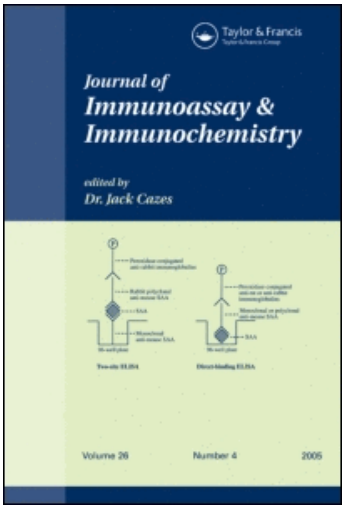


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# AN ENZYME IMMUNOASSAY FOR QUANTITATION OF A TYPE 2 PNEUMOCYTE-SPECIFIC SURFACTANT-ASSOCIATED ANTIGEN IN BRONCHOALVEOLAR LAVAGE FLUID

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## ABSTRACT

A sensitive, specific and reproducible assay for quantitation of a surfactant-associated antigen in mouse bronchoalveolar lavage fluid is described. The procedure employed was a non-equilibrium competitive enzyme immunoassay in which detection of unreacted antibody was significantly enhanced by the use of a second antibody and peroxidase-antiperoxidase as the reporter label. The sensitivity of the assay was such that bronchoalveolar lavage fluid had to be diluted 15 to 30-fold prior to assay. No interference by serum proteins or soluble tissue proteins was observed. This assay may provide *in vitro* quantitative assessment of the functional status of type 2 pneumocytes.

KEY WORDS: Surfactant, Type 2 pneumocytes, Bronchoalveolar lavage, Enzyme immunoassay.

## INTRODUCTION

Type 2 pneumocytes are active participants in the cellular response of the lung to environmental injury. Following alveolar damage these cells function as a reserve population from which regeneration of type 1 pneumocytes occurs (1). In addition, hypertrophy and hyperplasia of type 2 pneumocytes is frequently



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observed in interstitial lung diseases, and may be associated with abnormal surfactant synthesis and secretion (2,3). The extent and significance of these latter responses in the evolution of chronic lung diseases remain uncertain because of the difficulties of assessing alterations in the functional status of type 2 pneumocytes. Demonstration of altered morphology by electron microscopic studies or of altered surfactant secretion by analysis of phospholipids in bronchoalveolar lavage fluid are comparatively inefficent and technically difficult. However, the development of specific antibodies to surfactant-associated proteins secreted by type 2 pneumocytes has made possible an alternative approach to the analysis of the responses of these cells, via immunoassay of these proteins in brochoalveolar fluids.



We have recently reported the preparation of a monospecific antibody to a fraction from lung homogenate enriched in lamellar bodies of type 2 pneumocytes (4). This antibody, which specifically labels type 2 pneumocytes in immunohistochemical studies, is directed against an antigen which is also associated with pulmonary surfactant. In this paper we report the development of an enzyme immunoassay which detects this surfactant-associated antigen in bronchoalveolar lavage fluid with high sensitivity and specificity. The procedure is a non-equilibrium competitive assay in which the sample and a limiting amount of specific antibody are preincubated and the unreacted antibody is subsequently allowed to bind to microplate wells coated with delipidated lamellar body proteins. Detection of the bound antibody is significantly

enhanced by the use of a second antibody and horseradish peroxidase-antiperoxidase (PAP) complex. This assay may provide *in vitro* quantitative assessment of the functional status of type 2 pneumocytes.

## MATERIALS AND METHODS

### Antibodies

The preparation of a monospecific antibody to a lamellar body-enriched fraction of lung homogenate has been previously reported (4). Briefly, mouse lung parenchymal tissue was homogenized in 1.2 M sucrose and centrifuged in a discontinuous density gradient at 80,000 x g for 180 min. Material from the interface between the 0.25 M and 0.75 M sucrose layers, which was highly enriched in intact lamellar bodies, was recovered and used to immunise rabbits. Antiserum was absorbed with normal mouse serum and with pooled membrane fractions of homogenates of mouse liver, kidney, spleen and brain. The absorbed antibody preparation was monospecific by immunodiffusion against delipidated lamellar body proteins. Immunoperoxidase staining of tissues revealed specific labelling of alveolar type 2 pneumocytes, together with staining of the surfactant layer lining small and medium-sized airways, in mouse, rat and human lung. No cross-reactivity was detected with any other cell type in mouse liver, spleen, kidney, brain, heart, intestine and salivary gland, and the type 2 pneumocyte-specific staining was completely abrogated by absorption of the primary antibody with mouse lung

homogenate. For use in the enzyme immunoassay, an immunoglobulin fraction of the antiserum was prepared by precipitation with 40% saturated ammonium sulphate.

Swine anti-rabbit immunoglobulins and rabbit PAP were purchased from commercial sources (Dako Immunoglobulins, Copenhagen, Denmark).

## Antigen

A standard antigen preparation was obtained by delipidation of the lamellar body-enriched fraction recovered from the sucrose gradient. Extraction in chloroform-methanol was performed as previously reported (4) and the protein recovered from the aqueous-organic solvent interface was resolubilised and assayed by the Bio-Rad Protein Assay Kit (Bio-Rad, Richmond, CA) microassay procedure using bovine serum albumin as the standard. Although this antigen preparation contained a number of contaminating serum proteins, principally albumin, the content of specific antigen per microgram of protein was extremely reproducible from batch to batch, varying by no more than $\pm$10%. Thus this preparation was satisfactory for use as a reference antigen for generating standard curves. For the purposes of this assay, 1 unit of specific antigenic protein was defined as the amount contained in 1 ng of the standard antigen preparation.

## Collection of bronchoalveolar lavage fluid

Female BALB/c mice, approximately 12 weeks old, were obtained from the Animal Breeding and Holding Unit, University of New South

Wales. Animals were killed by exsanguination under ether anaesthesia and the thoracic viscera exposed. A #18 needle was inserted into the right ventricle and the pulmonary vascular bed was perfused with 0.9% saline under a pressure of 60 cm of water for 60 sec at which time the lungs appeared completely white. The trachea was then cannulated with a modified intravenous cannula (Medicut, Sherwood, St. Louis, MO) and 5 x 1 ml lavages were performed with 0.9% saline. Typical recovery of lavage fluid was 3.6-4.4 mls i.e. 70-85%. The fluid was centrifuged at 100 x g for 10 min to remove cells and was then assayed for specific antigen content.



Immunoassay procedure

Flat-bottomed 12-well strips for enzyme immunoassay (Immulon-2 Removawell Strips, Dynatech, Alexandria, VA) were assembled in rigid holders to form a microplate. Each well was coated with 30 ng of the standard antigen preparation in sodium carbonate buffer, pH 9.6, at 37° C for 4 h and then at 4° C for 18-20 h. In a separate V-well microplate (Linbro, Flow Laboratories, Sydney, Australia), triplicate samples containing 60 ul each of standards (0-256 units/ml) or unknowns and 60 ul of antibody (1.67 ug/ml of immunoglobulin protein) were incubated at 4° C for 18-20 h. The optimum amounts of antigen and antibody had been empirically determined by a series of preliminary experiments. All dilutions were performed in PBS containing 0.05% Tween-20 (PBST): at least two different dilutions of each unknown

were tested. Wells containing diluent alone constituted an antibody blank.

Following the overnight incubation, the wells of the antigen-coated plate were washed 4 times with 0.9% saline containing 0.05% Tween-20 using a Titertek Handiwash (Flow). Unoccupied protein-binding sites were blocked by addition of 200 ul of a 1% solution of casein hydrolysate (Oxoid, Sydney, Australia) in PBS and incubation at 37$^{o}$ C for 60 min (5). After a further 4 washes, 100 ul of the antigen-antibody mixture were transferred from each well of the V-well plate to the corresponding well of the antigen-coated plate, using a multichannel pipette. The plate was incubated at room temperature for exactly 30 min, the wells were again washed 6 times, and 100 ul of a 1:2000 dilution of swine anti-rabbit immunoglobulins were added to all wells. Following incubation for 60 min and a further 6 washes, 100 ul of a 1:2000 dilution of PAP were added and incubation continued for 30 min. The wells were finally washed another 6 times and 100 ul of chromogenic substrate added (2,2'-azino-di-(3-ethyl benzthiazoline sulfonic acid) (ABTS) in citrate buffer, pH 4.0, containing 0.03% hydrogen peroxide). The plate was incubated for 30 min at room temperature in the dark and the colour developed was read on a Titertek Uniskan plate reader (Flow) at 405 nm. Data reduction was performed using a computer programme in BASIC written by one of us. Mean absorbance for each sample less the absorbance of the antibody blank was calculated and expressed as a percentage of the absorbance of the zero

antigen standard. The percentage absorbance of the standards was plotted against the logarithm of the antigen concentration to yield a standard curve. The equation for the regression line of best fit was calculated for the linear portion of the curve and used to determine the antigen concentration for the unknown samples.

## RESULTS

### Optimization of the assay system

To optimize the assay, we employed a protocol similar to that recommended by Rennard et al. (6). Standard curves for antigen in the range 2-1024 units/ml were generated using combinations of a range of limiting amounts of specific antibody (0.5-5.0 ug/ml) and coating antigen (15-120 ng/well). Some representative curves are plotted in Figs. 1 and 2 to demonstrate the effect of varying each of these parameters. The optimum combination was that which provided the best compromise of sensitivity, a high zero antigen absorbance, and a long, relatively steep linear segment of the standard curve. This proved to be 1.67 ug/ml of antibody and 30 ng/well of coating antigen and these values were chosen for the assay. In initial experiments, we employed horseradish peroxidase-labelled sheep anti-rabbit immunoglobulins (Dako) as the reporter enzyme-conjugated label. However, in subsequent experiments we adapted the second antibody and enzyme-antienzyme sandwich, as routinely employed in immunohistochemistry, for use as the reporter label. We found that, using empirically determined

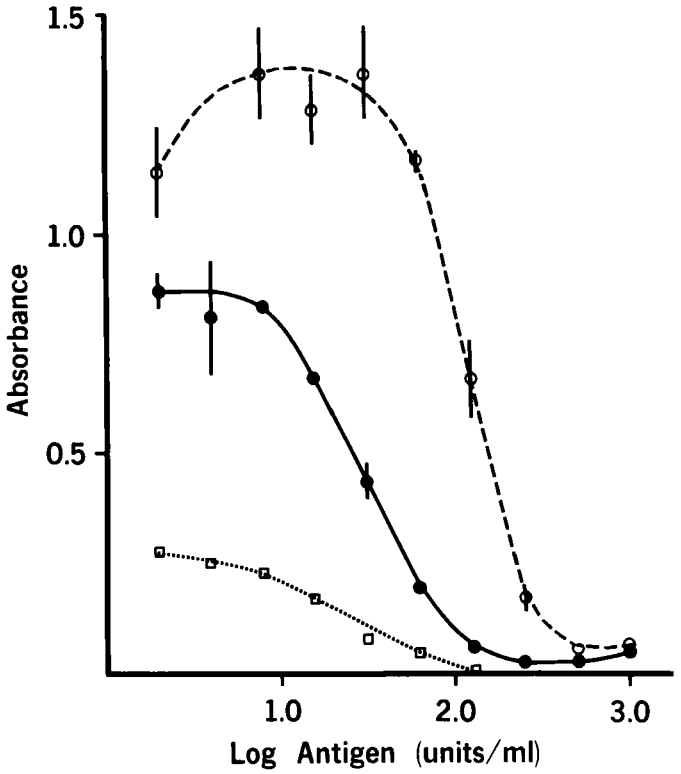


FIGURE 1. Effect of varying the limiting amount of specific antibody upon the shape and slope of the standard curve. From above downwards, the curves were generated with 5.0, 1.67 and 0.5 ug/ml of ammonium sulfate-precipitated antibody protein, respectively. Wells were coated with 60 ng of the standard antigen preparation for all curves. Error bars represent standard deviations of triplicate values and are plotted wherever these are larger than the symbol.

dilutions of 1:2000 for both the second antibody and PAP, the sensitivity of detection of bound specific antibody was increased 4 to 8-fold. This system was therefore incorporated into the optimized assay.

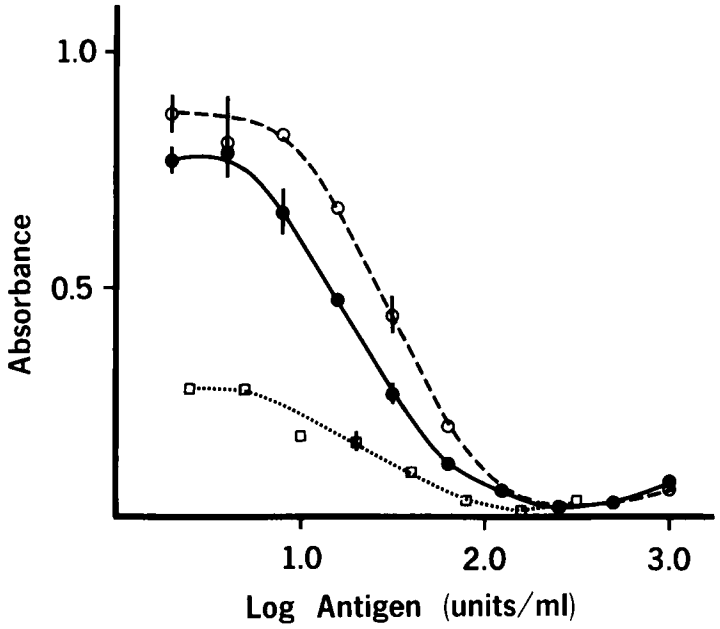


FIGURE 2. Effect of varying the amount of coating antigen upon the shape and slope of the standard curve. From above downwards, the curves were generated with 60, 30 and 15 ng/well of the standard antigen preparation. An antibody concentration of 1.67 ug/ml was used for all curves. Error bars represent standard deviations of triplicate values and are plotted wherever these are larger than the symbol.



Characteristics of the optimized assay

The optimized assay for surfactant-associated antigen proved to be extremely sensitive as well as highly specific and very reproducible. A typical standard curve is depicted in Fig. 3. The standard curve was reproducibly linear from 8-128 units/ml and the standard deviation of individual points on this portion of the curve was frequently too small to plot. A consistent artefact was the appearance of a "hook effect" in the range 2-8 units/ml: this

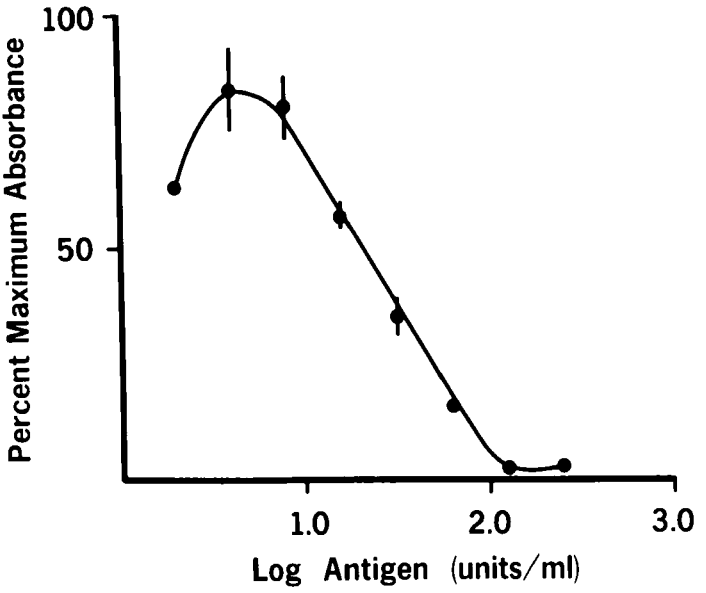


FIGURE 3. A typical optimized standard curve in which standards and unknowns were incubated with 1.67 ug/ml of specific antibody and unreacted antibody was detected using 30 ng of coating antigen per well. Each point has been plotted as a percentage of the absorbance of the zero antigen standard less the absorbance of the antibody blank. Error bars represent standard deviations of triplicate values and are plotted wherever these are larger than the symbol.



phenomenon is well recognised in enzyme immunoassay systems (7). Unknown samples having a mean absorbance greater than that of the 2 units/ml standard were deemed to be below the reliable detection range of the assay because of the occurrence of this effect. Thus in practice the lower limit of detection of antigen was in the range 8-16 units/ml. The upper limit for accurate quantitation of antigen in unknown samples was deemed to be a mean sample absorbance not less than 5% of that of the zero antigen standard, giving a useful working range of approximately 15-100 units/ml.

The specificity of the assay was examined by testing for interference or false positive results with (a) normal mouse, rat, human and fetal calf sera and (b) a mixture of soluble mouse tissue proteins, prepared by thoroughly homogenizing mouse liver, kidneys, spleen and brain and collecting the supernatant after centrifugation. At protein concentrations of 1-5 mg/ml, i.e. more than $10^5$ times the protein concentration of the standard antigen preparation that yielded a reliable positive result, none of these samples produced any false positives in the assay.

Intra- and inter-assay reproducibility was high. The coefficient of variation of replicates within a given assay rarely exceeded 5% and was often considerably less. Unknown samples tested at various dilutions on separate occasions yielded a coefficient of variation of less than 10%.

The standard antigen preparation was stable upon storage at $-70^{\circ}C$ for at least 3 months and the antibodies were stable indefinitely at $4^{\circ}C$. Antigen-coated plates stored at $-20^{\circ}C$ for 1 month gave satisfactory results in the assay; however, the slope of the standard curve was steeper when using these plates. Because coating of the plates could conveniently be performed in parallel with the incubation of standards and unknown samples with antibody, freshly coated plates were used in all the experiments.



Surfactant-associated antigen in bronchoalveolar lavage fluid

The optimized assay was a sensitive and reliable tool for quantitating the presence of surfactant-associated antigen in mouse bronchoalveolar lavage fluid. The results of

TABLE 1

Surfactant-associated antigen in mouse bronchoalveolar lavage fluid

| Animal No. | Antigen content (units/ml) |
|---|---|
| 1 | 489 ± 24 |
| 2 | 474 ± 77 |
| 3 | 543 ± 85 |
| 4 | 524 ± 91 |
| 5 | 519 ± 70 |
| 6 | 486 ± 46 |
| 7 | 445 ± 7 |
| 8 | 541 ± 98 |
| Mean ± s.d. | 503 ± 32 |

Values for each animal represent mean ± range of two determinations at different dilutions (1:15 and 1:30).



surfactant-associated antigen determinations on lavage fluid samples from 8 mice (each sample tested at dilutions of 1:15 and 1:30) are presented in Table 1. The assay yielded a relatively narrow normal range for surfactant-associated antigen in bronchoalveolar lavage fluid. Multiple determinations of the antigen content of individual samples of bronchoalveolar lavage

fluid on separate occasions yielded a coefficient of variation of less than 10%.

Pooled bronchoalveolar lavage fluid was centrifuged at 10,000 x g for 60 min to pellet surfactant lipids (8). The supernatant and pellet were recovered and the pellet resuspended in an equal volume of PBST. Assay of these samples revealed that approximately 65% of the antigen was spun down in the pellet, confirming that the antigen was associated with the surfactant lipids.

## DISCUSSION

On the basis of the studies described, we have developed a specific, sensitive and reproducible assay for surfactant-associated antigen in bronchoalveolar lavage fluid. The assay is not subject to interference by the presence of more than 1 mg/ml of serum proteins or soluble tissue proteins and exhibits a very high degree of specificity for the surfactant-associated antigen. The sensitivity of the assay has been significantly enhanced by the use of a second antibody and PAP to amplify the detection of the bound antibody: the advantages of using the second antibody-PAP system have recently been confirmed by other workers describing an immunoassay for elastin peptides (9). Our assay for surfactant-associated antigen reliably detects 15 units/ml of antigen i.e. 15 ng/ml of the standard antigen preparation, which is estimated to contain less than 10% specific antigenic protein (unpublished data).

Five other groups of workers, who have reported the preparation of specific antibodies to surfactant-associated

antigens from various species, have developed immunoassays for these antigens in bronchoalveolar lavage fluid, lung homogenate or amniotic fluid (10-14). The assay described by Sugahara et al. (10) is a Laurell rocket immunoelectrophoresis method which only detects antigen in the concentration range 5-25 mg/ml. This technique therefore necessitates considerable preparative work to concentrate and extract the sample for assay. Gikas et al. (11) have described an equilibrium competitive radioimmunoassay that requires highly purified antigen and tracer-labelled antigen. It is also relatively insensitive and detects antigen in the concentration range 0.2-20 ug/ml. The enzyme immunoassays reported by Katyal and Singh (12) and by Kuroki et al. (13) are more sensitive. Both of these are two-site sandwich assays: the former requires antibodies raised in two different species whilst the latter uses two different monoclonal antibodies. We initially expended considerable effort in attempts to develop a sandwich immunoassay based upon adsorption of specific antibody to a solid phase, binding of standards or unknowns, and detection of bound antigen by enzyme-labelled specific antibody. However, in our hands this approach yielded a less sensitive assay which had a narrow useful working range and was poorly reproducible. Our competitive assay system appears to be several-fold more sensitive than the sandwich assays reported by these workers. Finally, the enzyme immunoassay reported by Shelley et al. (14) for surfactant-associated antigens in human amniotic fluid is very similar in principle to the assay we have developed.

Unfortunately, it is not possible to assess the level of sensitivity they obtained, because results were reported relative to a pool of amniotic fluid samples obtained at term, and no absolute reference standard was employed. However, our technique is likely to be considerably more sensitive because detection of bound antibody has been significantly improved by the use of a second antibody-PAP reporter label. In addition, we have adapted the system to microplates, making smaller sample volumes possible as well as conserving reagents, and have developed a fairly simple procedure for preparation of a reference standard antigen.



The assay technique has proved to be useful for quantitating surfactant-associated antigen directly in mouse bronchoalveolar lavage fluid. We also attempted to quantitate the antigen in samples of rat and human bronchoalveolar lavage fluid. However, we were unable to detect the antigen in these samples with this assay (data not shown). The primary antibody has been shown immunohistochemically to label type 2 pneumocytes in rat and human lung tissue (4). It is possible that the failure to detect the antigen in bronchoalveolar lavage fluid might reflect species differences in the molecule bearing the antigenic determinant, which would lead to reduced antibody avidity and thus affect the performance of a competitive assay.

Our data clearly demonstrate that the antigen in mouse bronchoalveolar lavage fluid is associated with the surfactant lipids. We have shown immunohistochemically (4) that the antigen localizes to the cytoplasm of type 2 pneumocytes and the

surfactant layer lining small and medium-sized airways. Thus we infer that the antigen is a product of type 2 pneumocytes and is secreted as a part of the surfactant complex. We hypothesize that changes in type 2 pneumocyte function may be reflected in alterations in the content of surfactant-associated antigen in bronchoalveolar lavage fluid.

The assay is so sensitive that samples of bronchoalveolar lavage fluid had to be diluted 15 to 30-fold so that the absorbance values fell within the linear portion of the standard curve. This is despite the fact that bronchoalveolar lavage fluid is itself a relatively dilute sample. We believe that this is the first successful application of an immunoassay for a surfactant-associated antigen to detection directly in bronchoalveolar lavage fluid: previous studies have either required concentration of the lavage fluid and extraction of the antigen for assay (10,13) or collection of undiluted tracheal fluid (11). The much greater sensitivity of the present assay will make possible simple and direct determination of surfactant-associated antigen in samples of bronchoalveolar lavage fluid and may help to provide *in vitro* quantitative information about the responses of type 2 pneumocytes.



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