Rational Basis for Foam-Stability Assays of Amniotic Fluid Surfactant

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We evaluated the relative contributions of various phospholipids to the formation of foam in the amniotic fluid "foam stability test," by use of an artificial system of saline, ethanol, and dipalmitoyl 3-sn-phosphatidylcholine to determine the relationship of formation of stable foam. At dipalmitoyl 3-sn-phosphatidylcholine concentrations of 20, 40, and 60 mg/liter, the threshold ethanol volume fraction was 0.46, 0.48, and 0.51, respectively. We similarly evaluated the ability of other phospholipids to form stable foam at various concentrations and ethanol volume fractions and found: bovine brain sphingomyelin > dipalmitoyl 3-sn-phosphatidylcholine > egg sphingomyelin > egg lecithin > phosphatidylglycerol. The corresponding propensities of different chemical species of phosphatidylcholine to form foam at 40 mg/liter were: dipalmitoyl 3-sn-phosphatidylcholine > dioleoyl phosphatidylcholine > dimyristoyl phosphatidylcholine > distearoyl phosphatidylcholine.

Additional Keyphrases: fetal status • lipids • hyaline membrane disease • respiratory distress syndrome • pregnancy • Foam Stability Index test

Hyaline membrane disease (also called the "neonatal respiratory distress syndrome") is caused in part by inadequate synthesis, by the lung, of materials that lowers surface tension (surfactant). Recent attempts at prenatal detection of inadequate surfactant synthesis have focused on evaluation of the functionality of surfactant that is expelled via the fetal trachea into the amniotic fluid.

In 1972, Clements et al. introduced the amniotic fluid foam stability test ("shake test") for assessing fetal pulmonary maturity, an assay designed to measure functionality, i.e., the presence or absence of surfactant phospholipids (1). Surprisingly simple, the assay involves shaking 1 ml of amniotic fluid with 1 ml of ethanol/water (95/5 by vol). Thus the ethanol volume fraction in the final assay mixture is 50% of 0.95, or 0.475. The ethanol inhibits foam formation caused by certain constituents such that at an ethanol volume fraction of 0.475 (as in the shake test) only surface-active phospholipids can sustain a stable foam at the air–liquid interface. Because dipalmitoyl 3-sn-phosphatidylcholine (dipalmitoyl lecithin, DPL) is the principal surfactant phospholipid produced by the lung, the assay of Clements et al. is largely an index to the concentration of DPL in amniotic fluid. In 1973, Edwards and Bailie introduced a modification of the original shake test, in which they used absolute (i.e., 100%) rather than 95% ethanol as the reagent (2).

These two "shake tests" have attracted much attention and have had a mixed reception (3–5). Some investigators claim excellent reliability, but there are many reports noting that the absence of foam formation may be associated with mature pulmonary status. In addition, there seems to be a general reluctance to credit a method that is so simply performed, perhaps in part because the principles underlying the method are not appreciated and understood. The analyst may not be aware of the relationship of foam formation to ethanol volume fraction, to the type of phospholipids present in the assay mixture, and to the concentrations of the phospholipids.

We have used an artificial system of ethanol and saline in various ratios to investigate foam formation by several phospholipids, namely phosphatidylcholine, sphingomyelin, and phosphatidylglycerol, that are commonly present in amniotic fluid. We also assayed several "non-physiological" phosphatidylcholines, to assess the effects of fatty-acid carbon length and degree of saturation on foam formation.

Methods

Shake test. The shake test was performed by adding the stated amount of ethanol/water (95/5 by vol) to an aqueous phase, either saline in the case of the artificial system or amniotic fluid in the dilution study. Phospholipid standards were prepared at a concentration of 1.0 g/liter in ethanol/water (95/5) in 10-ml solutions. The following phospholipids were obtained from Sigma Chemical Co., St. Louis, Mo. 63178: dipalmitoyl 3-sn-phosphatidylcholine (dipalmitoyl lecithin, DPL, cat. no. P6138); egg lecithin, repurified from Sigma preparation (cat. no. P5513), dimyristoyl lecithin (cat. no. P0888), dioleoyl lecithin (cat. no. P1013), distearoyl lecithin (cat. no. P1263), egg sphingomyelin (cat. no. S0756), chick yolk phosphatidyl glycerol (cat. no. P0514), and bovine brain sphingomyelin (cat. no. S7004). The purity of each compound was checked via thin-layer chromatographic migration by spotting, developing, and exposing thin-layer chromatography plates to iodine vapors. Where necessary, about 20 μl of chloroform was first added, to facilitate solubilization of the phospholipid in ethanol. This amount of chloroform added to the DPL did not significantly affect results of the assays.

In all cases, after addition of ethanol, tubes were capped with an air-tight plastic cap and vigorously shaken for 30 s, allowed to stand for 15 s, and then closely observed for the...
formation of a ring of foam at the meniscus. The meniscus of the tubes was evaluated as either "negative" or "positive" (Figure 1), the absence of bubbles, or an incomplete ring of bubbles, constituting a "negative" result and the presence of a complete ring of bubbles, multiple rings, or layers a "positive" result. The threshold ethanol volume fraction—that is, the highest ethanol volume fraction at which a single ring of bubbles persists—was the endpoint of each series of experiments for a given amount of phospholipid. All assays were done at 25 °C.

Foam stability assays of pure phospholipids. A known amount of each phospholipid analyzed in the artificial system of saline and ethanol was added with a micro-syringe into a series of 13 × 75 mm plastic tubes containing ethanol and saline at ethanol volume fractions ranging from 0.42 to 0.55, in a final volume of about 1.0 ml. The tubes were then capped, shaken, and evaluated as described above. Amounts of phospholipids evaluated ranged from 15 to 250 μg per assay. For each amount, the ethanol volume fraction at the threshold of foam formation was recorded.

Amniotic fluid studies: effect of dilution. Two amniotic fluid specimens were obtained at cesarean-section delivery of mature fetuses, and centrifuged (1000 × g, 5 min). The supernate of each amniotic fluid was decanted and thoroughly mixed. Six dilutions of the fluid with isotonic saline were prepared just before the shake tests were done. On two occasions—one by one technologist, and once by two technologists—the diluted amniotic fluid specimens were independently evaluated at various ethanol volume fractions for the formation of stable foam. In all cases, 0.50 ml of amniotic fluid (undiluted or diluted) was added to 15 × 75 mm plastic tubes, along with volumes of ethanol/water (95/5) to give ethanol volume fractions in the range of interest. Tubes were then capped, shaken, and evaluated as described previously. We did not test dilutions of amniotic fluid with saline greater than fivefold.

Results

Foam formation as a function of DPL concentration and ethanol volume fraction. Figure 2 illustrates the foam threshold curve separating foam formation from no foam formation in the artificial system DPL, saline, and ethanol. The curve represents "threshold" ethanol volume fractions, i.e., the highest ethanol volume fraction that will permit stable foam to occur. In all subsequent figures, the curves shown represent the threshold ethanol volume fractions for the indicated phospholipid.

Foam threshold curves for various phospholipids. Figure 3 depicts threshold curves for five phospholipids: bovine brain sphingomyelin, DPL, egg sphingomyelin, egg lecithin, and phosphatidylglycerol. The threshold curve for DPL is included for comparison. DPL and bovine brain sphingomyelin show ability to form stable foams (at concentrations as low as 20 mg/liter) at an ethanol volume fraction of 0.475, whereas the other phospholipids have much lower threshold curves.

Figure 4 illustrates threshold curves for four species of phosphatidylcholine. We found that, among the di-saturated species, the ability to form stable foam at higher ethanol volume fractions improves as the number of carbons of the α-
β-fatty acids increases from 12 (lauroyl) to 14 (myristoyl) to 16 (palmitoyl).

Figure 5 shows the threshold ethanol volume fractions as a function of the degree of dilution of an amniotic fluid specimen with saline. The undiluted amniotic fluid had a threshold ethanol volume fraction of 0.515. The actual data points obtained by three technologists are presented, to give an impression of the variability of the results.

Figure 6 shows threshold curves obtained upon dilution of two amniotic fluid specimens, each with a different initial threshold ethanol volume fraction when undiluted (see Methods). Figures 5 and 6 demonstrate that a curve similar to that observed on using DPL in the artificial system (Figure 2) can be obtained with amniotic fluid specimens obtained at term. If DPL were the only factor in amniotic fluid that affects foam formation, then the estimated DPL concentration of the two amniotic fluids would be about 180 and 250 mg/liter of amniotic fluid, respectively, or 90 and 60 mg/liter in the assay mixture. (See note added in proof, p 1984.)

Discussion

Many substances can form foam at an air-liquid interface after vigorous agitation. Foam formation is a complex process related to the viscosity, solubility, and concentrations of the substances involved and the temperature and surface tension of the solution. The ability of a given substance to form a stable foam is largely related to its own surface-tension characteristics. For example, if the surface tension of a bubble formed from protein shaken in water is lower than that of the existing air-liquid interface, then the bubble structure may remain stable for an extended period of time. Conversely, if it is higher, the bubble tends to collapse. Thus, formation of foam by any pure compound or any combination of compounds appears to be an indication that for that compound (or mixture) at that concentration and temperature, the surface tension of the bubble structure is lower than that of the air-liquid interface, allowing the bubble to persist.

Ethanol is a nonfoaming competitive surfactant that lowers the surface tension of a water-ethanol mixture (1). As the volume fraction of ethanol is increased, the surface tension decreases (Figure 7). At low ethanol volume fractions—e.g., 0.10—proteins, fatty acids, lyso- and glycerophospholipids can form stable foams. However, at ethanol volume fractions exceeding 0.40, only glycerophospholipids can form stable foams. In aqueous ethanol at 25 °C, as the ethanol volume fraction is increased from 0.40 to 0.50, the surface tension is reduced by 0.22 μN/m (1N = 106 dynes), going from 3.11 μN/m at an ethanol volume fraction of 0.40, to 2.89 μN/m at an ethanol volume fraction of 0.50 (Figure 7). Substitution of amniotic fluid for water or addition of other phospholipids might alter the magnitudes of these values somewhat.

Whether the very small difference in surface tension going from an ethanol volume fraction of 0.40 to 0.50 may explain the profound differences in "DPL threshold values" is highly speculative. Whether or not increasing ethanol concentrations change (e.g., the solubility of the phospholipids or the viscosity of the solution must also be considered.

Recently, we proposed a quantitative assay of the amniotic fluid surfactant based on the ability of an amniotic fluid-ethanol mixture to produce stable foam after the mixture had been shaken vigorously (6). In the method, a series of graded
ethanol volume fraction, in 0.01 ethanol volume fraction increments, is used to arrive at the threshold ethanol volume fraction (highest ethanol volume fraction that permits stable foam to be formed) of the amniotic fluid specimen. This assay, which we call the “Foam Stability Index (FSI)-Test,” has demonstrated reliability in predicting fetal pulmonary maturity (6). The FSI value is synonymous with the term “threshold ethanol volume fraction” as used here.

In establishing the FSI Test, we pointed to the relationship of the threshold ethanol volume fraction to the concentration of DPL in an artificial system (6). Because we dilute 0.500 ml of amniotic fluid to give a final total volume of about 1 ml, the concentration of DPL in the final assay mixture (per constant volume) should be about 50% of the DPL concentration (or DPL equivalents) in the amniotic fluid. Table 1 shows the relation between threshold ethanol volume fraction value (FSI value) and concentration of DPL (or DPL equivalents). We find that an amniotic fluid FSI value of 0.46 or greater in the great majority of cases renders assurance that the fetus has an adequate amount of pulmonary surfactant and that an FSI value of 0.48 or greater gives complete assurance of fetal pulmonary maturity. An FSI value of 0.46 corresponds to an approximate DPL concentration (or DPL equivalent) of 20 mg/liter in the final assay mixture, or approximately 40 mg/liter in the amniotic fluid specimen; an FSI value of 0.48 would correspond to a DPL concentration of 40 mg/liter in the final assay mixture, or 80 mg/liter in the amniotic fluid specimen.

Figure 1 illustrates the quantitative relationship of ethanol volume fraction and DPL concentration to the presence (or absence) of foam formation. Note that the range of DPL concentrations that is of interest in evaluating fetal maturity (20 to 100 mg/liter in amniotic fluid corresponding to 10 to 50 mg/liter in the assay) is in a region where the slope of the curve is the greatest. Thus, information on the threshold ethanol volume fraction can be used to estimate the “DPL equivalents” present in the amniotic fluid (see Table 1).

The final ethanol volume fraction is critical in determining the outcome of the shake test (1). As noted in Figure 2, even minute errors in pipetting either ethanol or amniotic fluid can influence the outcome of a shake test, insofar as this system simulates a shake test with amniotic fluid. Figures 5 and 6 suggest that amniotic fluid behaves similarly. Because DPL is the most-synthesized phospholipid as the fetus matures, it is clear from Figure 2 that foam formation in the range of DPL concentrations of clinical importance depends very critically on the final ethanol volume fraction. This may account for some of the discrepancies reported concerning the reliability of the shake test.

We evaluated stable foam formation by each of several

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<th>Table 1. Dipalmitoyl 3-sn-Phosphatidylcholine (DPL) Concentration as a Function of Threshold Ethanol Volume Fraction (TEVF)</th>
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*a Approximate.

*b This value equals the approximate concentration of DPL in the assay divided by the aqueous volume fraction.
phospholipids (Figures 3 and 4). At the ethanol volume fractions used in the Clements' test (0.475) or in the Edwards and Baillie modification (0.50) only bovine brain sphingomyelin, egg sphingomyelin, and DPL can form stable foams at concentrations below 100 mg/liter. The much lower threshold curve for egg sphingomyelin than for bovine brain sphingomyelin is probably attributable to the different fatty acid compositions of the two. Table 2 lists percentages of fatty acids found in the bovine brain sphingomyelin and in the chick yolk sphingomyelin that we used in our assays, as well as percentages for human serum sphingomyelin fatty acids. The predominance of palmitate (16:0) in chick yolk sphingomyelin compared to the relative predominance of longer-chain fatty acids in bovine brain sphingomyelin suggests that increasing fatty acid chain length may enhance stable foam formation in our artificial assay system. Human serum sphingomyelin contains a significant amount of longer chain fatty acids, and may contribute to the formation of foam in “false positive” shake tests observed with amniotic fluid specimens contaminated with blood (see Table 2). The composition of sphingomyelin fatty acids in amniotic fluid has not been reported to our knowledge.

Figure 3 also indicates that phosphatidylglycerol of egg origin has no ability to form a stable foam at an ethanol volume fraction of 0.475 at concentrations up to 250 mg/liter. Since amniotic fluid phosphatidylglycerol contains a large amount of disaturated species, we attempted to enrich egg phosphatidylglycerol with dipalmitoyl phosphatidylglycerol to give a 2:1 ratio by weight. Although these components did not completely dissolve at a concentration of 2 g/liter in 95% ethanol, it was possible to assay the suspension, which upon dilution and shaking, went into solution. No change in the threshold curve was observed. Thus, while the composition of sphingomyelins clearly affected foam formation, no such effect was noted for egg phosphatidylglycerol enriched with dipalmitoyl phosphatidylglycerol.

We evaluated several synthetic lecithins with respect to stable foam formation in ethanol–saline (Figure 4). Among the three disaturated species, the increase in fatty acid chain length from 12 carbons to 16 carbons clearly enhances the ability to maintain stable foam at higher ethanol volume fractions. Distearoyl lecithin presented some solubility problems and could not be evaluated under these conditions. Dioleoyl lecithin also shows ability to form stable foam, but even at concentrations as great as 300 mg/liter in the assay mixture it cannot form foam if the ethanol volume fraction exceeds 0.475.

At term, amniotic fluid contains, per liter, about 50–200 mg of lecithins (mainly DPL), 10–40 mg of sphingomyelins, and up to 20 mg of phosphatidylglycerol. In practical terms, lecithins other than DPL appear to contribute little, if any, to stable foam formation in saline–ethanol mixtures at an ethanol volume fraction of 0.500. The threshold curve for egg lecithin suggests that even at concentrations of 100 mg/liter (50 mg/liter of the assay mixture), which is approximately the total lecithin concentration in a mature amniotic fluid, the lecithins present in egg do not produce stable foam formation at an ethanol volume fraction of 0.475. It should be noted that no DPL is present in egg lecithins.

**Note added in proof:** After submitting this manuscript, we quantitated DPL after osmium tetroxide treatment of lipid extracts of several amniotic-fluid specimens. The observed DPL in each case was much lower than the theoretical concentration based on the threshold ethanol volume fraction, indicating that DPL is not the only amniotic fluid constituent capable of forming a stable foam at EVFs above 0.48. We are currently investigating this observation.

**References**


