Intravital microscopic observations of 15-μm microspheres lodging in the pulmonary microcirculation

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Lamm, Wayne J. E., Susan L. Bernard, Wiltz W. Wagner, Jr., and Robb W. Glenny. Intravital microscopic observations of 15-μm microspheres lodging in the pulmonary microcirculation. J Appl Physiol 98: 2242–2248, 2005. First published February 10, 2005; doi:10.1152/japplphysiol.01199.2004.—Vascular infusions of 15-μm-diameter microspheres are used to study pulmonary blood flow distribution. The sites of microsphere lodging and their effects on microvascular perfusion are debated but unknown. Using intravital microscopy of the subpleural surface of rat lungs, we directly observed deposition of fluorescent microspheres. In a pump-perfused lung model, ~0.5 million microspheres were infused over 30 s into the pulmonary artery of seven rats. Microsphere lodging was analyzed for the location in the microvasculature and the effect on local flow after lodging. On average, we observed 3.2 microspheres per 160 alveolar facets. The microspheres always entered the arterioles as singletons and lodged at the inlets to capillaries, either in alveolar corner vessels or small arterioles. In all cases, blood flow continued either around the microspheres or into the capillaries via adjacent pathways. We conclude that 15-μm-diameter microspheres, in doses in excess of those used in typical studies, have no significant impact on pulmonary capillary blood flow distribution.

METHODS

Animal preparation. All animal work was approved by the University of Washington Animal Care and Use Committee and follows the guidelines for animal experimentation provided by the National Institutes of Health (14). Seven Sprague-Dawley rats weighing 395 ± 29 g (mean ± SD) were anesthetized with pentobarbital sodium (10–15 mg/kg intraperitoneal injection), intubated via a tracheostomy and ventilated with 5% CO2 in air via a Harvard rodent ventilator (model 683, Holliston, MA). The tidal volume, respiratory rate, and positive end-expiratory pressure (PEEP) were set at 3 ml, 30 breaths/min, and 4 cmH2O, respectively. The rats were placed on a heating pad adjusted to maintain a core body temperature of 38°C, and the chest was opened by a midline sternotomy. They were heparinized with 500 units by intracardiac injection and exsanguinated by cardiac puncture. The collected blood was mixed with equal parts of lactated Ringer plus 4% bovine albumin buffered to a pH of 7.4 and placed into the perfusion circuit (Fig. 1). The perfusion circuit consisted of an adjustable-height heated venous reservoir, Cole/Parmer dual-head roller pump (model 7013, Vernon Hills, IL), and a 40-μm in-line filter (Pro-Fuel Motorcycle no. 823, Purolator, Brentwood, TN), which also served as a windkessel to dampen the pulse pressure and trap air bubbles. A 2-mm-diameter (OD) catheter was placed into the main pulmonary artery via the right ventricle, and a 2.5-mm-diameter (OD) catheter was placed in the left atrium. The pulmonary artery catheter had two side ports to allow for the injection of microspheres and measurement of pulmonary arterial pressure (Ppa). Care was taken to avoid introducing any air bubbles into the perfusion circuit. The lungs were initially perfused at ~0.01 ml·min⁻¹·g body wt⁻¹. The left chest wall was removed, and the right pulmonary artery was tied off to ensure that the entire perfusion went to the visualized lung. Physiological variables were monitored and recorded by use of a Mac G3 (Apple Computer, Cupertino, CA) with a MacLab8S (AD Instruments, Castle Hill, NSW, Australia). Airway pressure, Ppa, and pulmonary venous pressure (Ppv) were continuously measured by use of Abbott Critical Care Systems transducers (N. Chicago, IL) and were referenced to the level of the observation field. Because pressures were not recorded from the very tips of the catheters, pressures were corrected for tubing resistance. Samples of the perfusate were taken at the beginning and during the study to monitor pH, PCO2, PO2 (ABL 5, Radiometer, Copenhagen), and hematocrit.

Video microscopy. We used a Zeiss AXIOTECH vario 100 HD microscope (reflected-light dark field) and reflected light with a fluorescent rhodamine filter illuminated by a mercury/xenon lamp (200 W) passed through heat-absorption and green-interference filters. The microscope was fitted with both Epiplan-Neofluar ×10 and ×20 lenses and Video Zoom ×0.33–×1.6. Attached to the video zoom was an Iris LCL-902K (Watec America) video camera with autointensity gain. For color photography, an AutomaticCam video camera (Mi-
croimage Video Systems, Boyertown, PA) and a Nikon E995 digital camera (Nikon, Tokyo, Japan) with a Thales Digital Camera Coupler (Thales Optem, Fairport, NY) could also be mounted. The scope and stand were mounted on a TMC Micro-g vibration isolation table (Peabody, MA) to isolate the system from room vibrations. The camera signal was fed into a Sony SVHS video recorder (model SVO-5800). Figure 1 shows a schematic of the intravital microscope and perfusion-ventilation system. Figure 2A shows the microscope lens and a cross section of the pleural window. The pleural window was constructed with a 6-mm-diameter windowpane (Fig. 2B) mounted within a suction ring that was attached to a micromanipulator (David Kopf Instruments, Tujunga, CA). The suction ring (−20 to −25 cmH₂O) gently immobilized the lung surface (21, 22). The dimensions of the pleural window are presented in Fig. 2C. Once the window was in place, the microscope was focused on the surface of the lung. Because of a very narrow depth of field, only capillaries and microspheres immediately under the pleural surface remained in focus. We therefore saw alveolar walls and septal vessels within the pleural window. We could not see the alveolar walls and septal vessels that were on the opposing side of the alveoli.

Perfusion within the circuit was slowly increased to ∼0.03 ml·min⁻¹·kg body wt⁻¹. We stopped ventilation briefly at end expiration during all video recordings. The video image area was 1.25 × 0.95 mm of the subpleural surface. We could view the lung either with reflected light using dark or bright field with a rhodamine filter (546/590 nm, excitation/emission). The video imaged area was first visualized by dark field and the image recorded. The rhodamine filter was then substituted for the dark field, allowing visualization of red fluorescent microspheres (peak emission 595 nm).

While viewing and videotaping, we infused a bolus of 0.4 million red, 0.025 million yellow, and 0.025 million green fluorescent 15-μm microspheres into the pulmonary artery over 30 s. The microspheres had a mean diameter of 14.7 μm with a SD of 0.3 μm (8). The yellow and green microspheres were included for later visualization in the excised lung with an Imaging CryoMicrotome (Barlow Scientific, Barlow Scientific)
Olympia, WA). Before injection, the microspheres were sonicated, vortexed, and mixed in the same syringe. After microsphere infusion, we returned to dark-field imaging and recorded blood flow for 15–20 s. Using a frame grabber (ProTV, Formac, Berkeley, CA), we printed a picture mapping the location of the red microspheres in the field of view. Switching to a higher magnification (×340), we briefly recorded a video image of flow around each red microsphere. This was repeated for three randomly selected additional nonoverlapping areas.

Image analysis. A lighting artifact created by the conical shaped light beam reflecting off of the pleural surface produced an easily identified outline of each alveolus (Fig. 3A). Alveolar facets were defined as the surfaces of the alveoli against the windowpane. We replayed the recorded images and analyzed the initial area as well as three other nonoverlapping areas by dark-field imaging to determine the number of alveoli, the number of red microspheres per unit area, and whether the microspheres lodged in clusters of two or more. We counted only those microspheres that lodged at the pleural surface. By reviewing the video recording, we could determine whether the deposited red microspheres entered the arterioles as singlets or clusters. From the high-magnification images, we determined the location of all lodged red microspheres within the microcirculation (i.e., arteriole, corner vessel, or alveolar capillary) as well as any effect of microspheres on local blood.

Red blood cell movement in the pulmonary microcirculation is easily seen with intravital microscopy but is difficult to document and convey to those not familiar with the techniques. To clearly demonstrate the flow of red blood cells, we labeled red blood cells with carbocyanine as previously reported (20), with minor modifications. Cells were washed with phosphate-buffered saline, and incubation was carried out at 4°C to improve cell preservation. After thorough washing, cells were suspended to a hematocrit of 30% with 0.9% NaCl for injection. At 12–24 h before the study, a Sprague-Dawley rat weighing between 350 and 450 g was injected with 1–2 ml of labeled cells through a tail vein. Red cell fragments and stiff cells were removed from the circulation by the recipient rat. Blood was then procured from this rat and used in the pump-perfused lung. Flow cytometry confirmed that 1–2% of red blood cells were labeled.

Evaluation of flow in observed areas. We were concerned that the suction surrounding the window may alter the flow beneath it. To evaluate this, we used the Imaging CryoMicrotome that determines the spatial location of every microsphere in the lung with very high spatial resolution (4). At the completion of the study, the lungs were filled with optimal cutting temperature compound (Tissue-TeK, Sakura Finetek, Torrance, CA) via the airways and frozen. The surface of the lung previously in contact with the pleural window was marked to allow later identification of the window location. The Imaging CryoMicrotome counted microspheres through a series of thresholding techniques (4). We injected too many red fluorescent microspheres to permit the analysis program to accurately count microspheres because of doublet and triplet clusters. We therefore simultaneously injected two other colors of microspheres in numbers we knew we could faithfully count to measure regional pulmonary blood flow. We analyzed two rat lungs with the Imaging CryoMicrotome to determine whether flow to the region under the pleural window was different than flow to other regions of the lung. We estimated local flow at each microsphere location by determining the distance to the 10th nearest neighboring microsphere. High-flow regions would have more microspheres in a given lung volume and

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Fig. 3. Time sequence of microsphere infusion into the lung at the same pleural area. A: dark-field image, preinfusion. B: fluorescent (rhodamine filter) image, preinfusion. C: fluorescent image, midway into the infusion; microspheres lodging below the pleural surface appear large and out of focus. D: fluorescent image, postinfusion. Note specific example of microspheres (C and D) aggregating at the same location. Microspheres below the pleural surface appear as large indistinct glows, whereas microspheres at the pleural surface appear sharply defined.
hence smaller distances to the 10th nearest neighbor. The distance to the 10th nearest neighbor was therefore inversely related to local flow. We analyzed 10 random locations within each of five lung regions. The first region was directly under the pleural window and within 100 μm of the pleural surface. Three other regions, well away from the pleural window and within 100 μm of the pleural surface, were also measured (one region at the level of the pleural window, one region more cephalad, and one region more dorsal). The last region included parenchyma deep within the lung and well away from the pleural surface.

Statistics. All results are presented as means ± SD. Statistical comparisons were made by using a two-tailed, paired t-test. Statistical significance was accepted at P < 0.05.

RESULTS

Hemodynamics. Ppa dropped slightly after microsphere injection, 21.6 ± 5.2 to 20.5 ± 4.7 cmH2O (P < 0.05). Ppv averaged 4.9 ± 1.2 cmH2O, whereas peak airway pressure and PEEP averaged 10.8 ± 1.9 and 3.5 ± 0.4 cmH2O, respectively. There were no significant changes in Ppv, peak airway pressure, or PEEP after microsphere injection. The blood gases and pH from the venous reservoir were normal, and hematocrit averaged 19 ± 1%. These values were constant over the course of the experiment. Time from the start of lung perfusion to infusion of microspheres averaged 40 ± 10 min. The remainder of the study required an average of 75 ± 30 min to complete.

Video analysis of microsphere deposition. We observed 3.2 ± 3.1 red microspheres/mm² viewing area. Each square millimeter area contained 160 ± 18 alveolar facets, which would correlate to a diameter of ~80 μm per alveolar facet, a value equal to that found by Presson et al. (17) in a similar rat preparation. From the video images, we determined that microspheres always (33 out of 33 observations) entered the preparation. From the video images, we determined that microspheres always entered (33 out of 33 observations) entered the arterioles as singlets (Fig. 3). Figure 3 shows periods during the time sequence of injection of microspheres. The first view (Fig. 3A) is preinjection using dark field for a clear visualization of the field. The second view (Fig. 3B) is the same field preinjection using the rhodamine filter. The third view (Fig. 3C) is midway through the microsphere injection; several microspheres are present, but of particular interest is a microsphere in the lower left-hand corner. There are also a number of microspheres lodging below the pleural surface that appear large and out of focus. The final view (Fig. 3D) is postmicrosphere infusion, and now there are two additional microspheres lodged against the one identified in the previous view (Fig. 3C). The subsequent arrival of the second and third microsphere demonstrates that flow continued in the region of the initial microsphere. There are also many more microspheres out of focus in the background of Fig. 3D.

We found that, over time, microspheres collected in vessels as singlets (37%), doublets (29%), triplets (24%), and quadruplets (11%). Figure 4 shows a representative view of microspheres aggregated in the microvasculature just under the pleural surface. Of the total 112 microspheres observed, 38% were located in arterioles, 62% in corner vessels, and <1% in capillaries within alveolar facets. In all cases, blood flow continued in the vicinity of the microspheres in arterioles and corner vessels. Microspheres appeared to lodge at capillary inlets to alveolar facets but did not stop blood flow in alternate pathways across the alveolar surfaces (see video clip 1 at http://jap.physiology.org/cgi/content/full/01199.2004/DC1). As previously defined (12), alveolar septal vessels and corner (manifold) vessels were classified depending on the location within or between alveoli; that is, vessels were considered to be corner vessels if they were not located within an alveolar facet. Microspheres were never seen to pass completely through the microcirculation to the venous side. Despite injecting 20 times more microspheres than usually injected in living animal studies, we saw no change in vascular resistance and virtually no alteration in regional blood flow. Although we injected many more microspheres than usual, we observed only 1 microsphere per 50 alveolar facets, illustrating the sheer number of alveoli in the lung that produce the remarkable vascular reserve.

We also directly observed red blood cells flowing past lodged microspheres (see video clip 1 of blood flow around a lodged microsphere).

Imaging CryoMicrotome. By determining the location of all green and yellow fluorescent microspheres with the Imaging CryoMicrotome, we found no difference in regional blood flow below the pleural window compared with other pleural lung regions (Table 1). We therefore concluded that the viewing window did not affect the blood flow in this subpleural region. Furthermore, subpleural blood flow was shown to be ~30% less than parenchymal blood flow deeper in the lungs (Table 1), as shown previously by Short and coworkers (19).

DISCUSSION

We studied the sites of microsphere lodging and their effects on microvascular perfusion in the pulmonary microcirculation. Using videomicroscopy of the subpleural surface of pump-perfused rat lungs, we found that microspheres always entered the arterioles as singlets. They lodged at the inlets to capillaries, in either alveolar corner vessels or small arterioles. In all cases, blood flow continued unabated either around the microspheres or into the alveolar capillaries via adjacent capillary pathways. We conclude that 15-μm microspheres, in doses typically used in physiological studies, have an inconsequential impact on local pulmonary capillary blood flow.

Several issues need to be considered in interpreting the data from the present study. First, we assumed that subpleural capillaries are perfused in the same general manner as internal capillaries. There are at least two significant differences between internal and subpleural capillaries. First, subpleural capillaries are 30% larger in diameter than internal capillaries...
is an estimated 20 fluorochromatic microspheres into the left lung only. Because lodging per 50 alveolar facets after an injection of 0.4 million capillary vessels, we doubt that the microspheres would make it all of the way through the septal vessel bed in an intact animal, although we have observed that the microspheres have on blood flow.

A unique opportunity to more directly study differences in blood flow to surface and interior lung regions was present in this study. To show that the suction window did not alter the blood flow beneath it, we compared blood flow beneath the window to the rest of the pleural surface (Table 1). By locating every yellow and green microsphere with the Imaging Cryo-Microtome, we demonstrated that blood flow per lung volume was similar beneath the window and at distance from it. Furthermore, we confirmed that the blood flow distribution to the pleural region of the lung in the rat is similar to that previously reported in rats, dogs, and rabbits (11, 19) and is ~30% less than parenchymal blood flow.

The second issue is that the lungs in this study were pump perfused, and it is well recognized that the resistance of pump-perfused lungs is higher than in intact animals. As pressure drops along the vascular tree from arterioles to capillaries, driving pressures from the capillary to the left atrium will be less in pump-perfused compared with intact animals that have similar arterial pressures. Hence the capillary pressure and thus the driving pressure propelling these microspheres is likely to be lower than that of an intact animal. We would expect that the microspheres could penetrate further into the septal vessel bed in an intact animal, although we doubt that the microspheres would make it all of the way through the myriad capillary vessels.

In this study, we observed an average of 1 microsphere lodging per 50 alveolar facets after an injection of 0.4 million fluorescent microspheres into the left lung only. Because there is an estimated 20 × 10⁶ alveoli per rat (13) and assuming that 40% of blood flows to the left lung, we would estimate 1 microsphere per 20 alveoli. Our lower count of 1 to 50 is reasonable considering the above-mentioned reduced pleural flow and that we are only counting microspheres on one facet of each alveolus.

We did not observe an increase in pulmonary arterial pressures after the microsphere injections. Our prior study predicted that we should have seen an increase in Ppa in the pump-perfused model (9). The difference between the two studies is that the prior study utilized a fully dilated isolated perfused rat lung, which would not allow for any vascular recruitment. In this study, the pulmonary vasculature of our rats was not fully dilated, allowing recruitment of other pathways when microspheres lodges within the microcirculation. This recruitment would explain the lack of change in pulmonary vascular resistance and pressure after microsphere injection.

The microsphere method for measuring regional organ perfusion is an established and well-accepted technique (18). It is based on the fundamental principle that labeled microspheres lodge within capillary beds in proportion to local blood flow. The microsphere method has been validated in the lung using both radiolabeled red blood cells (3) and a “molecular microsphere” (1). A basic tenet is that injected microspheres are inert and do not alter local flow or resistance. It is argued that microspheres occlude only a fraction of capillaries and do not, therefore, significantly change local vascular resistance. However, this has never been directly substantiated. Indirect support for this contention has been obtained in a pump-perfused rat lung with a fully dilated circulation, where pulmonary vascular resistance was found to increase by only 0.8% for every 100,000 microspheres injected (4). There have been only a few studies to indirectly demonstrate that the microspheres given in appropriate numbers and methods do not alter regional organ perfusion (1, 15). Hales and Cliff (10) provided video evidence of microspheres lodging in rabbit ear arterioles and the persistent blood flow around the microspheres. The present study is the first to dynamically visualize microspheres lodging in the pulmonary microcirculation and to show the minimal effects that microspheres have on blood flow.

We found that microspheres lodged mainly in the arterioles and corner vessels at the entrances to the alveolar septal capillaries. This observation is similar to the findings of Hales and Cliff (10) in the rabbit ear microvasculature. They found that microspheres lodged in the arteriole at the mouth of fine nutrient vessels and midstream in the arterioles. The nutrient vessels are analogous to the septal capillaries in our rat lungs. Hales and Cliff also observed microspheres within the nutrient vessels, which was rare in our observations of the septal capillaries in the pump-perfused lungs. The higher pressure of the systemic system may explain the difference in how far microspheres are pushed into the capillaries. Furthermore, Hales and Cliff reported microspheres moving within the viewing window as well as microspheres leaving the window altogether (attributed to arteriole to venous shunts) (10). We did not observe microspheres moving from their original lodged location under the pleural window but only watched their behavior for 15–30 min. In addition, we did not find any arteriole-to-venous shunts.

Intravital microscopy allowed us to directly visualize subpleural blood flow and the deposition of microspheres in the pulmonary microcirculation. We visualized microspheres entering the pulmonary circulation as singlets with subsequent microspheres lodging adjacent to previously deposited microspheres. Because microspheres are carried by blood flow, this observation demonstrates that blood flow persists in the vicinity of lodged microspheres. In fact, subsequent microspheres entering the microcirculation appeared to preferentially flow into prealveolar capillaries in which a prior microsphere had lodged. This demonstrates that there are preferential pathways for blood flow at the microcirculatory level and that a single lodged microsphere does not prevent subsequent microspheres from taking the same preferential pathways (see video clip 2, at http://jap.physiology.org/cgi/content/full/01199.2004/DC1). We also directly observed red blood cells flowing around the deposited microspheres, demonstrating that microspheres do not completely occlude the prealveolar capillary in which they are lodged.
We can imagine a number of possible ways in which blood flow can persist around lodged microspheres. One possibility is that the microspheres are not perfectly round and do not completely fill the circular cross section of a prealveolar capillary or arteriole. Using scanning electron microscopy, we have previously demonstrated that the fluorescent microspheres used in this study are uniformly round (5), making this explanation unlikely. Another potential consideration would be that prealveolar capillaries or arterioles are not perfectly circular in cross section and may allow space for blood to flow past round microspheres. Red blood cells are very deformable (5, 6) and able to squeeze through conduits only a few micrometers in diameter. An alternate explanation is that some of the microspheres reaching the capillaries may become lodged within the “sheets and posts” proposed by Fung and Sobin (7). Microspheres would become stuck against posts, allowing blood to continue to flow through the sheets supported by other posts. Another mechanism could be that microspheres stick at bifurcations of vessels where the cross section is not circular, allowing blood flow to continue into nonoccluded vessels. It is also possible that a microsphere could completely occlude a vessel and that blood flow is then diverted to another vessel that reconnects downstream to the microsphere, allowing the blood to detour around the obstruction. Finally, and least likely, microspheres could become adherent to arteriolar walls through interactions with endothelial cells and their glycoca-lyces. The fluorescent microspheres used in this study do not have any reactive sites on their surfaces and are unlikely to bid to endothelial surfaces. Furthermore, we never observed microspheres transiently stopping within an arteriole and then moving at a later time, providing further evidence that the microspheres must lodge within a lumen smaller than their diameters. Although we do not have definitive proof, we speculate that the microspheres become lodged within noncircular lumens and that blood is able to flow through small spaces between the microspheres and vessel walls.

Before this present study, the dynamic arrival of microspheres in the pulmonary microcirculation had not been visualized. If the locations of microspheres are determined after a large number of microspheres have lodged, bunches of microspheres will be identified within the same vessel. One reasonable conclusion might have been that the microspheres arrived at their destination as a group. Our study clearly demonstrates that this conclusion is not correct and that, when handled appropriately, microspheres arrive in the pulmonary microcirculation individually.

Microspheres are distributed within the microcirculation in proportion to the flow to each region. Because the driving pressure from pulmonary artery to left atrium is the same throughout all vessels, the total resistance within a given pathway determines the flow to a region. Microspheres are preferentially distributed to the regions of the microcirculation with the least total resistance from pulmonary artery to left atrium. If a microsphere lodging in a capillary bed does not appreciably change the resistance of the pathway feeding and draining that region, additional microspheres will continue to be distributed to the same region through the same arteriole and microspheres will lodge within the same prealveolar capillary or arteriole (see video clip 2).

The possibility that microspheres may alter the local physiology of the pulmonary microcirculation has been a decades-long concern of investigators and reviewers. How is it possible that several hundred thousand microspheres can be injected into a finite circulation and not affect organ perfusion resistance? This paradox is now resolved by our observations showing that when microspheres enter the microcirculation of the lung and wedge therein, they do not significantly impede the movement of red blood cells in their local vicinity. The video clip demonstrates that red cells can flow around the lodged microsphere within the extra-alveolar capillary. Again, it should be emphasized that although large numbers of microspheres (400,000) were injected into a single lung, on average only one microsphere lodged per 50 alveolar facets. These mechanisms leave plenty of reserve for the microcirculation to remain undisturbed. We think that this is clear evidence that, when injected in large numbers, the microspheres present no significant impediment to blood flow. This observation suggests that serial injections of microspheres faithfully represent pulmonary blood flow when measured to lung regions as small as a few microliters in volume. This impressive capacity for the microcirculation to sieve foreign bodies, including small blood clots and fat emboli from broken bones while maintaining a low resistance and large gas-exchanging surface area, highlights one of the central design features of the pulmonary circulation.

REFERENCES


