Flow Cytometric Analysis of the Hermes Homing-Associated Antigen on Human Lymphocyte Subsets

By Juan de los Tojos, Sirpa Jalkanen, and Eugene C. Butcher

The homing of lymphocytes is controlled by interactions with high endothelial venules (HEV), specialized vessels that define sites of lymphocyte extravasation into lymph nodes and inflamed tissues. In humans, lymphocyte-HEV binding involves a lymphocyte surface glycoprotein (GP) of 85 to 95 kd (CD44, H-CAM), defined by monoclonal antibody (MoAb) Hermes-1. To define the expression of this homing-associated adhesion molecule during human lymphocyte development, we performed two-color immunofluorescence analyses of human bone marrow (BM), thymus, peripheral blood (PB), and tonsillar lymphocytes. The highest levels of Hermes-1 antigen are displayed by circulating B and T cells in the blood, which are uniformly positive and bear roughly twice the level of antigen present on mature lymphocytes within organized lymphoid tissues and BM. "Immature" CD4^+ CD8^- T cells in the thymus are Hermes-1 positive, whereas thymocytes of mature phenotype CD4^- CD8^- are positive. The Hermes-1 antigen is present at high levels on the same population of thymocytes that bears high surface levels of CD3, a component of the T-cell antigen receptor complex, suggesting that levels of T-cell homing and antigen receptors characteristic of mature peripheral T cells appear coordinately during thymocyte maturation/selection. Essentially all T cells in the periphery are Hermes-1^+, including T blasts, and the homing-associated antigen is maintained at high levels on T cells stimulated in vitro by phytohemagglutinin (PHA) and interleukin-2 (IL-2) maintained T-cell clones and lines. In contrast, although most resting IgD^- B cells are positive, a significant fraction of B cells in tonsils are Hermes-1^+; these cells are predominantly PNA^+ IgD^- and CD20^+, a phenotype characteristic of sessile, activated B cells in germinal centers. In all lymphocyte populations examined, there is a linear correlation in staining for Hermes-1 and Hermes-3, an antibody that defines a distinct functionally important epitope on this molecule. The results demonstrate a precise regulation of this homing-associated antigen during lymphocyte differentiation.

Mature lymphocytes migrate between various lymphoid organs and other tissues in a process that is believed to facilitate immune response by rendering the full repertoire of clonal lymphocyte specificities available throughout the body and to be important in determining the tissue-specific distribution of effector and memory lymphocyte populations.1-4 Lymphocyte recirculation and homing requires lymphocyte interactions with endothelial cells at sites of exit from the blood. Circulating lymphocytes have the capacity to recognize and bind to the endothelial cells lining specialized venules, especially (although not exclusively) the postcapillary high endothelial venules (HEV) in lymphoid tissues, subsequently migrating through the vessel wall into surrounding lymphoid or inflammatory tissues.1-5 Numerous studies in animal model systems suggest that the mechanisms involved in lymphocyte-endothelial cell recognition play an important role in regulating the access of lymphocyte subsets to extravascular sites, and hence in determining the characteristics of local immune responses.1,6 The molecules involved in lymphocyte-endothelial cell interaction, and the expression of these molecules at various stages of lymphocyte ontogeny, development, and antigen-dependent differentiation, are therefore of interest.

We previously described a human lymphocyte glycoprotein of 85 to 95 kd, defined by monoclonal antibody (MoAb) Hermes-1, that is involved in human lymphocyte interactions with HEV.4-6 Antibody Hermes-3 against this "gp90Hermes" inhibits lymphocyte binding to HEV in mucosal lymphoid organs,8 but not to HEV in peripheral lymph nodes or synovium, suggesting a specific role for the Hermes antigen in recognition of tissue-specific endothelial cell determinants in mucosal organs. Indeed, gp90Hermes from mucosal HEV-binding lymphoid cells binds saturably and reversibly to purified mucosal vascular addressin, a tissue-specific endothelial cell molecule selectively expressed at sites of lymphocyte extravasation in mucosal lymphoid and extralymphoid tissues.9,10 Recognition of synovial and lymph node HEV also appears to involve gp90Hermes in that a polyclonal antiserum produced against the isolated antigen blocks all three classes of lymphocyte-HEV interaction. Antibody Hermes-1 itself does not interfere with HEV binding.8-10 Cross-precipitating immunoprecipitation analyses demonstrate that Hermes-1, Hermes-3, and the polyclonal anti-gp90 serum recognize the same 85- to 95-kd species.8 These findings suggest an important role for gp90Hermes in control of human lymphocyte traffic. Molecules related or identical to lymphocyte Hermes antigen are expressed by a wide variety of cell types, suggesting that this molecule (H-CAM) may have broader roles in cell adhesion as well11; the Hermes antigen was recently identified with CD4412 and ECAMRIII.13 However, the ability of gp90Hermes to bind an HEV addressin,11 and the inhibitory effects of antibodies to the Hermes antigen on lymphocyte-HEV binding, imply that gp90Hermes is intimately involved with the mechanisms of HEV recognition/adhesion and

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suggest an important role for this homing receptor class in control of human lymphocyte traffic.

In the present study, we used two-color immunofluorescence/flow cytometric analyses to examine expression of the Hermes antigen on human lymphocyte subsets. The results provide a framework for consideration of the role of this molecular class in the physiology of human lymphocyte migration and trafficking in vivo.

MATERIALS AND METHODS

Tissues and cells. Human tonsils, thymuses, and bone marrow (BM) were from surgical or biopsy specimens determined to be pathologically normal. Tonsilys were from infants undergoing cardiac surgery, and tonsils were from 3- to 12-year-old individuals. Peripheral blood (PB) samples were from normal healthy adults aged 21 to 34 years.

BM mononuclear cells, and PB lymphocytes (PBLs) were isolated from whole BM and blood with Ficoll-Hypaque (Histopaque-1077, Sigma, St Louis). Tonsil and thymus cells were suspended by mincing and teasing in RPMI containing 25 mmol/L HEPES, pH 7.3, and 5.0% fetal calf serum (FCS) and were centrifuged over Ficoll-Hypaque to remove dead cells before immunofluorescence staining was performed.

Approval was obtained from the institutional review board for these studies. Patients or volunteers were informed that blood or BM samples were obtained for research purposes and that their privacy would be protected. In some experiments, Ficoll-isolated PBLs were stimulated to blastogenesis by incubation at 37°C with 0.1% PHA B (Difco) for three days.

Antibodies. The MoAbs used are shown in Table 1. FITC-conjugated anti-Leu-2 (CD8), Leu-3 (CD4), Leu-4 (CD3), Leu-6 (CD1), Leu-M3 (CD14), Leu-11a (CD16), HLA-DR, and transferrin receptor antibodies were obtained from Becton Dickinson (Mountain View, CA). Anti-B1 (CD20)14 was from Coulter (Hialeah, FL). 6A4, an anti-B-lineage marker,15 and L3B12, anti-human leukocyte common antigen (CD45)16 were gifts from R. Warnke and R. Levy (Stanford), respectively, and were used as cell culture superantigens. Hermes-1 (a rat IgG2a) and Hermes-3 (mouse IgG2a) have been described previously.17 30G12, a rat IgG2a anti-mouse T20018 was used as a control for Hermes-1. Hermes-1, Hermes-3, and 30G12 were used as purified antibodies or as biotin conjugates. All antibodies were titered and used at saturation for staining cell suspensions.

F(ab), fragments of sheep anti-mouse IgG FITC conjugated were obtained from Sigma (St Louis), and Texas red-conjugated avidin was obtained from Cooper Biomedical (Malvern, PA). FITC-PNA was obtained from EY Laboratories, San Mateo, CA.

Immunofluorescence staining and flow cytometry. To assess the immunologic phenotype of Hermes-1-defined lymphocyte subsets, two-color immunofluorescence staining was performed with a 2-step procedure. Approximately 10^6 cells were incubated in 96-well microtiter plate wells in 60 μL medium containing a mixture of first-stage antibodies (various FITC-conjugated MoAbs, admixed with biotinylated Hermes-1 or control antibodies) and subsequently with 20 μL second-stage Texas red avidin. Each incubation step was performed on ice for 30 minutes with resuspension and two washings after each stage. All reagents contained 0.1% sodium azide.

Two-color staining with unconjugated mouse antibodies was performed by sequential incubation of cells with the mouse antibody, FITC-anti-mouse IgG containing 5% normal human serum, biotinylated Hermes-1 or 30G12 (control), and Texas red avidin. All incubations and washes were performed as described above. Staining with FITC-PNA was carried out as described previously.18

Stained cells were resuspended in 2% formalin in 1.25x phosphate-buffered saline (PBS) and maintained at 4°C in the dark until analyzed on a modified Becton Dickinson FACS.19 Four parameters (green and red fluorescence, and forward and oblique light scatter) were collected from each cell. Thirty thousand cells were analyzed from each sample. Clumped or dead cells and cell debris were excluded from the recorded data by appropriate electronic gates.19 Light-scatter gates were used to select lymphocytelike cells for detailed analysis, excluding monocytes, neutrophils, and dead cells, which were essentially as defined by Parks and Herzenberg,20 and Loken et al.21 T-cell clones. Human T-cell clones were obtained from three independent sources. Several antigen-reactive and alloreactive T cells of both cytotoxic/suppressor and helper phenotypes were tested. Alloreactive interleukin-2 (IL-2)-producing (helper) T-cell clones T4-31, CWN, C2-2, and 22 through 27 (from J. Goronzky and C. Weyand, Stanford) were grown in minimal amounts of recombinant IL-2 (rIL-2 CETUS, Emeryville, CA). Allospecific cytotoxic T-cell clones AH7 and F1 (from C. Clayberger and A. Krenskey, Stanford)22 were grown in IL-2 obtained from the supernatant of phytohemagglutinin (PHA)-stimulated lymphocytes. Purified protein derivative-specific helper T-cell clones 115, 15, 113, and the suppressor clone II127 were also grown in IL-2 obtained from supernatant of PHA-stimulated lymphocytes. These cells have been described previously22: 15 is from cell line 5, II13 is from cell line 13.

<table>
<thead>
<tr>
<th>Table 1. Antibodies Used</th>
<th>Major Cell Reactivity</th>
<th>Source or Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antibody</strong></td>
<td><strong>Antigen or CD Cluster</strong></td>
<td><strong>Immature thymocytes</strong></td>
</tr>
<tr>
<td>Anti-Leu-6</td>
<td>CD1</td>
<td>CD4</td>
</tr>
<tr>
<td>Anti-Leu-4</td>
<td>CD3</td>
<td>Mature T cells</td>
</tr>
<tr>
<td>Anti-Leu-3</td>
<td>CD4</td>
<td>T subset and monocytes</td>
</tr>
<tr>
<td>Anti-Leu-2</td>
<td>CD8</td>
<td>T and NK subsets</td>
</tr>
<tr>
<td>Anti-B1</td>
<td>CD20</td>
<td>B cells</td>
</tr>
<tr>
<td>L3B12</td>
<td>LCA</td>
<td>Leukocytes</td>
</tr>
<tr>
<td>6A4</td>
<td>—</td>
<td>B cells</td>
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<tr>
<td>IgD</td>
<td>—</td>
<td>B subset</td>
</tr>
<tr>
<td>Anti-transferrin receptor</td>
<td>—</td>
<td>Activated blasts</td>
</tr>
<tr>
<td>Anti-HLA-DR</td>
<td>HLA-DR</td>
<td>—</td>
</tr>
<tr>
<td>Anti-Leu-M3</td>
<td>CD14</td>
<td>Mature monocytes/macrophages</td>
</tr>
<tr>
<td>Anti-Leu-11a</td>
<td>CD16</td>
<td>NK cells, neutrophils</td>
</tr>
<tr>
<td>Hermes-1</td>
<td>gp85-95 (CD44)</td>
<td>—</td>
</tr>
<tr>
<td>Hermes-3</td>
<td>gp85-95 (CD44)</td>
<td>—</td>
</tr>
</tbody>
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Abbreviation: BD, Becton Dickinson.
RESULTS

**PBLs.** PBL subsets were analyzed by two-color immuno-fluorescence for staining with Hermes-1. Forward and obtuse light-scattering parameters were used to exclude monocytes and the few remaining polymorphonuclear leucocytes in the Ficoll-isolated preparation. Antibodies against B-cell and T-cell antigens, the natural killer cell (NK)-associated antigen CD16 (Leu-11a),22 and other antigens were used. Eight specimens were analyzed; three were analyzed in detail with each of the antibodies discussed below. FACS plots from a representative study are shown in Fig 1.

Essentially all PBLs bear the Hermes-1 antigen; 91% to 99% (n = 8) were Hermes-1* (>4 fluorescence units). In three experiments analyzed in detail, 97% to 99% were positive, >96% staining at very high levels (>12 fluorescence units). One hundred percent of T cells, identified by CD3 expression, stained with Hermes-1, and the CD4 and CD8 T-cell subsets bore comparable levels of the antigen. B cells, identified by monoclonal anti-CD20,19 or by the B-lineage-specific antibody 6A413 were also intensely stained, 98% to 100% being Hermes-1*. The B cells of one individual stained significantly less intensely than T cells in the same sample (roughly half as bright, modal fluorescence), but in the other PBL specimens examined, B cells and T cells bore equivalent levels of the Hermes-1 antigen. Only 1% to 9% of lymphocyte-gated PBLs were Hermes-1+ (n = 8). In the three samples analyzed in detail, <2% of this population were conventional B cells or CD3+ T cells. In two samples, this Hermes-1+ fraction was significantly enriched in cells bearing CD8 (26% or 52% positive in the two experiments) and the NK-associated antigen CD16 (55% or 61% positive), suggesting that the NK population may be enriched in Hermes-1 dull cells. (This observation is being explored further in separate studies). PB immunoblasts, defined as the 1% to 3% of lymphocytes exhibiting the highest forward light scattering, were nearly all Hermes-1+ (96% of CD20+ B blasts, and 99% of CD3+ T blasts were >25 fluorescence units).

**Tonsils.** Immunofluorescence plots from a representative experiment are shown in Fig 2, and the deduced phenotype of the Hermes-1+ and Hermes-1- subpopulations is summarized in Fig 3. A majority of tonsillar lymphocytes (66% to 78%, n = 3) were Hermes-1+ (>4 fluorescence units), with a modal fluorescence about half that of circulating PBLs stained in parallel. Nearly all tonsillar T cells, whether CD4+ or CD8+, were positive. T-cell blasts, defined by forward light scatter and CD3 expression, constituted 2.2% to 2.8% of T cells, and were 91% to 98% Hermes-1+ (n = 3).

In contrast, tonsillar B cells are divided into discrete Hermes-1+ and Hermes-1- fractions (Fig 2). (We prefer the designation Hermes-1+ for these tonsillar lymphocytes because although there is extensive overlap with negative control staining these cells exhibit a slight shift in FACS profile in comparison with cells stained with irrelevant class-matched antibody.) Hermes-1+ B cells displayed modal Hermes-1 fluorescence similar to that of tonsillar T lymphocytes and were enriched for cells bearing surface IgD and expressing intermediate levels of CD20 and only low levels of PNA receptor (Figs 2 and 3). This phenotype is characteristic of mantle zone B cells.

Ninety percent of Hermes-1+ tonsillar lymphocytes bore B-lineage markers. This Hermes-1+ B-cell subset was enriched for IgD+, PNA+, and CD20+ cells (Fig 2), a phenotype associated with germinal center cells.14 However, although it is not apparent in standard plots (Fig 2), a small subset of IgD+ B cells is also Hermes-1+ (2% to 7% in different experiments). Both Hermes-1+ and Hermes-1- fractions bore equivalent levels of HLA-DR and the B-lineage antigen defined by MoAb 6A4. The large cell
fraction of B cells, representing 3% to 12% of total tonsillar cells in different experiments, consisted of discrete Hermes-1\(^{b0}\) and Hermes-1\(^{b0}\) populations as well (21% to 45% Hermes-1\(^{b0}\), n = 3).

**Thymus.** Most thymocytes are Hermes-1\(^{b0}\) (<6 fluorescence units), yielding a fluorescence profile only slightly shifted in comparison with the class-matched negative control (illustrated by representative FACS plots in Fig 4). Approximately 15% of thymocytes (12% to 17%, n = 4) bear levels of the Hermes-1 antigen comparable to those of tonsillar lymphocytes, roughly twofold lower than those on PB leukocytes. (One thymus, excluded from consideration in this study, was atypical in that most cells were Hermes-1\(^{b0}\) and of mature phenotype; this may have been due to stress-induced involution of the cortex.)

Staining with Hermes-1 correlated with expression of surface CD3 (Fig 4, first panel, and Fig 5): Most cells expressing low or intermediate levels of surface CD3 were Hermes-1\(^{b0}\), whereas thymocytes bearing CD3 at levels comparable to those of circulating immature PBL T cells were largely Hermes-1\(^{b0}\). Most thymocytes of mature "single-positive" phenotype with respect to CD4 and CD8 expression (ie, CD4\(^{+}\), CD8\(^{+}\) or CD4\(^{+}\), CD8\(^{+}\)) were Hermes-1\(^{b0}\). Indeed, ~49% of Hermes-1\(^{b0}\) cells stained for CD8, and 60% stained for CD4 (mean n = 3). Ninety-five percent were stained with a combination of anti-CD4 and anti-CD8 antibodies. Thus, ~46% of Hermes-1\(^{b0}\) cells were CD4\(^{+}\), CD8\(^{+}\), and 35% were CD4\(^{+}\), CD8\(^{b0}\). Conversely, the major Hermes-1\(^{b0}\) thymocyte population was primarily double positive (Figs 4 and 5), with both anti-CD4 and anti-CD8 reagents each staining most Hermes-1\(^{b0}\) cells.

Staining with Hermes-1 correlated inversely with expression of CD1 and receptors for PNA: Hermes-1\(^{b0}\) cells tended to be CD1\(^{b0}\) and PNA\(^{b0}\), whereas the major Hermes-1\(^{b0}\) population bore high levels of CD1 and PNA receptors (Fig 4). Thus, the predominant phenotype of Hermes-1\(^{b0}\) thymocytes is CD3\(^{b0}\), single-positive (CD4\(^{+}\) or CD8\(^{+}\)), CD1\(^{b0}\) and PNA\(^{b0}\); whereas that of Hermes-1\(^{b0}\) thymocytes is CD3\(^{b0}\), CD4\(^{+}\), CD8\(^{+}\), CD1\(^{b0}\), and PNA\(^{b0}\).

A small population of "double-negative" (CD4\(^{-}\), CD8\(^{-}\)) cells was also identified and were characterized in two representative thymuses. About 1.9% (mean n = 3) of cells failed to stain with a mixture of anti-CD4 and anti-CD8 antibodies; these cells were divided into Hermes-1\(^{b0}\) (0.65% of thymocytes) and Hermes-1\(^{b0}\) (1.2%) subsets. However, simultaneously staining for CD4, CD8, and Bl (CD20)
Fig 4. Dual-fluorescence analysis of human thymocytes. Hermes-1<sup>hi</sup> thymocytes, which constitute ~15% of the entire population, are enriched for cells of mature phenotype: CD3<sup>+</sup>, CD8<sup>-</sup> or CD4 single positive, PNA<sup>hi</sup>, and CD1<sup>+</sup>. Hermes-1<sup>lo</sup> thymocytes express the immature phenotype PNA<sup>lo</sup>, CD1<sup>lo</sup>, CD3<sup>lo</sup> and are positive for both CD4 and CD8. CD4 + CD8 (right top panel) indicates staining with anti-CD4 and anti-CD8 antibodies combined. The background staining of thymocytes in the fluorescein channel is less than that of tonsillar lymphocytes or marrow cells, but we consider that intensity must exceed one unit to be meaningful.

revealed that some of these were CD20<sup>+</sup> (B cells). Only 1.2% of thymocytes were CD4<sup>-</sup>, CD8<sup>-</sup>, and CD20<sup>+</sup>: 30% of these were Hermes-1<sup>hi</sup> and 70% were Hermes-1<sup>lo</sup>. A significant subset (about one fourth of the double-negative Hermes-1<sup>lo</sup> fraction) lacked surface leukocyte common antigen (LCA) and could represent RBCs or other nonlymphoid elements. Thus, only ~0.8% to 1% of thymocytes appear to represent LCA<sup>-</sup>, non-B, CD4<sup>-</sup>, CD8<sup>-</sup> lymphocytes. This fraction of thymocytes contains both Hermes-1<sup>hi</sup> and Hermes-1<sup>lo</sup> cells in roughly equal numbers (estimated as 40% Hermes-1<sup>hi</sup>, 60% Hermes-1<sup>lo</sup>). The CD4<sup>-</sup>, CD8<sup>-</sup>, CD20<sup>+</sup> population consisted predominantly of small lymphocytes, as determined by forward light scattering.

Based on forward light-scattering measurements, Hermes-1<sup>hi</sup> thymocytes are slightly larger on average ("medium"-sized) than the bulk of (small) Hermes-1<sup>lo</sup> cells. Two percent to 4% (mean 3, n = 4) of thymocytes were large, defined arbitrarily as displaying forward light-scattering intensity >channel 95. These presumed blasts were predominantly (82%) Hermes-1<sup>lo</sup>, displaying relative proportions of Hermes-1<sup>-</sup> and Hermes-1<sup>hi</sup> cells roughly similar to that of the total thymus. The Hermes-1<sup>lo</sup> cells in this gate were also CD3<sup>+</sup>

BM. Because BM is a heterogeneous tissue, comprising diverse cell lineages at various stages of development, the immunofluorescence and light-scattering patterns of BM populations are more complex. In addition, the cellular composition of the marrow varies significantly from patient to patient. For the present studies, three representative specimens were studied in detail. RBCs and most polymor-
phonuclear leukocytes were removed from the samples by Ficoll separation. For the integrations discussed below, we used a Hermes-1 fluorescence gate of 8.3 fluorescence units, slightly higher than that used to define Hermes-1 cells in the other tissues: this gate was selected to discriminate between the levels of Hermes-1 antigen characteristic of the bulk of lymphocytes (Hermes-1\textsuperscript{hi} fraction) and those expressed on the major population of transferrin receptor-bearing lymphocytelike cells (Hermes-1\textsuperscript{lo}, described below). A lymphocytelike light-scattering gate, which includes primarily lymphocytes and nucleated erythroid precursors, is used. These lymphocytelike cells constituted 33% to 52% (mean 40%, n = 3) of the total Ficoll-purified samples; 28% of these were Hermes-1\textsuperscript{hi}, and 72% were Hermes-1\textsuperscript{lo}.

The Hermes-1\textsuperscript{lo} fraction in two of the three BM samples contained a major subset of T cells (~60% CD3\textsuperscript{+}, Fig 6), but a third sample lacked significant numbers of T cells. Most of the T cells were CD4\textsuperscript{+} (ratio of CD4/CD8 = 2:1). All three samples contained a significant subset of B cells (8% CD20\textsuperscript{+} or 6A4\textsuperscript{+}) among the Hermes-1\textsuperscript{lo} fraction.

BM T cells were Hermes-1\textsuperscript{-} (96%) bearing somewhat lower levels of Hermes-1 antigen than PBLs (~55% as bright). Most BM B cells (CD20\textsuperscript{+}) were Hermes-1\textsuperscript{-}; their modal fluorescence was ~30% lower than that of the T cells, and the mean fluorescence was only half (46%, mean n = 3) of that of marrow T cells (and ~25% of that of PBLs).

The Hermes-1\textsuperscript{lo} fraction was highly enriched for cells bearing transferrin receptors. Conversely, cells bearing transferrin receptors were predominantly Hermes-1\textsuperscript{hi}, although they displayed a rather wide range of staining with Hermes-1. These transferrin receptor-expressing cells are likely to be normoblasts, nucleated erythroid precursors whose light-scattering properties overlap those of lymphocytes. Consistent with this assumption, they represent a major fraction in all lymphocyte-gated plots but do not express any of the lymphocyte-specific differentiation antigens examined.

HLA-DR\textsuperscript{+} cells constituted roughly 4% of BM (2% to 7%, n = 3), and were divided into discrete Hermes-1\textsuperscript{hi} (43%) and Hermes-1\textsuperscript{lo} (57%) subsets (Fig 6). Staining with anti-LCA defined three populations bearing distinct levels of LCA. As shown in Fig 6, LCA\textsuperscript{hi} cells (9% of the gated cells, which are believed to represent mature lymphocytes\textsuperscript{24}) were Hermes-1\textsuperscript{hi}. Cells expressing intermediate levels of LCA were mostly Hermes-1\textsuperscript{lo}, as was the LCA\textsuperscript{-} fraction (primarily normoblasts).

**Coexpression of Hermes-1 and Hermes-3 epitopes.** MoAb Hermes-3 blocks lymphocyte binding to HEVs in mucosal lymphoid organs (eg, the appendix), and defines an epitope on the Hermes-1 antigen distinct from that recognized by Hermes-1 itself. Immunofluorescence studies of numerous cell lines and immunohistologic analyses of many non-Hodgkin's lymphomas have revealed a close correspondence between staining with Hermes-1 and Hermes-3.\textsuperscript{24} Further, Hermes-3 and Hermes-1 cross-precipitate each other in immunoprecipitations from iodinated PBLs.\textsuperscript{25} To assess coexpression of the Hermes-1 and Hermes-3 epitopes by normal lymphocyte populations, we analyzed Hermes-1\textsuperscript{-} vs Hermes-3 staining with two-color immunofluorescence. We found a consistent linear correlation in staining for these two epitopes on lymphocytes of each of the tissues examined, including tonsils, thymus, BM, intestinal lamina propria lymphocytes, and PBLs (data not shown). This indicates that within the limits of the approach the Hermes antigen expressed by lymphocytes at all stages of lymphocyte differentiation appears to bear both the nonblocking Hermes-1 epitope and the function-associated Hermes-3 epitope.

**Hermes-1 antigen expression by in vitro activated lymphocytes.** To assess the effect of in vitro blastogenesis on the expression of the Hermes-1 antigen, we stimulated PBLs with PHA and analyzed them by immunofluorescence. After 72-hour stimulation, ~70% of cells were blasts (assessed morphologically). Almost all remained brightly stained with Hermes-1. Figure 7 shows Hermes-1 and control immunofluorescence staining profiles of the blasts in the culture.
The importance of the Hermes antigen in lymphocyte recognition of HEV and in lymphocyte homing is clear, however. Antibodies against one epitope of gp90\(^{\text{Hermes}}\) selectively inhibit lymphocyte binding to mucosal HEV,\(^8\) and a polyclonal anti-gp90\(^{\text{Hermes}}\) inhibits binding to all HEV classes.\(^8\) Furthermore, preliminary studies indicate a direct role for the lymphocyte Hermes antigen in binding to vascular addressins, tissue-specific endothelial cell molecules involved in directing lymphocyte extravasation: Energy transfer techniques have been used to detect specific molecular binding of the Hermes antigen isolated from KCA, a mucosal HEV-binding cell line, to affinity-purified mucosal vascular addressin,\(^9\) a 58- to 66-kd GP involved in lymphocyte recognition of Peyer's patch HEV.\(^10\) These findings,

(identified by high forward-light scattering properties); 90% of the blasts were CD3\(^+\) T cells, and 86% expressed surface transferrin receptors, confirming their activated state. As shown, essentially all of these T blasts were Hermes-1\(^+\), with modal fluorescence approximately twofold greater than that of control PBLs analyzed in parallel. Some of this increased intensity undoubtedly relates to cell size. A subset of the blasts was CD20\(^+\) B cells (14%), and 95% of these were also Hermes-1\(^+\), with modal fluorescence comparable to that of the T blasts (Fig 7).

We also assessed the expression of the Hermes-1 antigen on IL-2-maintained T-cell clones and lines. As shown in Table 2, all clones and lines examined were Hermes-1\(^+\), and most displayed levels of Hermes-1 antigen greater than those on PBLs. In addition to the eight clones shown in Table 2, two T cell clones derived from dermal T cells and several uncloned T-cell lines from skin, synovium, or lamina propria were assessed by immunofluorescence microscopy: all were intensely positive.

**DISCUSSION**

First, some aspects of the biology of the Hermes-defined glycoprotein (GP) class warrant discussion. This class of GPs is expressed not only on lymphocytes, but also on neutrophils, monocytes, and other leukocytes.\(^6,28,27\) Closely related molecules are also expressed on some nonhematopoietic cell types, including fibroblasts, glial cells, keratinocytes, follicular dendritic cells, and others.\(^11-13\) This widespread distribution leads to the hypothesis that the Hermes antigen(s), which on lymphocytes is involved in multiple organ-specific endothelial cell recognition events, may subserve distinct recognition or adhesion events in other cell types. Indeed, evidence shows that gp90\(^{\text{Hermes}}\) from fibroblasts (through its apparent identity with extracellular matrix receptor III),\(^1,3\) and from lymphocytes can bind extracellular matrix components including collagen and fibronectin (S. Jalkanen, personal communication, January 1989), suggesting that like other cell adhesion molecules gp90\(^{\text{Hermes}}\) may have domains capable of interacting specifically with a number of distinct ligands. Furthermore, recent cloning of cDNAs to gp90\(^{\text{Hermes}}\) demonstrates that an N-terminal extracellular domain of this molecule is homologous to cartilage proteoglycan core and link protein domains believed to be involved in interactions both with glycosaminoglycans and with proteins that participate in cartilage formation.\(^29\) These considerations mitigate that the functional significance of expression of this class of molecules on any given cell, as defined simply by staining with Hermes-1 and Hermes-3, be interpreted with caution. This caveat, of course, applies formally to any use of antibodies to define the distribution of functionally important molecules, in that the presence of antigen per se does not necessarily imply that the antigenically defined molecule is functional or displays the same function in every cellular context.

**Table 2. Hermes-1 Staining of T-Cell Clones**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Phenotype</th>
<th>Specificity</th>
<th>Hermes-1 Fluorescence*</th>
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<tr>
<td>T4-31</td>
<td>Th CD4*</td>
<td>PPD</td>
<td>105</td>
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<td>I5</td>
<td>Th CD4*</td>
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<td>PPD</td>
<td>70</td>
</tr>
<tr>
<td>II27</td>
<td>Tc/s CD8</td>
<td>PPD</td>
<td>70</td>
</tr>
<tr>
<td>F1</td>
<td>Tc CD4*</td>
<td>DR3</td>
<td>102</td>
</tr>
<tr>
<td>A17</td>
<td>Tc CD8*</td>
<td>HLA-A2</td>
<td>110</td>
</tr>
<tr>
<td>A18</td>
<td>Tc CD8*</td>
<td>HLA-A2</td>
<td>105</td>
</tr>
<tr>
<td>PBLs</td>
<td>-</td>
<td>-</td>
<td>50</td>
</tr>
</tbody>
</table>

*Cells were incubated with saturating levels of Hermes-1 followed by an FITC-rabbit anti-rat Ig. Control staining with second-stage only was negligible (1 U). Modal fluorescence is given in arbitrary linear units.

Abbreviations: Th, T-helper cell; Tc, cytotoxic T cell.
combined with the correlation between Hermes expression we define and the HEV binding ability of mature lymphocyte populations,35 suggests that the gp90 Hermes on lymphocytes are homing receptors for HEV. Hermes probably acts in concert with other lymphocyte surface GPs [eg, the lectin-homologous bleeding] lymph node homing receptor gp90 HEL.14 (or its human equivalent), the integrin-related Peyer's patch homing receptor LPAM-1/VLAA.32 and other adhesion molecules] to control lymphocyte-HEV interactions and lymphocyte trafficking in humans.

We used two-color immunofluorescence to study the cell surface expression of the Hermes antigen on defined lymphocyte subsets. The results are discussed in relation to expression of this homing-associated antigen in the major lymphocyte lineages.

**T cells.** Immature T cells undergo a complex process of differentiation and selection within the thymus, leading to export of mature, circulating, immunocompetent T cells to the periphery. 12% to 17% of thymocytes expressed surface Hermes-1 antigen at levels comparable to those of mature peripheral T cells in tonsils (described below). These Hermes-1 T thymocytes are characteristically of small or medium size and exhibit a "mature" phenotype (CD4⁺ or CD8⁺, CD1⁻, PNA⁻). Immunohistologic studies indicate that these Hermes-1⁺ cells are primarily located in the medulla, with a few scattered throughout the cortex.7

Correlation of Hermes-1 antigen with CD3, part of the T-cell antigen receptor complex, was of particular interest. Consistent with the observations of Lanier et al,33 we observed tridomial staining with CD3, with negative, weakly positive, and brightly positive subpopulations. Expression of the Hermes-1 antigen correlated closely with expression of high levels of surface CD3. CD3 is believed to be transported to the cell surface only in conjunction with the T-cell receptor for antigen.44 The correlation between high CD3 and Hermes-1 antigen levels therefore suggests a coordinate upregulation during T-cell maturation of at least two cell surface recognition systems required for T-cell function in the periphery, the T-cell antigen recognition complex and lymphocyte receptors for endothelium. Thus, in the context of intrathymic differentiation, the expression of this homing-associated adhesion molecule occurs on a late, phenotypically mature stage of thymocyte differentiation.

Most thymocytes express an immature phenotype (CD4⁺, CD8⁺, PNA⁺, and CD1⁻) and are believed to represent a terminal stage of thymocyte differentiation: Thymocytes of this phenotype appear to die within the thymus without contributing to the mature peripheral T-cell pool.35,36 These thymocytes lack putative homing receptors, bearing only low to undetectable levels of surface Hermes-1 antigen.

In the mouse, a CD4⁺, CD8⁺ population of immunoblasts, readily identified by FACS analysis, is believed to represent an immature thymic stem cell population capable of differentiating into each of the major T-cell phenotypes.35,36 Attempts to define expression of the Hermes-1 antigen on a comparable population in the human thymus were unsuccessful, because very few cells of this phenotype were present in the thymuses studied. Approximately 2% of thymocytes were double negative (CD4⁻, CD8⁻), but half of these either bore the CD20 B-lineage antigen or were negative for the leukocyte common antigen and thus could not be confidently defined as lymphocytes. The remaining CD4⁺, CD8⁺ thymocytes were divided into Hermes-1⁺ and Hermes-1⁻ populations, but they were predominantly small, containing few if any immunoblasts as defined by forward light scattering. Furthermore, thymic blasts, which were divided into Hermes-1⁺ and Hermes-1⁻ fractions in proportions similar to that of total thymus, were not enriched for double-negative cells. These results argue for caution in interpreting CD4⁺, CD8⁺ cells in human thymus as being analogous to double-negative mouse thymocytes.

Essentially all peripheral T cells are Hermes-1⁺. Circulating T cells in the PB bear two- to three-fold higher levels of antigen than medullary thymocytes or resident T cells in tonsils. Because this finding was reproducible, it was probably not due to differences in cell handling or preparation and may reflect a selective enrichment in the most actively recirculating lymphocyte pool of cells expressing high levels of this homing receptor. No differences in mean Hermes-1 antigen expression by CD4⁺ vs CD8⁺ T-cell subsets were observed. The phenotype of T cells in BM was similar to that of tonsillar T cells. These presumably represent mature peripheral T cells, known to be present in variable numbers in human marrow.

T immunoblasts, whether identified by forward light-scattering properties in PBLs or tonsils, generated in vitro by PHA-induced mitogenesis, or exemplified by IL-2-driven T-cell clones or lines, uniformly bore high levels of the Hermes-1 antigen. The expression of this homing receptor antigen on T-cell clones is in keeping with our previous finding that human T-cell clones bind efficiently to HEVs in the Stamper-Woodruff in vitro frozen section assay.37

**B cells.** Although most BM B cells (CD20⁺ lymphocytes) were Hermes-1⁺, their mean fluorescence was somewhat lower than their nodal fluorescence or the fluorescence of marrow T cells, indicating the presence of a Hermes-1⁻ subset in the CD20⁺ population. Heterogeneous expression of surface Hermes-1 antigen may reflect a contribution of both mature B cells and of immature, developing pre-B lymphocytes to the CD20⁺ population.

Nearly all B cells in PB, and most surface IgD⁺ B cells in tonsils bear high levels of Hermes-1 antigen, in most instances at levels comparable to those of T cells from the same source. (In one PB sample, B cells were significantly duller than T cells. The significance of this is unknown.) In contrast to the uniform expression of Hermes-1 antigen by T cells, however, there is a major subpopulation of Hermes-1⁻ B cells within tonsils. Tonsils are an immunologically activated lymphoid organ, with prominent germinal centers within the B-cell domains. Germinal centers are sites of proliferation of antigen-specific B cells,38 believed to be responding to their cognate antigen presented by follicular dendritic cells within the germinal center. As a population, germinal center cells undergo immunoglobulin heavy chain class-switching during proliferation in the germinal center microenvironment and are believed to give rise to specific memory B cells.39,41 Consistent with immunohistologic studies, most of the Hermes-1⁻ B cells in...
HOMING-ASSOCIATED ADHESION MOLECULE

tonsils display a phenotype associated with this activated
germinal center population (ie, IgD⁺, PNA⁺, CD20⁺, and
enriched for large cells as defined by forward angle scatter).
B cell blasts in PHA-stimulated cultures of PBLs remained
Hermes-1⁺, suggesting that suppression of Hermes antigen
expression may not be a necessary consequence of mitogene-
sis (or that it may require longer than the three days of in
vitro stimulation we performed). Such suppression may be a
result of specific differentiation signals associated with pro-
iferation in the germinal center microenvironment in vivo.

The expression of Hermes-1 antigen by migratory-compet-
tent, IgD⁺ B cells but not by germinal center cells closely
parallels observations of MEL-14-defined lymph node hom-
ing receptor expression by B cells in the mouse. In
keeping with their loss of these antigens, mouse germinal
center cells are unable to interact with HEV or to migrate
into HEV-bearing organs in vivo. In that antigen-specific
germinal center cells must arise from blood-borne, migrating
precursors and are believed to export migratory-competent
memory cells, the results support a complex regulation of
these two homing receptor classes during antigen-dependent
B-cell responses with specific and presumably transient
downregulation during the antigen-driven proliferation and
differentiation of B cells within the germinal center microen-
vironment.

Other populations in the BM. In other studies, we
showed that the Hermes-1 antigen is expressed by mature
monocytes and granulocytes, and is also present at high levels
on pluripotent stem cells in human BM. We now show
that presumptive nucleated erythroid precursors in BM, cells
exhibiting lymphocyte light-scattering properties and bear-
ing cell surface transferrin receptors (but not lymphocyte
differentiation antigens), display low levels of Hermes-1
antigen, suggesting that the antigen, which is present only at
very low levels on mature RBCs is downregulated during
maturation of the erythroid lineage. This is in keeping with
the fact that RBCs are normally retained in the vascular
compartment and do not actively extravasate like nucleated
leukocytes. In this context, it may be relevant that platelets,
which interact with vascular endothelium in the setting of inflam-
lation but do not extravasate actively, are Hermes-1⁻ (J.
Toys, unpublished observations, 1987).

In conclusion, the current studies illustrate unique levels of
Hermes antigen expression by lymphocytes representing
different stages of antigen-independent and antigen-depen-
dent lymphocyte differentiation. The findings imply a pre-
cise regulation of this adhesion molecule/homing receptor
during lymphocyte differentiation. On lymphocyte popu-
lations in secondary lymphoid organs, expression of the
Hermes antigen appears to correlate with (and is probably
one important determinant of) the capacity of cells to
interact with specialized endothelium at sites of lymphocyte
extravasation, and to participate in the recirculating lympho-
cyte pool. The role of the Hermes antigen on cells within
primary lymphoid organs, particularly on populations that
are not believed to recirculate, remains to be determined and
could reflect involvement of the Hermes antigens in other
cell and/or substrate adhesion events important in position-
ing of these lymphocyte subsets within their local microenvi-
ronments.

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