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# Immunoregulatory properties of pulmonary surfactant: effect of lung lining fluid on proliferation of human blood lymphocytes

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ABSTRACT Human pulmonary surfactant has not been shown to have immunoregulatory properties. A study was designed to examine the effect of whole surfactant (lyophilised bronchoalveolar lavage fluid) and purified surfactant lipids obtained from normal lungs of three species (man, pig, rabbit) on the lymphoproliferative responses of human peripheral blood lymphocytes to mitogens and alloantigens. Whole surfactant and purified surfactant lipids suppressed the proliferative response to phytohaemagglutinin in a dose dependent manner. The purified lipid fraction appeared to cause more suppression than whole surfactant in all the species. There was significant suppression of the proliferative response to B cell mitogens, T cell mitogens (pokeweed mitogen and phytohaemagglutinin), and alloantigens as tested by allogeneic mixed lymphocyte cultures. These results suggest an immunoregulatory role for pulmonary surfactant, most likely due to the lipid fraction.

Although the normal lung is continuously exposed to potential allergens and irritants, the mononuclear cells lining the alveolus do not normally mount any appreciable immune response. They retain, however, the capacity to recognise and phagocytose inhaled foreign material. Control mechanisms must therefore exist to protect the alveoli from potentially damaging immune reactions.[1]

The dominant cell in the fluid lining the alveolus is the alveolar macrophage, a cell that has been shown to be relatively inefficient in presenting antigen to T helper cells.[2,3] Alveolar macrophages reside in a lipid rich alveolar lining fluid containing pulmonary surfactant. Several classes of serum lipoproteins suppress lymphocyte proliferation,[4] and limited studies have shown that commercial preparations of some phospholipids do so as well.[5] In addition, canine pulmonary surfactant has immunosuppressive properties[6]; but the effect of human pulmonary surfactant on immune responses is unknown.

We considered whether surfactant might down regulate immune responses in the airspaces of normal lungs. To explore this we lyophilised bronchoalveolar lavage fluid and extracted the lipid fraction from human, rabbit, and pig lungs, and examined the in vitro effects of these preparations on the responses of human peripheral blood lymphocytes to mitogens and alloantigens.

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## Methods

### PREPARATION OF SURFACTANT

Six 8 week old pigs were exsanguinated under barbiturate anaesthesia. The trachea was isolated and intubated, and the lungs lavaged with 200 ml phosphate buffered saline (PBS). Three rabbits (aged 9, 14, and 16 months) were killed by carbon dioxide inhalation and lavaged as above with 50 ml PBS. Bronchoalveolar lavage was performed[7] in three patients (aged 30, 40, and 56 years) undergoing diagnostic fibreoptic bronchoscopy for investigation of a non-inflammatory condition (cough in two cases and minor haemoptysis in one) when the airways were macroscopically normal. None of the patients was a current smoker. Subjects gave informed consent to the study, which was approved by the Brompton Hospital ethics committee.

The lavage fluid was immediately centrifuged at 300 *g* for 10 minutes to remove the cells. The acellular supernatant was dialysed against distilled water for 48 hours, lyophilised, and stored under nitrogen at 4°C. For convenience this mixture of lung lining fluid components was called whole surfactant. For use in cell culture "whole surfactant" was reconstituted in

RPMI 1640 containing 25 mM Hepes buffer and 2 mM L-glutamine (Gibco) in a known dry weight for volume. The lipid fraction was obtained by methanol-chloroform extraction.[8] The resultant lipids were resuspended in RPMI by ultrasonication in a known dry weight for volume.

### PREPARATION OF PERIPHERAL BLOOD LYMPHOCYTES

Peripheral venous blood from healthy adult volunteers was defibrinated and diluted with an equal volume of minimum essential medium containing 25 mM Hepes buffer (Gibco). This was layered in 30 ml aliquots over 15 ml lymphocyte separation medium (Flow Laboratories) in 50 ml polypropylene tubes (Falcon), and centrifuged at 500 *g* for 30 minutes. The cell layer at the interface was aspirated and washed twice with minimum essential medium, then reconstituted at $0{\cdot}5 \times 10^6$ cells/ml in RPMI 1640 containing 25 mM Hepes buffer and 10% heat inactivated fetal calf serum, which had previously been absorbed with autologous erythrocytes at 37°C for 30 minutes to remove isoantibodies.[9]

### LYMPHOCYTE CULTURES

All cultures were performed in triplicate in 6 ml polypropylene tubes (Falcon 2063). Each culture contained $0{\cdot}5 \times 10^6$ cells in 1 ml of RPMI with 10% fetal calf serum in the presence of penicillin 100 IU/ml and streptomycin 100 μg/ml (Gibco). Tritiated thymidine (3HTdR, New England Nuclear) was added 18 hours before harvesting. At 72 hours the cell suspension was divided into aliquots of 200 μl in 96 well microtitre plates and harvested with an Ilacon harvester. When tritiated thymidine was incorporated in triplicate cultures the results varied by less than 15% from the mean value. Results were expressed as a percentage of the control value for cultures without surfactant. Viability was assessed by trypan blue exclusion. Lymphocyte responsiveness was assessed in the following ways:

(1) The effect of "whole surfactant" on lymphocyte response to the T cell mitogen phytohaemagglutinin was examined. Cultures were performed with lymphocytes from six normal non-smoking subjects in the presence of an optimal concentration (0·5 μg/ml) of phytohaemagglutinin (PHA-P, Wellcome), and including varying concentrations of pooled whole surfactant (0·05, 0·1, 0·2, 0·4 mg/ml RPMI) from three pigs, three rabbits, and three human subjects. As pooled surfactant was used to obtain sufficient standardised material for these experiments, we also set up simultaneous cultures in the presence and absence of whole pig surfactant from six separate animals using the same blood lymphocytes and at optimal phytohaemagglutinin concentration to determine the range of immunosuppression for material from different animals.

(2) The effects of a range of phytohaemagglutinin concentrations from suboptimal to supraoptimal was investigated by means of dose-response studies on blood cells from six subjects in the presence and absence of whole surfactant from pigs and rabbits (phytohaemagglutinin concentrations: 0, 0·05, 0·1, 0·25, 0·5, 1·0, 5, 10 μg/ml; surfactant concentration 0·025 mg/ml).

(3) The effects of purified surfactant lipids were studied in cell cultures from six subjects in the absence and presence of doubling concentrations of purified surfactant lipids (0·025, 0·05, 0·1, 0·2 mg/ml) from all three species at optimal phytohaemagglutinin concentration (0·5 μg/ml).

(4) The effects of purified surfactant lipids on the response to B cell and T cell mitogens and to allogeneic antigen stimulation were compared in lymphocyte cultures from nine subjects in the presence of purified pig surfactant lipids (0·05 mg/ml) and either phytohaemagglutinin at optimal concentration, pokeweed mitogen (Gibco) at 1:100 dilution, or allogeneic mixed lymphocyte culture. Mixed lymphocyte cultures were set up by adding peripheral blood lymphocytes from unrelated donors. Before being added to the test responder lymphocytes, the donor cells were resuspended at $1 \times 10^6$ cells/ml RPMI with 10% fetal calf serum and incubated in the presence of mitomycin C (Sigma, 50 μg/ml) at 37°C for 30 minutes. They were washed twice and resuspended in RPMI to a concentration of $1 \times 10^6$ cells/ml. The final cultures were performed by adding $0{\cdot}5 \times 10^6$ donor cells (stimulator cells) to $0{\cdot}5 \times 10^6$ responder cells in 1 ml of RPMI containing 10% fetal calf serum and antibiotics as above. After seven days cultures were harvested. The pokeweed mitogen cultures were also harvested after seven days.

### DETERMINATION OF THE PHOSPHOLIPID PROFILE OF THE PURIFIED SURFACTANT LIPIDS

The lavage fluid phospholipid composition of each sample from pig, rabbit, and man used in the above experiments was determined. Individual phospholipids were separated by one dimensional thin layer chromatography using the chloroform-methanol extracts and phospholipid standards.[10] The silica gel plates were charred and the proportions of each phospholipid present were determined by means of a Shimadzu Cs-920 reflectance densitometer.

### ANALYSIS OF RESULTS

The Wilcoxon matched pairs signed ranks test for pairs of related samples was used to determine significance levels.

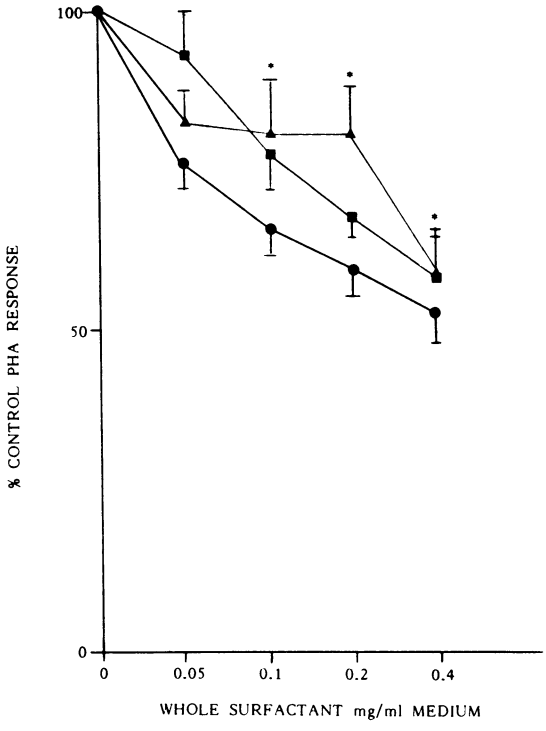


Fig 1 *Dose dependent suppression of the proliferative response of human peripheral blood lymphocytes from six individuals to phytohaemagglutinin (PHA) at optimal concentration (0·5 μg/ml), induced by whole surfactant from three species. Results are expressed as % control phytohaemagglutinin response (means with standard errors) for cultures without surfactant. ■ Human; ▲ pig; ● rabbit. *Significant difference (all species) from surfactant free control ($p \leq 0{\cdot}05$, Wilcoxon matched pairs signed ranks test).*

## Results

### EFFECT OF WHOLE SURFACTANT ON LYMPHOCYTE PROLIFERATION IN RESPONSE TO OPTIMAL PHYTOHAEMAGGLUTININ

Whole surfactant from all three species (pig, rabbit, and man) significantly suppressed the proliferative response of lymphocytes to stimulation by phytohaemagglutinin at optimal concentration in a dose dependent manner (fig 1). No loss of cell viability was observed. The degree of immunosuppression induced by whole surfactant from six individual pigs on the same test cells, at optimal phytohaemagglutinin concentration, ranged from 65% to 86% (mean 75% (SD 9%)) of the control response.

### EFFECT OF WHOLE SURFACTANT FROM PIG AND RABBIT ON LYMPHOCYTE PROLIFERATION IN RESPONSE TO A RANGE OF PHYTOHAEMAGGLUTININ CONCENTRATIONS

Whole surfactant (0·025 mg/ml) significantly suppressed lymphocyte proliferation at concentrations of phytohaemagglutinin ranging from suboptimal to supraoptimal (fig 2). No loss of cell viability was observed.

### EFFECT OF PURIFIED SURFACTANT LIPIDS ON LYMPHOCYTE PROLIFERATION

Purified surfactant lipids from all three species significantly suppressed the proliferative response to phytohaemagglutinin at optimal concentration in a dose dependent manner (fig 3). No loss of cell viability was observed.

### COMPARISON OF EFFECTS OF PURIFIED PIG SURFACTANT LIPIDS ON RESPONSE TO DIFFERENT MITOGENS AND ALLOANTIGENS

Purified surfactant lipids induced a variable but significant suppression of the proliferative responses of lymphocytes to both mitogens tested (phyto-

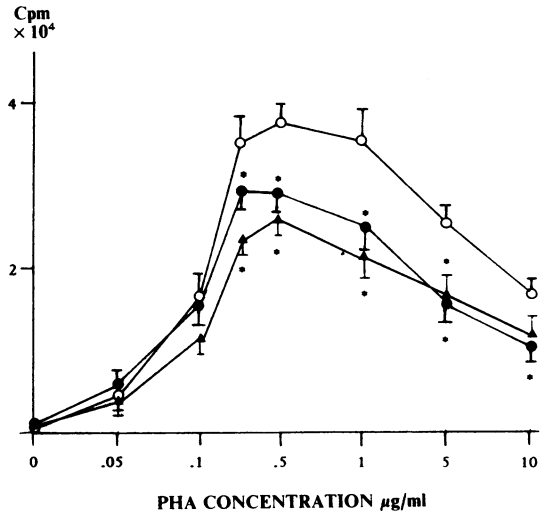


Fig 2 *Effect of whole pig and rabbit surfactant on the response of human peripheral blood lymphocytes (six subjects) to various concentrations of phytohaemagglutinin (PHA), showing significant suppression of proliferation at suboptimal, optimal, and supraoptimal concentrations of phytohaemagglutinin. Values are means and standard errors. ○ Phytohaemagglutinin response without surfactant; ▲ phytohaemagglutinin response in the presence of pig surfactant (0·025 mg/ml); ● phytohaemagglutinin response in the presence of rabbit surfactant (0·025 mg/ml). *Significant difference from surfactant free control ($p = 0{\cdot}05$, Wilcoxon matched pairs signed ranks test). Cpm—counts per minute.*

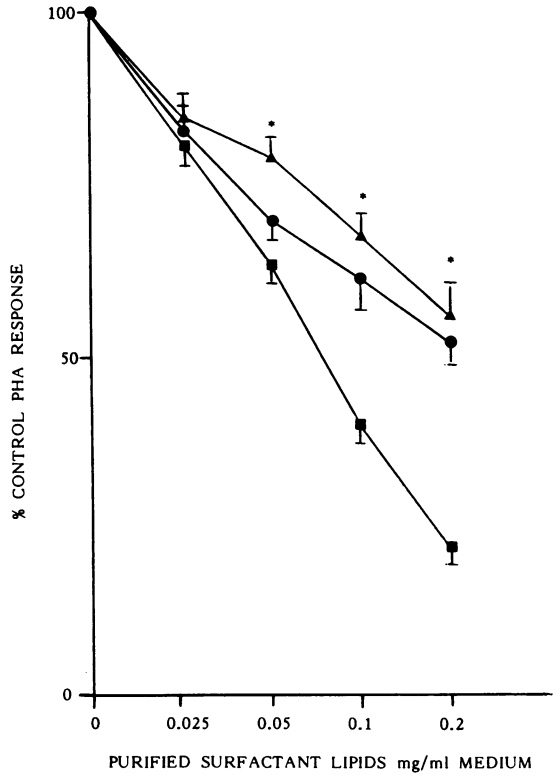


Fig 3 *Dose dependent suppression of the proliferative response of human peripheral lymphocytes from six individuals to phytohaemagglutinin (PHA) at optimal concentration (0·5 μg/ml), induced by purified surfactant lipids from three species. Results are expressed as % control phytohaemagglutinin response (means with standard errors) for cultures without surfactant lipids.* ■ *Human;* ▲ *pig;* ● *rabbit.* **Significant difference for all species compared with lipid free control (p = 0·05, Wilcoxon matched pairs signed ranks test).*

haemagglutinin and pokeweed mitogen) and to alloantigens in mixed lymphocyte cultures (fig 4).

PHOSPHOLIPID PROFILES OF SURFACTANT FROM THE THREE SPECIES

There was little difference in the relative proportions of the major phospholipid components of surfactant between individuals or between species. The dominant phospholipid present was phosphatidylcholine (man 62%, rabbit 56%, pig 57%), with lesser amounts of phosphatidylglycerol (man 18%, rabbit 15%, pig 13%) and phosphatidylinositol (man 6%, rabbit 11%, pig 5%), in proportions similar to those previously reported for normal surfactant.[11]

## Discussion

Various serum lipids have immunoregulatory properties[4] and much interest has centred on the effects of low density lipoprotein.[12 13] The role of pulmonary surfactant lipids in modulating immune responses remains relatively unexplored. Ansfield *et al* showed that canine lung surfactant suppressed lymphocyte proliferation in response to mitogens and alloantigens[6] and that various commercial preparations of phospholipids, including the dominant classes found in surfactant, had immunosuppressive properties.[5]

In this study we sought to determine whether human pulmonary surfactant (lyophilised lavage fluid and purified lipid fraction) possessed immunosuppressive properties. Whole surfactant and purified surfactant lipids from not only human but also pig and rabbit lung suppress in vitro proliferative responses to phytohaemagglutinin in a dose dependent fashion. Like us, Ansfield *et al* did not observe any cell toxicity from protein free purified surfactant lipids.[5]

It could be argued that our lyophilised preparation is not truly surfactant as lavage fluid also contains bronchial secretions. Purified lavage fluid lipids do, however, contain proportions of the dominant surfactant phospholipids similar to those found in surfactant

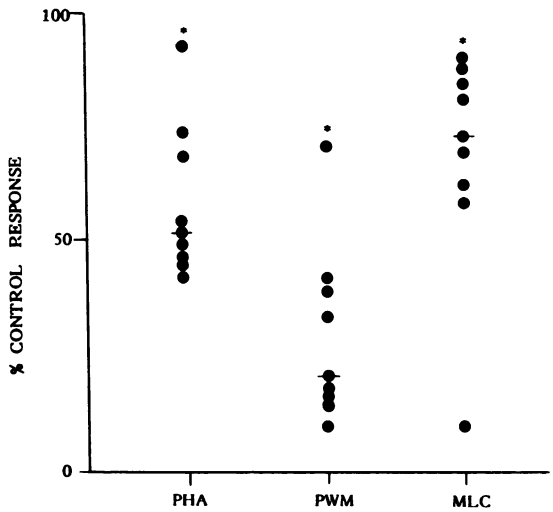


Fig 4 *Comparison of the levels of suppression of human peripheral blood lymphocyte responses (nine subjects) to different mitogens and alloantigens induced by purified pig surfactant lipids (0·5 mg/ml). Results are expressed as % control responses compared with the lipid free controls for each mitogen or alloantigen. PHA—Phytohaemagglutinin (0·5 μg/ml); PWM—pokeweed mitogen (1:100 dilution); MLC—allogeneic mixed lymphocyte cultures (0·5 × 10⁶ donor plus 0·5 × 10⁶ responder cells/ml).* **Significant difference from lipid free control (p = 0·01, Wilcoxon matched pairs signed ranks test).* — — — *Median value.*

purified by differential and density gradient centrifugation, and as secreted by in vitro cultures of type II alveolar cells.[11 14] Moreover, surfactant lipids prepared by chloroform-methanol extraction, which we have used, possess surface tension lowering properties.[15] We do not, however, infer that only the components with surface active properties are implicated in the immunosuppression induced by pulmonary surfactant.

We observed significant suppression of the proliferative response to B and T cell polyclonal activators and also to allogeneic antigen stimulation using surfactant lipids, which raises questions about the mode of immunosuppression. Preincubation of lymphocytes with serum low density lipoproteins followed by their removal before the addition of mitogen produces inhibition of proliferation, but this effect is lost if lipids are added more than 19 hours after the addition of mitogen.[16] Our own provisional results show that pulmonary surfactant has a similar effect. This suggests that surfactant lipids exert their bioregulatory effect at the inductive phase of cell triggering rather than at the stage of cell proliferation.

Shinitzky *et al* have shown that incubation of lymphocytes with in vitro prepared phosphatidylcholine-cholesterol liposomes results in strong inhibition of their mitogenic response to concanavalin A.[17] This was associated with change in cell membrane fluidity, which is determined in part by the cholesterol: phospholipid ratio. We may reasonably postulate therefore that exogenous surfactant phospholipids may alter lymphocyte membrane fluidity, with the effect of down regulating signal triggering and the subsequent synthesis of DNA.

Given that we have demonstrated a possible immunoregulatory role for pulmonary surfactant, it is interesting to compare our findings with the reports that alveolar macrophages function relatively poorly in antigen presentation.[1] Although, like blood monocytes and other antigen presenting cell types, alveolar macrophages express MHC class II antigens normally, we suggest that the lipid milieu in which they reside might interfere with their capacity to present foreign antigens effectively to T helper cells within the alveolar air spaces. If this were so, conditions in which there is an alteration in the normal surfactant composition might lead to enhanced antigen presenting capacity of alveolar macrophages. The consequences in an environment rich in inhaled antigens would be readily apparent. Perpetuation of the inflammatory response in the alveolus is a characteristic of interstitial lung disease. We propose the hypothesis that imbalance in the proportions of the immunoregulatory components of pulmonary surfactant may well contribute to a continuing alveolitis that eventually results in lung fibrosis. We have recently obtained evidence in support of this hypothesis.[18 19]

In conclusion, we have shown that human, rabbit, and pig pulmonary surfactant suppresses the lymphoproliferative responses to various stimuli, suggesting that surfactant may have an important role in down regulating immune reactions in the normal lung.

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