The Behavior of Sonicated Albumin Microbubbles Within the Microcirculation: A Basis for Their Use During Myocardial Contrast Echocardiography

Mark W. Keller, Steven S. Segal, Sanjiv Kaul, and Brian Duling

The purpose of this study was to determine whether the behavior of sonicated albumin microbubbles accurately mimics red blood cell flow in the microcirculation and is thus consistent with their use as in vivo tracers of red blood cell flow during myocardial contrast echocardiography. Accordingly, microbubbles prepared from fluorescein-conjugated albumin and fluorescently labeled red blood cells were injected intravascularly in eight golden hamsters. Their intravascular distribution, velocities, arteriolar-to-venular transit times, and flux ratios at branch points were determined in the microcirculation of the cheek pouch. Albumin microbubbles (mean diameter, 4.9±3.6 μm) and red blood cells displayed a similar frequency of distribution across the arteriolar lumen (33% in the central 20% of the arterioles), and their arteriolar velocities were also similar (2.5±0.7 mm/sec and 2.3±0.7 mm/sec, p=NS). The mean velocities of microbubbles correlated well with those of red blood cells at baseline and after adenosine application (r=0.97 and r=0.89, respectively), as did the calculated maximum velocity (r=0.98 and r=0.80, baseline and adenosine, respectively). The velocity profiles across the lumen of the vessels for albumin microbubbles and red blood cells were similar at baseline and after adenosine-induced velocity changes. The flux ratios at branch points also correlated well (r=0.92, p<0.001). Arteriolar-to-venular transit times of albumin microbubbles were similar to those of red blood cells in vessels ranging in size from 22 μm to 45 μm. We conclude that the behavior of albumin microbubbles in the microcirculation mimics that of red blood cells and supports their use as intravascular tracers of red blood cell flow during myocardial contrast echocardiography. (Circulation Research 1989;65:458-467)

Flow patterns of intracellular microbubbles can be traced by two-dimensional echocardiography. It has been proposed that application of the principles of indicator dilution to flow patterns of the microbubbles may enable one to make a noninvasive assessment of regional myocardial blood flow in vivo. Time-intensity curves derived from regions of the myocardium during transit of sonicated contrast agents suggest that microbubbles produced by sonication may behave as true intravascular tracers. The use of microbubble suspensions as myocardial echo contrast agents has been limited by the deleterious effects produced by the contrast agents on left ventricular function, systemic hemodynamics, and coronary blood flow. We have recently demonstrated that human albumin may be an ideal material for the production of microbubbles because, unlike other echo contrast agents, it does not produce significant changes in coronary blood flow, left institutes of Health. S.K. is the recipient of the Clinical Investigator Award (K08-HL-01833) and the FIRST Award (R29-HL-38345) of the National Institutes of Health. Presented in part at the 37th Annual Scientific Session of the American College of Cardiology in Atlanta, March 1988, and in part at the Annual Meeting of the Southern Society of Clinical Investigation in New Orleans, February 1988, for which M.W.K. was awarded the Burroughs-Wellcome Young Investigator Award. Address for correspondence: Mark W. Keller, MD, Department of Medicine, Box 158, University of Virginia Health Sciences Center, Charlottesville, VA 22908. Received May 12, 1988; accepted January 31, 1989.
ventricular function, or systemic hemodynamics. A major limitation to wider use of the technique is that the adequacy of albumin microbubbles as an index of red blood cell flow has not been validated by direct observation. Therefore, we developed a method to prepare fluorescently labeled albumin microbubbles that permitted us to test the hypothesis that the rheologic behavior of albumin microbubbles is similar to that of red blood cells in the intact microcirculation. Our findings support our hypothesis and suggest that albumin microbubbles may be useful in measuring regional myocardial blood flow during two-dimensional echocardiography.

Materials and Methods

Animal Preparation

Eight male golden hamsters (mean weight, 107 ± 15 g) were anesthetized with pentobarbital sodium (70 mg/kg i.p.; Abbott Laboratories, North Chicago, Illinois). A tracheotomy was performed to ensure a patent airway, and the right femoral vein was cannulated for continuous infusion (420 μl/hr) of 0.9% saline containing 1 mg/ml pentobarbital sodium for replacement of respiratory fluid loss and maintenance of anesthesia. The left external carotid artery was cannulated to permit intra-arterial injection of albumin microbubbles in high enough concentrations to allow visualization of an adequate number of microbubbles within the cheek pouch. The animal was placed on a platform, and the cheek pouch was prepared for visualization of the microcirculation as described previously.

The cheek pouch preparation was superfused continuously at approximately 5 ml/min with a bicarbonate-buffered physiological salt solution containing (mM) NaCl 131.9, KCl 4.6, CaCl₂ 2.0, MgSO₄ 1.2, and NaHCO₃ 20. The pH of the superfusate was maintained at 7.4 by bubbling it with 5% CO₂ in nitrogen. The cheek pouch was maintained at 37 ± 1°C by controlling the temperature of the superfusate solution, while esophageal temperature was maintained at 37–38°C with conducted heat.

Microvessels of the cheek pouch were viewed with an intravital microscope (model ACM, Zeiss Optical Corporation, Thornwood, New York) equipped with an immersion objective (×16, n.a. 0.50; Plan-Neuflcorr, Zeiss). Transillumination was performed with a Ploem illuminator (model 468031, Zeiss) coupled to a video camera equipped with a Newvicon tube (series 68, Dage-MTI, Michigan City, Indiana). An interchangeable fluorescence cube (model W480, Zeiss) was used during fluorescent microscopy in conjunction with a strobe illuminator (model 126, Chadwick Helmhuth, El Monte, California) at a flash rate of 30/sec. The strobe was synchronized to the video vertical frame rate of an SIT camera (series 66, Dage-MTI). The overall magnification of this system was ×770 as presented on a high resolution monitor (model HR2000, Dage-MTI). The video images were calibrated with a stage micrometer (100×0.01 = 1 mm, Graticules Ltd., Tonbridge, Kent, United Kingdom) and recorded on videotape with either a Panasonic (model NV-8950, Matsushita Electrical Industrial, Japan) or a video recorder (model AV-3650, Sony Corporation, Park Ridge, New Jersey). A character generator (model G-77, Odetics, Anaheim, California) provided time in hundredths of seconds on the recorded images.

Microbubble Preparation

Human albumin (5% solution, New York Blood Center, New York, New York) was labeled with dichlorotetrazoliumylaminofluorescein 1 dihydrochloride (DTAF) (Research Organics, Cleveland, Ohio) as previously described. Sixti micromoles DTAF was added to 20 ml phosphate-buffered human albumin solution, and the solution was stirred at room temperature for 1 hour. Eight milliliters labeled albumin was treated with a sonicator equipped with a ½-inch tip (model W-375, tip #200, Heat Systems Ultrasonic, Plainview, New York) at a power output of 120 W for 40 seconds in a 10-ml plastic syringe (Becton-Dickson, Rutherford, New Jersey).

Forty-five seconds after sonication, coarse surface bubbles were removed, and the remaining solution was allowed to stand for 4–8 hours, at which time a thin layer of foam containing labeled microbubbles had formed on the surface. The microbubbles present in this foamy layer were resuspended twice in 10 ml unlabeled 5% albumin solution to wash excess free label. After the second wash, the microbubbles were resuspended in 1 ml albumin.

Production of Fluorescent Red Blood Cells

Red blood cells from a separate donor animal were labeled by two methods. In one animal, tetramethylrhodamine isothiocyanate (TRITC, Research Organics) was used as the labeling agent, and in three animals, calcein (C-0875, Sigma Corporation, St. Louis, Missouri), which binds to intracellular calcium, was used. TRITC-labeled red blood cells could be readily distinguished from the DTAF-labeled microbubbles by the difference in the wavelengths of their fluorescent emissions; however, these red blood cells were quickly cleared from the circulation and were only visible on the first pass through the microvessels. Calcein-labeled red blood cells were stable in the circulation. This stability allowed simultaneous comparison of microbubble and red blood cell behavior throughout the course of an experiment after a single bolus injection at the initiation of the protocol. Because the wavelengths of the fluorescent emissions of calcein-labeled cells and DTAF-labeled microbubbles are the same, they were distinguished from each other on the basis of the more variable size distributions and irregular shapes of the microbubbles.

Protocol

Microbubble size distributions were obtained from in vitro observations. In one animal, in vivo size
distributions were also obtained. Arterioles and venules were identified that exhibited normal tone and reactivity as assessed by responses to topical application of 0.1 mM adenosine and elevation of superfusate oxygen concentration to 10 or 21%. The microscope image was focused to limit observations to the median plane of the vessel.

Injections of labeled microbubbles and red blood cells were performed during observations of the tissue under stroboscopic illumination and fluorescence microscopy. In the first four animals, from 0.1 to 0.3 ml labeled microbubbles were injected over a period of 3–10 seconds. In a second set of animals, direct comparison of the rheology of microbubbles and red blood cells was made possible by simultaneous injection of labeled microbubbles and red blood cells. In one animal, TRITC-labeled red blood cells were injected before each injection of microbubbles. In three animals, a 0.1-ml bolus of calcine-labeled red blood cells was injected at the beginning of the experiment. Observations were recorded at second, third, and fourth order arterioles and neighboring venules.

Topical application of approximately 0.1 μM adenosine was used to alter vessel diameter and velocity. After the vessel size and flow response had stabilized, microbubbles were reinjected and recordings were repeated.

Image Analysis

Recorded videotape images were digitized and analyzed off-line (Mipron system, Kontron Electronics, Eching, West Germany) in a 340×240×8 format. The computer was calibrated in the x-y planes using a recorded image of the calibration reticule. The diameters of 100 microbubbles were determined in vitro from a sample of the stock solution used in each animal. In one animal, similar measurements were made in vivo. Only microbubbles and red blood cells that were in sharp focus on single frame review were analyzed to limit observations to the horizontal plane at the center of the vessel. With a ×16 objective (n.a., 0.5) and an overall magnification of approximately ×770, the optical section should be less than 10 μm. Velocity profiles were assessed by reference to a transparent acetate sheet placed on the video screen to limit observations to the median plane of the vessel. Recorded videotape images were digitized and analyzed off-line (Mipron system, Kontron Electronics, Eching, West Germany) in a 340×240×8 format. The computer was calibrated in the x-y planes using a recorded image of the calibration reticule.

In five of these vessels, repeat measurements were made after adenosine application. Sites of microbubbles and red blood cells were measured in the vessel for calculation of distribution and velocity profiles across the vessel lumen. The fluxes (the number of microbubbles and/or red blood cells passing per second through each arteriole, its branches, and a neighboring venule, where applicable) were determined to calculate microbubble and red blood cell tissue transit times. The flux ratios at branch points were calculated from the ratio of branch flux to flux in the feed vessel.

Data Analysis

All data were compiled and analyzed on a minicomputer (VAX 8200, Digital Equipment Corporation, Maynard, Massachusetts) with commercially available software (either RST/1, Bolt, Beraneck and Newman, Cambridge, Massachusetts, or BMDP, University of California Press, Los Angeles, California). Unless otherwise noted, all data are expressed as mean±1 SD. All data pertaining to direct comparisons between microbubbles and red blood cells were derived from observations made in the final four hamsters in which both microbubbles and red cells were injected. Data from each of these four animals are represented in the analyses of intravascular distributions, velocities, velocity profiles, and the flux ratios at branch points. Although our findings were similar for microbubbles in the first four animals, limited data are shown from these animals because we felt it was important to have direct comparisons between microbubbles and red blood cells whenever possible. Red blood cell transit times were calculated for only three of the four animals because in one of the animals the bolus injection of red blood cells was not recorded.

Microbubble and red blood cell distributions were measured in eight vessels ranging from 18 to 54 μm in diameter during control conditions. Based on the knowledge of the precise intravascular position within the 10 μm optical section relative to the vessel center and its walls, the normalized radial position was determined for microbubbles and red blood cells. The distance from the vessel center to vessel wall was then divided into fifths, and the percentages of albumin microbubbles and red blood cells lying within each region were calculated.

For the determination of velocity profiles across the vessel lumen, the velocities and radial positions of microbubbles and red blood cells were measured in eight vessels ranging in diameter from 18 to 45 μm. In five of these vessels, repeat measurements were made after adenosine application. Sites within the vessels were selected in which no upstream branch points existed for at least two vessel diameters. To define the velocity profile across the vessel lumen, curves were fit to the plots of intravascular position versus intravascular velocity by nonlinear regression. Velocity profiles were calculated for microbubbles and red blood cells for direct comparison. To describe the velocity profiles within individual arterioles, the following equation was used to determine their relative positions in the vessel for calculation of distribution and velocity profiles across the vessel lumen. The fluxes (the number of microbubbles and/or red blood cells passing per second through each arteriole, its branches, and a neighboring venule, where applicable) were determined to calculate microbubble and red blood cell tissue transit times. The flux ratios at branch points were calculated from the ratio of branch flux to flux in the feed vessel.
was used: \( V(r) = V_{\text{max}} \{1 - [a(r/R + b)]^K\} \), where \( V(r) \) is the velocity at radial position \( r \), \( V_{\text{max}} \) is the maximum velocity at the center of the vessel, \( a \) is a scaling factor that allows a positive intercept with the vessel wall, \( R \) is the radius of the vessel, \( b \) is a factor that allows a shift of the peak of the profile away from the vessel center, and \( K \) is an exponent that defines the shape of the curve.\(^{15}\) If \( K=2 \), then the profile is parabolic. As \( K \) increases, the curve becomes more flat. \( V_{\text{mean}} \) was calculated by integration of the velocity profile equation. Because the values for \( b \) were small, \( V_{\text{mean}} \) was approximated by: \( V_{\text{max}} \{K+2-2(a)^K\}/(K+2) \). The ratio of \( V_{\text{max}}/V_{\text{mean}} \) was calculated by using the following equation: \( V_{\text{max}}/V_{\text{mean}} = (K+2)/(K+2-2(a)^K) \).\(^{15}\) This ratio defines the degree of blunting of the profile with a value of 2 for parabolic flow and 1 for plug flow.\(^{15}\) For each vessel, the mean velocity of microbubbles and red blood cells was also determined from the mean of the velocities of the individual microbubbles and red blood cells actually measured in each vessel.

Mean arteriolar and venular transit times (the average time required for the indicator particles to flow from the point of injection to the recording site) were calculated for eight injections from plots of arteriolar and venular fluxes by the following equation: \( t = \Sigma y_i/\Sigma y_i \), where \( t \) is the mean transit time and \( y_i \) is the flux of indicator particles at time \( t_i \).\(^3\) The areas under the curves of arteriolar and venular fluxes were calculated from: \( A = \Sigma y_i \), where \( A \) is the area and \( y_i \) is as defined above. To find the arteriolar to venular transit time, \( t_a \) is the mean arteriolar transit time, and \( t_v \) is the mean venular transit time.

**Statistical Analysis**

The median size of albumin microbubbles in vitro was compared with that measured in vivo by the Mann-Whitney test for nonparametric data. Comparisons of the distribution of albumin microbubbles and red blood cells within the same vessels under basal conditions and after adenosine provocation were performed by analysis of variance. The mean velocities of microbubbles and red blood cells within the same vessels were compared by Student's \( t \) test. Values for the measured mean arteriolar velocities, the calculated \( V_{\text{max}} \), the calculated mean arteriolar velocities, and the flux ratios at branch points of albumin microbubbles and red blood cells were compared by linear regression analysis. The values for \( a \), \( K \), and ratios of \( V_{\text{max}}/V_{\text{mean}} \) for albumin microbubbles and red blood cells in five vessels at baseline and after adenosine administration were compared by analysis of variance.

**Results**

**In Vivo Appearance of Microbubbles and Red Blood Cells**

The mean in vitro size of the microbubbles used for these experiments was 4.9±3.6 \( \mu \)m with a range of 1-12 \( \mu \)m. The size of albumin microbubbles was found to be slightly larger in vivo than in vitro (median values of 5 \( \mu \)m and 4 \( \mu \)m, respectively; \( p=0.01 \)). Figure 1A illustrates an arteriole observed under transillumination, and Figure 1C illustrates the presence of DTAF-labeled albumin microbub-
FIGURE 2. Panel A: A 17-μm arteriole branching into two 12-μm arterioles under transillumination. Panel B: Calcein-labeled red blood cells during their first pass through the vessel and its branches. Panel C: Dichlorotriazinylaminofluorescein 1 dihydrochloride-labeled microbubbles and calcein-labeled red blood cells in the same vessels. The magnification is ×770 in all three photomicrographs. The vessel is in the basal state in all three photomicrographs.

bles within the same vessel viewed during epifluorescent microscopy. Figure 1B illustrates an arteriole under transillumination, and Figure 1D illustrates a TRITC-labeled red blood cell within the same vessel during fluorescent microscopy. TRITC-labeled red blood cells were observed only during their first pass because they were promptly removed from the circulation. Figure 2A depicts a 17-μm arteriole branching into two 12-μm arterioles under transillumination, and Figure 2B illustrates calcein-labeled red blood cells during their first pass through these vessels under fluorescent microscopy. Figure 2C depicts both calcein-labeled red blood cells and DTAF-labeled microbubbles within the same vessel and its branches under fluorescent microscopy. Unlike the TRITC-labeled red blood cells (Figure 1D), the calcine-labeled red blood cells recirculated throughout the experiment. The calcine-labeled red blood cells had a characteristic biconcave discoid shape and a bright, uniform fluorescence that enabled them to be readily distinguished from the DTAF-labeled microbubbles that had a varied size distribution, assumed irregular shapes in vivo, and had a less bright fluorescence. Because velocities were measured from the center of mass of the microbubbles and red blood cells, and labeled microbubbles and labeled red blood cells were seen in apparent contact with the vessel wall, it was possible to measure velocities within 2.5 μm of the vessel wall (for a particle 5 μm in diameter). Occasionally, microbubbles came in contact with the vessel wall and stuck to the endothelium. In arterioles, this was a transient phenomenon, usually lasting less than 1 second, and occurred only at branch points. However, a small number of microbubbles became permanently attached to the vessel walls in the collecting venules. Microbubbles were not observed to occlude or obstruct arterioles, venules, or capillaries.

Intravascular Distribution of Microbubbles and Red Blood Cells

Under control conditions, both albumin microbubbles and red blood cells were found in significantly higher numbers in the central fifth of the vessel (32±10% and 34±15%) than in the outer fifth (8±8% and 9±7%, p<0.01; Figure 3). The distribution of microbubbles was similar to that of red blood cells at baseline. Repeat measurements were made in five of the vessels after adenosine-induced vasodilation. The percentage of albumin microbubbles in the central fifth of the vessel was higher than that of red blood cells after adenosine administration (45±10% and 33±6%, respectively, p=0.04; Figure 3).

Velocities of Microbubbles and Red Blood Cells

The data derived from the velocity profile equation are shown in Table 1. The number of microbubbles and red blood cells counted per vessel was 27±10 and 21±5, respectively. The mean of the measured velocities for all 13 vessels (eight vessels at baseline and five after adenosine application) was 2.5±0.7 mm/sec for albumin microbubbles and 2.3±0.7 mm/sec for red blood cells, respectively, and demonstrated a good correlation: y=0.94x+0.3 (r=0.92, p<0.0001; Figure 4). The measured mean velocities correlated well with Vmax, calculated from the integral of the velocity profile equation: y=0.98x+0.5, (r=0.86, p<0.001). The Vmax calculated for microbub-
bles and red blood cells also correlated well: $y = 0.8x + 0.5$ ($r = 0.85, p < 0.0001$; Figure 3).

In the five vessels that were examined after adenosine application, the mean measured velocities and calculated $V_{\text{max}}$ for albumin microbubbles were compared with those obtained for red blood cells before and after adenosine. In three vessels, adenosine-induced vasodilation resulted in an increase in the velocities of microbubbles and red blood cells, whereas in two vessels the velocities decreased. At baseline, the mean velocity demonstrated a good correlation: $y = x - 0.1$, ($r = 0.91, p = 0.005$) as did the $V_{\text{max}}$: $y = 0.8x + 0.4$, ($r = 0.98, p = 0.003$). After adenosine application, the mean velocities correlated well: $y = 0.9x + 0.6$, ($r = 0.98, p = 0.03$) as did $V_{\text{max}}$: $y = 0.7x + 0.7$, ($r = 0.8, p = 0.09$). The slopes of the regression equations for the mean velocities and $V_{\text{max}}$ were less steep after adenosine application than at baseline. This change indicated that adenosine produced less of a velocity change for microbubbles than for red blood cells. There were no significant differences in the values of $a$, $K$, or $V_{\text{max}}/V_{\text{mean}}$ obtained at baseline and after adenosine provocation.

Velocity profiles were generated from data collected in the eight vessels at baseline and in five of these vessels after adenosine application (Figure 5). The velocity profiles of microbubbles (open circles) and red blood cells (filled circles) were found to be similar at baseline and after adenosine administration (Figure 5). Adenosine slowed velocity in two vessels and increased velocity in three vessels. Therefore, for each vessel, the velocity profiles were divided into a fast and a slow group (Figure 5).

**TABLE 1. Velocity Profile Data**

<table>
<thead>
<tr>
<th>Injection</th>
<th>Radius (μm)</th>
<th>$V_{\text{max}}$ (mm/sec)</th>
<th>$K$</th>
<th>$a$</th>
<th>$V_{\text{max}}/V_{\text{mean}}$</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>MB RBC</td>
<td>MB RBC</td>
<td>MB RBC</td>
<td>MB RBC</td>
<td>MB RBC</td>
</tr>
<tr>
<td>1</td>
<td>25</td>
<td>3.1 2.2</td>
<td>2.0 4.0</td>
<td>0.9 1.0</td>
<td>1.6 1.5</td>
</tr>
<tr>
<td>2</td>
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<td>1.0 1.0</td>
<td>1.3 2.1</td>
</tr>
<tr>
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<td>1.4 1.5</td>
</tr>
<tr>
<td>4 ADO</td>
<td>22</td>
<td>2.3 3.1</td>
<td>2.1 2.7</td>
<td>0.8 0.8</td>
<td>1.3 1.3</td>
</tr>
<tr>
<td>5</td>
<td>15</td>
<td>4.5 4.8</td>
<td>1.4 1.7</td>
<td>0.5 0.7</td>
<td>1.3 1.4</td>
</tr>
<tr>
<td>5 ADO</td>
<td>23</td>
<td>2.5 2.3</td>
<td>2.0 1.0</td>
<td>1.0 0.6</td>
<td>2.0 1.6</td>
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<td>6</td>
<td>16.5</td>
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<tr>
<td>6 ADO</td>
<td>21</td>
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<tr>
<td>7</td>
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<tr>
<td>7 ADO</td>
<td>18</td>
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<tr>
<td>8 ADO</td>
<td>10</td>
<td>2.7 2.6</td>
<td>2.2 1.0</td>
<td>0.7 0.5</td>
<td>1.5 1.3</td>
</tr>
</tbody>
</table>

$V_{\text{max}}$: maximum velocity at the center of the vessel; $K$: exponent that defines the shape of the curve; $a$: scaling factor that allows a positive intercept with the vessel wall; $V_{\text{mean}}$: mean velocity; MB, microbubble; RBC, red blood cell; ADO, adenosine application.
Velocity profiles were similar for microbubbles and red blood cells in the fast and slow groups. The values for \( V_{\text{max}} \) and \( V_{\text{mean}} \) were significantly higher in the fast group than in the slow group for both microbubbles and red blood cells \((p=0.01\) and \(p=0.01\), respectively\). The means of the ratio \( V_{\text{max}}/V_{\text{mean}} \), which defines the shape of the velocity profile, were not significantly different, with values of 1.6±0.3 for microbubbles and 1.6±0.2 for red blood cells in the slow group and 1.5±0.2 for microbubbles and 1.4±0.1 for red blood cells in the fast group \(p=0.5\).

Transit Times for Microbubbles and Red Blood Cells

A representative plot of the time course of microbubble flux in an arteriole and venule is shown in Figure 6. The mean arteriolar, mean venular, and the arteriolar-to-venular transit times for eight vessels are shown in Table 2. The number of microbubbles and red blood cells counted per injection were 306 and 175, respectively. The mean arteriolar-to-venular transit time was 14.6±7.2 seconds for all eight vessels and ranged from 4.0 seconds for a 22-\(\mu\)m arteriole to 27.4 seconds for a 44-\(\mu\)m arteriole. In three experiments, the transit times for bolus injections of labeled red blood cells were also determined just before determination of microbubble transit time (Table 2B) and were similar to the transit times obtained for microbubbles in the same vessel (21.4 vs. 21.0 seconds for microbubbles and red blood cells, respectively). The areas under the
arteriolar and venular curves were calculated to provide a measure of overall vessel flux. The ratio of the areas under the curves for arteriolar flux to venular flux was 2.6 for microbubbles and 3.9 for red blood cells.

Behavior of Microbubbles and Red Blood Cells at Branch Points

The flux ratios at branch points (the number of particles per second passing down a branch vessel divided by the number of particles per second passing down the feed vessel) were measured in eight vessels ranging from 17 to 54 μm in diameter, which bifurcated into branches ranging from 12 to 45 μm (Figure 2). The microbubbles and red blood cells behaved similarly at branch points (Table 3). A good correlation was noted between the flux ratios of microbubbles and red blood cells at branch points: y=0.99x+2.82 (r=0.92, p<0.001).

<table>
<thead>
<tr>
<th>Table 2. Arteriolar-Venular Transit Times</th>
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<tbody>
<tr>
<td>Arteriolar diameter (μm)</td>
</tr>
<tr>
<td>---------------------------</td>
</tr>
<tr>
<td>Mikroebbles only</td>
</tr>
<tr>
<td>33</td>
</tr>
<tr>
<td>22</td>
</tr>
<tr>
<td>33</td>
</tr>
<tr>
<td>31</td>
</tr>
<tr>
<td>45</td>
</tr>
<tr>
<td>Mean</td>
</tr>
<tr>
<td>Microbubbles and red blood cells</td>
</tr>
<tr>
<td>34</td>
</tr>
<tr>
<td>22</td>
</tr>
<tr>
<td>44</td>
</tr>
<tr>
<td>Mean</td>
</tr>
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</table>

Transit times are given in seconds.

Discussion

In this study, we observed for the first time the intravascular behavior of fluorescent albumin microbubbles and red blood cells during their sequential passage through the hamster cheek pouch. We found that the behavior of albumin microbubbles in the microcirculation closely parallels, but is not identical to, that of fluorescently labeled red blood cells. Using microbubbles comparable in mean size with red blood cells, we found similarities in their distribution, velocity, velocity profiles, arteriolar to venular transit times, and branch point flux within vessels. Although directionally similar, the changes in velocities induced in microbubbles after pharmacological interventions were smaller than that of red blood cells.

Physical Characteristics of Microbubbles

The mean diameter of the albumin microbubbles produced by sonication was 4.9±3.6 μm, which is similar to that of hamster red blood cells. However, there was a wide range of microbubble sizes in comparison with red blood cells. Since microbubbles are spherical in shape, they have a larger

<table>
<thead>
<tr>
<th>Table 3. Flux Ratio at Branch Points</th>
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<tbody>
<tr>
<td>Vessel</td>
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Flux ratio is given as branch flux/feed-vessel flux.
volume than biconcave red blood cells of comparable diameter. This difference in volume (72 vs. 59 \(\mu m^3\) for a 3-\(\mu m\) diameter particle), as well as the wider range of microbubble sizes, may have led to differences in rheology within the microcirculation because particle volume is an independent determinant of intravascular rheology.\textsuperscript{16,17}

Because it was possible to attach a fluorescent label to albumin microbubbles and wash them repeatedly, it is likely that the albumin microbubbles are composed of an albumin shell with an air-filled center. This shell undoubtedly imparts to microbubbles physical properties that are different from those of red blood cells that have deformable membranes. Furthermore, the mass of an albumin microbubble is certainly different than the mass of a red blood cell. These differences in deformability and mass are also likely to produce some of the differences observed in the rheologies.\textsuperscript{16,17}

**Intravascular Distribution of the Microbubbles**

Microbubbles and red blood cells were distributed in a similar manner across the lumen of microvessels at baseline with both showing a significantly higher concentration in the center of the vessel than at the edge. These data are supported by in vitro and in vivo experiments demonstrating a central crowding of red blood cells within vessels due to axial migration.\textsuperscript{16} At higher velocities, the microbubbles were found at higher concentrations in the vessel center. This central streaming may be due to the physical differences between microbubbles and red blood cells.\textsuperscript{16} We assume that the microbubbles and red blood cells observed in these experiments were contained in a relatively thin optical section in a plane passing through the center of the vessel.\textsuperscript{14} At an overall magnification of approximately \(x770\) with a \(x16\) objective (n.a., 0.50), the optical section should have been less than 10 \(\mu m\). The sections of the vessel adjacent to the endothelium have a slightly smaller volume due to the curvature of the wall, but this has been shown to produce less than a 10% error.\textsuperscript{17}

**Velocity of Microbubbles**

The mean velocities of microbubbles measured in individual vessels were similar to those of red blood cells measured in the same vessels. These data are in agreement with a preliminary report describing the velocities of sonicated Renografin-76 (diatrizoate meglumine and diatrizoate sodium) microbubbles and red blood cells within the microcirculation.\textsuperscript{15,18-21}

The velocity profiles were similar for microbubbles and red blood cells. The ratio \(V_\text{max}/V_\text{mean}\), which describes the shape of the velocity profile, was not different for microbubbles and red blood cells and was intermediate between parabolic and plug flow, which is in concordance with previously described velocity profiles.\textsuperscript{15,20} Alterations in red blood cell velocity produced by adenosine-induced vasodilation produced directionally similar, but unequal, changes in microbubble velocity that may, in part, be related to the differences in volume and rigidity of microbubbles and red blood cells.\textsuperscript{16} It has been demonstrated in vivo that spherocytes in anemic mice do not display the increase in velocity seen with normal red cells. Perhaps this occurrence is attributable to greater volume and rigidity of spherocytes.\textsuperscript{21}

**Tissue Transit Time**

The tissue transit times of microbubbles and red blood cells were found to be similar when measured in adjacent arterioles and venules. The longest transit time observed for microbubbles was 27.4 seconds from a first order arteriole to the neighboring venule. Progressing down the vascular tree, shorter transit times were noted; the shortest was 4.0 seconds from a fourth order arteriole to a collecting venule. These data compare favorably with arteriolar to venular transit times previously reported across capillary beds.\textsuperscript{22-25}

The areas under the curves of the arteriolar fluxes were consistently larger than the areas under the venular fluxes. Some of this disparity might be explained by microbubble trapping or decay. Although the microbubbles did not occlude microvessels, a small percentage (not quantitated) of microbubbles was observed to stick to the walls of collecting venules. The most obvious explanation is that each arteriole feeds several parallel venules within any single segment of tissue.\textsuperscript{26} Once again, it is possible that the physical characteristics of microbubbles led to streaming into different vascular channels and contributed to the discrepancies seen in the arteriolar and venular fluxes.\textsuperscript{16}

**Branch Point Flux Ratio**

The behavior of microbubbles and red blood cells at branch points was not significantly different with both demonstrating nearly identical fluxes. The branch point flux ratio is determined by the shape, size, and rigidity of the red blood cell\textsuperscript{27} and by the hematocrit of the feed vessel, vessel diameter, and flow-rate distribution.\textsuperscript{28} In vivo data suggest that the red blood cell flux ratio is also related to red blood cell flow in the microcirculation.\textsuperscript{29-31} The close relation between microbubble and red blood cell flux ratios in our study indicates that, despite differences in shape, microbubble flow at branch points may be identical to that of red blood cell.

**Differences Between Microbubbles and Red Blood Cells**

The physical differences between albumin microbubbles and red blood cells are likely to be the cause of the differences observed in the rheologies of microbubbles and red blood cells. Microbubbles have higher concentrations in the center of the vessel after adenosine application, their velocity changes are less after adenosine vasodilation, and they have a tendency to stick to the endothelium. Furthermore, although the arteriole-to-venule tran-
sit times were similar for microbubbles and red blood cells in this study, the transit time of fluorescent red blood cells may not represent the true transit time of native red blood cells because labeling may lead to slowing in the capillaries. Shorter arteriole-to-venule transit times have been reported with dextran-saline injections. This finding implies that a proportional relation, rather than an equality, may exist between the rheological behavior of microbubbles and that of native red blood cells.

Conclusions

We have demonstrated that DTAF-labeled albu-
min microbubbles behave in a manner that is closely related to that of TRITC- and calcein-labeled red blood cells within the microcirculation although minor differences in the behavior of the two do exist. These findings may serve as a foundation for using albumin microbubbles as intravascular tracers of red blood cell flow. Our data suggest that albumin microbubbles could be used during myocardial contrast echocardiography to measure regional myocardial blood flow in vivo.

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