Characterization of Amniotic Fluid Lamellar Bodies by Resistive-Pulse Counting: Relationship to Measures of Fetal Lung Maturity

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Resistive-pulse counting studies of amniotic fluid lamellar bodies are presented and demonstrate a strong concordance with the predictions of accepted measures of fetal lung maturity. Uncentrifuged as well as centrifuged specimens could be evaluated, because cells and debris are rejected electronically. The technique is not affected by bilirubin or debris of lysed whole blood, and only mildly by meconium. Lamellar body number density and mean lamellar body volume were determined for 161 uncentrifuged and 241 centrifuged specimens. Number density maturity criteria (40 000/μL and 26 000/μL, respectively) were shown to be highly concordant with established measures of fetal lung maturity; mean lamellar body volume did not extend this concordance. Since electronic cell counters are generally available 24 h per day and the approach requires neither centrifugation nor subjective interpretation and is rapid and inexpensive, it is proposed that determining lamellar body number density by resistive-pulse counting may be a useful initial screen for the assessment of fetal lung maturity.

This investigation describes a new approach to the initial screening of amniotic fluid for the assessment of fetal lung maturity (FLM): characterization by resistive-pulse counting of the lamellar bodies (LBs), the surfactant-containing, lamellated structures that are secreted by the type II pneumocytes and are associated with pulmonary maturity (1, 2). The traditional approach to FLM testing involves the determination of the lecithin–sphingomyelin ratio (L/S) (3) and percent phosphatidylglycerol (PG) (4) by thin-layer chromatography (TLC). The technical difficulty, time, and expense of TLC has led to the development of many alternative methods (5). Some of these are themselves quite expensive; further, they are better predictors of pulmonary maturity (“negative” result) than of respiratory distress syndrome (“positive” result) (6). Thus, a “cascade” concept involving serial testing has been proposed (7). An example of such a cascade is the use of the “foam stability index” (FSI) (8) as an initial screen, with a negative result terminating the testing. If the screen result is positive, another rapid test is used, fluorescence polarization (9). Similarly, only those specimens still positive by this second test must finally be examined for L/S and PG. Such an approach has been shown to reduce substantially both turnaround time and costs (7, 10). The present work demonstrates that the determination of the lamellar-body number density (LBND) is a rapid, inexpensive, and effective initial screen in such a cascade approach.

One of the heralds of FLM is the increase in the L/S and PG of the LBs. Moreover, there is a concomitant dramatic increase in their number density. Because of their very large molecular mass, a striking increase in light scattering occurs and, therefore, in amniotic fluid turbidity. This is the physical basis for the “A455” method (11), which currently is the most rapid and least expensive approach to the determination of FLM. However, the presence of interfering chromogens such as hemoglobin, methemoglobin, and bilirubin can artifactually increase the absorbance at 650 nm and thus give a false impression of maturity. A differential light-scattering technique has been described (12), which eliminates the effect of such interferences with some concomitant loss in analytical sensitivity. This differential approach demonstrated, moreover, a simple relationship between absorbance at 650 nm and LBND, leading to the present study. Determination of LBND in this work does not require centrifugation, because cellular components and other debris are rejected electronically. This eliminates a major source of inter-laboratory and inter-method variation caused by the centrifugation process (13, 14). The technique provides a direct quantification of what is assessed indirectly by the A455 approach, and it is not subject to the interferences with that method. This study describes the measurement of the LBND, the mean lamellar body volume (MLBV), L/S, PG, and FSI on a large number of amniotic fluid specimens, the concordance of predictions of FLM among these methods, and the effects of interfering substances.

Materials and Methods

Resistive-pulse counting assesses particle volume by measuring the resistance increase caused by a particle partly occluding a pore between two conducting media. The number of such pulses allows the determination of particle number density. By rejecting pulse heights above and below those of the particle in question, other particles present in the specimen are not counted. In these studies, I used the platelet channel of a commercial cell counter (S+IV; Coulter Electronics, Hialeah, FL) to determine LBND and mean volume. The instrument also generates a graphical display of the fraction of the total LBND vs particle volume. The counter rejects particles below 2.0 μL in volume (1.56 μm diameter), because these are unlikely to be platelets. Because LB size is in the 1–5 μm range (1), with most being nearer 2 μm in diameter (2), LBND may be slightly underestimated by the use of such a platelet counter. This effect was quantified by using polystyrene latex spheres of diameters 1.40 μm, 1.78 μm, 2.28 μm, and 3.1 μm with quoted uncertainties (1 SD) in diameter of 0.04 μm, 0.07 μm, 0.03 μm, and 0.04 μm, respectively (Polysciences, Inc., Warrington, PA). The manufacturer’s quoted concentration (2.5 g/dL) was verified for the 3.1-μm spheres by direct hemacytometric counting (“Bright-Line”; Reichert, Buffalo, NY) of a 10-fold dilution of the stock solution, taking the
mass density of polystyrene latex spheres as 1.05 g/cm³ (16). The polystyrene latex spheres were studied by the resistive pulse technique at dilutions of 200-fold for the 3.1-μm spheres, 500-fold (2.28-μm spheres), and 1000-fold (1.78-μm and 1.40-μm spheres). Bilirubin interference was determined with commercial control material (chemTRAK Level 3 Bilirubin Control; Medical Analysis Systems, Inc., Camarillo, CA). Effects of hemolysis were assessed by using uncentrifuged osmotically lysed human blood. Interference due to meconium was determined by obtaining fresh meconium from the nursery and resuspending it in saline (NaCl, 154 mM, 4°C). Commercial control material was used for day-to-day precision controls (Coulter Diagnostics). The counter itself was calibrated to manufacturer's specifications (S-CAL Calibrator; Coulter Diagnostics). Amniotic-fluid specimens obtained by amniocentesis and submitted to the Clinical Laboratory of Cedars-Sinai Medical Center were well-mixed by numerous inversions of the container and then divided into two aliquots. One, for L/S and PG determinations by TLC (Fetal-Tek 200; Helena Laboratories, Beaumont, TX), FSI (Lumidex-FSI; Beckman Diagnostics, Brea, CA), and resistive-pulse counting as described above, was centrifuged at 500 × g for 5 min. The other, uncentrifuged, was studied only by resistive-pulse counting. The aliquots typically had been refrigerated for 12 to 24 h at 7°C before counting. Each was allowed to equilibrate at room temperature for 1 h and then continuously mixed on an oscillating rocker for at least 5 min before being aspirated into the counter.

**Results**

Using a 10-fold dilution of the 3.1-μm polystyrene latex spheres, I counted 610 spheres in the 0.2 mm × 0.2 mm × 0.1 mm volume of the hemacytometer, implying a particle number density of 1.53 × 10^9/μL. This agrees exactly with what is calculated for spheres of this diameter at the quoted concentration of 2.5 g/dL. When these spheres were studied by the resistive-pulse approach at the 200-fold dilution, the measured number density was 8500/μL, compared with the expected value of 7640/μL, for a recovery of 111%. The measured particle volume was 12.8 fl, from which the diameter of the particle is calculated to be 2.90 μm, 94% of the expected value. Thus, the counter is able to characterize 3.1-μm polystyrene latex spheres adequately, a size that is in the midrange of what is usually quoted for the LBNDs themselves. For the 2.28-μm polystyrene latex spheres, the recovery was 89% and the measured 6.02 fl volume yields a calculated diameter of 2.26 μm, 99% of the expected value. Recovery declined to 67% for the 1.78-μm spheres, and from the measured volume of 4.82 fl the diameter of 2.1 μm is calculated, 18% higher than expected. Finally, using the 1.4-μm polystyrene latex spheres, the recovery decreased abruptly to 12%, and the measured volume of 4.06 fl corresponds to a diameter of 1.98, an error of 41%. The performance of the counter is thus demonstrated to be consistent with its specifications.

Figure 1 displays the distribution by particle volume of the lamellar bodies in an uncentrifuged specimen of near-term amniotic fluid. The other assessments of FLM for this specimen included an L/S ratio of 1.9, PG of 15%, and FSI of 48. Note that the values for PG, FSI, and, as described below, LBND, are consistent with maturity, while that of L/S is borderline. Such range of assessment is commonly seen among all the methods of assessing FLM, and this point will be addressed further in the Discussion. In this uncentrifuged specimen the LBND was determined to be 170 000/μL, while in the centrifuged aliquot of the same material the LBND was 102 000/μL. The mean lamellar body volume (MLBV) values for the two specimen types were 6.34 fl and 6.06 fl, respectively, yielding corresponding volume-averaged particle diameters of 2.30 μm and 2.26 μm. The distribution graphically demonstrates that particles smaller than 2 fl are not counted. Because this distribution is not what is expected for platelets, the instrument does not accept the result as a platelet count. The values for LBND and MLBV are thus obtained by manual override. If it is assumed that the curve declines smoothly to the origin from its value at the 2-fl cutoff, then about 10% of the area of the lamellar body distribution curve (that is, about 10% of the total mass of the lamellar bodies) is not assessed by the instrument when it is calibrated according to vendor specification.

I investigated 241 specimens by resistive-pulse counting, of which 161 were studied before centrifugation and all 241 were studied after centrifugation. The L/S ratio and percentage phosphatidylglycerol were determined for each of these specimens, and the FSI was determined for 199 of those centrifuged. This number included 132 of the specimens that had been studied by resistive-pulse counting before centrifugation. These data allowed comparison of measures of fetal lung maturity in general and, in particular, the relation between LBND and FSI.

Table 1 shows the concordance between LBND and FSI. The criteria for maturity were as follows: L/S ≥ 2.0; PG ≥ 3%; FSI ≥ 47%; LBND ≥ 40 000/μL (uncentrifuged) or LBND ≥ 26 000/μL (centrifuged).

Given a mature or immature result for LBND or FSI, Table 2 summarizes the probability that the standard measures of FLM indicated maturity or immaturity. Criteria for maturity are the same as described for Table 1 above.

Only three of the specimens studied here came from mothers whose newborns subsequently developed respiratory distress syndrome. All three of these specimens were determined to be immature by the criteria of LBND, L/S, and PG. One of the three was immature by FSI as well, but the FSI could not be measured for the other two, owing to staining with meconium.

The LBND of the centrifuged specimens was highly correlated with that of specimens studied without centrifugation (correlation coefficient r = 0.932, slope = 0.60, and intercept = −80/μL). This 40% loss in LBND is also
Table 1. Comparison of the Fetal Lung Maturity Predictions of LBND and FSI

<table>
<thead>
<tr>
<th></th>
<th>Uncentrifuged (n = 132)</th>
<th>Centrifuged (n = 199)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Mature by LBND and FSI</td>
<td>78</td>
<td>123</td>
</tr>
<tr>
<td>II. Immature by LBND and FSI</td>
<td>38</td>
<td>54</td>
</tr>
<tr>
<td>III. Mature by LBND and Immature by FSI</td>
<td>12*</td>
<td>16*b</td>
</tr>
<tr>
<td>IV. Immature by LBND and mature by FSI</td>
<td>4c</td>
<td>6d</td>
</tr>
</tbody>
</table>

* All 12 mature by L/S and (or) PG. b Nine mature by L/S and (or) PG; seven immature both by L/S and PG. c One mature by L/S and (or) PG; three immature both by L/S and PG. d One mature by L/S and (or) PG; five immature by both L/S and PG.

reflected in an average 7.1% decrease in mean lamellar body volume after centrifugation, a modest effect but indicating that larger particles are lost preferentially during centrifugation. On the other hand, the MLBV was nearly independent of the LBND (r = 0.265, slope = 0.003 fl/μL⁻¹ and intercept = 6.16 fl for the uncentrifuged specimens, while r = 0.311, slope = 0.004 fl/μL⁻¹, and intercept = 5.71 fl after centrifugation). This very slight dependence of MLBV on LBND resulted in a rather narrow range being observed in the value for MLBV for the entire group of specimens studied: 6.37 ± 0.60 fl (mean ± 1 SD) for uncentrifuged specimens and 5.92 ± 0.55 fl after centrifugation. Below very-low values for LBND (about 25 000/μL for uncentrifuged and 15 000/μL for centrifuged specimens) there was about a 10 to 20% decrease in these values. Above these rather low thresholds, MLBV was essentially independent of LBND.

Addition of 10 mg of bilirubin per liter increased LBND by less than 1000/μL. This is less than 2.5% of the maturity cutoff value for uncentrifuged material, even at a bilirubin concentration only rarely reached in amniotic-fluid specimens. Similarly, when osmotically lysed human blood was added to amniotic-fluid specimens with a resulting hemoglobin concentration of 10 g/L, LBND was again observed to increase by less than 1000/μL. This is a small effect for the degree of hemolysis likely to be encountered clinically and represents the worst-case analysis—that is, all the debris of the cell destruction is still present, because the lysed blood was not centrifuged before it was added to the amniotic fluid.

Finally, I assessed the effect of meconium as follows. Sufficient meconium was added to amniotic-fluid specimens to increase the measured absorbance at 390 nm (the biliverdin absorption peak) by 0.5 A. This corresponds to prominent meconium staining, but it increased measured LBND by less than 5000/μL. If the contaminated specimen was then centrifuged in the usual manner, the increase was less than 3000/μL. On the other hand, as described above, this same 40% decrease would typically be seen in the true LBND produced by the lamellar bodies themselves. Hence, no advantage would be anticipated from centrifugation, even in meconium-stained specimens.

I assessed day-to-day variation in results, using commercial platelet controls. Using the 4C₄⁺ Abnormal Low Platelet Control (77 000 platelets/μL, mean platelet volume 9.9 fl), I observed a coefficient of variation (CV) of 4.2% for particle number density and a CV of 2.0% for mean particle volume. Similarly, the 4C₄⁺ Normal Platelet Control (232 000 platelets/μL, mean platelet volume 9.8 fl) showed CVs of 2.5% and 1.9%, respectively. Within-day precision was assessed by determining LBND 10 consecutive times for a series of amniotic-fluid specimens characterized by LBND that spanned the range of clinical interest. The mean values obtained for LBND (and their respective CVs) were: 4100/μL (12%); 11 900/μL (7.1%); 30 500/μL (2.3%); 45 500/μL (5.5%); 73 400/μL (4.0%); and 94 400/μL (4.3%). The respective MLBV values for these same specimens (and their CVs) were: 4.9 fl (5.5%); 6.1 fl (4.0%); 6.6 fl (3.8%); 6.1 fl (2.5%); 6.5 fl (3.0%); and 6.1 fl (3.4%).

Amniotic-fluid specimens (both centrifuged and uncentrifuged) could be stored at room temperature for at least three days and still have results within the above-described precision. This could be prolonged for at least two weeks for specimens stored at 7 °C. For centrifuged specimens held at −20 °C for five to 10 weeks, the measured LBND showed a somewhat wider variation (3% ± 9%). Not surprisingly, the variation was greater for frozen uncentrifuged material, because some of the many cellular components, including epithelial cells, would be lysed, producing debris having a range of sizes, including some in the range counted as LBs. For this case the LBND variation was 3% ± 30%. Freezing changed the MLBV relatively little: 3% ± 5% for centrifuged material, 6% ± 6% for uncentrifuged specimens.

Table 2. Probability with Which Resistive-Pulse Counting and Foam Stability Index Predict Other Criteria of Fetal Lung Maturity

<table>
<thead>
<tr>
<th>Technique</th>
<th>Result</th>
<th>At least L/S</th>
<th>At least L/S and PG</th>
<th>At least L/S and (or) PG</th>
<th>At least L/S and PG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resistive-pulse counting</td>
<td>Mature (n = 111)</td>
<td>65%</td>
<td>69%</td>
<td>51%</td>
<td>84%</td>
</tr>
<tr>
<td></td>
<td>Immature (n = 50)</td>
<td>8%</td>
<td>6%</td>
<td>4%</td>
<td>10%</td>
</tr>
<tr>
<td>Centrifuged</td>
<td>Mature (n = 164)</td>
<td>67%</td>
<td>77%</td>
<td>57%</td>
<td>87%</td>
</tr>
<tr>
<td></td>
<td>Immature (n = 77)</td>
<td>9%</td>
<td>10%</td>
<td>4%</td>
<td>16%</td>
</tr>
<tr>
<td>Foam stability index</td>
<td>Mature (n = 130)</td>
<td>65%</td>
<td>81%</td>
<td>59%</td>
<td>87%</td>
</tr>
<tr>
<td></td>
<td>Immature (n = 69)</td>
<td>18%</td>
<td>12%</td>
<td>6%</td>
<td>25%</td>
</tr>
</tbody>
</table>

For the entire group of specimens studied (n = 241), 116 were mature by the L/S criterion, of which 97 were also mature by PG (84%). Similarly, of specimens mature by PG (n = 135), 97 were also mature by L/S (72%).

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Discussion

The work presented here demonstrates that the resistive-pulse counting technique can be used to characterize the number density and mean volume of amniotic fluid LBs. Thus, a physical description of the LBs can be obtained rapidly by using the platelet channel of the type of commercial cell counter that is available 24 h per day in most medical centers. On the other hand, such instruments are calibrated to reject particles under 2.0 fl in volume (because such are unlikely to be platelets), so the LBND is underestimated. This fraction represents about 10% of the total mass of the LBs in Figure 1. In the differential light-scattering assessment of LBND (12) in centrifuged material, a cutoff criterion for maturity was found to be 32 000/µL, compared with 26 000/µL in the present study. The light-scattering method detects the smaller particles, so it appears that the missing 10% of the area under the curve (mass) represents about 20% of the total number of the LBs. Because it has already been pointed out that the MLBV depends only weakly on the LBND, this partial loss would be expected to be similar for all specimens, and thus it does not adversely affect the use of resistive-pulse counting, which is a much simpler technique than the light-scattering approach. Nonetheless, it is noteworthy that these two approaches, based on completely different physical principles, yield such similar values for the maturity criterion.

Moreover, Table 1 shows that determination of LBND by resistive-pulse counting provides an impression of fetal lung maturity very similar to that given by the FSI. Indeed, in the case of uncentrifuged specimens, the two techniques agreed in 116 of 132 cases, or 88% of the time. In those cases in which the LBND indicated maturity and the FSI indicated immaturity, all 12 examples were mature by the criteria of L/S or PG, or both. In the four cases classified as immature by LBND and mature by FSI, only one was mature by L/S and (or) PG, whereas three were immature by both criteria. Thus, when there was disagreement between the two approaches, the LBND was more likely to be consistent with TLC results than was FSI. Similar results were obtained for centrifuged specimens, again as indicated in Table 1.

Table 2 continues this comparison. Note that specimens considered mature by the LBND criterion, whether centrifuged or uncentrifuged, had associated likelihoods of maturity by the various TLC criteria that were very similar to those of the specimens predicted to be mature by FSI. That is, LBND and FSI tended to identify as mature the same type of specimen so characterized by TLC. On the other hand, it is seen in Table 2 that FSI is more likely to classify as immature those specimens that are mature by the TLC criteria. For example, 25% of the 69 specimens classified as immature by FSI were mature by the criteria of L/S and (or) PG, while only 10% of the specimens classified as immature by LBND (uncentrifuged) were mature by L/S and (or) PG. This increased to 16% for centrifuged specimens classified as immature by the LBND approach. Thus, when a specimen is predicted to be immature by LBND, used as screening test, it is less likely that the specimen will be considered mature by TLC than if the screening test had been the FSI.

It is important to note here that no measures of FLM give uniform concordance in their predictions. For example, as noted at the bottom of Table 2, only 84% of the specimens mature by L/S were also mature by PG. Similarly, only 72% of those mature by PG were mature by the L/S criteria. This lack of agreement even among the "gold standards" of FLM assessment demonstrates the fundamental difficulty of the laboratory assessment of FLM in the present state of the art.

Although specimens with very low values for LBND had values for the MLBV that were 10–20% less than the average value for all specimens, this observation provides no additional discriminant ability in assessing FLM, because such specimens would already be classified as immature, owing to their low values of LBND. However, the somewhat lower value for MLBV for very immature specimens might be of value in discriminating against interferences found in other specimen sources not evaluated here—vaginal pool material for example. Similarly, although immaturity was correctly predicted by LBND, L/S, and PG as determined on the three specimens of amniotic fluid obtained from mothers whose newborns subsequently developed respiratory distress syndrome, this number is too small for a meaningful picture of the full use possible for MLBV to emerge. The MLBV may be of greater use as a discriminant tool when larger numbers of amniotic-fluid specimens from mothers with newborns with respiratory distress syndrome, as well as specimens not obtained by amniocentesis, have been accumulated.

Of the 241 specimens studied here, the FSI test was done on only 199. The major reason for this difference is that the FSI cannot be used on specimens that have hemoglobin or meconium staining or have significant cellular debris even after centrifugation, as is often the case with contamination with blood in a prior amniocentesis, or in the presence of substantial numbers of neutrophils. The FSI is not affected by bilirubin. The resistive-pulse technique, on the other hand, has been shown above to be nearly immune to interferences by bilirubin, hemoglobin, and most cellular material, even uncentrifuged, as well as after centrifugation, for the degrees of such interferences likely to be encountered in any clinical setting. Further, even pronounced meconium staining did not change the measured value of LBND sufficiently to alter the impression of maturity or immaturity in most settings. Thus, it is far less likely that the LBND approach will not be applicable to a given specimen than is the case with FSI.

It is also significant that FSI requires 3 mL of amniotic fluid for the complete study, whereas LBND requires only about 0.15 mL adequately to fill the aliquot for aspiration of the 0.1 mL required by the counter. The preliminary centrifugation required for FSI, and other laboratory assessments of FLM, is not required for LBND, further decreasing the sources of error and variation, as well as the time required for the study. Additionally, the endpoint of the LBND assay is an objective number, the cutoff criterion: 40 000/µL for uncentrifuged material and 26 000/µL for centrifuged specimens. This is to be contrasted with reading a complete ring of foam in the FSI approach, a somewhat subjective visualization with unavoidable user-to-user variation.

As indicated in the Results section, only three newborns of the 241 in this study developed respiratory distress syndrome, and all three had amniotic-fluid specimens classified as immature by LBND, L/S, and PG. One was immature by FSI as well, whereas FSI was not performed on the remaining two because of meconium staining. These numbers are too small to make fully useful calculations of sensitivity, specificity, predictive value for positive and negative results, and diagnostic efficiency. For completeness, however, these values are 100%, 69%, 4%, 100%, and 69%, respectively. It is noteworthy that none of the newborns who were to
develop respiratory distress syndrome was missed by any of these approaches. These percentages are comparable with the values seen for other assessments of FLM (10), although the predictive value of an "immature" result (4%) is particularly low. On the other hand, the predictive value of a "mature" result is very high, equal to that of all other methods, which typically approach 100%; this is the case in any disease in which the prevalence is low. Thus, as in all screening tests, the price of detecting the true positive (here, the newborn who will develop respiratory distress syndrome) is the false prediction for many who will in fact not develop respiratory distress syndrome (i.e., a high false-positive rate).

In conclusion, I propose that resistive-pulse counting of amniotic-fluid LBs in the platelet channel of a commercial cell counter is a suitable initial screening test in the cascade approach for assessment of FLM. The method offers speed, economy, very small specimen requirement, 24-h availability, and the objectivity of a numerical result, and does not require centrifugation.

References