GERMINAL CENTER B CELLS LACK HOMING RECEPTORS NECESSARY FOR NORMAL LYMPHOCYTE RECIRCULATION*

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Germinal centers are discrete sites of lymphoid blastogenesis that arise within the follicles of lymphoid organs in response to antigenic stimulation. They consist predominantly of dividing B cells (1), but also contain interspersed macrophages and antigen-presenting follicular dendritic cells (2, 3), as well as small numbers of T cells (4) of helper (Lyt-1^+,2^-) phenotype (5). The differentiation pathway(s) and the nature of the B lymphoblasts in germinal centers have been a mystery due to the absence of a specific cell surface marker that would allow their study in cell suspensions. However, such a marker for germinal center lymphoid cells (GCLC)[1] has recently been identified: Peanut agglutinin (PNA), a plant lectin with specificity for terminal galactosyl residues on cell surface oligosaccharides, binds selectively to GCLC in antigen-stimulated mouse lymphoid organs (1, 6, 7). Hence, GCLC can be identified in cell suspensions as a distinct "PNA^{hi}" B cell population, binding 10–30 times as much PNA as other B cells and most T cells, which are therefore designated "PNA^{lo}" (1, 7). Most plasma cells and plasmablasts are also PNA^{lo} (1, 7).

We have recently used PNA in conjunction with other markers to study the surface phenotype of GCLC (1). Peyer's patch (PP) GCLC bear low levels of surface immunoglobulin (Ig), with IgA being the major isotype expressed (1). The surface IgA^+, IgM^-, IgD^- phenotype of most GCLC in this site is strikingly different from the IgA^-, IgM^+, IgD^+ phenotype of the smaller PNA^{lo} PP B cells, but perhaps more than coincidentally similar to that characterizing IgA plasma cell precursors as studied in rabbit PP (8) and rat thoracic duct lymph (9). GCLC in immunized peripheral lymph nodes are also IgD^- (7), and are thought to contain progenitors of IgG plasma cells (7, 10, 11). Germinal centers in these nodes can be induced with specified antigens, allowing us to better characterize the responses taking place within the germinal center. Our studies, consistent with earlier investigations (12), have shown that many GCLC are specific for the immunizing antigen (7). In addition, we have presented evidence suggesting that GCLC undergo antigen-driven differentiation (specifically,

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[1] *Abbreviations used in this paper:* CSM, cell-suspending medium; FITC, fluorescein isothiocyanate; GCLC, germinal center lymphoid cells; HEV, high endothelial venules; MNL, mesenteric node lymphocytes; PBS, phosphate-buffered saline; PNA, peanut agglutinin; PNL, peripheral (axillary, brachial, and/or inguinal) node lymphocytes; PP, Peyer's patch, PPL, Peyer's patch lymphocytes; RAR, relative adherence ratio; SAR, specific adherence ratio; TRITC, tetramethylrhodamine isothiocyanate.

heavy chain class switching upon reexposure to antigen) under the influence of the germinal center microenvironment (7). Finally, this microenvironment has been implicated as a site for the generation of memory B cells (10, 11); this possibility is now being tested using purified PNA^{hi} cells in adoptive transfer experiments.

The present studies extend our examination of GCLC to another aspect of their differentiative state—their migratory capability. The vast majority of mature peripheral lymphocytes migrate continuously between various lymphoid organs, traveling from the lymph into the systemic vasculature, then leaving the bloodstream to reenter lymphoid tissues (13). This recirculation pattern presumably allows the whole spectrum of lymphocytes with predetermined specificities to encounter and react to localized antigen deposits. The traffic of lymphocyte populations can be directed preferentially through either mucosal or nonmucosal lymphoid organs by virtue of selective recognition of organ-specific determinants on high endothelial venules (HEV), the specialized vessels in lymphoid organs that serve as major sites of lymphocyte emigration from the blood (14, 15). If PP GCLC are precursors of IgA plasma cells, as their expression of surface IgA seems to suggest (1, 16), then it might reasonably be expected that they would be preprogrammed to migrate selectively to mucosal-associated tissues (i.e., sites of IgA secretion). Such selective migration might be directed by the expression of recognition receptors for organ-specific determinants associated with endothelial cells in mucosal tissues, as proposed for gut-homing mesenteric node and thoracic duct immunoblasts (14, 17). On the other hand, a variety of experiments have indicated that "germinal center cells stand as islands amidst the general flow and traffic of migrating cells" (18). Although GCLC and certain immunoblasts are thought to be capable of entering germinal centers when applied locally (19, 20), cellular exchange between germinal centers and the surrounding pool of lymphocytes is in general remarkably difficult to detect (18). This apparent segregation of germinal centers suggests that GCLC may not be competent to recirculate, and may in fact lack the normal migratory mechanisms that most small lymphocytes possess.

The studies reported here support the view that germinal centers harbor B cells undergoing a nonmigratory stage of antigen-activated differentiation. Our findings indicate that GCLC are unable to migrate normally into lymphoid tissues, and suggest that this migratory deficiency is due in large part to their inability to recognize and bind to the post-capillary HEV that normally mediate the passage of recirculating blood lymphocytes into lymphoid organs.

Materials and Methods

Animals. 2–3-mo-old BALB/c and BALB.K ($H\text{-}2^k$) mice were bred and maintained in our colony.

Preparation of Cell Suspensions. Cell handling procedures and cell suspending medium (CSM) have been described (15, 21).

Enrichment for GCLC by Panning. GCLC, which comprise up to 20–30% of the lymphocytes in PP (1, 6), were isolated from suspensions of PP lymphocytes (PPL) by taking advantage of their unique IgD^-, PNA^{hi} phenotype. A two-stage "panning" (22) procedure was used. First, petri dishes (100 × 15 mm, 1029; Falcon Labware, Oxnard, CA) were coated with a mouse monoclonal antibody directed against IgD (anti-Igh-5a, clone H10-4-22 [23], purified by adsorption and elution from a protein A-Sepharose column) at 50 μg/ml in 0.15 M NaCl, 0.05 M Tris-HCl, pH 9.5. 3×10^7 PPL per plate were incubated at 4°C for 40 min, after which the plates were gently swirled and allowed to incubate for another 30 min. The nonadherent IgD^-

cells (predominantly T cells and GCLC) were then recovered and positively selected by incubation on dishes coated with PNA (E. Y. Laboratories, San Mateo, CA) at 50 μg/ml. The plates were decanted and washed gently, and the bound PNA^{hi} cells were then eluted by incubating the dishes for 15 min at room temperature with 0.2 M galactose in phosphate-buffered saline (PBS) containing 2% newborn calf serum. A pasteur pipette was used to squirt the plates vigorously to further release the adherent cells. Finally, the purified cells (90–95% PNA^{hi}, 90–100% viable) were washed through serum and resuspended in CSM. Roughly 2×10^6 purified GCLC were recovered per plate, representing a 25–35% recovery of PNA^{hi} cells.

Suspension Staining for Identification of GCLC IgD^+ B Cells, and T Cells. Using techniques described previously (15), cell suspensions were analyzed by fluorescence microscopy to detect surface markers characteristic of different lymphocyte subpopulations. Fluoresceinated PNA (fluorescein isothiocyanate (FITC)-PNA) (E. Y. Laboratories) was used as a marker for GCLC, while IgD^+ B cells were detected using purified, biotinylated H10-4-22 (see above) as a first stage, followed by an FITC-avidin second stage (Vector Laboratories, Inc., Burlingame, CA). T cell reagents included first stage monoclonal rat antibodies directed against Thy-1.2 (clone 30-H12) and Lyt-1 (clone 53-7.3) (24), followed by an affinity-purified, highly specific fluoresceinated rabbit anti-rat Ig second stage. For simultaneous staining of T cells and IgD^+ B cells (a procedure that identifies GCLC by their lack of fluorescence), anti-IgD, anti-Thy-1.2, and anti-Lyt-1 reagents were simply mixed together.

Tissue Section Staining. Brachial lymph nodes were removed 7 d after front footpad immunization with $1–2 \times 10^7$ sheep erythrocytes. Frozen sections of these antigen-stimulated lymph nodes and of PP were air-dried for 5 min, fixed in cold acetone for 2 min, and then incubated successively with (*a*) 150 μg/ml MEL-14, a rat monoclonal antibody directed against the putative lymphocyte receptor for peripheral node HEV (25), (*b*) a second stage rabbit anti-rat Ig at 200 μg/ml, and (*c*) a third stage horseradish peroxidase-coupled swine anti-rabbit Ig antiserum (Dako Corp., Santa Barbara, CA; used at 1:2). IgD^+ B cells and/or germinal centers were defined in serial sections using biotinylated anti-IgD (H10-4-22) and biotin-conjugated PNA (E. Y. Laboratories), respectively, followed by horseradish peroxidase-conjugated avidin (Vector Laboratories, Inc.) as a second stage. Incubations and development of staining with substrate were carried out as described elsewhere (1).

Direct Fluorescent Labeling of Cells. Using methods reported previously (15), lymphocytes were labeled with FITC or tetramethylrhodamine isothiocyanate (TRITC) under conditions which produced intense cellular fluorescence without altering cell viability, migratory capacity, or detectability of cell surface antigens.

In Vivo Homing Studies

Two different protocols were used to study the localization of GCLC in vivo:

HOMING STUDIES USING ^{51}CR-LABELED LYMPHOCYTES. Sample populations (PNA^{hi} PPL, PNA^{lo} PPL, unseparated PPL, or mesenteric node lymphocytes [MNL]) were labeled by incubation for 1 h at 37°C in 100 μCi Na $^{51}CrO_4$/2×10^7 cells/ml CSM. After washing through a large cushion of serum, labeled cells from each sample population ($2–5 \times 10^6$ cells carrying 20,000–35,000 cpm) were injected intravenously into each of two to three syngeneic recipients. Radioactivity in recipient organs was determined 2 h later. The following host organs were removed and analyzed: pooled lymph nodes (axillary, brachial, inguinal, and mesenteric nodes), PP, small intestine, spleen, lung, and liver. Results are expressed as the percent of injected cpm retained by an organ: [(organ count − background)/(total counts injected)] × 100.

HOMING STUDIES USING TRITC-LABELED LYMPHOCYTES. 2×10^7 TRITC-labeled unseparated PPL or antigen-stimulated peripheral node lymphocytes (PNL) were injected intravenously into syngeneic recipients. 2 and 24 h later, separate cell suspensions were made from recipient lymphoid organs (peripheral lymph nodes, PP, mesenteric node, spleen, and bone marrow). Aliquots of these suspensions were counterstained with fluoresceinated reagents to determine the proportions of GCLC and other lymphocyte subpopulations among the localized rhodaminated sample cells. These proportions were then compared with the proportion of the corresponding lymphocyte subpopulation in the injected sample population.

In Vitro Assay of Lymphocyte Binding to HEV. This technique, modified from the system of

Stamper and Woodruff (26), has been described previously in detail (21). Briefly, lymphocytes in Hanks' balanced salt solution containing 1% bovine serum albumin were incubated with mild agitation for 30 min at 7°C on freshly cut frozen sections of lymph nodes or PP. After incubation, adherent cells were fixed to the sections by placing the slides in cold PBS containing 1% glutaraldehyde. After fixation, the nonadherent cells were gently rinsed off.

Two variations of this in vitro assay were performed to determine the HEV-binding properties of GCLC: (*a*) Unseparated PPL were incubated on PP and peripheral node sections. The proportion of PNA^{hi} and PNA^{lo} cells bound to HEV was then determined by directly staining the sections and adherent cells with FITC-PNA. As described elsewhere (15, 21), thinner sections (6 instead of 10 μm) and a milder fixing procedure were used to minimize autofluorescence and to render PNA^{hi} and PNA^{lo} cells easily distinguishable from one another. (*b*) PNA^{hi} and PNA^{lo} PPL, fractionated by panning as described above, were incubated on separate sets of frozen sections. To allow quantitative comparisons, an internal standard population of FITC-labeled MNL was mixed in roughly equal proportion with each sample population before incubation. The ratio of viable sample to standard cells (R_I) was determined by fluorescence-activated cell sorter (B-D FACS Systems, Becton, Dickinson & Co., Sunnyvale, CA) analysis as previously described (21). Following incubation, the ratio of sample to standard cells bound to HEV (R_{HEV}) was determined. Data were pooled from multiple sections. A specific adherence ratio (SAR) was calculated for each sample ($SAR = R_{HEV}/R_I$). To allow direct comparisons between sample populations, the HEV adherence of the PNA^{hi} and PNA^{lo} PPL was related via a relative adherence ratio (RAR) to the HEV adherence of a reference population of unseparated PPL that had been incubated on a separate set of sections in each experiment. For example, the RAR (of PNA^{hi} to unseparated PPL) equals $SAR_{PNA^{hi}PPL}/SAR_{\text{unseparated PPL}}$. The RAR is equivalent to the calculated number of sample cells bound to HEV per reference cell bound under the same conditions.

Fluorescence Microscopy. A Zeiss microscope equipped with HBO 50 W mercury vapor lamp epi-illumination and exciter/barrier filter combinations for rhodamine (546 nm excitation) and fluorescein (440–490 nm excitation) was used.

Statistical analyses. Calculation of standard errors of cell suspension counting data (e.g., the proportion of PNA^{hi} cells in unseparated PPL) was based on the binomial distribution. Standard errors of ratios (e.g., RAR) were calculated using the delta method (27).

Results

In Vivo Localization of GCLC after Intravenous Injection. For technical reasons, we have chosen to focus our attention primarily on the migratory properties of PP rather than peripheral node GCLC. Due to the continuous exposure of PP to a variety of intestinal antigens, germinal centers can regularly be found in this site, with GCLC comprising 10–30% of the lymphocyte population (1, 6). Furthermore, GCLC can be confidently identified in PP cell suspensions solely on the basis of their PNA^{hi} phenotype, whereas PNA must be used in conjunction with other markers to identify GCLC in lymph nodes and spleen because these organs contain a significant number of PNA^{hi} T cells (7).

As shown in Table I, ^{51}Cr-labeled PNA^{hi} PPL home very poorly to lymph nodes, PP, and the spleen in comparison with PNA^{lo} PPL, unseparated PPL, and MNL. The small amounts of radioactivity that were detected in the lymph nodes and PP of PNA^{hi} PPL recipients could be explained by the expected localization pattern of the 10% T cell and IgD^+ B cell contaminants in the panned PNA^{hi} fraction. PNA^{hi} PPL also migrate to the gut wall in very small numbers, as do the small lymphocyte populations studied, whereas other investigators have shown that many immunoblasts from the thoracic duct lymph and mesenteric node home rapidly and specifically to this site (reviewed in 28). One factor which may contribute to the migratory deficiency

TABLE I

*In Vivo Localization Pattern of ^{51}Cr-labeled PNA^{hi} PPL (GCLC) in Comparison with other Lymphocyte Populations**

Recipient organ	Mean percent of injected cpm localized ± SE‡ after injection of			
	PNA^{hi} PPL	PNA^{lo} PPL	Unseparated PPL	MNL
Lymph nodes§	0.23 ± 0.03	2.8 ± 0.4	2.1 ± 0.3	7.0 ± 0.9
PP	0.17 ± 0.02	1.1 ± 0.2	1.1 ± 0.2	2.3 ± 0.3
Small intestine¶	0.32 ± 0.04	0.33 ± 0.05	0.54 ± 0.08	0.54 ± 0.08
Spleen	8.7 ± 1.1	19 ± 2.9	18 ± 2.7	31 ± 3.9
Lung	7.1 ± 0.9	5.3 ± 0.8	5.3 ± 0.8	2.1 ± 0.3
Liver	32 ± 4.1	21 ± 3.3	19 ± 3.0	17 ± 2.2
Number of recipients	3	2	2	3
Cpm injected per recipient	32,400	20,300	34,700	33,400

* ^{51}Cr-labeled lymphocytes were injected intravenously into syngeneic recipients, and the radioactivity in recipient organs was determined 2 h later. Sample populations included (*a*) PNA^{hi} PPL (90 ± 1.3% PNA^{hi}) purified using the two-stage panning procedure described in Material and Methods, (*b*) unseparated PPL (18 ± 1.7% PNA^{hi}), and (*c*) MNL. PNA^{lo} PPL, recovered by decanting the unbound PPL fraction after panning with PNA alone, were included primarily as a control for cell handling. Removal of PNA^{hi} cells was incomplete, with the PNA^{lo} fraction still containing 6 ± 1.0% PNA^{hi} cells.

‡ Standard error = $\bar{x}$ $(0.221/\sqrt{n})$, where $\bar{x}$ is the mean percent of injected cpm localized in a recipient organ, n is the number of recipients, and 0.221 is the result of an analysis of variance performed on log-transformed data.

§ Pooled axillary, brachial, inguinal, and mesenteric lymph nodes.

¶ After removal of PP.

TABLE II

*PPL Localizing in Lymphoid Tissues are Predominantly Non-GCLC**

Recipient Organ	Phenotype of localized donor PPL			
	Percent PNA^{hi}‡		Percent IgD^+§	Percent Thy-1.2^+, Lyt-1^+, or IgD^+§
	2 h	24 h	24 h	24 h
Peripheral lymph nodes¶	0.0 (<0.7)	0.0 (<0.7)	45 (2.2)	99 (0.4)
PP	0.0 (<0.7)	0.0 (<0.7)	88 (1.4)	99 (0.5)
Mesenteric lymph nodes	0.0 (<0.7)	0.0 (<0.7)	65 (2.1)	99 (0.5)
Spleen	1.0 (<2.3)	0.8 (<2.0)	68 (2.1)	98 (0.6)
Bone marrow	1.6 (<3.1)	1.2 (<2.6)	70 (2.1)	94 (1.3)

* 2×10^7 unseparated TRITC-labeled PPL (17 ± 1.7% PNA^{hi}, 45 ± 2.2% IgD^+, and 76 ± 1.9% staining with anti-Thy-1.2, anti-Lyt-1, and anti-IgD combined) were injected intravenously into each of four syngeneic recipients. At both 2 and 24 h after injection, cell suspensions were prepared from similar organs pooled from two recipients. The cells were counterstained with anti-IgD, anti-IgD plus anti-T cell, and/or fluorescein-indicated PNA reagents. For each organ and staining condition, 500 localizing donor lymphocytes were detected as red cells under a fluorescence microscope, and were assessed for staining with the fluoresceinated reagents by switching filter sets to visualize green fluorescence.

‡ Upper limits of 95% confidence intervals are given in parentheses.

§ Standard errors are given in parentheses.

¶ Unless otherwise indicated, "peripheral lymph nodes" refers to pooled axillary, brachial, and inguinal nodes.

of GCLC may be their preferential entrapment by the liver; many PNA^{hi} PPL localized in this organ despite the fact that these cells were >95% viable when injected.

One potential problem in interpreting these results is that the PNA^{hi} cells could have suffered some damage or alteration by being bound to the PNA-coated plates, thereby rendering them migratory incompetent and increasing their susceptibility to sequestration by the liver. However, confirmatory results were obtained in a complementary experiment in which unseparated TRITC-labeled PPL were injected intravenously into syngeneic recipients, with the surface phenotype of the localizing cells determined 2 and 24 h later (Table II). 17% of the injected PPL were PNA^{hi}, whereas none of the cells localizing in PP or lymph nodes, and only 1% of the TRITC-labeled cells found in the spleen bound high levels of PNA (500 localized cells were counted from each organ). It is possible that the GCLC actually were homing to lymphoid organs in these experiments, but were rapidly losing or masking their PNA-binding sites in vivo and therefore were not detected when counterstained. Since GCLC can also be identified as IgD^- B cells (1), this possibility was tested by counterstaining aliquots from recipient organ cell suspensions simultaneously with anti-IgD and anti-T cell (Thy-1.2 and Lyt-1) monoclonal antibodies, thus identifying GCLC by their lack of green fluorescence. 98–99% of the TRITC-labeled lymphocytes that localized in lymph nodes, PP, and spleen were stained by this procedure, indicating that T cells and IgD^+ B cells, but not GCLC, were migrating to these sites. In another experiment, GCLC in antigen-stimulated peripheral nodes exhibited a similar inability to home: 2 h after injection of PNL containing 13% PNA^{hi} B cells, few if any GCLC localized in HEV-bearing organs. In peripheral lymph nodes, only 3/700 localized cells scored were PNA^{hi}, whereas 495/500 stained with anti-T cell or anti-IgD reagents. In recipient PP, 0/600 were PNA^{hi}, and 497/500 stained with anti-IgD or T cell antibodies.

HEV-binding Ability of GCLC. The migratory insufficiency of GCLC could be due either (*a*) solely to the entrapment of these cells in the liver or other organs before they have an opportunity to migrate into lymphoid sites, or (*b*) to a lack of functional homing receptors, as might be evidenced by the diminished capacity of these lymphocytes to recognize and to adhere to the HEV which normally direct lymphocyte exit from the bloodstream. We tested the ability of GCLC to bind in vitro to HEV in frozen sections of PP and lymph nodes. In an experiment examining the in vitro binding of a PPL population containing 19% PNA^{hi} cells, only 2/500 cells adhering to PP HEV, and 1/500 cells bound to peripheral node HEV, were PNA^{hi}. In a complementary experiment, purified GCLC were shown to bind to HEV only 10% as well as unseparated PPL, a level of binding that could be caused by residual T cell and IgD^+ B cell contaminants. These results are presented in Table III. This in vitro analysis demonstrates that GCLC fail to recognize and bind to HEV, and thus lack an ability that appears strictly necessary for normal lymphocyte migration.

GCLC Fail to Express the Putative Homing Receptor Determinant Recognized by MEL-14. The failure of GCLC to adhere to peripheral node HEV might be explained at the biochemical level by their lack of expression of a surface "receptor" for peripheral node endothelial cells. Evidence supporting such a lack of receptor expression has been obtained using MEL-14, a monoclonal antibody that recognizes a cell surface determinant that either is closely and universally associated with, or is in fact on the lymphocyte receptor for peripheral node HEV (25). Immunohistologic studies of

TABLE III
GCLC Fail to Bind to HEV

Organ section	Relative binding to HEV (RAR)*			
	Counterstained PNA^{hi} PPL‡	Purified PNA^{hi} PPL§	PNA^{lo} PPL¶	Unseparated PPL
Peripheral lymph nodes	0.0 (<0.06)	0.1 (0.02)	0.9 (0.1)	Unity
PP	0.0 (<0.07)	0.1 (0.02)	0.8 (0.1)	Unity

* RAR is the calculated number of sample cells that would bind to HEV per unseparated PPL binding if an equal number of each were incubated on the sections under the same conditions.

‡ After incubation of unseparated PPL on the sections, PNA^{hi} cells were detected by counterstaining HEV-adherent PPL with FITC-PNA. 19 ± 1.8% of the unseparated PPL were PNA^{hi}. Upper 95% confidence limits are in parentheses.

§ These cells were purified using the two-stage panning procedure, and were 94 ± 1.2% PNA^{hi}. Standard errors are in parentheses.

¶ Nonadherent PPL recovered from PNA-coated plates (10 ± 1.4% PNA^{hi}) were included as a control for cell handling. Standard errors are in parentheses.

frozen sections of PP (Fig. 1) and antigen-stimulated peripheral nodes (Fig. 2) reveal that the vast majority of GCLC (PNA^{hi} lymphocytes) are MEL-14^-, whereas most other B and T cells in these sites are MEL-14^+. In Fig. 2 B, note that virtually the only cells in stimulated nodes which fail to stain with MEL-14 besides GCLC are those that reside within medullary areas. The nonstaining of these zones might have been predicted, since medullary areas consist predominantly of a delicate meshwork of resident plasma cells and reticular cells, and not of unstimulated, recirculating lymphocytes.

Discussion

We have demonstrated that GCLC home very poorly to lymphoid tissues in comparison with other lymphocyte populations. The principal features of these lymphoblasts that may render them migratory incompetent are (*a*) their inability to recognize and bind to HEV, which almost certainly precludes their migration into lymphoid tissues via the bloodstream, and (*b*) their possession of high levels of surface-binding sites for PNA—presumably terminal galactosyl residues (29)—which may make them susceptible to sequestration by the liver. These characteristic features of GCLC merit further discussion.

A likely explanation for the failure of GCLC to adhere to HEV when incubated on either PP or peripheral node sections is simply that these cells may lack the surface molecules necessary for lymphocyte-endothelial cell recognition. In fact, both PP and peripheral node GCLC fail to express detectable levels of the cell surface determinant recognized by MEL-14, a determinant that is on or near the lymphocyte surface receptor for peripheral node endothelial cells. An in-depth characterization of the distribution and the nature of the determinant(s) recognized by MEL-14 will be presented in a subsequent manuscript.[2]

We must reconcile the nonmigratory nature of GCLC with the concept that these lymphoblasts must eventually leave the germinal center microenvironment in order

[2] W. M. Gallatin, I. L. Weissman, and E. C. Butcher. A cell surface molecule involved in organ-specific homing of lymphocytes. Manuscript submitted for publication.

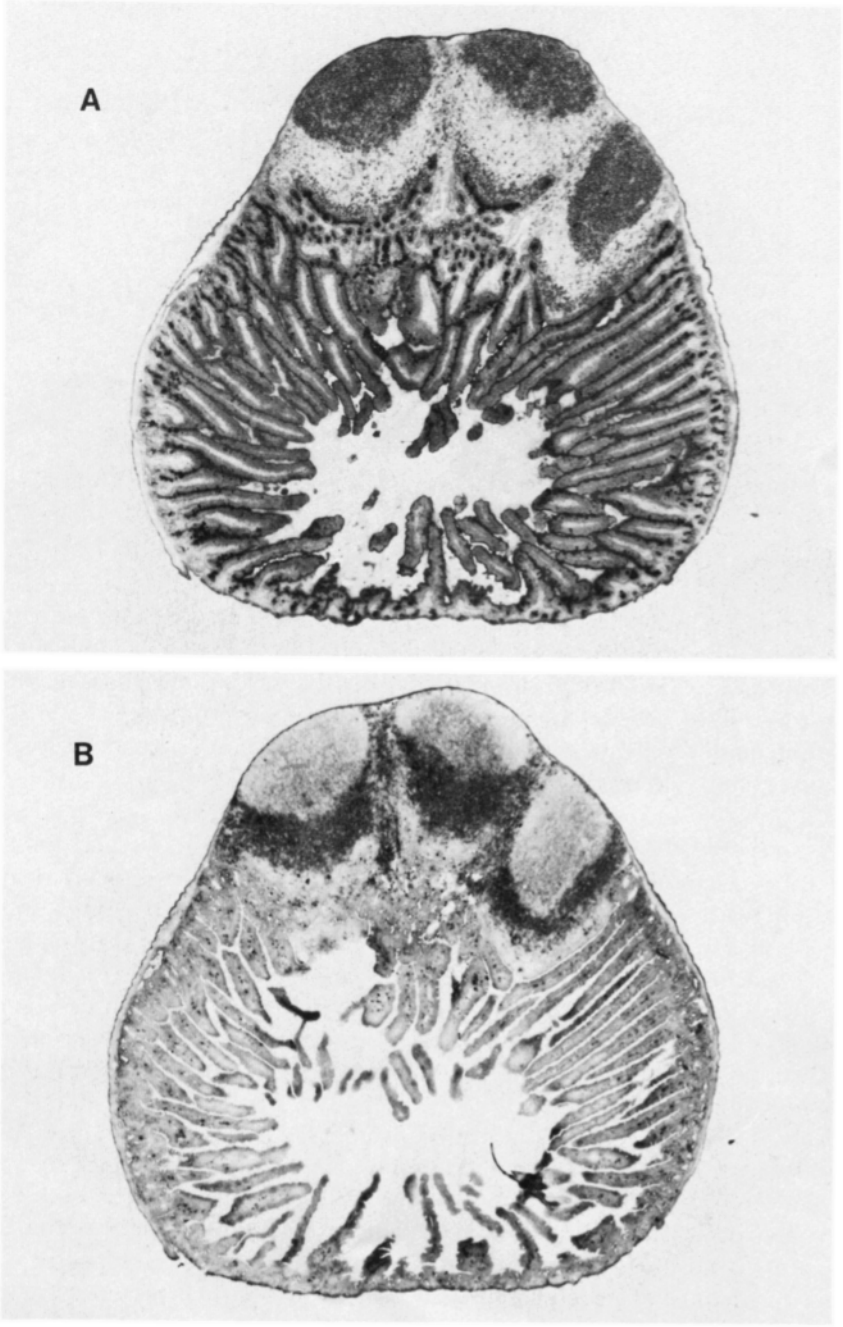

FIG. 1. Immunoperoxidase analysis of the cellular distribution of the putative receptor for peripheral node HEV in murine PP. A and B are serial frozen sections of a PP as it is found in situ in the wall of the small intestine, with A stained with PNA to define germinal centers, and B stained with MEL-14, a monoclonal antibody directed against a determinant associated with the lymphocyte receptor for peripheral node HEV (see text). The figure illustrates that PNA^{hi} lymphocytes (GCLC) and MEL-14-staining cells are largely mutually exclusive populations.

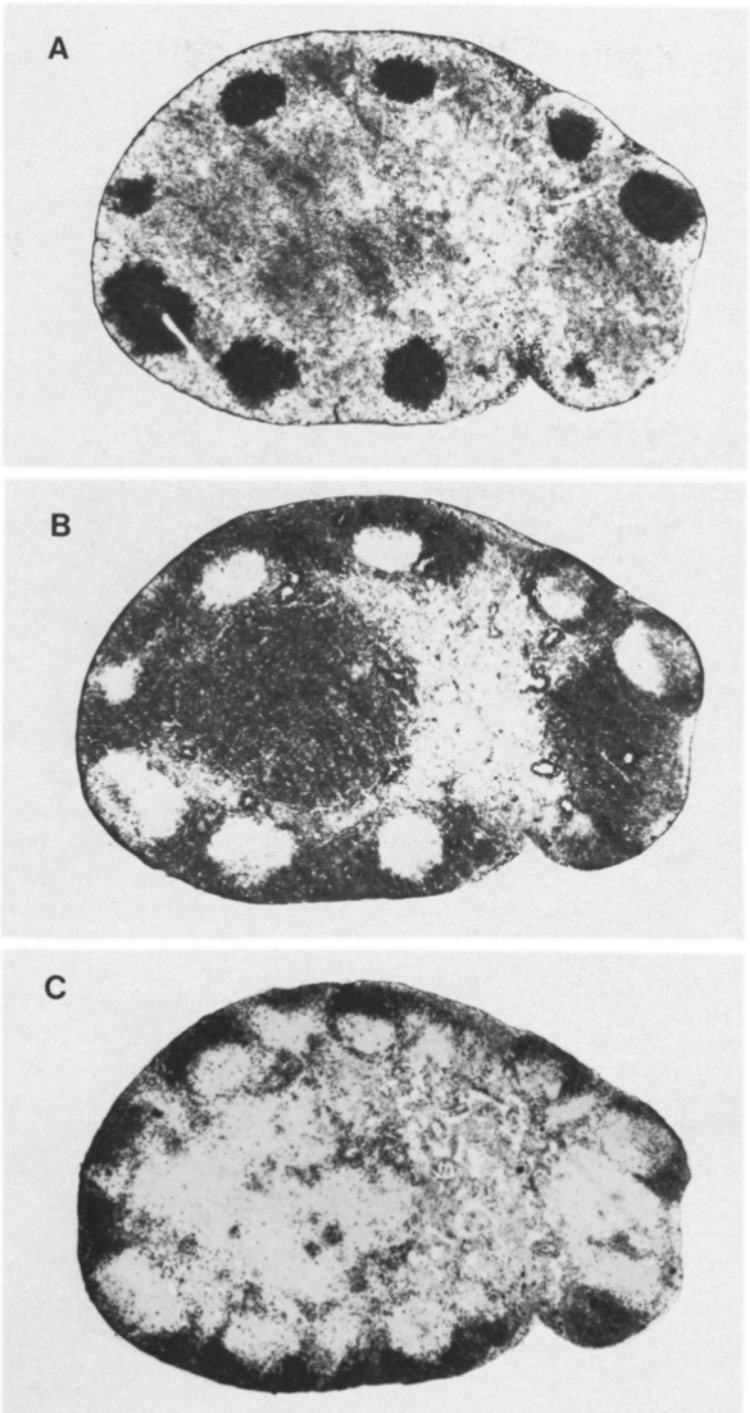

FIG. 2. Additional immunoperoxidase studies using serial sections of a sheep erythrocyte-stimulated brachial lymph node. The section in panel A has been stained with PNA and has several PNA^{hi} germinal centers. MEL-14 staining of the section in panel B reemphasizes that most GCLC fail to express the determinant recognized by MEL-14 (also note the nonstaining medullary area—see text). C illustrates the distribution of cells bearing surface IgD, and is included to demonstrate that GCLC are IgD^-, and to define IgD^+ B cell domains and the nonstaining T cell areas. Comparison of B and C confirms that MEL-14 stains the vast majority of T cells and IgD^+ B cells in antigen-stimulated lymph nodes.

to fulfill their putative effector functions as memory B cells and/or plasma cells. For example, circumstantial but fairly convincing evidence suggests that PP germinal centers harbor precursors of the IgA-secreting plasma cells that populate the lamina propria of the gut and other mucosal sites. PPL generate IgA-containing cells in the spleen and lamina propria of adoptive transfer recipients (30–32) and yield cells that possess cytoplasmic IgA when stimulated in vitro by pokeweed mitogen (33). Studies in rabbits have shown that the precursors of these cells are a surface IgA^+, IgM^- subpopulation of PPL (8). Because most surface IgA^+ cells (70–85%) and almost all IgM^- B cells in mouse PP are GCLC (1), IgA plasma cell precursors are probably largely contained in the germinal center B cell population. While in PP these plasma cell precursors (GCLC or otherwise) contain little or no cytoplasmic Ig (16), and take up to 7–14 d to generate IgA plasma cells in adoptive transfer recipients (30, 32). The lag time for plasma cell generation suggests that these precursor cells require a period of further differentiation, perhaps in the host's mesenteric lymph node or spleen (16, 32). By contrast, IgA plasma cell precursors found in mesenteric node and thoracic duct lymph exhibit more advanced characteristics: Many of these surface IgA^+, IgM^- immunoblasts, thought to be derived from the PP precursors, possess cytoplasmic as well as surface IgA (16), and are capable of migrating rapidly and specifically to mucosal sites where they can differentiate into plasma cells as early as 24 h after transfer (16, 31).

In light of these observations, the inability of GCLC to recognize HEV is viewed as a transient phenomenon related to a specific phase of B cell differentiation that occurs within the confines of the germinal center microenvironment. We propose that PP GCLC eventually migrate out of the germinal center and, at that time or during a subsequent period of maturation, reexpress organ or tissue-specific receptors for endothelial cells in mucosal-associated tissues. These newly acquired homing receptors would then selectively direct the migration of these cells to mucosal sites, where they would differentiate into IgA-secreting plasma cells, thereby accounting for the observed limitation of IgA-secreting cells to these areas. It is possible that the migration of lymphocytes to the gut wall is initially directed by lymphocyte recognition of an endothelial determinant similar to (or perhaps the same as) that expressed on PP HEV (14, 17). Consistent with this hypothesis, many gut-homing mesenteric node immunoblasts do in fact express receptors specific for PP HEV (14, 17). Adoptive-transfer experiments with purified populations of PP GCLC are now in progress, and should provide more insight into the origin of the mucosal IgA response. Although less well characterized, some IgG plasma cell precursors are thought to reside within peripheral node germinal centers (7, 10, 11), where we would expect similar considerations to apply (e.g., eventual induction of organ or tissue-specific recognition mechanisms leading to the distribution of IgG plasma cells to nonmucosal lymphoid organs and tissues).

The transient nonmigratory phase of differentiation undergone by GCLC may be a general feature of antigen-activated lymphocytes. Earlier investigations have shown that lymphocytes capable of recognizing an immunizing antigen are functionally absent from the recirculating lymphocyte pool for 1–2 d after intravenous administration of that antigen (34, 35). Antigen-reactive cells gradually return to the circulation as dividing, activated lymphocytes after a period of local proliferation in lymphoid tissues (34, 35). Thus, antigen-activated lymphocytes may require a phase

of local proliferation and differentiation, whether in germinal centers or other sites of blastogenesis, during which alterations of their migratory capabilities and/or specificities may occur.

One of the most characteristic and perhaps functionally significant features of GCLC is their expression of high levels of PNA-binding sites. These sites, thought to be terminal galactosyl residues (29), are normally masked by sialic acids on the majority of circulating lymphocytes (36), thus presumably providing the molecular basis for the distinction between PNA^{hi} and PNA^{lo} lymphocytes. Neuraminidase treatment of glycoproteins or lymphocytes removes terminal sialic acids and reveals the normally subterminal galactosyl residues. Several investigators have demonstrated that these desialylated glycoproteins (37) and lymphocytes (36, 38) are rapidly cleared from the circulation due to sequestration in the liver. Neuraminidase-treated lymphocytes can be released after resynthesizing cell surface carbohydrates, which indicates that the liver is not scavenging these cells simply because of nonspecific damage (38). The enzymatically altered lymphocytes can still recognize HEV (39), implying that their hepatic entrapment is not caused by an impairment of the cells' normal migratory mechanisms. Rather, the presence of galactose-specific, lectin-like molecules on the surfaces of hepatocytes (40, 41) and/or Kupffer cells (42, 43) may mediate the localization of these cells in the liver. In support of this suggestion, both rat hepatocytes and Kupffer cells have been shown to spontaneously aggregate with desialylated lymphocytes and erythrocytes (42). This in vitro rosette formation can be inhibited by the addition of galactose, which suggests that the cell-cell interaction is mediated by the terminal galactosyl residues of the desialylated cells (42). Whatever the mechanism, the hepatic clearance of desialylated cells may also function in the trapping of naturally occurring PNA^{hi} lymphocytes (e.g., GCLC and cortical thymocytes), since these cells also have abundant terminal galactosyl residues. It seems likely that Kupffer cells, which are in direct contact with the sinusoidal blood, may play more of a role in the entrapment of desialylated cells than hepatocytes, since the fenestrated endothelium lining the sinusoids of the liver serves as a functional barrier between hepatocytes and blood-borne cells.

The presence of endogenous, galactose-specific molecules on liver cells makes it tempting to speculate that lectin-like molecules with similar specificity may also be present on stromal elements in germinal centers and the thymic cortex, where they could retain PNA^{hi} cells at particular stages of differentiation. Such a model of lymphocyte retention may provide a specific recognition mechanism to help explain reports that certain blasts in the lymph of stimulated lymph nodes are able to migrate into germinal centers when applied locally to the next node in a lymph node chain (19), and that rabbit appendix cells contain a population of blasts (presumably GCLC) that are able to enter spleen germinal centers following intravenous injection (20). These reports of blasts migrating into germinal centers should not be interpreted as contradictory to our results, because (*a*) the autoradiographic techniques used in these experiments could detect the localization of a minor lymphocyte subpopulation that would be quantitatively insignificant in our assays, (*b*) local application of blast cells bypasses HEV and therefore does not address the problem of blast cell migration from blood into HEV-bearing lymphoid organs, and (*c*) we have indeed found that a small proportion of GCLC localize in the spleen.

The realization that exposed cell surface galactosyl residues may influence migra-

tory behavior has led us to speculate further on the significance of lymphocyte expression of PNA-binding sites. It has been suggested that PNA binding may be a marker for immature cell types (44, 45). However, our phenotypic analysis suggests that PNA^{hi} PPL are fairly well-developed lymphocytes, (the majority of these cells express surface IgA, an isotype not expressed until relatively late in B cell differentiation [1]). We suggest instead that PNA may serve as a marker for nonmigratory lymphocytes proliferating and/or differentiating under local microenvironmental influences. The major PNA^{hi} lymphocyte populations (GCLC, cortical thymocytes [45], and possibly a subpopulation of bone marrow cells [46]) are all geographically restricted, rapidly differentiating lymphocytes. Because lymphocytes can resynthesize and modify cell surface glycoproteins, a probable step in switching from a sessile, migratory-incompetent cell to a circulating, migratory-competent cell may involve not only the acquisition of receptors for HEV, but also the masking of PNA-binding sites by the attachment of sialic acids. Galactosyl residues would no longer be exposed, thereby providing "exit visas" for these cells and allowing them to migrate free from possible entrapment in the liver by the galactose-specific receptors. Studies are now in progress to determine the extent of correlation between migratory incompetence and lymphocyte expression of high levels of PNA-binding sites.

Summary

Germinal center B cells (GCLC) are a discrete population of antigen-activated lymphoblasts that lack surface IgD and express abundant cell surface binding sites for peanut agglutinin (PNA). These phenotypic features render GCLC easily distinguishable from nearly all plasma cells, T cells, and unstimulated B cells, and have enabled us to identify and isolate GCLC from antigen-stimulated murine lymphoid organs. We have examined the migratory properties of these lymphoblasts in (*a*) short-term in vivo homing studies, and (*b*) an in vitro assay of lymphocyte binding to postcapillary, high endothelial venules (HEV) in frozen sections of Peyer's patches and peripheral lymph nodes. In the in vivo experiments, intravenously injected GCLC failed to migrate in significant numbers to peripheral lymphoid organs in comparison with T cells or IgD^+ B cells. In the in vitro binding assay, GCLC did not adhere to HEV in either Peyer's patch or peripheral node sections. A variety of factors, such as preferential sequestration in the liver, may operate in vivo to influence the localization of these cells. However, their nearly total failure to migrate into lymphoid organs can best be explained by their inability to recognize and adhere to the specialized HEV which normally mediate the emigration of recirculating lymphocytes from the blood into these sites. The concept that GCLC fail to express functional homing receptors for HEV has been further supported by studies using MEL-14, a monoclonal antibody that appears to recognize the lymphocyte surface receptor for peripheral node HEV: In contrast to most peripheral lymphocytes, GCLC fail to bind MEL-14. These migratory and endothelial-recognition properties of GCLC, when viewed in the context of the possible role of these cells as precursors of plasma cells and/or memory B cells, have led us to propose that the inability of GCLC to recognize HEV may be transient and related to a phase of sessile B cell differentiation.

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