Surfactant changes during experimental pneumocystosis are related to Pneumocystis development

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ABSTRACT: Pneumocystosis-related surfactant changes have been reported in both humans and corticosteroid-treated experimental hosts. As corticosteroids induce an increase in pulmonary surfactant, some findings could be considered as controversial. The aim of this study was to investigate whether the surfactant composition changes during experimental pneumocystosis were related to the Pneumocystis development.

In this work two corticosteroid-untreated animal models were used: rabbits, which develop spontaneous pneumocystosis at weaning; and severe combined immunodeficiency mice, which were intranasally inoculated with *Pneumocystis carinii*. Surfactant phospholipid and protein content was explored by bronchoalveolar lavage. The *in vitro* effect of surfactant on *P. carinii* growth was also explored.

Further studies are needed to determine how *Pneumocystis* induces the reported early modifications of the surfactant, and why the parasite development is inhibited by pulmonary surfactant.


*Pneumocystis carinii* pneumonia (PCP) is a major cause of morbidity and mortality in patients suffering from acquired immunodeficiency syndrome (AIDS), receiving chemotherapy or immunosuppressive drugs for organ transplantation or other pathological conditions [1]. If immunodeficiency represents the main aetiological factor, the basic mechanism of PCP pathogenesis remains poorly understood. Current concepts suggest that the initial stages of PCP involve attachment of *P. carinii* organisms to the alveolar epithelium [2, 3]. Histological studies, using transmission electron microscopy (TEM), have demonstrated that *P. carinii* attaches preferentially to type I pneumocytes and rarely binds to type II cells [2, 4]. Consequently, *P. carinii* can interact closely with pulmonary surfactant and modify its composition. Pulmonary surfactant is a complex material containing phospholipids and specific proteins, which are involved in host defence; therefore, changes of pulmonary surfactant composition may leave the lung vulnerable to invading microorganisms [5].

Indeed, biochemical and physiological studies of PCP have suggested an important role of the surfactant system in the disease pathogenesis [6–9]. The most important aspects are: 1) in AIDS patients with PCP, the composition of pulmonary surfactant is highly modified [6, 7, 10, 11]. A decrease in the surfactant phospholipid (PL) level and an increase of total protein (P) in the bronchoalveolar lavage fluid (BALF) have been reported. Moreover, pulmonary surfactant abnormalities were present before PCP onset and development of *P. carinii* enhances these disturbances [12]; 2) other investigators have found a marked increase in surfactant protein-A (SP-A) level in humans with PCP [13, 14] or corticosteroid-treated rats [15]; and 3) intratracheal surfactant instillation in rats with PCP, improved gas exchange and PCP-related pulmonary injuries [16]. These results suggest the natural surfactant participates in the host defence against PCP by inducing a negative effect on parasite growth. However, as corticosteroids induce an increase in surfactant lipids and proteins [15, 17, 18], results obtained by using corticosteroid-treated hosts, remain controversial.

Therefore, in the present work, two animal models developing PCP without corticosteroid administration were used: the rabbit which develops spontaneous PCP at weaning [19], and the severe combined immunodeficiency (SCID) mouse nasally inoculated with *P. carinii* [20–22]. The kinetic interaction between *Pneumocystis* growth and host surfactant was investigated by BALF analysis. In addition, in a second step, the influence of synthetic (Surfexo; GlaxoWellcome, Issy-les Moulineaux, France) or seminatural (Curosuri; Chiesi, Parma, Italy) surfactants on the growth of *P. carinii in vitro* was investigated.
Materials and methods

Rabbit model of pneumocystosis

It was previously found that untreated weaning rabbits are spontaneously and heavily infected with \textit{P. carinii} [19]. Usually, they develop PCP with a favourable outcome. In this study, 77 California-New Zealand hybrid rabbits, aged 1–70 days, were used in order to determine the kinetic patterns of the parasite growth and the surfactant phospholipid/protein ratio. These rabbits were purchased from a commercial supplier (Vasseur, Prouzel, France).

SCID mouse model of pneumocystosis

Ten week old female SCID mice (Iffa-Credo, Lyon, France) were used as experimental hosts to establish the kinetic pattern of the Pneumocystis infection. Mice from the same colony were used in previous \textit{P. carinii} experiments and were found free of latent \textit{P. carinii} infection [20, 22]. SCID mice were anaesthetized with a drug cocktail (ketamine hydrochloride 2mg + diazepam 0.03 mg + atropine 0.01 mg per animal) administered i.p., and then nasally inoculated with a fresh inoculum of \(3 \times 10^{6}\) mouse-derived \textit{P. carinii} organisms per animal in 30 µL culture medium. Parasites were obtained from Pneumocystis infected outbred mice (INSERM-U42). In order to obtain viable, pure parasite samples to infect SCID mice, parasite extraction was performed as previously described [23]. Briefly, lungs of the infected mice were removed aseptically and cut into small pieces in sterile Dulbecco modified Eagle’s medium (DMEM), (FO455-Biochrom KG, Berlin, Germany). Parasites were extracted by agitation of the lung pieces with a magnetic stirrer, and the resulting homogenate was poured through gauze. The pellet was then resuspended in DMEM and the suspension was filtered successively through 250–63 µm stainless steel mesh and through 8 µm filters (Nuc-lepore, Serlabo, France). Checking of samples for other pathogens and parasite quantitation were performed on air-dried smears stained with toluidine blue O (TBO) and RAL-555 (Réactifs RAL, Paris, France) as previously described [23].

A total of 112 SCID mice were infected and used in order to determine the kinetic patterns of the parasite growth and of the surfactant protein ratio from 1–60 days post-inoculation (p.i.).

Quantitation of \textit{P. carinii} organisms

The parasite quantitation on lung homogenate samples of rabbits or SCID mice was performed using a quicker method with a good recovery [20, 24]. Briefly, animal lungs were removed and cut into small pieces in sterile Hank’s balanced salt solution (HBSS) without \(\text{Ca}^{2+}\) and \(\text{Mg}^{2+}\) at 4°C. Pieces were homogenized using a Stomacher-400 blender (Prolabo-Vitré, France) for 10 min; large particles were removed by pouring the homogenate through sterile gauze. The filtrate was centrifuged at 2,900 x g for 10 min (4°C) and the pellet resuspended in HBSS. Quantitation of all stages of \textit{P. carinii} parasite was performed as mentioned above.

BALF

Animals were anaesthetized by i.p. injection of pento-barbitral. The trachea was then cannulated and the bronchoalveolar lavage (BAL) was performed. The lungs were rinsed five times with sterile NaCl 0.9% solution (the used volume was correlated to the animal body weight, from 0.5–20 mL). Fluid recovered (about 90% of total instilled volume) was pooled on crushed ice and centrifuged at 2,900xg for 10 min at 4°C to pellet cells. The supernatant volume was recorded and aliquots were taken for protein level determination.

Biochemical studies

Biochemical study of BALF was performed as described previously [7]. Briefly, total P amounts were measured by Lowry’s method, total PLs were extracted from the supernatant with chloroform/methanol according to Bligh and Dyer method, and total lipid phosphorus was quantified according to Böttcher. Individual PL classes were separated by bidimensional thin-layer chromatography (TLC) [7].

Total PL amounts are expressed in micrograms per milliliter BAL. Total Ps are expressed in milligrams per milliliter BAL. The changes in the surfactant composition were expressed, as previously described [7, 12] as PL/P ratio. This ratio was used in order to avoid technical variations inherent in dilution secondary to BALF recovery.

Influence of surfactant on in vitro \textit{P. carinii} development

The cultures were carried out using rat-derived Pneumocystis organisms and L2 rat alveolar epithelial cells (American Tissue Cell Collection (ATCC) CCL No. 149). The cells were grown in DMEM supplemented with 10% heat-inactivated foetal calf serum (FCS) on glass coverslips placed in the wells of 24 well flat bottom plates (Costar, Cambridge, USA). When cell monolayer cultures were subconfluent, the medium was removed and 1 mL of the parasite suspension (about \(3 \times 10^{6}\) parasites) was added to each well. Plates were incubated for 48 h in an atmosphere of 5% \text{CO}_2, at 37°C. After the incubation, coverslips were washed three times with phosphate buffered saline Dulbecco (PBS/D) medium at 37°C and \textit{P. carinii} organisms were microscopically counted after methanol-Giemsastaining [23].

We have evaluated the influence of synthetic (Surfexo) or seminatural (Curosurf) surfactants on the \textit{P. carinii} in vitro growth. Various concentrations of surfactants, 100–500 µg mL\(^{-1}\) Surfexo and 50–500 µg mL\(^{-1}\) Curosurf, were added to the medium and plates were incubated for 48 h. Control assays were carried out using freshly extracted parasites cultivated in the same conditions without surfactant. Finally, the effect of tyloxapol, a nonionic detergent present in the Surfexo composition (8 µg tyloxapol per 108 µg dipalmitoyl phosphatidylcholine (DPPC), was test-ed on the parasite cultures at concentrations varying 7–60 µg mL\(^{-1}\).
Statistical analysis

Significance of the results were determined by the non-parametric Mann and Whitney test. A p-value of less than 0.05 was considered statistically significant.

Results

Spontaneous PCP of rabbits at weaning: kinetic patterns of the parasite rate and of the surfactant changes

The kinetic of the Pneumocystis parasite rate in rabbits is shown in figure 1a. During weaning (from day 21 following birth), the parasite rate increased sharply, reaching the maximum value after day 30. The number of parasites then decreased over the next 5–6 weeks.

The PL/P ratio (fig. 1a) showed a slight increase from birth to the weaning period, followed by a rapid decrease up to day 42, mainly due to protein increase (fig. 1b). Later, this ratio tended to reach the control value, as it was recorded in germ-free rabbits of the same age (PL/P= 1.15 ±0.3 p<0.005). No significant changes in the relative proportion of different PL were detected during the PCP course in rabbits.

PCP in SCID mice: changes of surfactant composition

The kinetic pattern of the Pneumocystis growth in SCID mice, intranasally inoculated with mouse-derived parasites, is shown in figure 2a. After a 6 day latency period, in which the parasites were microscopically undetectable, the slope of the growth curve was constantly rising, showing a varying but persistent growth rate (fig. 2a). The Pneumocystis growth was more important at day 21 p.i.

The PL/P ratio (fig. 2a) showed a marked increase from day 4 p.i., reaching the maximum value at day 21. Thereafter, this ratio decreased relatively slowly to become closer to the control value (PL/P=0.3±0.13). The relative proportion of different PL was not significantly modified during SCID mouse PCP course.

The effect of surfactant on in vitro Pneumocystis growth

In order to evaluate the influence of surfactant on Pneumocystis, the effect of artificial (Surfexo) or seminatural (Curosurf) surfactants on the in vitro parasite growth in short-term Pneumocystis cultures, was tested. As shown in figures 3–5, the two surfactant therapeutic preparations...

Fig. 1. – Surfactant changes in spontaneous rabbit pneumocystosis (n=4–10). a) kinetic monitoring of the surfactant phospholipid/protein (PL/P) ratio in terms of parasite rates and b) kinetic monitoring of surfactant PLs and P absolute values. Values are mean±SEM. –■–: total parasites; –▲–: PL/P; –●–: total PLs; –❏–: total Ps. BAL: bronchoalveolar lavage.

Fig. 2. – Surfactant changes in severe combined immunodeficiency mouse pneumocystosis (n=3–4). a) kinetic monitoring of the surfactant phospholipid/protein (PL/P) ratio in terms of parasite rates and b) kinetic monitoring of surfactant PL and P absolute values. This is the number of days postinoculation. Values are mean±SEM. –■–: total parasites; –▲–: PL/P; –●–: total PLs; –❏–: total Ps. *: p<0.05; **: p<0.01; ***: p<0.001.
inhibited parasite growth. A 200 µg·mL⁻¹ Surfexo concentration induced a 50% growth inhibition. The same effect was obtained with only 100 µg·mL⁻¹ Curosurf concentration. Moreover, it was verified that 7–60 µg·mL⁻¹ tyloxda-pol, was not involved in the surfactant inhibition effect on the Pneumocystis growth.

**Discussion**

The aim of this work was to clarify possible interrelationships between pulmonary surfactant and Pneumocystis growth in two experimental hosts developing PCP without corticosteroid administration: the rabbit [25] and the SCID mouse. Surfactant analysis in young rabbits is problematic because the observed changes could be related to physiological maturation as well as to Pneumocystis development. So, even if mammal surfactant maturation is usually achieved within 1–2 days following birth [26], PCP-related surfactant changes recorded in young rabbits could be considered somewhat controversial. For this reason, the PL/P ratio was also studied in a second

PCP model, the SCID mouse. As SCID mice have been intranasally inoculated with the parasite, the start date, the inoculum origin and size as well as the kinetics of this experimental infection are well known. Actually, this model has been used for many years [21] and was recently agreed by the European Concerted Action on Pneumocystis Research [27]. Of interest, in these two models, natural PCP course can be monitored before its onset and until spontaneous recovery in rabbits or in severe pulmonary involvement in SCID mice.

The present work clearly showed that surfactant changes were linked to Pneumocystis growth. Similar changes were observed in BALF from both rabbits and SCID mice.
When the parasite rate increases and PCP develops, PL/P ratio decreases mainly because of high protein levels. In SCID mice PL/P ratio remains low while the parasite increase goes on and PCP progresses (fig. 2). On the contrary, in rabbits, when parasites become undetectable by spontaneous healing of the infection, PL/P ratio increases again tending to the control value. This control value was confirmed in 100 day old rabbits (data not shown). This kinetic pattern is similar to that observed in AIDS-related PCP in humans [7, 12], where studies on BALF have found a decrease in the surfactant PL level and an increase in total P. These changes were present before PCP and enhanced with Pneumocystis proliferation.

The main finding of the present work was that the changes in pulmonary surfactant occurred also before noticeable proliferation of Pneumocystis. Thus, a significant increase in PL/P ratio, mainly due to increased PL amounts, is recorded whereas parasites are barely detectable in rabbit lungs or even microscopically undetectable in SCID mice.

The pathogenic mechanisms involved in the observed changes remain unclear. When the parasite rate is high, surfactant abnormalities probably result from transudation of plasma proteins into the alveoli. Pneumocystis attachment to the alveolar epithelium and parasite proliferation cause an increase in the alveolocapillary membrane permeability, which has been well documented [4]. Subsequently, an injury to the alveolar epithelium occurs, ultimately leading to the denudation of the basement membrane [28]. However, a low number of parasites can modify the composition of the alveolar lining fluid at an earlier stage. This finding suggests that the parasite could influence the turnover of surfactant by type II alveolar epithelial cells. This interaction between Pneumocystis and type II pneumocytes was shown in vitro [8]. Moreover, the SP-A level is markedly increased in both human [13, 14] and rat PCP [15]. Interestingly, this surfactant specific protein, which belongs to the collectin family, on the one hand interacts specifically with mannose residues of Pneumocystis major surface glycoprotein (MSG) and, on the other hand inhibits the PL secretion by type II pneumocytes and stimulates its clearance [29]. In spite of these findings, the relationship between the early Pneumocystis growth and the surfactant metabolic abnormalities remains hypothetical.

In order to determine whether pulmonary surfactant alteration was only a marker of the infection course of whether it could play a role in the development of PCP, the effect of exogenous surfactants on Pneumocystis growth was evaluated in vitro (figs. 3–5). Exogenous surfactants exhibited a significant inhibitory effect on Pneumocystis growth suggesting that pulmonary surfactant reduction could be an additive mechanism in PCP pathogenesis. Two, recent reports could support this hypothesis. One by EIDING et al. [16], who observed improvement of rat PCP after surfactant replacement, and the other by SLATER et al. [30], who observed a dramatic improvement with adjunctive surfactant therapy for PCF in an infant with acute lymphoblastic leukemia.

In conclusion, although the relationship between Pneumocystis development and pulmonary surfactant are not fully understood, some lines of evidence suggest that surfactant could play a role in the pathogenesis of Pneumocystosis. Further studies should be conducted in animal models without corticosteroids to determine how Pneumocystis induces early changes in surfactant composition, as well as why the parasite growth is inhibited by normal endogenous or exogenous surfactant.

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