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The Roles of L-Selectin, β7 Integrins, and P-Selectin in Leukocyte Rolling and Adhesion in High Endothelial Venules of Peyer’s Patches

Eric J. Kunkel,* Carroll L. Ramos,* Douglas A. Steeber,† Werner Müller,‡ Norbert Wagner,‡ Thomas F. Tedder, † and Klaus Ley2*

Lymphocyte trafficking into Peyer’s patches requires β7 integrins and L-selectin. Here, we use intravital microscopy to examine leukocyte rolling and adhesion in Peyer’s patch high endothelial venules (HEV) of wild-type, L-selectin-deficient (L−/−), β7 integrin-deficient (β7−/−), and β7/L−/− mice. Although the leukocyte rolling flux fraction was reduced by 70%, Peyer’s patches in L−/− mice were of normal size and cellularity. In β7−/− mice, the rolling flux fraction was normal, but the number of adherent leukocytes in HEV was greatly reduced. The median leukocyte rolling velocity was reduced in L−/− mice, suggesting that β7 integrin-deficient and L-selectin mediate rolling in Peyer’s patch HEV at different velocities. β7/L−/− exhibited both a low rolling flux fraction and low adhesion and had severely reduced Peyer’s patch size and cellularity. The residual rolling in these mice was completely blocked by a P-selectin mAb. A significant P-selectin component was also detected in the other genotypes. Twenty-six percent of B and T lymphocytes isolated from Peyer’s patches of wild-type mice expressed functional ligands for P-selectin, and this fraction was increased to 57% in β7−/− mice. Peyer’s patch HEV were found to express P-selectin under the conditions of intravital microscopy, but not in situ. Our data suggest a novel P-selectin dependent mechanism of lymphocyte homing to Peyer’s patches. In situ, β7 integrins and L-selectin account for all lymphocyte homing to Peyer’s patches, but P-selectin-dependent rolling, as induced by minimal trauma, may support trafficking of effector T lymphocytes to Peyer’s patches.


Peyer’s patches, one component of the gut-associated lymphoid tissue, are located on the wall of the small intestine and play an important role in the mucosal immune response (particularly an IgA response). Lymphocytes continuously recirculate through Peyer’s patches to sample sequestered Ag and further differentiate into mucosal effector T and B lymphocytes. The first step in lymphocyte recirculation through Peyer’s patches is the movement of these cells from the Peyer’s patch high endothelial venules (HEV) into the surrounding lymphoid tissue. As in many other lymphoid and nonlymphoid tissues, lymphocyte recruitment to Peyer’s patches is thought to require several steps: initial attachment to the endothelium from the blood (capture), rolling along the endothelium, firm adhesion to the endothelium in response to activating substances, and subsequent emigration out of the venule (reviewed in Ref. 1).

Several molecules have been implicated in mediating lymphocyte recruitment into Peyer’s patches. L-selectin is a Ca2+-dependent mammalian lectin expressed constitutively on granulocytes and monocytes as well as on most circulating lymphocytes, including naive and certain effector T cells (2). Although L-selectin-deficient (L−/−) mice have morphologically normal Peyer’s patches, they exhibit a significant reduction in short term homing of exogenous lymphocytes to Peyer’s patches (3). Subsequent data have shown that this reduction in homing is most likely the result of a deficit in leukocyte rolling when L-selectin function is blocked, because lymphocytes treated with an L-selectin mAb and injected i.v. fail to roll in Peyer’s patch HEV (4), and functional blockade of L-selectin with a mAb in vivo reduces the number of rolling leukocytes in Peyer’s patch HEV by up to 90% (5). However, the normal size and cellularity of Peyer’s patches in L−/− mice (3) suggest that other important adhesion molecules can bypass the requirement for L-selectin in lymphocyte recruitment to Peyer’s patches. In fact, the absence of L-selectin delays, but does not prevent, lymphocyte homing to Peyer’s patches (3).

The most important adhesion molecule in lymphocyte recruitment to Peyer’s patches is α4β7, an integrin expressed at low levels on naive T and B cells and at high levels on effector and memory T cells within the gut (6). This integrin can mediate rolling interactions in vitro independent of L-selectin (7). Treatment of lymphocytes with a mAb specific for the α4 chain of this integrin results in a twofold increase in the rolling velocity of exogenous lymphocytes passing through HEV of wild-type Peyer’s patches in vivo (4). Recently, β7 integrin-deficient (β7−/−) mice have been generated (5) to address the role of α4β7 under physiologic conditions. These mice have severely reduced Peyer’s patch cellularity.

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3 Abbreviations used in this work: HEV, high endothelial venule; L−/−, L-selectin-deficient; β7−/−, β7 integrin-deficient; β7/L−/−, β7 integrin-L-selectin double knock-out.

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and decreased lymphocyte adhesion to Peyer’s patch HEV. However, the number of leukocytes rolling in Peyer’s patch HEV of β
t2−/− mice was normal, although the leukocyte rolling velocity was increased. Lymphocyte rolling in Peyer’s patch HEV of both wild-type and β
t2−/− mice was inhibited by >90% with an L-selectin mAb (5). Here, we examine β7 integrin/L-selectin double mutant (β7L−/−) mice that lack both adhesion molecules known to be important in lymphocyte homing to Peyer’s patches. We hypothesize that in the absence of these two adhesion molecules, other, previously unknown, adhesion mechanisms in Peyer’s patch HEV may be unmasked.

Materials and Methods

Animals

Mice deficient for L-selectin (3) or β7 integrin (5) expression were generated as described previously. Mice deficient for both L-selectin and β7 integrin were generated by breeding homozygous L−/− mice and homozygous β7−/− mice and testing the progeny for both mutated genes by Southern blotting. The absent cell surface expression of L-selectin and β7 integrin was confirmed using two-color immunofluorescence flow cytometry4,5. β7−/− mice were generated on a C57BL/6 background, and L−/− mice were at least a fifth generation backcross onto a C57BL/6 background. Control experiments were performed in age-matched C57BL/6 mice bred at the University of Virginia (breeders purchased from Hilltop Farms, Scottsdale, PA). All experiments were performed on healthy mice that were at least 8 wk of age. All mice were housed in a conventional facility. All animal experiments were approved by the institutional animal care and use committee.

Monoclonal Abs

The P-selectin mAb RB40.34 (rat IgG1, 30 μg/mouse) was a gift from Dr. Dietmar Vestweber at the University of Munster (Munster, Germany). This Ab blocks P-selectin-dependent adhesion in vitro (8), P-selectin-dependent leukocyte rolling in vivo (9), and P-selectin-dependent leukocyte recruitment in vivo (10). The P-selectin mAb MEL-14 (rat IgG2b; 100 μg/mouse) was purified from hybridoma supernatant (American Type Culture Collection, Manassas, VA). This Ab blocks L-selectin-dependent lymphocyte homing to peripheral lymph nodes (10) and L-selectin-dependent leukocyte migration across high endothelial venules of mouse Peyer’s patches requiring L-selectin and β7 integrin expression. L-selectin and β7 integrin synergistically mediate lymphocyte migration to mesenteric lymph nodes. Submitted for publication.

Initial attempts at staining Peyer’s patch HEV with indirect immunofluorescence of peroxidase-conjugated secondary Abs yielded unsatisfactory results, most likely because of low expression of P-selectin (data not shown). Therefore, we developed a new staining technique employing fluorescent beads. Recombinant protein G (Sigma) was covalently coupled to fluorescent-carboxylated (2-μm diameter) YG microspheres (Polysciences, Warrington, PA) using a carbodiimide coupling kit according to the manufacturer’s directions (Polysciences). Beads were stored in the storage buffer provided and used within 1 wk. Thirty minutes before observation of the Peyer’s patch, 200 μl of protein G-coupled beads were mixed with 200 μg of either a nonbinding rat IgG (Pierce, Rockford, IL) or the P-selectin mAb RB40.34. The mixture was allowed to incubate for 30 min before the beads were washed in isotonic saline with 1% BSA three times. The beads were resuspended in isotonic saline with 1% BSA and vortexed to break up any aggregates. Animals were injected with three sequential 50-μl boluses of the bead/g-coupled beads at 5-μm intervals following a 5-min post-injection period to allow for removal of any circulating beads. Subsequently, the RB40.34-coupled beads were injected in the same manner, and any bound beads were observed by intravital fluorescence microscopy.

Determination of P-selectin ligand activity on Peyer’s patch lymphocytes

Indirect immunofluorescence flow cytometry was used to examine binding of a recombinant human P-selectin-IgG chimera (gift from S. R. Watson, Genentech, South San Francisco, CA) to suspensions of Peyer’s patch lymphocytes. Human P-selectin can bind to mouse P-selectin (14). Peyer’s patches were isolated from wild-type, β7−/−L−/−, and β7L−/− mice and suspended in PBS, pH 7.4, with 2 mM CaCl2, 0.02% sodium azide, and 5% goat serum. Lymphocytes from Peyer’s patches were then released by gentle syringe aspiration. A multivalent P-selectin complex (15) was preformed by mixing recombinant P-selectin-IgG chimera and FITC-conjugated goat Fab’2, anti-human IgG (Fc-specific) Ab (CalTAG, Burlingame, CA) in 1/2(w/w) ratio followed by incubation at 4°C for 30 min. Aliquots of the P-selectin-IgG FITC complex (2 μg P-selectin-IgG/4 μg goat anti-human IgG FITC) were added to 1 x 104 cells and incubated for 20 min on ice. Lymphocytes in the Peyer’s patch preparation were identified by coinoculation with 1 μg each of phycoerythrin-conjugated mAb against the CD3 complex on T cells (clone 17A2, rat anti-mouse IgG2b, and CD45R/ B220 on B cells (clone RA3-6B2, rat anti-mouse IgG2a, Pharmingen, San Diego, CA). Selectin-specific adhesion was confirmed by inhibition of P-selectin complex binding to Peyer’s patch lymphocytes by treating cell
suspensions with 5 mM EDTA. Cell suspensions were analyzed on a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ) using CellQuest software (version 1.2).

Data analysis

Microvessel diameters were measured using a digital image processing system (16). Centerline blood flow velocity was determined after i.v. injection of 2-μm diameter fluorescent YG microspheres (Polysciences, Warrington, PA) by measuring frame-to-frame displacement (three microspheres per venule). Average blood flow velocity was determined by dividing the measured centerline velocity by a factor of 2. The rolling leukocyte flux fraction was determined as the number of rolling leukocytes expressed as a percentage of all leukocytes passing through the venule per unit time. In Peyer’s patch HEV, the number of rolling leukocytes was consistently higher (by ~60%) than the product of the flow rate and the systemic leukocyte concentration, a parameter used in previous studies (9, 11) as the denominator for the rolling flux fraction. An increased concentration of leukocytes in microvessels can occur as a consequence of multiple flow partition at bifurcations in the microvascular network (17, 18), leading to leukocyte accumulation in the most distal branches of the arteriolar tree. Consequently, venules fed by these branches also carry blood containing a higher leukocyte concentration. Therefore, the flux fraction in wild-type mice was set at 100%, and the flux fractions in the other genotypes were adjusted accordingly. The leukocyte rolling flux fraction was also corrected for variations in centerline velocity and venular diameter as described previously (9). Individual leukocyte rolling velocities were measured from video recordings by analyzing 5 to 15 leukocytes/venule and measuring the time necessary to travel a fixed distance (~50–80 μm) using a digital image-processing system (16).

Statistical analysis

All statistical comparisons were conducted using an analysis of variance with a post-hoc Student-Newman-Keuls multiple comparison procedure. SPSS software (SPSS, Chicago, IL) was used for all statistical analyses. Statistical significance was set at p < 0.05.

Results

General observations

All mice used in this work appeared healthy and of normal size and weight for their ages. During the intubation surgery, no enlarged cervical lymph nodes were observed in any mice. Similar to what has been previously reported, the systemic leukocyte counts in L−/− mice (3) were slightly higher than those in wild-type mice (Table I). Although previous work has not identified an increase in circulating counts in β2−/− mice (5), we found that circulating counts in these mice were also higher than those in wild-type mice (Table I). Similarly, the circulating leukocyte counts in β2/L−/− mice were elevated (Table I). The percentages of granulocytes and mononuclear leukocytes were similar in each genotype (Table I).

Peyer’s patch histology

We first examined the morphology of Peyer’s patches from wild-type, L−/−, and β2/L−/− mice (Fig. 1). The number of Peyer’s patches visible along the small intestine was reduced from ~8 to 10 in wild-type mice to ~1 to 2 in both the β2−/− and β2/L−/− mice (data not shown). The Peyer’s patches that could be located using a dissecting microscope on the wall of the small intestine in β2/L−/− mice were small and hypocellular (Fig. 1C) compared with wild-type Peyer’s patches (Fig. 1A). The number of Peyer’s patches in L−/− mice was not different from that in wild-type mice, and they were similar in size and cellularity to those in their wild-type counterparts (Fig. 1B). Concomitant with reduced Peyer’s patch size in β2−/− mice, the number of HEV observed through intravital observation was reduced compared with that in wild-type mice (data not shown) and the average diameter of those seen was slightly smaller (Table II).

![Figure 1](http://www.jimmunol.org)
Leukocyte rolling in Peyer’s patch HEV

We used blocking mAb to determine which adhesion molecules were mediating the leukocyte rolling in each genotype (Fig. 2). As shown previously (4, 5), leukocyte rolling in Peyer’s patches from wild-type mice was predominantly mediated by L-selectin, since the L-selectin function-blocking mAb MEL-14 blocked most of the observed rolling (Fig. 2A). However, injection of the mAb RB40.34 significantly reduced the rolling flux fraction by about 29% in wild-type mice (Fig. 2A). Interestingly, the percentage of granulocytes in the wild-type mice investigated here was about 29 to 38% of the total circulating leukocyte count (Table I), suggesting that many of the cells rolling using P-selectin may be granulocytes. Neutrophils are known to express a constitutively functional ligand for P-selectin called PSGL-1 (19). However, effector T cells also express functional PSGL-1 (20), and using P-selectin may be granulocytes. Neutrophils were noted, however, that the rolling flux fraction in L<sup>−/−</sup> mice was mediated by P-selectin (23).

Consistent with the mAb blocking data, the leukocyte rolling flux fraction in L<sup>−/−</sup> mice was only about one-third of that found in wild-type mice (Fig. 2B). Residual rolling in these mice was largely mediated by P-selectin (the mAb RB40.34 to P-selectin blocked almost all leukocyte rolling), but the rolling flux was unaffected by the mAb PS/2 to the α<sub>4</sub> integrin chain (Fig. 2B). Similar to wild-type mice and consistent with previous results (5), leukocyte rolling in β<sub>7</sub><sup>−/−</sup> mice was mediated by L-selectin (Fig. 2C). P-selectin blockade with the mAb RB40.34 in β<sub>7</sub><sup>−/−</sup> mice led to a slight (~10%), but not significant, reduction in the rolling flux fraction (Fig. 2C). β<sub>7</sub>/L<sup>−/−</sup> mice showed a low rolling flux fraction similar to that found in L<sup>−/−</sup> mice. This residual leukocyte rolling was totally P-selectin dependent (Fig. 2D).

Leukocyte rolling in vivo is known to be affected by geometric and hemodynamic conditions such as venular surface-to-volume ratio and average blood flow velocity (9, 22). Therefore, we measured several parameters that could affect leukocyte rolling in these experiments (Table II). Fluorescent beads were used to measure the centerline blood flow velocity in each observed venule. The data from all genotypes were stratified for venules with similar average wall shear rates.

Leukocyte rolling velocities

Adhesion molecules involved in leukocyte rolling may change the total number of rolling leukocytes, their rolling velocity, or both parameters (11). Therefore, we also analyzed the velocities of rolling leukocytes in each genotype (Fig. 3). In HEV of wild-type mice, leukocyte rolling velocities ranged from <10 to >100 μm/s (Fig. 3A) with a median velocity of 43 μm/s (Fig. 3E and Table III). In L<sup>−/−</sup> mice, the population of faster rolling leukocytes was missing (Fig. 3B), which significantly reduced (p < 0.05 vs wild-type mice) the median rolling velocity to 30 μm/s (Fig. 3E and Table III). Conversely, slow rolling leukocytes were missing in β<sub>7</sub><sup>−/−</sup> mice (Fig. 3C), which significantly increased (p < 0.05 vs wild-type and L<sup>−/−</sup> mice) the median velocity to 85 μm/s (Fig. 3E and Table III). Taken together, these findings indicate that the leukocyte rolling velocity histogram in wild-type mice is composed of two distinct populations: slower β<sub>7</sub> integrin-dependent rolling and more rapid L-selectin-dependent rolling. It should be noted, however, that the rolling flux fraction in L<sup>−/−</sup> mice is much lower (Fig. 2). This suggests that L-selectin may be important in capturing leukocytes from the flowing blood in Peyer’s patches in addition to mediating rolling. It therefore appears that efficient leukocyte attachment requires L-selectin even in the presence of β<sub>7</sub> integrin. In β<sub>7</sub>/L<sup>−/−</sup> mice (Fig. 3D), only a residual population of rolling leukocytes was detected (Fig. 2), which rolled at an intermediate velocity with a median of 56 μm/s (Fig. 3E and Table III), not significantly different from the velocity distribution in wild-type mice. Rolling in β<sub>7</sub>/L<sup>−/−</sup> mice was mediated by P-selectin (Fig. 2D). The observed velocity distribution is similar to that seen in venules of the mouse cremaster muscle where rolling is dominated by P-selectin (23).

Table II. Hemodynamic parameters of observed HEV

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Venules (n)</th>
<th>Diameter (μm)</th>
<th>Centerline Velocity (μm/s)</th>
<th>Wall Shear Rate (s&lt;sup&gt;−1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>6</td>
<td>25</td>
<td>19.2 ± 0.9</td>
<td>1907 ± 103</td>
</tr>
<tr>
<td>L&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>5</td>
<td>27</td>
<td>22.1 ± 1.5</td>
<td>2110 ± 188</td>
</tr>
<tr>
<td>β&lt;sub&gt;7&lt;/sub&gt;&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>5</td>
<td>34</td>
<td>17.9 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1918 ± 146</td>
</tr>
<tr>
<td>β&lt;sub&gt;7&lt;/sub&gt;/L&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>7</td>
<td>33</td>
<td>16.0 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2096 ± 197</td>
</tr>
</tbody>
</table>

<sup>a</sup> Significantly smaller diameter than in wild-type and L<sup>−/−</sup> mice (p < 0.05).

![FIGURE 2. Leukocyte rolling flux fractions in Peyer’s patch HEV from wild-type, L<sup>−/−</sup>, β<sub>7</sub><sup>−/−</sup>, and β<sub>7</sub>/L<sup>−/−</sup> mice.](http://www.jimmunol.org/)

The number of leukocytes rolling through Peyer’s patch HEV was counted, and the rolling flux fraction was calculated in wild-type (A), L<sup>−/−</sup> (B), β<sub>7</sub><sup>−/−</sup> (C), and β<sub>7</sub>/L<sup>−/−</sup> (D) mice. mAb to P-selectin (RB40.34), L-selectin (MEL-14), or α<sub>4</sub> integrin (PS/2) were injected i.v. * indicates significantly lower than no mAb in same genotype (p < 0.05). # indicates significantly lower than wild-type with no mAb (p < 0.05). † indicates significantly lower than both no mAb and plus RB40.34 in the same genotype (p < 0.05). ▼ indicates significantly lower than L<sup>−/−</sup> mice with RB40.34 (p < 0.05). Data shown are the mean ± SEM of 2 to 8 mice and 8 to 34 venules.
The velocities of the residual rolling leukocytes was also examined after several blocking mAb treatments. Functional blockade of P-selectin with the mAb RB40.34 in wild-type mice caused a significant increase \((p < 0.05)\) in the median leukocyte rolling velocity from about 43 to about 96 \(\mu m/s\) (Table III). Since blocking P-selectin in wild-type mice also causes a \(>30\%\) reduction in the rolling flux fraction (Fig. 2A), the velocity increase may be due to the lack of a population of leukocytes that rolls more slowly using P-selectin. In \(\beta_7^{-/-}\) mice, addition of RB40.34 did not significantly alter the rolling flux fraction (Fig. 2B) and also did not affect the median rolling velocity (Table III). As shown above, P-selectin mediated almost all the rolling in \(L^{-/-}\) mice (Fig. 2C). Interestingly, the median velocity of the few remaining rolling leukocytes in these mice was very low (Table III).

### Table III. Median leukocyte rolling velocities by genotype and mAb treatment

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. Leukocytes</th>
<th>Median Rolling Velocity (\mu m/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>6 146</td>
<td>43</td>
</tr>
<tr>
<td>Wild type + RB40.34</td>
<td>2 32</td>
<td>96*</td>
</tr>
<tr>
<td>(L^{-/-}) + RB40.34</td>
<td>2 20</td>
<td>30*</td>
</tr>
<tr>
<td>(\beta_7^{-/-}) + RB40.34</td>
<td>5 165</td>
<td>76*</td>
</tr>
<tr>
<td>(\beta_7^{-/-}) + RB40.34</td>
<td>2 49</td>
<td>84*</td>
</tr>
<tr>
<td>(\beta_7/L^{-/-})</td>
<td>7 80</td>
<td>56</td>
</tr>
</tbody>
</table>

* Significantly higher median velocity than wild-type and \(L^{-/-}\) mice \((p < 0.05)\).
* Significantly lower median velocity than wild-type and \(\beta_7^{-/-}\) mice \((p < 0.05)\).
LEUKOCYTE ROLLING IN PEYER’S PATCH VENULES

FIGURE 4. P-selectin expression in Peyer’s patch HEV. Two-micron fluorescent beads coupled to rat IgG (control mAb) or RB40.34 (P-selectin mAb) were injected i.v. and allowed to bind to the venular endothelium in the Peyer’s patch. Very few control beads bound to Peyer’s patch HEV (A), whereas the number of RB40.34-coated beads that bound to the endothelium was approximately 10-fold greater (B). Binding was specific, since pretreatment of the mouse with an i.v. injection of RB40.34 prevented the binding of RB40.34-coated beads to the endothelium (C). As a positive control, RB40.34-coated bead binding to the venular endothelium in the acutely exposed mouse cremaster muscle is shown (D). Free flowing beads (double exposure due to the stroboscopic illumination) are shown by arrows. Bar = 50 μm.

(Fig. 4B). Binding was specific for P-selectin on the endothelium because i.v. pretreatment of the mouse with RB40.34 reduced the binding of the RB40.34-coated beads to levels comparable to that of the control beads (Fig. 4C). As a positive control, the binding of RB40.34-coated beads to the venular endothelium of the acutely exteriorized mouse cremaster muscle was investigated. Under these conditions, cremaster muscle venules, but not arterioles, express P-selectin (24). RB40.34-coated beads bound avidly to the venular (Fig. 4D), but not arteriolar (data not shown), endothelium in the mouse cremaster muscle. HEV in Peyer’s patches of L−/− and β7/−/− mice also bound RB40.34-coated beads (data not shown), consistent with the observed P-selectin-dependent rolling in these vessels (Fig. 2).

P-selectin expression is known to be induced by surgical trauma in several models of inflammation (9, 24–26). We injected both control and RB40.34-coated beads into intact mice before surgery. After waiting 20 min (the time necessary after an injection to clear any circulating beads), the Peyer’s patch was exteriorized and observed with intravital microscopy. Very few beads were bound to the endothelium (data not shown). In contrast, when the same number of beads was injected i.v. 5 to 10 min later (after exteriorization), extensive binding was observed. These data suggest that the P-selectin expression was increased as a consequence of the manipulation of the small intestine during intravital microscopic viewing.

Peyer’s patch lymphocytes express P-selectin binding activity

Because we observed that P-selectin was involved in leukocyte rolling in all genotypes (Figs. 2 and 3) and was expressed on the endothelium of Peyer’s patch HEV (Fig. 4), we determined whether purified Peyer’s patch lymphocytes from each genotype exhibited P-selectin ligand activity. A multivalent P-selectin complex was formed by cross-linking a P-selectin-IgG chimera with a FITC-conjugated secondary Ab (15) and was used to investigate P-selectin binding activity of purified Peyer’s patch lymphocytes from wild-type, L−/−, β7/−/−, and β7/−/−/− mice by flow cytometry (Fig. 5). Approximately 26% of wild-type Peyer’s patch lymphocytes bound multivalent P-selectin (Fig. 5A). A similar number (~32%) of Peyer’s patch lymphocytes from L−/− mice also stained positively (Fig. 5C). In contrast, 45% of lymphocytes from β7/−/− mice (Fig. 5B) and 57% of lymphocytes from β7/−/−/− mice (Fig. 5D) stained positively for P-selectin ligand activity. Binding was selectin specific, as shown by complete inhibition in the presence of 5 mM EDTA (dotted lines in Fig. 5). As a positive control, we used HL-60 cells, a myeloid precursor cell line known to bind to P-selectin (27, 28). All HL-60 cells stained positively with the P-selectin complex (data not shown).

Discussion

We have analyzed leukocyte-endothelial interactions in mice deficient for two molecules deemed very important for lymphocyte recruitment to Peyer’s patches: L-selectin and β7 integrin. Using epifluorescence intravital microscopy of Peyer’s patches in these mice, we have determined that in the absence of L-selectin and β7 integrin, endothelial P-selectin can mediate a low level of leukocyte rolling. We show expression of P-selectin on the endothelium of Peyer’s patch HEV after surgical manipulation and P-selectin binding activity of lymphocytes isolated from Peyer’s patches. We hypothesize that although P-selectin is probably not involved in recruitment of naive lymphocytes to Peyer’s patches, this molecule may be very important for recruitment of effector T cells to sites of mucosal inflammation.

Although we observed P-selectin-dependent leukocyte rolling in all mouse genotypes studied in this work (i.e., wild-type, L−/−, β7/−/−, and β7/−/−/−), P-selectin has never before been implicated in participating in the recruitment of lymphocytes to Peyer’s patches. In fact, no evidence exists that suggests that P-selectin is even expressed on Peyer’s patch HEV. P-selectin has, however, been shown to be expressed on HEV in inflamed as well as noninflamed palantine and adenoid tonsils (29, 30). Additionally, P-selectin expression has been detected in the small intestine using 125I-labeled RB40.34 (31), but was not localized to any specific region (e.g., Peyer’s patches or lamina propria). By using fluorescent beads coupled to the P-selectin mAb RB40.34, we show that P-selectin is indeed expressed on the
vascular endothelium of Peyer’s patch HEV under the conditions studied. Our data represent the first evidence for P-selectin expression in Peyer’s patch HEV. More importantly, we also show that P-selectin is functional in these vessels and can mediate rolling after minimal trauma (exteriorization). P-selectin expression is known to occur in response to mediators such as histamine (26, 32) or during inflammation in response to cytokines such as TNF-α and IL-1 (33, 34). Since RB40.34-coated beads did not bind to the vascular endothelium in Peyer’s patches before surgery was initiated, we conclude that this expression is due to up-regulation of surface expression of this molecule after surgical manipulation of the small intestine. Therefore, P-selectin may not be involved in the recruitment of lymphocytes to Peyer’s patches under in situ conditions, but may play a role in trafficking of effector T lymphocytes during trauma or inflammation.

Many types of leukocytes express P-selectin ligand activity. Neutrophils (19), monocytes (35), and eosinophils (36) express a constitutively functional P-selectin ligand (PSGL-1) that mediates at least 90% of the P-selectin binding activity during inflammation in vivo (37). Although all lymphocytes express PSGL-1 (38), only certain subsets, including both effector αβ and γδ T-cells (20) and memory T cells (39), can bind to P-selectin. PSGL-1 functionality is regulated by an inducible fucosyl transferase called FucT-VII (40). Thus, for a T cell to bind to P-selectin, PSGL-1 must have the proper post-translational modifications (41). P-selectin binding to PSGL-1 appears to mediate Th1 cell recruitment into inflamed skin in vivo (42). Of the leukocyte subsets present in the circulation under normal conditions, neutrophils, eosinophils, and effector T cells all can bind to and roll on P-selectin. We therefore suspect that neutrophils, eosinophils, monocytes, and effector T cells are rolling through Peyer’s patch HEV using P-selectin expressed on the HEV endothelium after surgical manipulation.

In vivo differentiation of leukocyte subsets in Peyer’s patch HEV is not achievable with current intravital microscopic tools, so we are unable to determine which types of leukocytes are rolling via P-selectin in this model. Ex vivo labeling of isolated lymphocytes would allow differentiation of lymphocytes from granulocytes, as shown previously (43). However, excessive handling and manipulation of isolated cells may lead to partial activation and phenotypic changes. We were interested in observing leukocyte-endothelial interactions under relatively unperturbed conditions, so isolated, labeled lymphocytes were not used in this study. Nevertheless, we found that a significant number of isolated Peyer’s patch lymphocytes from all genotypes express P-selectin ligand activity. Even though P-selectin expression in Peyer’s patch HEV is below the detection limit of our current methods under in situ conditions, significant numbers of T and B lymphocytes in the Peyer’s patch can bind to this molecule (26–57%). Naive lymphocytes may enter a Peyer’s patch through the use of L-selectin and β2 integrin and may acquire P-selectin binding activity after differentiation into Ag-specific effector lymphocytes. These T lymphocytes would then be able to traffic to mucosal inflammatory sites using P-selectin, similar to what has been observed for Th1 cell recruitment to inflamed skin (42). The increased fraction of P-selectin-binding lymphocytes found in Peyer’s patch preparations from β7−/− mice and β2/β−/− mice may be due to an enrichment in the number of effector T lymphocytes (which can bind to P-selectin) in Peyer’s patches of mice lacking β7 integrin, since fewer naive lymphocytes can enter this tissue when this molecule is absent. A similar enrichment in effector T lymphocytes has been observed in peripheral lymph nodes of L−/− mice, since few naive lymphocytes can traffic through this tissue in the absence of L-selectin (44). This interpretation is consistent with the recent observation of a shift in lymphocyte subsets in β2/β−/− mice (45).

Our data support a role for β7 integrin in mediating leukocyte rolling. This evidence comes from the shift in the rolling velocity distribution observed in the absence of β7 integrin. An increase in rolling velocity when αβ integrin has been blocked (4) or when β7 integrin is knocked out (5) has been reported. Our present analysis confirms and expands these data to show that at least three molecules are involved in modulating leukocyte rolling velocity in Peyer’s patches: L-selectin, β7 integrin, and P-selectin. The rolling velocity distribution in wild-type mice appears to be a superposition of three distributions: a population of leukocytes that rolls rapidly (~80 μm/s) mainly via L-selectin (seen in the β7−/− mice), a population of leukocytes that rolls more slowly (~30 μm/s) mainly using β7 integrin (seen in the L−/− mice), and a smaller population of leukocytes that

**FIGURE 5.** P-selectin ligand activity on Peyer’s patch lymphocytes. Suspensions of Peyer’s patch lymphocytes identified by expression of CD3 (T cell) or CD45R (B cell) from wild-type (A), L−/− (B), β2−/− (C), and β2L−/− (D) mice were incubated with a multivalent P-selectin complex. Immunofluorescence flow cytometry was used to detect P-selectin complex binding to lymphocytes. Compared with incubation of cell suspensions with secondary Ab alone (thin solid lines), positive staining with P-selectin complex (bold solid lines) was detected on lymphocytes from each mouse genotype. The percentage of (bold solid lines) was detected on lymphocytes from two to five mice.
rolls with an intermediate range of velocities via P-selectin (seen in the $\beta_2$-L-/E-dependent mice). These three distributions superimposed can replicate the wild-type distribution measured here. Pure $\beta_2$ integrin-dependent leukocyte rolling can be seen in L- and E-selectin-deficient mice treated with a blocking P-selectin mAb. In these mice, the residual rolling flux is only 5% of that in wild-type mice, suggesting that L-selectin is important for effi- cient leukocyte capture in Peyer’s patch HEV. The median rolling velocity in L- and E-selectin-deficient mice treated with a blocking P-selectin mAb is on the order of 20 $\mu$m/s. A small rolling flux fraction in conjunction with a low rolling velocity suggests that $\beta_2$ integrin mainly functions to strengthen rolling interactions (i.e., reduce the rolling velocity), but is unable to efficiently mediate leukocyte capture and initiate rolling.

In conclusion, we present evidence that P-selectin can be induced on Peyer’s patch venules endothelium but is probably not involved in the recruitment of naive lymphocytes to Peyer’s patches. Our data showing P-selectin ligand activity in lymphocytes isolated from Peyer’s patches suggests that effector lymphocytes can bind to P-selectin and may use this molecule for recruit- ment to mucosal sites of inflammation. Under physiologic conditions, L-selectin and $\beta_2$ integrins appear to account for all lymphocyte rolling and adhesion in Peyer’s patch HEV.

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References

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