Marrow Cell Egress: Specificity of the Site of Penetration Into the Sinus

By Jack K. Chamberlain and Marshall A. Lichtman

Blood cells exit the marrow through the wall of the marrow sinuses. Using quantitative electron microscopy of mouse marrow, we addressed two questions regarding the anatomic process of egress: Do leukocytes and reticulocytes exit at specific sites on the sinus wall or do they exit at random loci? Do leukocytes and reticulocytes in egress use a preformed pore or do they make the pore as they exit? We confirmed that exiting leukocytes and reticulocytes do not pass through intercellular junctions; rather, they penetrate endothelial cells. The distance of migrating cells from endothelial cell junctions (1.4 ± 0.1 μm) was significantly less (p < 0.001) than the distance of endothelial cell junctions from points selected randomly along the sinus wall (4.0 ± 0.4 μm). The initial stage of cell penetration is often associated with thinning and formation of pits in the endothelial cell. A close-fitting migration pore forms and allows passage of the cell to the sinus lumen. The migration pore of leukocytes reaches a maximum diameter as the middle third of the cell is in transit, whereas the migration pore of reticulocytes is maximal as the first third of the cell is in transit. In sinuses with leukocytes in transit, pores unassociated with migrating cells compose only 10%, whereas pores associated with migrating cells compose 90% of the 25 pores/mm of the sinus wall. In sinuses without cells in transit, pore frequency was only 2/mm. Pores without migrating cells were smaller and were sealed by abluminal adventitial cells. They may represent residues of recent transit. These studies provide quantitative support for a preferential migration site for cells at the parajunctional zone of the endothelial cell and support the concept that pores are made at the time of egress.

HEMOPOIETIC CELLS develop in the extravascular spaces of marrow. These spaces are penetrated by vascular sinuses that derive their blood from the arterial circulation of bone. The sinuses drain into a central sinus that enters the efferent vein of marrow and thereby the systemic circulation. Since the marrow circulation is closed and hemopoietic cells develop extravascularly, mature cells must traverse the walls of the vascular sinuses to gain entry into the circulatory system. Migration of mature cells has been shown to occur through the cytoplasm of sinus endothelial lining cells rather than between endothelial cells. Several observers have been impressed by the frequency with which cells exit near an endothelial cell junction.

The present studies were undertaken to examine quantitatively two questions regarding the anatomical process of granulocyte and reticulocyte egress: First, do granulocytes and reticulocytes exit at specific sites on the sinus wall or do...
they exit at random loci? Second, do cells in egress use a preformed channel or do pores develop as cells exit?

MATERIALS AND METHODS

Electron microscopy. Thirty female Swiss Webster mice weighing 30-35 g and four male Sprague Dawley rats weighing 150-200 g were used in this study.

After mice were given pentobarbital (0.05 mg/g), the right femur was removed, held vertically, and split longitudinally with a razor blade. The split femur was placed immediately in Karnovsky fixative for 2 hr at 4°C. The hardened marrow was then gently dissected from its bony cortex, floated into the fixative, and cut into 1-mm blocks. The tissue was kept in the fixative at 4°C for an additional 12–18 hr, following which it was washed in 0.1 M phosphate buffer pH 7.4 and post-fixed 90 min at room temperature in 1% osmium tetroxide buffered to pH 7.4 with phosphate buffer. The tissue was then rinsed in buffer, dehydrated in a series of graded alcohols, infiltrated with Durcupan, and placed in a Beem capsule for embedding. Sections (1 μm) were cut with glass knives on a Reichert OMU-3 ultramicrotome. These sections were stained with toluidine blue in order to identify the structures to be examined. The appropriate areas were cut into thin sections with Dupont diamond knives, stained with lead citrate and uranyl acetate, and examined with a JEOL 100B electron microscope.

Rat marrow was fixed by vascular perfusion and processed for scanning electron microscopy as previously described. Perfusion was done in order to empty the vascular sinuses of blood and facilitate examination of the sinus lumen. Specimens were examined with a Coates-Welter field emission scanning electron microscope.

Quantification of migration site. In order to test the relationship of the migration site to the endothelial cell junction the distance between the migration pore and its nearest endothelial cell junction was measured in 139 consecutive micrographs of marrow sections from 90 marrow blocks from 30 mice (three blocks/mouse) selected only for the presence of one or more migrating cells. To construct a random aperture for comparison we extended a perpendicular from the aperture through which migration was occurring to the opposite sinus wall. Using this point as the center, we marked a hypothetical aperture of the same diameter as the actual one on the opposite sinus wall. The distance from the margin of this hypothetical aperture to the nearest endothelial cell junction was measured and considered to be a random relationship (Fig. 1). The differ-
Quantification of aperture frequency. In order to test whether migration occurred through preformed pores or whether pores were made as migration occurred, we compared the frequency of apertures in the sinus wall of 155 sinuses with migrating cells with the frequency of apertures in 155 sinuses without cells in migration, using sections of mouse marrow. If migration of cells started through preformed pores, the frequency of apertures in the sinus wall should be similar whether or not cells were caught in the process of migration in the section under analysis.

RESULTS

Quantification of migration site. The results of an analysis made of 155 pore-junction relationships from 139 random sections of 30 mouse femurs is shown in Fig. 2. The abscissa represents the distance in μm from the migration pore to the endothelial cell junction. The ordinate shows the cumulative percentage of migration pores that were a given distance from a junction. The distribution of the true distances showed that 60% of apertures occurred within 1 μm of an endothelial cell junction. The frequency with which random apertures were positioned less than 1 μm from the nearest junction was 10%. The frequency distributions of the true relationship and the random relationship were highly significantly different (p < 0.001) and indicate that migration pores do not occur at random sites but are closer to an endothelial cell junction than would be expected by chance.

The effect of the plane of section may have accounted for some or all of the migration pores that were located greater than 1.0 μm from a junction. Thus in some cases apertures may have been sectioned in a plane that did not include the nearest cell junction but only a more distant margin. This is shown in Fig. 3. A section along the horizontal line would show the migrating leukocyte within 1 or 2 μm of the endothelial junction, whereas a section along the vertical line would not. In support of this possibility, the cumulative frequency curve between 50% and 100% was also significantly different from the random curve. This observation suggests that even those apertures more than 1 μm from a cell junction were not randomly distributed but were closer to a junction than would be expected by chance. The close relationship of the migration pore to the endothelial junction is shown in Fig. 4. A tongue of granulocyte cytoplasm largely free of organelles can be seen projecting into the sinus lumen. The migration pore is adjacent to an endothelial cell junction.
Quantification of pore frequency. Although the endothelial cell lining of the sinus lumen is considered to be continuous, especially when compared to the heavily fenestrated adventitial layer, occasional small gaps are present in specimens prepared for either transmission or scanning microscopy. Figure 5 is a scanning electron micrograph that shows several small defects in endothelial cells of a vascular sinus. Some of the circular defects in the endothelium are
closed by underlying tissue, probably representing reticular cells based on observations in transmission micrographs (see below). In order to determine if small pores might be permanent parts of the sinus structure forming the locus for the initiation of egress, we compared pore frequency in sinuses with and without migrating cells. In sinuses with migrating cells there were $25.1 \pm 1.0$ apertures/mm of wall compared to $1.7 \pm 0.4$/mm in sinuses without cells in migration (Table 1). Moreover, of the 25 apertures/mm, only $2.7 \pm 0.7$ or 10% did not have cells in migration. The diameters of apertures without cells in migration were significantly smaller than apertures with cells: $0.4 \pm 0.07$ μm compared to $1.5 \pm 0.12$ μm ($p < 0.02$). Also, apertures without cells were found to be underlain by the cytoplasm of reticular cells. Figure 6 shows the discontinuity of the endothelial cell layer undercoated by the cytoplasm of an adventitial reticular cell.

The initial stage of cell penetration may be associated with thinning and pit

Table 1. Frequency of Apertures in Marrow Sinus Wall

<table>
<thead>
<tr>
<th></th>
<th>Sinuses With a Migrating Cell</th>
<th>Sinuses Without a Migrating Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>With cell in pore</td>
<td>$22.4 \pm 1.0$</td>
<td>0</td>
</tr>
<tr>
<td>Without cell in pore</td>
<td>$2.7 \pm 0.7$</td>
<td>$1.7 \pm 0.4$</td>
</tr>
<tr>
<td>Total pores</td>
<td>$25.1 \pm 1.0$</td>
<td>$1.7 \pm 0.4$</td>
</tr>
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Data represent mean ± SE in number of apertures per mm sinus wall.
formation in the endothelial cell. Figure 7 shows thinning of the cytoplasm of an endothelial cell. The thinning of endothelium just above an abutting mature granulocyte and adjacent to an endothelial cell junction may represent the beginning of endothelial cell discontinuity and pore formation.

When cells were found in egress, a close fitting migration pore had formed through which the cell entered the sinus lumen. Figure 8A shows that the migration pore of leukocytes is small initially, in this case about 0.5 µm. The pore reaches a maximal diameter as the middle third of the cell is in transit (Figure 8B). In both examples an endothelial cell junction is present near the migration pore.
Fig. 8. TEM of marrow sinuses. (A) Granulocyte is in apparent early migration through narrow aperture. Endothelial cell junction is adjacent to aperture (arrow). (B) Granulocyte is in middle third of migration through a pore that measures about 1.7 μm. Endothelial cell junction lies above and adjacent to pore (arrow). Note constriction of migrating cell in both cases and relative exclusion of organelles in intraluminal pseudopod.

In the case of reticulocytes, the migration pore usually is large initially and decreases in diameter as the cell progresses into the lumen. In Fig. 9A a reticulocyte is in a crowning representation with a near-maximal pore diameter. In Fig. 9B the migration pore is narrower as the middle third of a reticulocyte is in passage. We quantified the size of the migration pore when granulocytes and reticulocytes exited the marrow. Figure 10 is a histogram with the diameter of the migration pore plotted against the percentage protrusion of the migrating cell in the lumen. Reticulocyte migration pores reach their mean maximum diameter of 1.6 μm during the first third of cell passage, whereas leukocyte migration pores reach their mean maximal diameter of 2.3 μm during the second third of passage.

DISCUSSION

Our observations support the supposition that marrow cells do not exit the hematopoietic compartment at random loci in the sinus wall. They enter the lumen of the vascular sinus through the parajunctional zone of the endothelial
Fig. 9. TEM of reticulocytes in migration. (A) Reticulocyte is in apparent early egress through 2.5-μm-diameter pore. (B) Reticulocyte is in apparent egress through 0.5-μm-diameter pore. Junctions noted by arrows. S, sinus lumen.

Fig. 10. Diameters of migration pores plotted against percentage protrusion of migrating cell. Bars, means ± SE of 6–10 measurements. Total of 73 cells, 38 leukocytes, and 35 reticulocytes were enumerated. We assumed that plane of section did not introduce significant error in this comparison.
cell with a frequency exceeding that expected by chance. This association may be the result of cell egress occurring at the site of least resistance. In this situation the endothelial cell has a passive role such that the migrating cell produces contact between basal and luminal endothelial cell plasma membranes to induce membrane fusion and aperture formation. The parajunctional zone is often the thinnest portion of the endothelial cell and as such could be a favored location for penetration. The presence of an adjacent dense endothelial cell junction could facilitate cytoplasmic perforation by providing support for the endothelial cell margin.

Selectivity in release of mature cells would be present because of the increased ability of mature cells to "bore" holes and translocate in comparison to immature cells. Since apertures are small and capable of only limited dilatation, increased deformability of mature granulocytes and erythrocytes would also favor egress. These characteristics can be inferred from the presence of tight-fitting migration pores and marked deformation of the migrating cell.

Alternatively, egress may be the result of a more specific interaction between the migrating cell and the abluminal surface of the endothelium. The finding of radial and circumferential microfilaments associated with apertures in endothelial cell cytoplasm suggests that the endothelial cell could participate actively in cell migration. In addition, migration of cells occasionally occurs through thick areas of endothelial cytoplasm. Most importantly, endothelial cell cytoplasm may be thin over broad distances, yet cell penetration occurs in the parajunctional zone, further supporting the hypothesis of specificity in the area of pore development. Initiation of aperture formation may be related to a specific interaction of the migrating cells with the endothelial cell. It has been postulated that cell-releasing factors gain entry into the hemopoietic compartment via endothelial cell fenestrae with diaphragms and that this determines the site of interaction with the migrating cell. We did not observe an increased incidence of these structures adjacent to endothelial cell junctions in the absence of cell migration, however. In view of the rapid endocytosis demonstrated in sinus endothelial cells and the thinness of the endothelial cell cytoplasm, it may not be essential to invoke fenestrae as the portals of entry for chemoattractants, since a releasing factor also could diffuse or be transported rapidly.

The low frequency of pores in areas of sinus wall where cell egress is not occurring suggests that pores do not preexist but are formed at the time of migration and seal when migration is complete. If migration of cells started through preformed pores, the frequency of apertures in the sinus wall should be similar whether or not cells were caught in the process of migration. That pores reseal is suggested by calculations of the number of cells that exit during the life of the sinus. Without resealing, the sinuses would accumulate enough pores to lead to their dissolution.

The number of reticulocytes seen by transmission electron microscopy in the hematopoietic compartment of marrow suggests that enucleation of erythroblasts usually precedes transit through the sinus wall, although pitting of the nucleus by the sinus wall can occur, rarely.

Differences in the pore size during leukocyte or reticulocyte migration likely relate to differences in cell deformability and a nucleus that imposes an added factor to cell transit.
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REFERENCES


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