Acellular hemoglobin solution enters compressed lung capillaries more readily than red blood cells

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Received 2 March 2000; accepted in final form 28 April 2000

Conhaim, Robert L., Lance A. Rodenkirk, Kal E. Watson, and Bruce A. Harms. Acellular hemoglobin solution enters compressed lung capillaries more readily than red blood cells. J Appl Physiol 89: 1198–1204, 2000.—High lung inflation pressures compress alveolar septal capillaries, impede red cell transit, and interfere with oxygenation. However, recently introduced acellular hemoglobin solutions may enter compressed lung capillaries more easily than red blood cells. To test this hypothesis, we perfused isolated rat lungs with fluorescently labeled diaspirin cross-linked hemoglobin (DCLHb; 10%) and/or autologous red cells (hematocrit, 20%). Septal capillaries were compressed by setting lung inflation pressure above vascular pressures (zone 1). Examination by confocal microscopy showed that DCLHb was distributed throughout alveolar septa. Furthermore, this distribution was not affected by adding red blood cells to the perfusate. We estimated the maximum acellular hemoglobin mass within septa to be equivalent to that of 15 red blood cells. By comparison, we found an average of 2.7 ± 4.6 red cells per septum in zone 1. These values increased to 30.4 ± 25.8 and 50.4 ± 22.1 cells per septum in zones 2 and 3, respectively. We conclude that perfusion in zone 1 with a 10% acellular hemoglobin solution may increase the hemoglobin concentration per septum up to fivefold compared with red cell perfusion.

DCLHb; blood substitutes; plasma volume expanders; pulmonary microcirculation; pulmonary perfusion; zone I lung

ACELLULAR HEMOGLOBIN SOLUTIONS are being developed as a replacement for whole blood in the treatment of hemorrhagic shock (5). These solutions have several practical advantages over whole blood, including the fact that they can be infused without regard to blood type and can be frozen and stored for up to a year.

Functionally, these solutions have oxygen binding properties that are similar to those of whole blood, but, unlike whole blood, they are not confined to the circulation. In a recent report, our laboratory showed that acellular hemoglobin filtered into both lung and soft tissue lymph of unanesthetized sheep (3). The lymph concentration of the solution was small (<1 g/dl), suggesting that it would not provide any significant convective oxygen transport to metabolizing tissue. However, the presence of such solutions within the interstitium means that they could act as a low-resistance oxygen diffusion pathway, facilitating diffusive oxygen transfer to tissue (14). Thus acellular hemoglobin solutions may contribute to transvascular oxygen flux in ways that red blood cells cannot.

In addition to their ability to filter from the circulation, acellular hemoglobin solutions may have access to intravascular spaces that exclude red blood cells. This may be particularly important within the lung. Pulmonary capillaries are susceptible to compression at high inflation pressures, including those commonly used to ventilate patients in critical-care settings (10). These pressures inhibit red cell flow and reduce pulmonary vascular perfusion (8, 11). Acellular hemoglobin may enter compressed capillaries more easily than red blood cells under these conditions.

The goal of the present study was to determine whether this was so. We specifically wanted to know whether acellular hemoglobin solutions could enter compressed lung capillaries (zone 1) more easily than red blood cells. To address this, we not only had to quantify acellular hemoglobin entry into compressed lung capillaries, but we also had to quantify red blood cell hemoglobin entry for comparison. In general, red blood cells are thought to be excluded from alveolar septal capillaries under zone 1 conditions (11, 19). However, the entry of even a few red blood cells might result in a significant mass of hemoglobin within the alveolar septum compared with that delivered by an acellular solution. Our approach was to perfuse isolated rat lungs under zone 1 conditions with either fluorescently labeled acellular hemoglobin solution or fluorescently labeled red blood cells. We used confocal fluorescence microscopy to quantify hemoglobin entry of either perfusate into septa. After completing these initial studies, we extended the red cell studies to determine how much additional red cell hemoglobin would enter septa under conditions of zones 2 and 3.

MATERIALS AND METHODS

We perfused isolated rat lungs with either fluorescently labeled hemoglobin solution or red blood cells, at various pressures. The lungs were fixed by rapid freezing, and histo-
logical samples were examined using confocal fluorescence microscopy. We prepared several groups of lungs to address specific questions (Table 1).

Rats (450–550 g) were anesthetized with intraperitoneal ketamine (40 mg/kg), xylazine (6 mg/kg), and acepromazine (1 mg/kg), and tied supine. After infusing 750 U/kg heparin into the femoral vein, the femoral artery was cut, allowing the animals to exsanguinate; the shed blood was saved. A polyethylene cannula (PE 190) was placed into the trachea and connected via a side arm to a tube through which high air flows were maintained. By adjusting the outflow resistance of this tube, tracheal pressure was set to 5–7 cmH₂O. This prevented the lungs from collapsing during lung harvest. We opened the chest with a sternum-splitting incision and tied polyethylene cannulas into the pulmonary artery and left atrium. The cannulated heart and lungs were then removed from the chest and placed, dorsal side down, into a small polyethylene foam container. The vascular cannulas were passed through the container walls.

We ventilated the lungs (25 breaths/min) with air using a piston pump (Harvard) and perfused them at the pressures shown in Table 2. The perfusate was placed into a reservoir determined the pulmonary artery pressure, which was referenced to the bottom of the foam container in which the lungs were perfused. Vascular and airway pressures were recorded using strain-gauge transducers (Statham) and an oscillograph (Grass). The perfusate was collected in a reservoir and ultrafiltered to remove any potential red cell or viral fragments. It was mixed with balanced electrolytes to produce a solution with a final hemoglobin concentration of 15%, a P₅₀ of 32 mmHg, an oxygen-carrying capacity of 1.39 ml/g, and a pH of 7.4 at 37°C (13). The methemoglobin concentration was <10%, and the endotoxin activity, as measured by Limulus amebocyte lysate assay, was <0.1 EU/ml.

After warming the frozen DCLHb solution to room temperature, we fluorescently labeled it by adding Evans blue to produce a DCLHb-Evans blue molar ratio of 4 to 1. We chose Evans blue because it forms a strong electrostatic bond to proteins that yields a bright, broad-spectrum, red fluorescence that is easily distinguished from the green autofluorescence of the tissue (2).

We fluorescently labeled rat autologous red blood cells using 5-(and 6)-chloromethyl SNARF-1, acetate (Molecular Probes). Each 50 μg of SNARF-1 was reconstituted with 8.5 μl of DMSO, to which we added 8.5 ml of phosphate-buffered saline (PBS: NaCl, 0.15 M; Na₂HPO₄, 0.003 M; Na₂HPO₄, 0.001 M; pH 7.4) in a light-protected container. Two to five milliliters of packed red blood cells were suspended in the SNARF-1 solution and incubated at 37°C for 15 min. The cells were pelleted, resuspended in PBS, and pelleted again. We repeated this until the supernatant was colorless. After the final spin, the cells were suspended in a measured amount of PBS to produce a hematocrit of 20. This red fluorescent red cell solution was then infused into isolated rat lungs.

We perfused the lungs for 10 min with labeled hemoglobin or red blood cells, then rapidly froze the lungs by flooding the foam container with liquid nitrogen. Just before freezing, we fluorescently labeled the lysine moieties of the alpha subunits (1, 15, 17). This stabilized the hemoglobin molecule as a tetramer, preventing degradation into nephrotoxic α-β dimers that do not transport oxygen. The extent of cross-linking was >99% (15). The solution was heat pasteurized and ultrafiltered to remove any potential red cell or viral fragments. It was mixed with balanced electrolytes to produce a solution with a final hemoglobin concentration of 10%, a P₅₀ of 32 mmHg, an oxygen-carrying capacity of 1.39 ml/g, and a pH of 7.4 at 37°C (13). The methemoglobin concentration was <10%, and the endotoxin activity, as measured by Limulus amebocyte lysate assay, was <0.1 EU/ml.

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We harvested frozen lung tissue blocks (4 × 4 × 4 mm; 5 per lung) and dehydrated them in cold ethanol (−70°C; 48–72 hr). The dehydrated blocks were embedded in resin (JB4, Polysciences), and thick sections (75 μm) were examined using a confocal fluorescence microscope equipped with a krypton-argon laser and solid state photodetectors (BioRad MRC 1024 ES).

### Table 1. Questions and perfusion conditions

<table>
<thead>
<tr>
<th>Question</th>
<th>Perfusate</th>
<th>Conditions</th>
<th>No. Lungs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Does acellular hemoglobin enter alveolar capillaries in zone 1?</td>
<td>Fluorescently labeled hemoglobin</td>
<td>Zone 1</td>
<td>5</td>
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<tr>
<td>Do red blood cells enter alveolar capillaries in zone 1?</td>
<td>Fluorescently labeled red blood cells</td>
<td>Zone 1</td>
<td>5</td>
</tr>
<tr>
<td>Do red blood cells interfere with acellular hemoglobin entry into alveoli in zone 1?</td>
<td>Fluorescently labeled hemoglobin + nonfluorescent red blood cells</td>
<td>Zone 1</td>
<td>3</td>
</tr>
<tr>
<td>Does more acellular hemoglobin enter alveolar capillaries in zone 2 than in zone 1?</td>
<td>Fluorescently labeled hemoglobin + nonfluorescent red blood cells</td>
<td>Zone 2</td>
<td>3</td>
</tr>
<tr>
<td>Do more red blood cells enter alveolar capillaries in zone 2 than in zone 1?</td>
<td>Fluorescently labeled red blood cells</td>
<td>Zone 2</td>
<td>3</td>
</tr>
<tr>
<td>Do more red blood cells enter alveolar capillaries in zone 3 than in zone 2?</td>
<td>Fluorescently labeled red blood cells</td>
<td>Zone 3</td>
<td>2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Question</th>
<th>Perfusate</th>
<th>Conditions</th>
<th>No. Lungs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Do more red blood cells enter alveolar capillaries in zone 3 than in zone 2?</td>
<td>Fluorescently labeled red blood cells</td>
<td>Zone 3</td>
<td>2</td>
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</tbody>
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### Table 2. Inflation and perfusion pressures

<table>
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<tr>
<th>Zone</th>
<th>P吸入</th>
<th>P呼気</th>
<th>P肺靜脈圧</th>
<th>P左房圧</th>
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<tr>
<td>Zone 1</td>
<td>25</td>
<td>15</td>
<td>15</td>
<td>0</td>
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<tr>
<td>Zone 2</td>
<td>15</td>
<td>5</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Zone 3</td>
<td>15</td>
<td>5</td>
<td>20</td>
<td>10</td>
</tr>
</tbody>
</table>

Pressures (P) are given in cmH₂O.
In a confocal microscope, structures above and below the plane of focus are not visible. Thus the specimen can be optically sectioned by focusing vertically (20). Individual optical sections measured $512 \times 512$ pixels. Sections were obtained at thicknesses of either 1.0 $\mu$m ($\times$20 objective) or 0.5 $\mu$m ($\times$40 objective). Each pixel had a gray-scale (fluorescence intensity) value that ranged from zero (black) to 255 (white). Evans blue-labeled DCLHb was imaged using the 647 nm laser line for excitation, and a 680 nm filter for detecting fluorescence. SNARF-1-labeled red blood cells were imaged using the 568 nm line for excitation, with a 598 nm detection filter. Excitation at 568 nm to view SNARF-1 also excited Evans blue-labeled DCLHb because of the broad excitation and emission spectra of Evans blue-labeled protein (12). For this reason, we did not infuse both Evans blue-labeled DCLHb and SNARF-1-labeled red blood cells into the same lung.

We measured DCLHb fluorescence gray-scale pixel values within septal images in individual optical sections (1.0 $\mu$m thickness) and used these data to compare acellular hemoglobin fluorescence among treatment groups. Our assumption was that fluorescence intensity was proportional to the thickness of DCLHb layer within each septum. The laser power, photodetector gain, and black-level (background) were set to the same values in all sections examined to ensure that gray-scale values were recorded comparably among treatment groups. We confirmed this by displaying the image of each section in a pseudo-color mode that identified those pixels that were darker than a gray-scale value of zero (green) and those that were brighter than a gray-scale value of 256 (red). The laser power, gain, and black-level were set so that <0.2% of all pixels appeared as either red or green. This ensured that the full sensitivity range of the photodetector was utilized to acquire each fluorescent image.

We used these images to quantify DCLHb fluorescence within septa by counting pixel gray-scale values in images of septal planes. Examples illustrating this technique are shown in Fig. 1. We completed the counts using public-

Fig. 1. Confocal fluorescence images of alveoli in lungs perfused with fluorescently labeled diaspirin cross-linked hemoglobin (DCLHb) in zone 1 (top left), or fluorescently labeled DCLHb plus nonfluorescent red blood cells in zone 2 (bottom left). The gray haziness in some alveoli is caused by DCLHb fluorescence within alveolar septa. We quantified this by measuring the fluorescence gray-scale value (0–256) in images of individual septa. Examples of areas measured are shown by the polygons in the enlarged images at right (asterisks lie adjacent to enlarged areas). Gray-scale fluorescence values were measurably greater in lungs perfused in zone 2 (see Table 2). Each image represents a tissue thickness of $\sim$1.0 $\mu$m.
domain software available for this purpose (NIH Image, version 1.61). We compared gray-scale values among lungs perfused with DCLHb to determine whether the perfusion conditions affected the mass of DCLHb that was present.

To quantify the mass of red cell hemoglobin present within septa, we counted red blood cells in stacks of optical sections. One hundred sections, spanning a tissue depth of 25 μm (50% optical section overlap), were acquired sequentially and stacked digitally to obtain a more complete septal view. Cells were counted using software to convert the image to a threshold view in which the only pixels visible were those caused by red cell fluorescence. The resulting image consisted exclusively of pixels with gray-scale values >100 (NIH Image, version 1.61). Within threshold view, we counted pixels occupied by images of several individual red blood cells (5–10 cells), then averaged these to obtain a value representing the average number of pixels per cell. Next, we counted the total number of threshold view pixels present within images of individual septal planes; these were defined using the methods illustrated in Fig. 1. We divided this septal pixel-count by the average number of pixels per red blood cell to obtain a value representing the average number of red blood cells per septum.

We also digitally reconstructed stacks of optical sections, obtained from lungs perfused in zone 1 with DCLHb, into three-dimensional alveolar images using commercial software designed for this purpose (Voxblast, version 1.0., Vaytek). We did this to gain a better understanding of the distribution of DCLHb and red blood cells within septa.

Statistics. Results are expressed as means ± SD. Data were compared using ANOVA and Fisher’s PLSD post hoc test (SAS Institute). Differences were considered to be significant at P < 0.05.

RESULTS

Perfusion with DCLHb in zone 1 produced flows that averaged 5.3 ± 1.6 ml/min at the start of the 10-min perfusion period (Table 3). By comparison, perfusion with red blood cells in zone 1 produced flows that averaged 3.9 ± 2.5 ml/min at the start of perfusion (not significant). Perfusion with red blood cells in zones 2 and 3 produced initial flows that were successively higher than those in zone 1, although the differences were not significant. After 10 min of perfusion, flows in all groups had declined (Table 3). Ten minutes of perfusion with acellular hemoglobin solution in zone 1 resulted in flows that were significantly higher than after 10 min of perfusion with red blood cells (Table 3).

<table>
<thead>
<tr>
<th>Table 3. Perfusate flows at beginning and end of 10-min perfusion</th>
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</thead>
<tbody>
<tr>
<td>Perfusate</td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td>DCLHb, * zone 1</td>
</tr>
<tr>
<td>RBC, zone 1</td>
</tr>
<tr>
<td>RBC, zone 2</td>
</tr>
<tr>
<td>RBC, zone 3</td>
</tr>
</tbody>
</table>

Values are means ± SD. DCLHb, diaspirin cross-linked hemoglobin; RBC, red blood cells. *No RBC in perfusate. †Significantly different from RBC zone 1 (P ≤ 0.05); ‡includes lungs perfused with both Evans blue-labeled DCLHb and unlabeled RBC, as well as lungs perfused only with SNARF-1-labeled RBC’s.

Measurement of DCLHb fluorescence in confocal septal images yielded gray-scale values of 93.3 ± 21.9 in lungs perfused in zone 1 with DCLHb (Table 4). Addition of red blood cells to the DCLHb perfusate in zone 1 produced gray-scale values of 109.8 ± 21.3. Septal fluorescence gray-scale values in lungs perfused in zone 2 with both DCLHb and red blood cells were 165.0 ± 37.4, which was significantly greater than either of the values obtained in zone 1. Distribution of DCLHb within a septum under zone 1 conditions can be better visualized in a three-dimensional reconstruction of DCLHb fluorescence (Fig. 2). Figure 2 shows that the hemoglobin solution is not uniformly distributed throughout the alveolus. The solution layer appears to be thinnest at the center of the septum, where the interstitial distribution is incomplete. Pixel analysis of this portion of the image revealed that 52% of the septal area was occupied by DCLHb. The solution appears as if it is not confined to capillaries, suggesting that it filtered from the microcirculation to fill the perimicrovascular interstitial space.

In lungs perfused with fluorescently labeled red blood cells, we found that the average number of cells per septum was 2.7 ± 4.6 in lungs perfused in zone 1 (Table 5). This value rose to 30.4 ± 25.8 in zone 2, and to 50.4 ± 22.1 in zone 3. These latter two values were not significantly different from each other, but they were significantly different from the value obtained in zone 1.

DISCUSSION

The goal of our study was to determine whether acellular hemoglobin could enter alveolar septa in zone 1 better than red blood cells. We found acellular hemoglobin to be present within alveolar septa in zone 1 (Table 4), but we also found that small numbers of red blood cells were present as well. To answer the question we originally raised, we had to determine whether the mass of acellular hemoglobin present within septa in zone 1 was greater than the mass of cellular hemoglobin present.

To address this, we calculated the grams of DCLHb likely to be present in an alveolar septum in zone 1, and compared it with the grams of hemoglobin present within red blood cells. We used the following assumptions. We assumed that an alveolar septum has a...
diameter of 75 μm, and assumed that the DCLHb layer within the septum in zone 1 is 1 μm thick. This latter assumption is supported by the cross-sectional views of septa shown in Fig. 2. Based on a hemoglobin concentration of 10%, we calculated that 4.4E⁻¹⁰ of hemoglobin were present within each septum in zone 1. To calculate the grams of hemoglobin in one red blood cell, we assumed that a hemoglobin concentration of 15 g/dl is equivalent to a red cell concentration of 5 E⁶ cells/μl. From these values, we calculated that 3 E⁻¹¹ grams of hemoglobin are present within each cell. By dividing this into the value we calculated for the mass of DCLHb in each septum, we estimated that 15 red blood cells would have to be present in each septum in zone 1 to equal the mass of hemoglobin in a 10% DCLHb solution.

Under clinical conditions it is unlikely that the plasma DCLHb concentration would ever reach 10%. Furthermore, the reconstructed view (Fig. 2) shows that only about one-half of the thinnest area of the center of the septum is filled with hemoglobin. Nonetheless, our calculations suggest that the addition of any acellular hemoglobin to plasma would likely augment hemoglobin delivery to septa in zone 1.

Our data provide no information on oxygen uptake by either form of hemoglobin during lung perfusion. Oxygen uptake depends on the rate at which hemoglobin flows through septa in zone 1, whether in cellular or acellular form. We cannot address this point because our studies were conducted with fixed tissue. However, it seems likely that acellular hemoglobin would be likely to flow through septa more readily than red blood cells under zone 1 conditions.

Table 5. Red blood cell distribution in alveolar septa

<table>
<thead>
<tr>
<th>Zone</th>
<th>Zone 1</th>
<th>Zone 2</th>
<th>Zone 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red blood cells per septum</td>
<td>2.7 ± 4.6</td>
<td>30.4 ± 25.8*</td>
<td>50.4 ± 22.1*</td>
</tr>
<tr>
<td>No. alveoli examined</td>
<td>37</td>
<td>30</td>
<td>29</td>
</tr>
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</table>

*Significantly different from zone 1.
this protective effect and increase the risk of pulmonary edema in injured lungs. This point is emphasized by the fact that we found DCLHb in the pulmonary microvascular interstitium, where it would surround septal microvessels (Fig. 2). Infusion of acellular hemoglobin solutions into trauma and critical care patients may enhance oxygen delivery, but the NO scavenging properties of these solutions might adversely affect the pulmonary microvascular filtration barrier.

Our data highlight an important point about differences in liquid and red cell distribution among pulmonary microvessels. We found significant inhomogeneities in septal red cell counts in zones 1 and 2, and the large SD about the mean values emphasize this point (Table 5). DCLHb was not uniformly distributed either, as can be seen in both the gray-scale values (Table 4), and in Fig. 3 (top). However, the DCLHb distribution appears to be significantly more uniform than the red cell distribution, suggesting that the acellular solution flowed throughout septa more freely than red blood cells in zone 1. The uniformity of the distribution is also emphasized by the three-dimensional reconstruction (Fig. 2).

Adding red blood cells in the DCLHb perfusate had no significant effect on DCLHb entry into septa (Table 4), suggesting that entrances to capillaries were not plugged by red blood cells. Alternatively, DCLHb may have entered the septal interstitium after filtration from the circulation. This hypothesis is suggested by the fact that DCLHb does not have the channel-like appearance within septa that red blood cells do (Fig. 3).

Our results also support an issue brought up several years ago by Wagner (18) and König and colleagues (9). These investigators pointed out that liquids and red blood cells may be distributed differentially within the pulmonary microcirculation, and our findings support this idea. In zone 1, we found fluorescent hemoglobin solution to be distributed relatively uniformly among alveolar septa, whereas red blood cells were nearly absent. Furthermore, including red blood cells in the perfusate did not restrict DCLHb entry into septa, suggesting that red blood cells did not provide any significant impediment to fluid entry, even in zone 1. This idea is important because it emphasizes that the concept of capillary perfusion may have to be considered differentially for liquids and cells. It also supports the point made by König and colleagues (9) that red blood cells and plasma can take different paths through the pulmonary microcirculation.

We did not count numbers of septa containing DCLHb or red blood cells because it is difficult to do this in confocal images. In individual optical sections, septa of some alveoli inevitably lie outside of the image plane (Fig. 1). This leads to an artificially low value for the fraction of septa that contain DCLHb or red blood cells. More septa are present in stacks of optical sections (Fig. 3), but obtaining accurate septal counts from these images is difficult because some septa are partially or completely hidden by others. Also, the septa of some alveoli lie outside of the stack. We found it more reliable to count red blood cells in septal images rather than to count numbers of septa. However, the impression given by Fig. 3 top is that most, if not all, alveolar septa contained at least some DCLHb.

The variation in the gray-scale value of septal DCLHb fluorescence (Table 4) does not reflect concentration differences, because the lungs were perfused with a 10% DCLHb solution. However, the variation must be due to differences in the thickness of the DCLHb layer within each septum. This would explain why the gray-scale value for zone 2 lungs was greater than that of zone 1. We were not able to use the gray-scale value to quantify the thickness of the hemoglobin layer within the septum because the septal layer...
was thinner than the resolving power of the microscope in the vertical (z) axis. Confocal resolution in this axis is one-half of that in the x and y axes (those within the plane of the page; 20).

Our fluorescent red cell studies offer some insight into the nature of septal perfusion during ventilation in zones 1, 2, and 3. In zone 1, we found that red blood cells were confined mainly to interalveolar corner vessels; few cells appeared within the septal plane (Fig. 3, Zn 1). Furthermore, vessels supplying septa appeared to be congested with fluorescence, suggesting inhibition of red cell entry into septal microvessels under zone 1 conditions. Zone 2 conditions significantly increased the number of cells entering septa, although the mean red cell count had a large SD (Table 5), suggesting nonuniform red cell distribution among septa. This is also evident in Fig. 3, Zn 2. In zone 3, larger numbers of cells were distributed throughout the septal plane than in zone 2, and the distribution appeared to be more uniform; the standard deviation was 44% of the mean, compared with 85% for the zone 2 values. However, differences between the zone 2 and 3 mean red cell counts were not significant, because of the standard deviations.

The main conclusion of our study is that acellular hemoglobin was able to enter compressed alveolar septal microvessels in zone 1 more extensively than hemoglobin confined to red blood cells. These results are consistent with previous findings from our laboratory that liquids can flow through septal microvessels in zone 1 (11), who found that red blood cells flowed through corner, but not septal, vessels in zone 1.

We thank Dr. Ken Burhop of Baxter Healthcare for kindly providing the DCLHb, and the Department of Veterans Affairs for use of their confocal microscope.

This work was supported by a grant from Baxter Healthcare and by National Heart, Lung, and Blood Institute Grant HL-49985.

REFERENCES


