPME beads. Lymphocytes were exposed to PPME beads (solid squares) or nonderivatized beads (open circles) in the presence of the indicated concentration of MEL-14 antibody. Bead binding (mean fluorescence) is expressed as a percentage of PPME-bead binding in the absence of MEL-14. The level of MEL-14 bound to independent samples of lymphocytes (open squares) was determined at varying MEL-14 concentrations. These values are expressed as a percentage of the fluorescence value of lymphocytes exposed to a saturating concentration of MEL-14. Bead and MEL-14 attachment were quantified by flow cytometry as described in Materials and Methods.

other lymphocyte cell-surface antigens did not affect PPME-bead binding (Fig. 2), despite the fact that several of these antibodies bound to the cell surface at higher levels than MEL-14 (see legend to Fig. 2). The negative antibodies included two that are directed against the lymphocyte function-associated antigen No. 1 (LFA-1) complex. This complex has been implicated in several immune adherence functions of lymphocytes, and the particular anti-LFA-1 antibodies are known to block T cell-mediated cytolysis (Davignon et al., 1981; Sarmiento et al., 1982; Springer et al., 1982).

The possibilities existed that MEL-14 prevented PPME-bead binding to lymphocytes by associating with the beads rather than the cells or as a consequence of a contaminant in the antibody preparation. These questions were addressed by comparing the effects of MEL-14 on PPME-bead binding to mouse and rat lymphocytes. The MEL-14 antibody, which was produced in rat, does not react with rat lymphocytes. However, PPME beads bind to the same degree and with the same apparent carbohydrate specificity to lymphocytes from both rat and mouse, as well as from humans (Stoolman, L. M., T. A. Yednock, and S. D. Rosen, manuscript submitted for publication). As shown in Fig. 3, MEL-14 inhibited bead attachment to mouse lymphocytes but had no effect on their attachment to rat lymphocytes. These results argue that MEL-14 exerts an inhibitory effect on PPME-bead binding by specifically reacting with its antigen on the mouse lymphocyte surface.

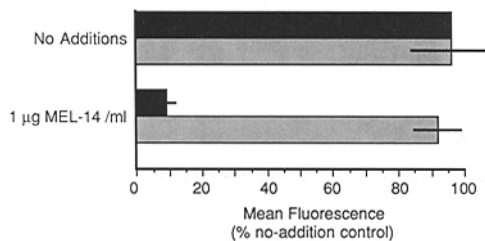
#### **Inhibition of Antibody Binding by Polysaccharides**

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

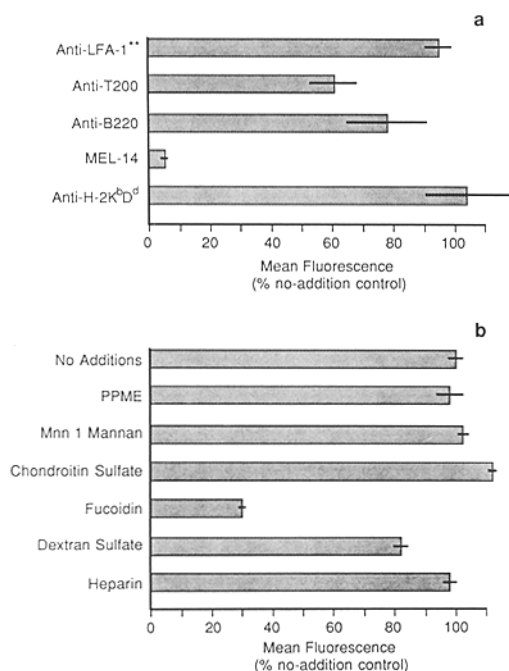


**Figure 2.** Effect of eight monoclonal antibodies on lymphocyte attachment to PPME beads. All antibodies were present at saturating concentrations 30 min before and then during exposure of lymphocytes to PPME beads (diluted 1:200). PPME-bead binding (in the absence of antibody) was 10 times that of nonderivatized-bead binding. The level of nonderivatized-bead binding was subtracted from all values to yield an index of specific PPME-bead binding. Fluorescence values (x-axis) were computed as percentages of specific PPME-bead binding in the absence of antibodies (set at 100%). The modal fluorescence (log-based scale such that an increase of 23 U corresponds to a twofold increase in fluorescence intensity) of peripheral lymphocytes (BALB/c) treated with the antibodies, and then with a second, fluorescent antibody (see Materials and Methods) were as follows: MEL-14 (rat monoclonal, IgG2a; Gallatin et al., 1983) - 130; Anti-T200 (30G 12, rat monoclonal, class-matched with MEL-14; Gallatin et al., 1983) - 150; Anti-LFA-1\* (FD 18.5, rat monoclonal; Sarmiento et al., 1982) - 130; Anti-LFA-1\*\* (FD 441.8, ATCC No. TIB 213, rat monoclonal; Sarmiento et al., 1982) - 130; Anti-H-2K<sup>k</sup> (11-4.1, ATCC No. TIB95, mouse monoclonal negative control, recognized by anti-rat second antibody) - 70; Anti-H-2K<sup>b</sup>D<sup>d</sup> (34.1.2S, ATCC No. HB79, mouse monoclonal, recognized by anti-rat second antibody) - 150; Hermes 1 (rat monoclonal negative control, class-matched with MEL-14, gift of Dr. S. Jalkanen [Stanford University, Stanford, CA]) - 60; Anti-B220 (RB6-6B2, gift of Dr. R. L. Coffman [DNAX Laboratories, Palo Alto, CA], rat monoclonal that recognizes B cell-specific form of the T200 glycoprotein family; Coffman, 1983) - 100; second antibody alone - 70. The anti-LFA-1 antibodies used here have been shown to block both antigen-specific T cell-mediated cytolysis and lectin-facilitated T cell-mediated cytolysis (Sarmiento et al., 1982).

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 preexposed to fucoidin (1 mg/ml) 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 fluorescent intensity of cells exposed to second antibody alone was subtracted from all values, and the level of specific binding of each antibody in the absence of fucoidin was set at 100%. (b) Lymphocytes were preexposed to the indicated polysaccharide at 1 mg/ml for 30 min at 4°C and then to 0.2 µg MEL-14/ml (slightly subsaturating) in the presence of the polysaccharide for an additional 30 min at 4°C. The cells were then washed and processed for immunofluorescence analysis. The fluorescence level of cells exposed to the second antibody alone was one-tenth that of cells first exposed to MEL-14 (in the absence of polysaccharide). This background signal was subtracted from all values to yield the level of specific MEL-14 binding. Values were computed as percentages of the control level of MEL-14 binding (in the absence of polysaccharide). None of the polysaccharides affected cell viability, as determined by trypan blue exclusion.

**Table I.** Six Mouse Lymphoma Cells 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

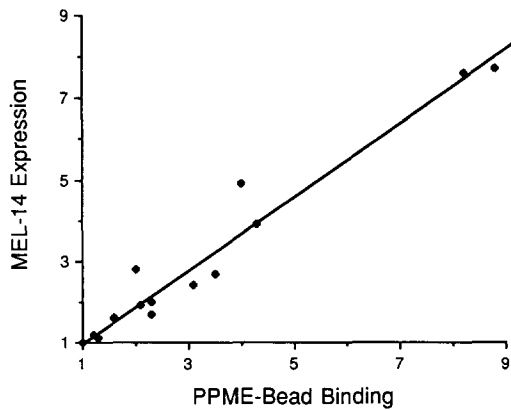
Cell type	Level of EDTA-sensitive PPME-bead binding	MEL-14 expression	PN HEV binding
	%	%	
Lymphocytes	100 ± 11	100	+
38C-13	426 ± 30	569	+
S49	23 ± 3	11	±
RAW 112	0	0	-
EL4	0	0	-
BW 5147	0	0	-
Sp20	0	0	-

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 Stamper-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  $2 \times 10^7$  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.

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

### Properties of Lymphoma Cell Lines

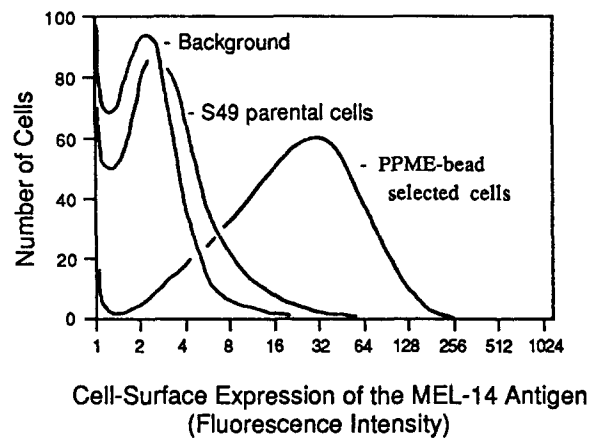
Six lymphoma cell lines were compared with normal mouse peripheral lymphocytes (isolated from mesenteric lymph nodes) for their ability to bind to PPME beads, their expression of the MEL-14 antigen, and their ability to bind to PN HEV. The attachment of normal lymphocytes to PPME beads as well as their 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 ~fourfold 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.



**Figure 5.** Correlation between the ability of PPME-bead-selected variants of the S49 cell line to bind PPME beads and expression of the MEL-14 antigen. S49 was subjected to a serial selection procedure for PPME-bead binding with FACS as described in the companion study. On 14 separate occasions during the course of selection, cell line isolates were tested concurrently for PPME-bead binding and MEL-14 antigen expression. In each case, the S49 parental line was also examined for use as a standard reference. Cells were incubated in the presence or absence of 0.5  $\mu\text{g}$  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).

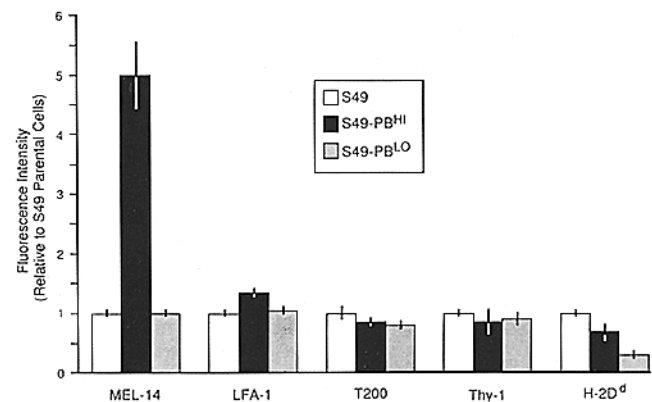
To investigate this correlation further, we examined S49 cells that had been subjected to a serial selection procedure for PPME-bead binding with FACS (see companion study, Yednock et al., 1987). During the course of this selection protocol, 14 cell-line isolates were evaluated for PPME-bead binding 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-PB<sup>HI</sup>, that was obtained after seven rounds of selection. S49-PB<sup>HI</sup> 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-PB<sup>HI</sup> cells expressed a



**Figure 6.** Binding of MEL-14 antibody to S49 parental cells and to variant S49 cells obtained after nine rounds of selection with PPME beads. Cells were incubated in the presence or absence of MEL-14 at 0.5  $\mu\text{g}/\text{ml}$  (a saturating concentration) for 30 min on ice and were then exposed to a fluorescent second antibody as described in Materials and Methods. Shown here are direct tracings of fluorescence histograms generated by analysis of the samples with flow cytometry. Fluorescence intensity (a logarithmic function) is plotted on the x-axis against the number of cells at each fluorescence level (y-axis). Mean fluorescence values for each sample were electronically integrated and were as follows: cells (S49 or the selected variant) exposed to second antibody alone (background), 2.0; S49 parental cells exposed to MEL-14, 2.9; variant S49 cells exposed to MEL-14, 19.9. After subtraction of the background value to determine the specific degree of MEL-14 expression, it was calculated that the variant cell line expressed a 20-fold higher mean level of the MEL-14 antigen than the parental S49 line.

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-PB<sup>HI</sup> cells was completely inhibited by MEL-14, whereas the binding of beads derivatized with heparin or



**Figure 7.** Expression of five cell-surface antigens by PPME-bead-selected variants of the S49 cell line. S49, S49-PB<sup>HI</sup>, and S49-PB<sup>LO</sup> were exposed to saturating concentrations of the indicated antibodies, and were then exposed to a second fluorescent antibody as described in Materials and Methods. The level of fluorescence was determined by flow cytometry and the fluorescence of cells exposed to second antibody alone was subtracted from all values. For each antibody, the level of binding to S49-PB<sup>HI</sup> or S49-PB<sup>LO</sup> is expressed relative to the level of binding to the parental S49 cell line (normalized to unity). Anti-Thy-1.2 (monoclonal antibody 30-H12, ATCC No. TIB 107) was a generous gift of Dr. A. L. Defranco (University of California, San Francisco). All other antibodies were obtained as described in the legend of Fig. 2.

**Table II. Effect of MEL-14 on the Attachment of S49-PB<sup>H1</sup> Cells to Beads Derivatized with PPME, Heparin, or Sulfated Galactan**

Bead type	Level of control binding to S49-PB <sup>H1</sup>	Level of binding to S49-PB <sup>H1</sup> treated with MEL-14
	%	%
PPME	100 ± 2	1 ± 1
Heparin	352 ± 5	334 ± 9
Sulfated galactan	84 ± 6	74 ± 7

S49-PB<sup>H1</sup> 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 sulfated galactan, k-carrageenan, was not affected (Table II). When S49-PB<sup>H1</sup> was subjected to a back selection procedure with PPME beads, the resulting cell line variant (S49-PB<sup>L0</sup>) was found to exhibit the low parental levels of PPME-bead binding and PN HEV attachment activities (Yednock et al., 1987). Furthermore, S49-PB<sup>L0</sup> cells expressed the original low level of the MEL-14 antigen (Fig. 7). In contrast to these changes in the MEL-14 antigen, neither the positive nor the back selection procedures with PPME beads affected the cell surface expression of the LFA-1, T-200, Thy-1, or H-2D<sup>d</sup> antigens (Fig. 7). These results establish that selection of cells for their ability to bind to PPME beads results in a specific and highly correlated change in the cell surface level of the MEL-14 antigen.

## Discussion

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-PB<sup>H1</sup> 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-

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

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