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Quantitation of Pulmonary Surfactant Protein SP-B in the Absence or Presence of Phospholipids by Enzyme-Linked Immunosorbent Assay

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We have developed an enzyme-linked immunosorbent assay (ELISA) that uses polyclonal or monoclonal anti-surfactant protein SP-B antibodies to quantitate purified SP-B in chloroform/methanol and in chloroform/methanol extracts of whole pulmonary surfactant at nanogram levels. This method has been used to explore the effect of the presence of different phospholipids on the immunoreactivity of SP-B. Both polyclonal and monoclonal antibodies produced reproducible ELISA calibration curves for methanolic SP-B solutions with protein concentrations in the range of 20–1000 ng/mL. At these protein concentrations, neither dipalmitoylphosphatidylcholine, dipalmitoylphosphatidylglycerol, nor phosphatidylcholine or phosphatidylglycerol from egg yolk had significant effects on the binding of antibodies to SP-B up to protein-to-lipid weight ratios of 1:20. Coating of ELISA plates with SP-B concentrations higher than 1 μg/mL produced a substantial decrease in the binding of antibodies to the protein that was prevented by the presence of negatively charged but not zwitterionic phospholipids. Characterization of the secondary structure of SP-B by far-UV circular dichroism showed that phospholipids induced pronounced changes on the conformation of SP-B when the solvent was evaporated and dry lipid–protein films were formed, a necessary step to expose protein to antibodies in ELISA. Under these conditions, negatively charged lipids, but not zwitterionic ones, induced a marked decrease on the ellipticity of SP-B that would be associated with a conformation that is significantly more exposed to antibodies.

Key Words: **thin films; monolayers; protein quantitation; epitope exposure; secondary structure; circular dichroism.**

The hydrophobic surfactant protein SP-B[1] is of critical importance for the specialized biophysical function of pulmonary surfactant (1, 2). The lack of an operative SP-B leads to lethal disorders due to severe respiratory failure (3, 4). SP-B is involved in the delivery of surface active material to the alveoli and in the formation of surface active phospholipid-enriched films which stabilize the respiratory alveoli (2, 5). Numerous studies during recent years have focused on the structure and lipid–protein interactions of SP-B in model bilayers (6–10) and monolayers (11–14). However, the mechanism by which SP-B modulates the biophysical behavior of the pulmonary surfactant complex still remains elusive. Several authors have reported specific interactions between SP-B and phosphatidylglycerol (PG), the main anionic phospholipid of surfactant (7, 9, 15). The interaction of the protein with acidic phospholipids has been proposed to aid in processes considered essential in surfactant dynamics such as tubular myelin formation (16, 17), interfacial phospholipid adsorption (5, 11), refining of surface film composition during compression (18), or protein-induced monolayer stabilization at high surface pressures (14, 19, 20).

The main objective of the present work was to develop an ELISA method to quantitate SP-B in organic solvents, using polyclonal or monoclonal anti-SP-B an-

[1] Abbreviations used: CCA, convex constraint algorithm; Chl, chloroform; DMAB, dimethylaminobenzoic acid; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)]; ECL, enzyme chemiluminescence; ELISA, enzyme-linked immunosorbent assay; LPC, 1-palmitoyl-2-lyso-*sn*-glycero-3-phosphocholine; MBTH, 3-methyl-2-benzothiazolinone hydrazone; MeOH, methanol; PBS, phosphate-buffered saline; PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine; PG, 1,2-diacyl-*sn*-glycero-3-phospho-*rac*-glycerol; SP-A, surfactant protein A; SP-B, surfactant protein B; SP-C, surfactant protein C; TFE, trifluoroethanol.

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tibodies, which is applicable in the absence or in the presence of surfactant phospholipids. We have used recognition of SP-B epitopes by polyclonal or monoclonal antibodies to probe specific structural features of the protein in the absence or presence of zwitterionic and anionic phospholipids.

MATERIALS AND METHODS

Materials

Chloroform (Chl) and methanol (MeOH) were HPLC-grade solvents from Scharlau (Barcelona, Spain). Sephadex LH-20 and LH-60 chromatography gels were from Pharmacia (Uppsala, Sweden). Egg yolk phosphatidylcholine (PC) and phosphatidylglycerol were from Sigma (St. Louis, MO) and dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG) were from Avanti Polar Lipids (Birmingham, AL). Substrates for enzyme immunoassays, 3-dimethylaminobenzoic acid (DMAB) and 3-methyl-2-benzothiazolinone hydrazone (MBTH), were also from Sigma. All other reagents and chemicals were from Merck (Darmstadt, Germany).

Surfactant Proteins

Surfactant proteins SP-B and SP-C were isolated from minced porcine lungs by an adaptation of the method of Curstedt *et al.* (21), which is described elsewhere (22). After isolation, solutions of purified SP-B in Chl/MeOH (2:1, v/v) were stored at −20°C until use. To coat microtiter ELISA plates with protein, SP-B solutions were first enriched in methanol by careful evaporation of chloroform from Chl/MetOH stock solutions of purified SP-B, under N_2, while methanol was continuously added to maintain the initial volume.

SP-A was isolated from purified pulmonary surfactant as previously reported (23).

Antibodies

Anti-SP-B polyclonal antiserum was raised in white New Zealand rabbits after five intramuscular inoculations at weekly intervals. A 100-μg dose of protein prepared in micelles of lysophosphatidylcholine in complete Freund's adjuvant was used for the first inoculation and 70 μg of protein in LPC micelles in incomplete adjuvant was used in subsequent injections. Serum was obtained from the inoculated rabbit 10 days after the last inoculation.

Anti-SP-B monoclonal antibody 8B5E, recognizing an epitope of human SP-B (24), was a generous gift of Dr. Yasuhiro Suzuki (University of Kyoto, Japan).

Western Blot Assay

Specificity of the polyclonal antiserum for porcine SP-B was determined by conventional Western blot assay as previously described (25) after electrophoresis of porcine SP-A, SP-B, and SP-C samples in 16% acrylamide gels and transfer to nitrocellulose filters using a Bio-Rad Trans-Blot cell. Detection of immunoreactive bands was achieved by enzyme chemiluminescence reaction.

Enzyme-Linked Immunosorbent Assay

The ELISA method developed in this work using either polyclonal or monoclonal anti-SP-B antibodies was carried out as follows. Each well of the plates (immunoplates Polysorp F96, Nunc A/S, Roskilde, DK) was filled with 100 μL of methanolic protein solution, containing a defined amount of SP-B as determined by amino acid analysis, either with or without phospholipids, and incubated at 37°C until the solvent had completely evaporated. The wells of the microtiter plates were then washed six times with a 0.05% (vol/vol) Tween 20 solution and blocked by treating each well for 1 h with 100 μL of 5% dried skim milk in phosphate-buffered saline (PBS) (137 mM NaCl, 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.6 mM KCl, pH 7.4). After being washed six times with 0.05% Tween 20, 100 μL of the primary antibody (1/500 dilution of the polyclonal antiserum or 20 μg/mL of the monoclonal antibody, in PBS containing 0.05% Tween 20) was added to each well and the plate was incubated for 1 h at 37°C. The plates were then washed again six times with Tween 20 and incubated for 1 h at 37°C with the secondary antibody, peroxidase-labeled anti-rabbit (when the primary was polyclonal) or anti-mouse (when the monoclonal antibody was used as primary) IgG antibody (Amersham International, Buckingshamshire, UK), diluted 1/500 in PBS containing 0.05% Tween 20. After being washed six times with Tween 20 solution, the plates were developed by adding 200 μL per well of the detection solution (80.6 mM DMAB, 1.56 mM MBTH, H_2O_2 0.0075% in 0.1 M phosphate buffer, pH 7). The reaction was stopped by the addition of 50 μL of 3 N H_2SO_4. Results were read spectrophotometrically at 570 nm in a Digiscan 340T microtiter reader (Asys Hitech Gmbh, Austria). Negative controls were always run using an anti-SP-A polyclonal antiserum as primary antibody to discount nonspecific signals.

To quantitate SP-B in surfactant organic extracts, aliquots of the samples, typically 5–10 μL containing 50–100 μg of phospholipids, were added into the first wells of the plates previously filled with 190 μL of methanol, and serial twofold dilutions with methanol were prepared. After total evaporation of the solvent by incubation at 37°C, the ELISA protocol was followed as described above.

Circular Dichroism

Far-UV circular dichroism spectra were obtained in a Jasco-715 spectropolarimeter equipped with a Xenon lamp. To obtain cd spectra of methanolic protein solutions, 200 μL of 50 μg/mL SP-B in methanol was placed in an 0.1-cm optical-path cell thermostated at 25°C, in the absence or in the presence of the desired amounts of phospholipids. Ten spectra were accumulated and averaged to improve signal-to-noise ratio. To measure the cd spectra of dry protein and lipid–protein films, 20 μL of 0.5 mg/mL SP-B in methanol or in chloroform/methanol (2:1, vol/vol), either in the absence or in the presence of 100 μg of phospholipids, was deposited on 25 $\times$ 25 mm quartz plates (Goodfellow, Cambridge, UK) and allowed to evaporate at room temperature. The plates were placed at the light path of the spectropolarimeter in different positions to sample and average cd spectra from eight defined points, in order to consider possible inhomogeneities in deposed samples on quartz plates. Five spectra were accumulated from each position of the quartz plates. To calculate ellipticity, the protein concentration was considered 0.5 mg/mL and the optical path length 0.032 mm, these values being the concentration and thickness of the protein organic solution layer deposited onto the plates.

Dichroism is presented in the figures as mean residue ellipticity, considering 110 as the mean molecular mass per amino acid in SP-B. Estimation of secondary structure from cd spectra in solution was approached using the convex constraint algorithm described by Perczel and co-workers (26) which allows decomposition of standard and sample cd spectra in any given number of independent components, corresponding to different structural elements. The cd spectra in the present study have been interpreted as a composition of four structural components: α-helix, β-sheet, β-turns, and random coil.

RESULTS

Figure 1 shows that the rabbit anti-porcine SP-B antiserum prepared in this work by immunization with SP-B/LPC suspensions specifically recognizes SP-B but not SP-A or SP-C in Western blots. In 16% acrylamide PAGE gels in the presence of SDS and under nonreducing conditions, the most prominent forms of SP-B and SP-C migrate with apparent mobilities of about 22 and 8 kDa, corresponding to SP-B dimers and SP-C monomers, respectively, as previously described (22). Purified and reduced porcine SP-A shows bands in the range of 28–42 kDa, due to glycosylation heterogeneity (23). The binding of polyclonal anti-SP-B antibodies to SP-B in the blots indicates that at least some epitopes in SP-B are preserved in the SP-B/SDS complexes.

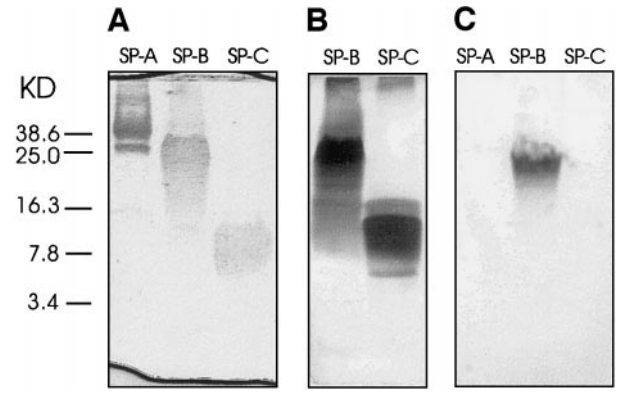

FIG. 1. PAGE analysis and Western blot of surfactant proteins using an anti-SP-B polyclonal antiserum. Electrophoresis of 5 μg of porcine surfactant proteins SP-A, SP-B, and SP-C was carried out in 16% acrylamide in the presence of SDS. SP-A samples were treated under reducing (DTT 50 mM) and SP-B and SP-C samples under nonreducing conditions. Gels were (A) stained with Coomasie blue, (B) silver stained, or (C) Western blotted after transfer to nitrocellulose using a rabbit anti-porcine SP-B polyclonal antiserum as primary antibody. A scale of molecular mass markers is presented to the left of the gels.

In our ELISA system this anti-SP-B polyclonal antiserum allows determination of SP-B in the range of 2–100 ng of protein per well (0.2–10 ng/mL) with excellent reproducibility, as shown in Fig. 2. For quantities of protein between 100 and 1000 ng (10–100 ng/mL), there is a consistently lower response of the polyclonal antibody in the ELISA reaction, observed as a valley in the OD curve. The polyclonal anti-SP-B antiserum does not recognize SP-A in amounts up to 10 μg, but recognizes SP-C in amounts higher than 1 μg. Analysis of isolated SP-C by electrophoresis and Western blot under overdeveloping conditions revealed the presence of trace amounts of SP-B (not shown) that were not detectable under the standard conditions used (Fig. 1). Our ELISA procedure is therefore also useful to evaluate the purity of SP-C preparations. Figure 2 also shows the calibration curve of SP-B using the anti-SP-B monoclonal antibody 8B5E (generously provided by Dr. Y. Suzuki, University of Kyoto, Japan) as the primary antibody. The binding of this monoclonal antibody to SP-B showed a linear response to the amount of SP-B coating the wells up to 1 μg of protein.

The biphasic behavior of the ELISA calibration curve of SP-B using polyclonal antibodies was reproducibly observed for four different preparations of SP-B from different lungs (Fig. 3). All preparations showed a marked decrease in the recognition of the protein by anti-SP-B antibodies for SP-B amounts in the well larger than 70–100 ng. Two SP-B preparations also showed such a biphasic response when using the monoclonal antibody.

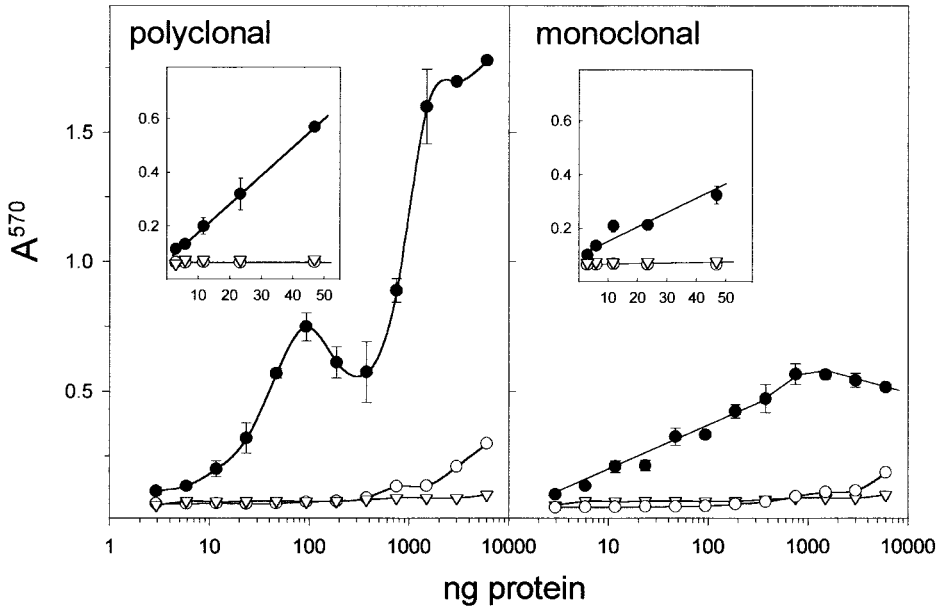

FIG. 2. ELISA calibration curves for porcine surfactant proteins using an anti-SP-B polyclonal antiserum (left) or the monoclonal anti-SP-B antibody 8B5E (right) as primary antibody. Variable amounts of SP-A (▽), SP-B (●), or SP-C (○) were used to coat the microtiter plate wells. Error bars represent standard deviations of three different experiments. Those bars not shown are within the symbol sizes. Insets: linear rescaling of plots.

Figure 4 shows the effect of the presence of different phospholipids, at a protein-to-lipid ratio of 1:20 by weight, on the recognition of SP-B by polyclonal and monoclonal antibodies. The calibration curve of SP-B was not affected by phospholipids when the protein amount per well was in the range of 5–50 ng, using either polyclonal or monoclonal primary antibodies (Fig. 4, inset). In contrast, when the amount of SP-B per well was larger than 100 ng, phospholipids had a pronounced effect on the recognition of epitopes in

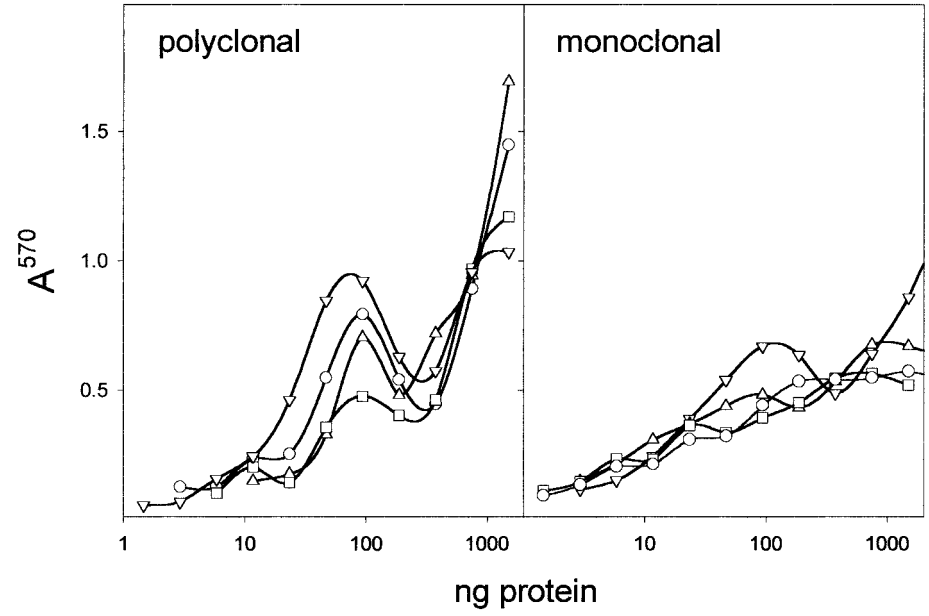

FIG. 3. Consistency of ELISA curves using anti-SP-B polyclonal (left) or monoclonal (right) primary antibody with different batches of SP-B. Symbols indicate each of four different experiments using protein purified from four different lungs.

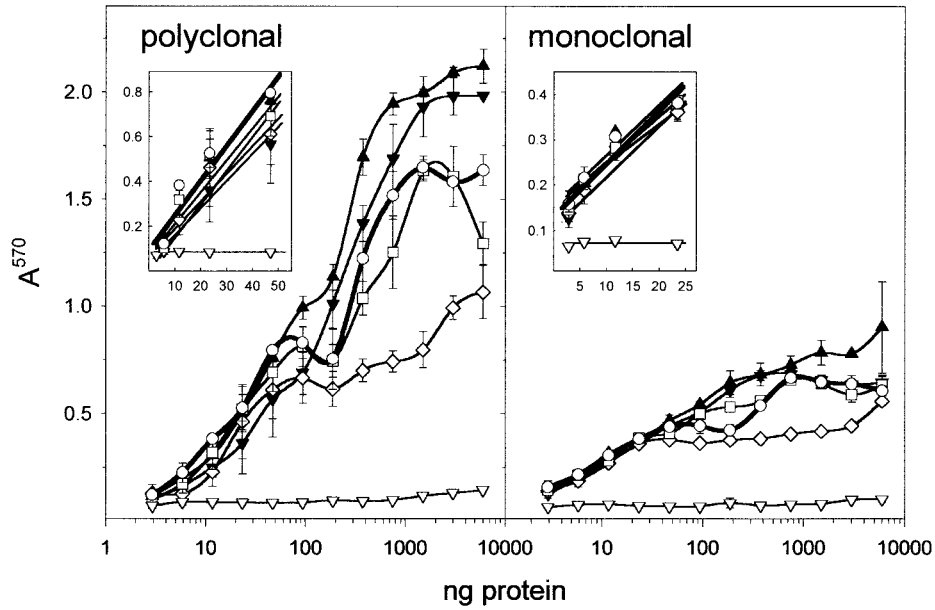

FIG. 4. Effect of phospholipids on the recognition of porcine SP-B by anti-SP-B polyclonal (left) or monoclonal (right) primary antibodies. Protein was exposed to antibodies in the absence (bold line, ○) or in the presence of egg yolk PC (◇), DPPC (□), egg yolk PG (▲), or DPPG (▼) at a protein-to-lipid ratio of 1:20 by weight. Controls (▽) for color development in the ELISA determinations were carried out using an anti-SP-A polyclonal antiserum as primary antibody. Error bars represent standard deviation after averaging three different experiments. Those bars not shown are within the symbol sizes. Insets: linear rescaling of plots.

SP-B by the primary antibodies. Under these conditions, the presence of negatively charged phospholipids, either DPPG or egg PG, led to the largest binding of antibodies, either polyclonal or monoclonal, to SP-B. When the wells were coated with lipids from methanol solutions, in the absence of SP-B, binding of the antibody was not detected, eliminating possible nonspecific adsorption of antibodies to negatively charged surfaces. The presence of DPPC did not substantially change the calibration curves of SP-B from those obtained in the absence of lipids. In contrast, the presence of egg PC caused lower binding of antibodies to SP-B for protein amounts larger than 70 ng per well. The ELISA calibration curves for SP-B in the presence of egg PG or DPPG showed a monophasic character up to protein amounts of 1 μg. The effect of the presence of the different phospholipids on the calibration curve of SP-B obtained with the polyclonal antibody was qualitatively similar to that observed using the monoclonal antibody. Calibration curves were also qualitatively and quantitatively similar for protein-to-lipid ratios of 1:5 and 1:10 by weight (data not shown).

The ELISA procedure has been used to quantitate SP-B in Chl/MeOH organic extracts obtained from different lavages of porcine lungs, containing the full surfactant lipid mixture and the two hydrophobic proteins SP-B and SP-C. Figure 5 shows the ELISA response of two of such extract samples progressively diluted with methanol in comparison with calibration curves obtained from defined amounts of purified porcine SP-B in the absence and in the presence of egg PG (1/20 protein to lipid by weight). Again, the presence of PG abolished the biphasic response to pure SP-B in the absence of phospholipids, allowing for a better estimation over a wider range of protein amounts. Interpolation of the sample absorbances at 570 nm in the linear portion of the calibration curves allowed estimation of the amount of SP-B present in the surfactant organic extracts. An amount of 14 $\pm$ 2.4 ng of SP-B per nmol of phospholipid (n = 7) in such extracts was determined, a value consistent with the content of SP-B previously found in pulmonary surfactant (27). Determination of the amounts of SP-B in several of the extracts by amino acid analysis, as previously described (25), yielded values that were always within $\pm$10% of the determinations by ELISA.

The binding behavior of the anti-SP-B antibodies, both in the absence and in the presence of different phospholipids, and depending on the amount of protein in the assays, suggests that there are potential concentration-dependent structural changes in SP-B with consequences for the accessibility of the protein epitopes to antibodies. To explore this possibility, we have analyzed the far-UV cd spectra of methanolic solutions of SP-B in the absence and in the presence of phospholipids (Fig. 6A). These spectra have been obtained at an SP-B concentration of 50 μg/mL, well above the protein concentration threshold that led to

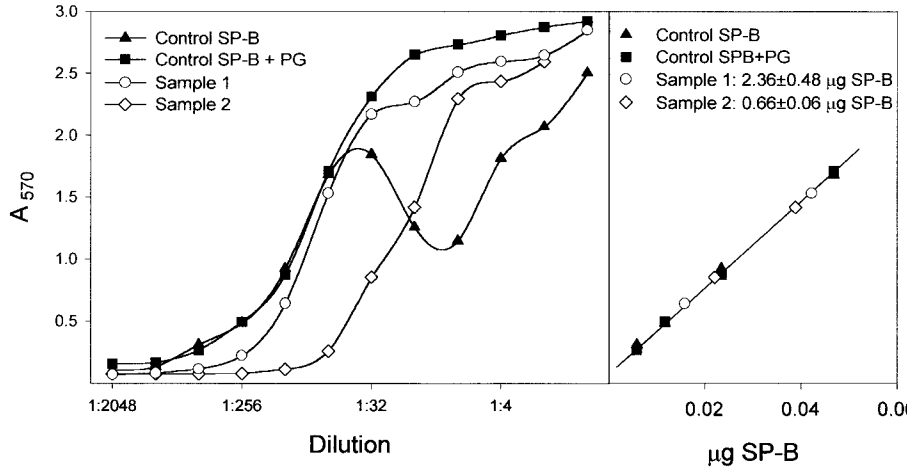

FIG. 5. Quantitation of SP-B in surfactant Chl/MeOH extracts by ELISA. (Left) ELISA response of two progressively diluted samples of surfactant organic extracts from different lungs (○, ◇), in comparison with standard samples of purified SP-B in the absence (▲) or presence (■) of egg PG (l/p ratio 20:1, w/w). Original concentration of standard SP-B, 0.3 μg/mL. (Right) Calibration line of SP-B in the absence or presence of PG, including interpolated values of A_{570} for the two test samples. Calculated amounts of SP-B in the samples are indicated.

differences in sensitivity from our ELISA method. All spectra were very similar, showing features consistent with a mainly α-helical conformation, including two minima of negative ellipticity at around 209 and 220 nm and a maximum at 195 nm. Deconvolution of these spectra into different independent components by the convex constraint algorithm (26) allowed estimation of the secondary structure contents presented in Table 1. In methanolic solution, SP-B was estimated to possess around 55–60% α-helix and 8–10% β-sheet, either in the absence or in the presence of phospholipids. In the presence of negatively charged phospholipids, the far-UV cd spectrum of SP-B had slightly lower ellipticity than in the absence of phospholipids or in the presence of phosphatidylcholine species (see inset in Fig. 6A). The associated structural differences are likely to be small. Quantitative analysis suggested that the variations in ellipticity could imply a lower percentage of random coil, 6–8% versus around 15%, and a higher amount of turns, 26–28% versus around 20%, in the presence of phosphatidylglycerol (also in Table 1).

The possibility that differences in SP-B exposure to antibodies in ELISA could come not from conformational differences in methanolic solution but from differences in epitope exposure influenced by the presence of phospholipids once the solvent is evaporated and dry lipid–protein films are formed must be considered. To explore this possibility we have analyzed far-UV cd spectra obtained from dry SP-B thin films, in the absence or in the presence of lipids, formed on quartz plates (Fig. 6B). The molar ellipticity of these spectra was calculated considering the concentration of protein or lipid/protein mixtures and the thickness of the liquid layer once the solution was delivered on the plate. These spectra were repetitive and well defined for wavelengths between 200 and 250 nm, but not for wavelengths lower than 200 nm. Cd spectra of dry films of pure protein were qualitatively and quantitatively different from those of dry lipid–protein films. In the absence of lipids, SP-B films produced a cd spectrum with a main minimum at around 222 nm and substantially lower ellipticity than the spectrum of SP-B in methanol. In the presence of DPPC, SP-B films had the spectrum with the largest negative ellipticity, and it was qualitatively and quantitatively comparable to those spectra in methanolic solutions containing DPPC. The presence of egg PC also shifted the features of the cd spectrum of dry SP-B films to those of the cd spectrum in methanolic solution, although with a somewhat reduced negative ellipticity. Cd spectra of dry films of SP-B containing negatively charged lipids,

TABLE 1

Secondary Structure of Surfactant Protein SP-B in Methanolic Solutions and in Dry Films, in the Absence and in the Presence of Different Phospholipids, Estimated from far-UV Circular Dichroism Spectra

	Methanolic solution				Dry film
	α	β	t	r	α
No lipid	53	8	23	16	58
Egg PC	55	11	18	16	66
DPPC	60	10	17	13	60
Egg PG	58	8	28	6	44
DPPG	56	10	26	8	30

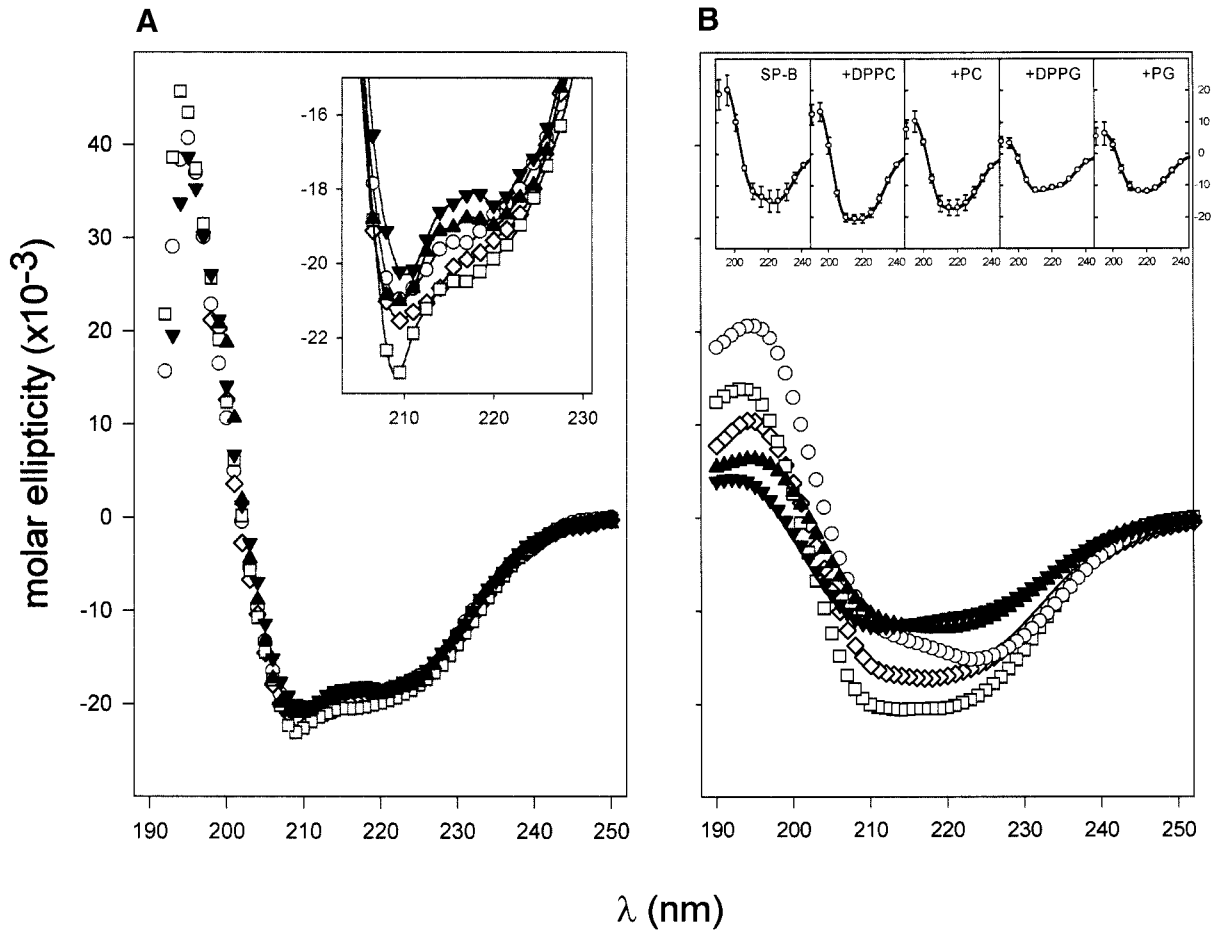

FIG. 6. Far-UV circular dichroism spectra of porcine SP-B in the absence and in the presence of phospholipids, in methanolic solutions (A) or in dry films (B). Symbols have the same meaning as in Fig. 4. Inset in (A): scale magnification. Insets in (B): individual spectra showing error bars after averaging ellipticities from three different preparations; lines represent theoretical cd spectra determined from the components of the secondary structure calculated in Table 1.

either DPPG or egg PG, were very similar and presented the lowest negative ellipticities.

Application of the convex constraint algorithm to the cd spectra of dry protein and lipid–protein films is not likely as reliable as using it for solution spectra, as the cd spectra of the dry thin films are probably affected by additional parameters such as the geometry of the sample film in the optical pathway (28), which cannot be compared with the standard spectra used by the method. The apparent percentages of α-helix estimated from those spectra from thin films have been included in Table 1 only for the purpose of a rough qualitative evaluation. Those calculated percentages of α-helix were similar to the values estimated by other deconvolution methods (29, 30). As occurred in methanolic solution, SP-B was calculated to possess about 60% α-helix in dry films, in the absence of lipids or in the presence of the two zwitterionic phospholipids, DPPC and egg PC. In contrast, the cd spectra of SP-B in the presence of phosphatidylglycerol yielded estimates of substantially lower α-helix content, around 44% in egg PG and 30% in DPPG.

DISCUSSION

Immunological methods such as Western blot analysis or ELISA are highly effective procedures for detecting and quantitating any given protein in complex biological samples. Numerous works have developed immunodetection methods to evaluate the level of pulmonary SP-A (see, for instance, Refs. 25, 31–33). Less numerous are studies using anti-SP-B antibodies to measure variations of this protein in surfactant samples (34, 35). SP-B is less immunoreactive than SP-A, in part because the hydrophobic protein is embedded in the surfactant lipid–protein complexes, probably reducing its accessibility to antibodies. It has been reported that the quantitation of SP-B in SP-B-contain-

ing samples by ELISA is much affected by the presence of phospholipids (35). We have recently shown that the level of insertion of SP-B in surfactant phospholipid bilayers is dependent on the method used to prepare lipid–protein samples (10), which might also lead to differences in exposure of SP-B epitopes. The main objective of the present work was therefore to standardize an ELISA which was useful to quantitate SP-B and also to determine the effects of the presence of different surfactant-relevant phospholipids on the recognition of SP-B by antibodies.

The ELISA procedure we have developed is useful for quantitating SP-B from organic solutions. Usually, protein quantitation in purified batches of SP-B has been carried out by amino acid analysis. Such methodology is not available in all laboratories and requires expending both a fair amount of protein and a relatively long time in sample processing. Given the accessibility of a microplate reader, usually a commonly available instrument, the ELISA determination described here can be used to estimate the amount of SP-B in multiple samples within few hours, requiring just a few nanograms of protein. This method shows high reproducibility with a good linear response, independently of the presence of phospholipids, for protein amounts in the range of 1–50 ng.

Above 70–100 ng of SP-B per well, the recognition of the protein by antibodies is influenced by both the amount of protein coating and the presence of different phospholipids. In the absence of lipids, the availability of adsorbed SP-B to antibodies shows a decrease above 70–100 ng protein per well. For protein amounts larger than 100 ng, the binding of antibodies, and the subsequent ELISA reaction, is significantly reduced. This feature is reproducible for SP-B preparations from different lungs and is likely therefore to originate in intrinsic properties of the protein. Coating the plates with densities of protein higher than a certain threshold probably leads to protein–protein interactions and possible conformational changes, resulting in a partial shielding of SP-B epitopes from the antibodies. Coating at high protein densities might have also differential effects on the relative affinity for binding to SP-B of the different antibodies in the polyclonal serum. However, we obtained similar qualitative changes in immunoreactivity when using a different anti-SP-B polyclonal antiserum (a gift from Dr. Jeff Whitsett from Children's Hospital Medical Center in Cincinnati, OH; data not shown). The effect is also present, although less markedly so, when using monoclonal antibodies, suggesting that the epitope recognized by the monoclonal antibody 8B5E could also have a different accessibility to antibodies depending on the coating density.

The presence of different phospholipids produces a marked effect on the sensitivity to ELISA at protein densities higher than 70 ng/well. This suggests that phospholipids somehow influence the postulated structural changes occurring in the protein at high protein density. Negatively charged lipids such as DPPG or egg PG produced a monotonic response to SP-B in the ELISA up to saturating amounts, without the discontinuity in OD observed in the absence of lipids at around 100 ng SP-B/well. In contrast, the presence of zwitterionic phospholipid species led to the lowest binding of antibodies to SP-B at high protein densities, suggesting that additional sequestration of certain SP-B epitopes was occurring.

Far-UV cd experiments show that the presence of phospholipids causes only small effects on the secondary structure of SP-B in methanolic solution. In this solvent, SP-B has about 55–60% α-helix, either in the absence or in the presence of phospholipids, a similar conformation to that previously observed in other solvents such as TFE (36). Phospholipids had more pronounced effects on protein conformation in dry lipid–protein films. The cd spectra of SP-B/phospholipid films containing negatively charged phospholipids showed a substantially lower ellipticity compared with the spectra in methanol, suggesting less α-helical content. Electrostatic interactions formed during coating could force more extended protein dispositions which are more exposed to antibodies than those in the absence of lipids or in the presence of zwitterionic lipid species. We previously showed that the conformation of SP-B was not extensively affected by zwitterionic lipids in bilayers (22). Other authors also reported no significant changes in the secondary structure of SP-B when the protein is associated with bilayers of DPPG or DPPC/PG (37). However, electron spin resonance studies with spin-labeled forms of SP-B showed that the protein has a larger rotational diffusion coefficient in DPPG than in DPPC bilayers, which only can be explained as a consequence of a more extended DPPG-induced protein conformation (38). This feature could explain our finding in the present work of a higher susceptibility of SP-B to binding of antibodies in the presence of negatively charged lipids, compared with neutral species.

One must also consider the interactions of the protein with the surface of the support. At low coating densities, the protein–support interactions would dominate, regardless of the presence or absence of the different phospholipids, leading to the phospholipid-independent behavior observed in the ELISA for protein coating lower than 90 ng/well. At protein coating densities higher than 90 ng/well, there would be a competition among protein–support, protein–protein, and protein–lipid interactions leading to the more complex behavior observed.

The quantitative method proposed here based on immunodetection of SP-B is a reliable and reproducible procedure, with potential applicability in the routine detection and evaluation of SP-B amounts in surfactant samples.

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