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HYPOBARIC HYPOXIA-RELATED IMPAIRMENT OF PULMONARY SURFACTANT PROTEINS A AND D DID NOT FAVOUR *PNEUMOCYSTIS CARINII* FRENKEL 1999 GROWTH IN NON-IMMUNOCOMPROMISED RATS

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Summary:

It has been suggested that patients with pulmonary surfactant impairment are more susceptible to *Pneumocystis* infection than healthy controls. Owing the fact that most patients with pulmonary surfactant impairment also suffer from hypoxia, we explored the effect of intermittent hypobaric hypoxia conditions on the ability of non-immunocompromised rats infected by endotracheal route with *P. carinii* to clear the infection from their lungs. Control rats, inoculated or not with *P. carinii*, were maintained in normobaric normoxic conditions, and were submitted or not to dexamethasone administration. It was found that even if hypobaric hypoxia weakened host immune mechanisms and impaired significantly the surfactant composition, mainly of surfactant proteins A and D, these changes were not enough to favour the *Pneumocystis* growth or to inhibit the clearing of *Pneumocystis* organisms from the lungs of non-immunocompromised rats. The potential influence of surfactant protein changes on *Pneumocystis* infection is discussed.

KEY WORDS : *Pneumocystis*, pneumonia, pulmonary surfactant, rat, altitude, hypobaric hypoxia.

Résumé : DES ALTÉRATIONS DES PROTÉINES A ET D DU SURFACTANT PULMONAIRE INDUITES PAR HYPOXIE HYPOBARE N'ONT PAS FAVORISÉ LE DÉVELOPPEMENT DE *PNEUMOCYSTIS CARINII* FRENKEL 1999 CHEZ LE RAT NON-IMMUNOCOMPROMIS

Il a été suggéré que les patients avec un surfactant pulmonaire altéré sont plus susceptibles à Pneumocystis *que les individus avec un surfactant normal. Comme la plupart des patients avec des altérations du surfactant présentent une hypoxie associée, nous avons exploré l'effet de conditions d'hypoxie hypobare intermittente sur la capacité de rats non-immunocompromis infectés avec* Pneumocystis carinii *par voie endo-trachéale, à éliminer leur infection. Les rats témoins, inoculés ou pas avec* P. carinii, *ont été maintenus dans des conditions de pression atmosphérique et d'oxygénation normales et traités ou pas par la dexaméthasone. Ces expériences ont montré que même si les conditions d'hypoxie hypobare altèrent significativement la composition du surfactant, notamment le niveaux de protéines A et D, et affectent les effecteurs de la réponse immune, elles ne facilitent pas la croissance de* P. carinii *ni inhibent les mécanismes d'élimination des parasites des poumons des rats non-immunocompromis. L'influence potentielle des changements des protéines du surfactant sur l'infection par* Pneumocystis, *est discutée.*

MOTS CLÉS : Pneumocystis, *pneumonie, surfactant, rat, altitude, hypoxie hypobare.*

INTRODUCTION

The opportunistic fungus *Pneumocystis jirovecii* Frenkel 1999 (Stringer *et al*, 2002) is a common cause of respiratory infection in immunocompromised patients (Sing *et al*., 1999a and b; Kovacs *et al*., 2001). However, asymptomatic colonization or sub-clinical *P. jirovecii* infection has been reported in apparently immunocompetent subjects or in patients with slight immunodepression but with lung disorders such as chronic obstructive pulmonary disease (COPD) (Cano *et al*., 1993; Calderon *et al*., 1996; Miller *et al*., 2001). Consistently, recent experimental research has shown that *Pneumocystis* organisms are able to replicate in the lung of immunocompetent hosts (Chabé *et al*., 2004) keeping intact their pathogenic power, as attested by the fact that *Pneumocystis* cells were able to infect susceptible hosts by airborne route in which they induced *Pneumocystis* pneumonia (PcP) (Dumoulin *et al*., 2000; Gigliotti *et al*., 2003; Chabé *et al*., 2004). Thus, though immunocompetent hosts are usually able to radically eliminate the parasites from their lungs (Sepkowitz *et al*., 1993), as long as they remain infected they are able to transmit the infection either to naive healthy hosts, which will develop a primary infection, or to immunosuppressed hosts, which may then develop PcP. In this manner, healthy hosts could behave

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as a dynamic reservoir to *Pneumocystis* species (Dei-Cas, 2000; Chabé *et al*., 2004).

Interestingly, oxygen is a regulator of pulmonary surfactant protein gene expression, especially in the lungs of animals exposed to hyperoxia (Nogee *et al*., 1991). Little information is available however on the effect of hypoxic conditions on either the pulmonary surfactant composition (Nardo *et al*., 2005) or the *Pneumocystis* development in the pulmonary alveolus. Synthesized by type II pneumocytes, pulmonary surfactant is a complex of protein (10 %) and lipid (90 %) molecules, which accumulates at the air/water interface and lowers the surface tension to prevent alveolar collapse (Dobbs *et al*., 1982; Robertson *et al*., 1992). Four specific surfactant-associated proteins (SPs) have been identified: hydrophilic proteins, SP-A and SP-D; and hydrophobic proteins, SP-B and SP-C (Possmayer, 1988; Ingenito *et al*., 1999; Haagsman & Diemel, 2001).

It is difficult to define the specific role of each surfactant protein because their respective effects tend to overlap one another. Nevertheless, it is commonly admitted that both SP-B and SP-C greatly increase the adsorption of surfactant lipids onto the surface film that lines the alveolus (Perez-Gil & Keough, 1998; Whitsett & Weaver, 2002). SP-C is the smallest (4.2-8 kDa) and the most hydrophobic surfactant protein (with 70 % of hydrophobic amino acids), and is only found in the lungs (Glasser *et al*., 2001). SP-B is an 8.7-kDa-protein component of tubular myelin, and enhances adsorption and re-spreading (Whitsett *et al*., 1995; Oviedo *et al*., 2001). SP-B is required for normal lung function (Melton *et al*., 2003; Nesslein *et al*., 2005).

SP-A and SP-D belong to the collectin family and they play a role in the innate immunity of the lung via their ability to bind various pathogens (Crouch & Wright, 2001; Wright, 2005). SP-A was the first surfactant protein to be identified, and is also the most abundant (Ingenito *et al*., 1999). Its molecular mass varies from 26 to 38 kDa. This protein also facilitates surface-active properties of the surfactant in the alveolus and regulates *in vitro* surfactant phospholipids synthesis (Kubchandani *et al*., 2001; Palaniyar *et al*., 2001). SP-D is a 43 kDa protein that has sequence homology to SP-A (Crouch, 2000); it is also involved in immune functions and participates to surfactant homeostasis (Crouch & Wright, 2001).

There are contrasting reports on the effect of hypobaric hypoxia on immune functions. Early work showed that exposure to hypoxia resulted in increased immunoglobulin levels with concomitant resistance to viral infection (Trapani, 1966). Chohan *et al.* (1975) reported increased serum IgG and IgA levels both in high-altitude natives and in sea-level natives inducted to high altitudes compared to sea-level residents. In contrast, Mirrakhimov & Kitaev (1979) and Kitaev & Tokhtabayev (1981) reported increased infant mortality due to respiratory infections among high-altitude natives. Meehan (1987) has shown that hypoxic exposure makes experimental animals more susceptible to bacterial infections. The latter observations suggest that the host defence mechanisms might be impaired under hypobaric hypoxia conditions. Nevertheless, cellular effectors of the immunity are particularly sensitive to oxidant stress because of the high content of polyunsaturated fatty acids in their plasma membranes (Coquette *et al*., 1986; Meydani *et al*., 1995). Moreover, these cells produce relatively high amounts of reactive oxygen intermediates as normal part of their specific defensive role. SaiRam *et al.* (1998) showed that hypoxia enhanced macrophage free radical production and induced an oxidative stress (decreased blood level of reduced glutathione and increased plasma malondialdehyde levels), leading to decreased T-cell activity.

In a previous study (Prévost *et al*., 1980), we demonstrated changes in surfactant phospholipids of rats exposed to hypobaric hypoxia. Furthermore, we have observed that in HIV infected patients, pulmonary surfactant abnormalities were present before PcP onset (Escamilla *et al.*, 1992, 1993; Prevost *et al.*, 1998). Likewise, in healthy young rabbits that develop currently benign PcP at weaning (Rajagopalan *et al.*, 1998; Dei-Cas *et al.*, 2006) marked surfactant changes occur before the development of PcP (Aliouat *et al*., 1998). We therefore hypothesized that surfactant modifications, especially of protein content, could promote *Pneumocystis* proliferation in the alveolar space of non-immunosuppressed hosts. The purpose of the present study was therefore to evaluate the influence of intermittent hypobaric hypoxia induced changes of pulmonary surfactant composition on the ability of *P. carinii* Frenkel 1999 to grow in the lungs of non-immunocompromised laboratory rats.

MATERIALS AND METHODS

Animal model, altitude simulator chamber and experimental design

Weaned male Wistar rats (Janvier, Le Genest St Isle, France) weighing 87.4 ± 4.6 g, were randomly divided in six groups, each one submitted to a specific condition. Group-1 rats (six rats) received dexamethasone in drinking water (2 g/L) three weeks before *P. carinii* inoculation and for the entire duration of the experiment. This group was used as control of the infectious power of the used *P. carinii* isolate. Rats of group 2 (six rats) were placed for four hours in an altitude simulator chamber designed by one of us (M.C.P, INSERM U563). In this system, atmospheric pressure can be decreased to 370 mm Hg

(corresponding to 6,000-m altitude) or increased to 760 mm Hg (sea level atmospheric pressure) in 20 min. There is not alteration of relative proportions of diverse gases that compose the atmospheric air. It results therefore a decrease of partial O_2 pressure from about 160 mm Hg at the sea level to about 78 mm Hg inside the chamber. The airflow rate inside the simulator was 420 L/h, corresponding to three renewals per hour of simulator atmosphere. The decrease of air pressure as well as the return to normobaric normoxic condition occurred at a rate of 17 mm Hg/min. After the 4-hour exposure, when pressure reached 760 mm Hg, group-2 rats were euthanatized. They were used as a control of the effect of hypobaric system on pulmonary surfactant in absence of *P. carinii*.

Rats of groups 3, 4 and 5 (six rats per group) were inoculated with *P. carinii* by intratracheal route (see below) and placed in the altitude simulator chamber for 4-hours/day sequential exposures five days/week for one, two or three weeks, respectively, and then euthanatized. Group-6 rats (six animals) were also inoculated with *P. carinii* but they were not exposed to hypobaric hypoxia (control group).

Source of *P. carinii* and intratracheal inoculation

The rats of all groups, except of group 2, were infected by non-surgical endotracheal route with *P. carinii* organisms (Ambrose *et al*., 2004) obtained from corticosteroid-treated Wistar rats (Dei-Cas *et al*., 1998). Parasite extraction from tissue lung and purification were performed as previously (Aliouat *et al*., 1993). These methods revealed efficient in obtaining viable and infectious *P. carinii* organisms (Anonyme, 1996; Durand-Joly *et al*., 2002). Uninfected control rats of group 2 were handled before *P. carinii* infected ones in order to avoid accidental airborne contamination, even in the altitude-simulating chamber, and were housed in a different room. Food and water were given *ad libitum*. Body weight and food uptake were recorded one and three times per week, respectively. The experimental protocol was approved by the local INSERM ethical scientific committee, according to the ethical principles laid down by the French Ministry of Agriculture. MCP holds the personal licence number 31-64 ("Certificat d'Autorisation d'expérimenter sur Animaux – Décret No 87-848 du 19 octobre 1987") establishing the authorisation to develop animal experimentations.

Assessing respiratory function

Breath frequency was measured in wide-awake unrestrained rats before and after hypobaric-hypoxia exposure using a plethysmograph, Datanalyst° and Iox° software's for *in vivo* data acquisition and real time analysis (AC 264, Emka Technologies, Paris, France). Arterial blood was taken on anesthetized rats (45 mg/kg Ketamin hydrochloride – Rhone Mérieux, Lyon, France) two days before hypobaric hypoxia exposure and immediately after. Femoral arterial blood sample was taken as soon as rats were returned to normobaric normoxic conditions. Blood gas analysis (PaO2, PaCO2, Hb-saturation), blood pH and acid-base balance assessing were performed within 10 min following the experiment on ABL-500 apparatus with specific electrodes (Radiometer, France).

Assessing the immunological function

At the time of euthanasia, two samples of heparinized blood were obtained from rat abdominal venous and used to assess cellular content and to isolate lymphocytes for flow cytometric analysis. Samples were treated with ACK buffer (Barten *et al*., 2001) (10 min at room temperature) to eliminate red blood cells, and incubated with anti-TCRaß-FITC (TCR = T cell receptor) (1/100), anti-CD4-biotine/streptavidine (1/400), or anti-B-lymphocyte-PE 1/200) antibody to label cells by immunofluorescence as previously described (Barten *et al*., 2001).

The influence of hypobaric hypoxia conditions on the susceptibility of peripheral lymphocytes to oxidative stress was evaluated by using the kit "Radicaux Libres" (KRL test, "Spiral" laboratories, Couternon, France), which was developed by Purpan-Hospital Biochemical Department (Toulouse, France) and previously applied to erythrocytes (Stocker *et al*., 2003; Lesgards *et al*., 2005). Briefly, the test assesses the capacity of cells to resist to lysis after a challenge with standardized oxidative radical source. Leukocytes were separated by centrifugation (20 min, 400 g) over lymphocyte separation medium (Eurobio, Les Ulis, France) from heparinized venous blood of rats diluted 1:2 in saline. Contaminating red blood cells were lysed with ammonium chloride lytic buffer. After washing, leukocytes were counted on Coulter T540 (Beckman/coulter), distributed in 96-well microplates and exposed, at 37° C, to the standardized release of free radicals resulting from the thermal decomposition of a 27-mmol/L 2,2'-azo-bis (2-amidinopropane) dihydrochloride solution (Stocker *et al*., 2003; Lesgards *et al*., 2005). Cell lysis rate was then assessed by measuring the optical density decay at 620 nm. Half slope mean is the time (minutes) when 50 % lysis occurs. Several experiments were made exposing each time six *P. carinii*-infected rats to hypobaric hypoxic conditions.

Collection of lung tissue samples

Lung tissue samples were divided in three parts. One part was used for *P. carin*ii detection and quantification (see below). A second part was immediately snap-frozen (in liquid nitrogen) and stored in deep freezer (– 80° C) until RNA extraction. The last part was used for protein quantification and analysis.

P. CARINII DETECTION AND QUANTIFICATION

Pneumocystis detection and quantification in lung tissue was performed on air-dried lung homogenate smears stained with RAL 555 (RAL, France). The total number of cystic forms (intermediate or late sporocytes and cysts) was calculated as previously described (Aliouat *et al*., 1993; Anonyme, 1996; Durand-Joly *et al*., 2002).

ISOLATION OF MRNA AND NORTHERN BLOT ANALYSIS

Total mRNA was isolated from 100-mg frozen lung tissue samples with Trizol method according to the manufacturer's instructions (Invitrogen, France). Precipitated mRNA was dissolved in sterile H_2O, quantified by absorbance at 260 nm and stored at – 80° C until analysis. Ten µg of mRNA from each sample was separated on formaldehyde gel, and capillary transferred on a nitrocellulose membrane.

SP-A, SP-B, SP-C and SP-D complementary DNA probes were made by transcription-polymerase chain reaction (RT-PCR) from RNA extracted from adult rat lung. The specific primers used were as follows: (a) for SP-A (557 bp), 299-CCT GGA GGA CGT GGA GAC AAG-320 and 856-TGC AGG GTT TTA CAG ACC AGA TG-833; (b) for SP-B (462 bp), 280-AAG TTC CTG GAA CAA GAA TGT GA-303 and 742-CCC AGC AGT GCA TCT AGT AGA AG-719; (c) for SP-C (377 bp), 178-ATG GGC CTT CAC ATG AGT CAG-199 and 555-TCT CCA CAC AAG GTG CTC ACA-534; for SP-D (498 bp), 639-ACA GAG GTG CTC CTG GAG ACA-660 and 1137-CTA GGG GCT CAG AAC TCA CAG A-1115.

The "AlkPhos Direct Labelling and Detection System" from Amersham Biosciences involves direct labelling of cDNA probe with a thermo-stable alkaline phosphatase enzyme by a covalently cross-linker, according to manufacturer instructions. For detection step, we used CDP-Star chemiluminescent reagent (Amersham Biosciences, France). A scanner and Image Quant software (Amersham Biosciences, France) were used to quantify mRNA band. Hybridization was also performed on the 18S rRNA to assess potential loading error on the reactive mRNA band.

PROTEIN EXTRACTION AND WESTERN BLOT ANALYSIS OF SURFACTANT PROTEINS

Rapidly, frozen lung tissue samples were thawed in ice cold RIPA buffer containing PMSF, Aprotin, Leuptin, then disrupted with an ultra-thurax and homogenized with a Dounce homogenizer at 4° C. Extracted proteins were quantified by Bradford micro dosage (Bradford, 1976). 50 µg proteins were separated on acrylamide gels. The reticulation of gels was function of protein molecular weight: 12 % for SP-A and SP-D and 16 % for SP-B and SP-C. We carried out electrophoresis in glycine buffer for SP-A and SP-D, and in tricine buffer for SP-B and SP-C (Biorad, France). Then we proceed to transfer (nitrocellulose for SP-A, SP-D and PVDF for SP-B, SP-C) in semi-dry electro blotting apparatus.

Proteins were detected after blotting with antibodies directed against SP-A and SP-D (Santa Cruz Biotechnology, France) and against pro-proteins SP-B and SP-C (Chemicon International, France, and Santa Cruz biotechnology France). A goat (Jackson Immuno Research laboratories inc., France) or rabbit (Promega, France) horseradish peroxidase (HRP) conjugated was used as secondary antibody. For detection, we used Chemiluminescence Luminol Reagent according to supplier protocol (Amersham Biosciences, France). Each blotting membrane was normalized by analysis with a monoclonal anti-β-actin antibody (Sigma, France). A scanner and Image Quant software (Amersham Biosciences, France) were used to assess the density of protein bands in relation to actin band density (Fig. 6). Uninfected non-immunocompromised rats maintained in normobaric normoxic conditions were used as source of control or normal surfactant samples.

STATISTICAL ANALYSIS

When appropriate, data were presented as mean±SEM. In statistical comparisons t Student test was used and $p < 0.05$ was considered as the limit of statistical significance.

RESULTS

CHARACTERIZING THE EXPERIMENTAL MODEL

Rat weight decreased significantly by 9.9 % ($p < 0.017$) after one-week, by 13 % ($p < 0.0027$) after two weeks, and by 12 % ($p < 0.016$) after three weeks of intermittent hypobaric hypoxia (Fig. 1). Under our experimental conditions, rats exposed to hypobaric hypoxia ate 13 % to 27 % less than controls. Weight decrease could also result from an increased elimination of body water secondary to both changes in vapour pressure inside the chamber and enhanced urine flow (Guiol *et al*., 1986).

Rats in the simulator chamber had impaired ventilation with a significant increase in respiratory rate (Table I). Analysis of arterial blood gases showed severe hypoxemia with a mean PaO_2 of 32.4 mm Hg after a 4-hour exposure. The high $PaCO_2$ values in control – as in experimental rats (Table I) could be related to the fact that animals were anaesthetized at the time of blood collection. The increase in haemoglobin level could be due rather to hemo-concentration than to hypoxemia. Actually, micro-haematocrit was found to be increased in animals exposed to hypobaric hypoxia conditions (data not shown).

Data	Control (n = 6)	4-h exposure to hypobaric hypoxia (n = 6)	1-week exposure to hypobaric hypoxia (n = 6)
Breath/min[b]	111.5 ± 6.2	144.2 ± 4.9*	146.2 ± 17.8*
pH	7.35 ± 0.1	7.35 ± 0.004	7.33 ± 0.1
PaO_2 (mm Hg)[c]	99.2 ± 6.8	32.4 ± 0.7*	64.8 ± 19.6
$PaCO_2$ (mm Hg)	46.9 ± 5.1	46.6 ± 1.05	43.7 ± 2.5
SaO_2 (%)	93.8 ± 1.6	38.0 ± 7.5*	76.4 ± 20.0
Hb (g/dL)	13.3 ± 1.0	15.5 ± 0.5	15.3 ± 0.2
HCO_3^- (mM/L)	25.5 ± 1.5	24.9 ± 0.4	22.4 ± 1.8
P. carinii cysts ($\times 10^6$)[d]	0.20 ± 0.1[d]	ND	0.30 ± 0.2

[a] Results are shown as mean ± SEM of six different animals. Measures were performed in triplicate separated by intervals of two min. The mean of three measures was considered as the basal value for one given animal.
[b] Breath frequency was measured in wide-awake unrestrained rats.
[c] Arterial blood was taken on anesthetized rats (see "Material and methods" section).
[d] *Pneumocystis* cyst counts per animal performed three weeks p.i.. Negative counts (microscopically undetectable parasites) were not included. Animals of this control group were inoculated with *P. carinii* and housed under normobaric normoxic conditions.
Hb = hemoglobin; PaO_2 and $PaCO_2$ = Partial O_2 and CO_2 pressures, respectively; SaO_2 = O_2-hemoglobin saturation; HCO_3^- = bicarbonate level in blood; ND = not done.
* Significant difference.

Table I. – Non-immunocompromised Wistar rats: hypobaric hypoxia impact on respiratory function and on the number of *Pneumocystis carinii* organisms in lungs[a].

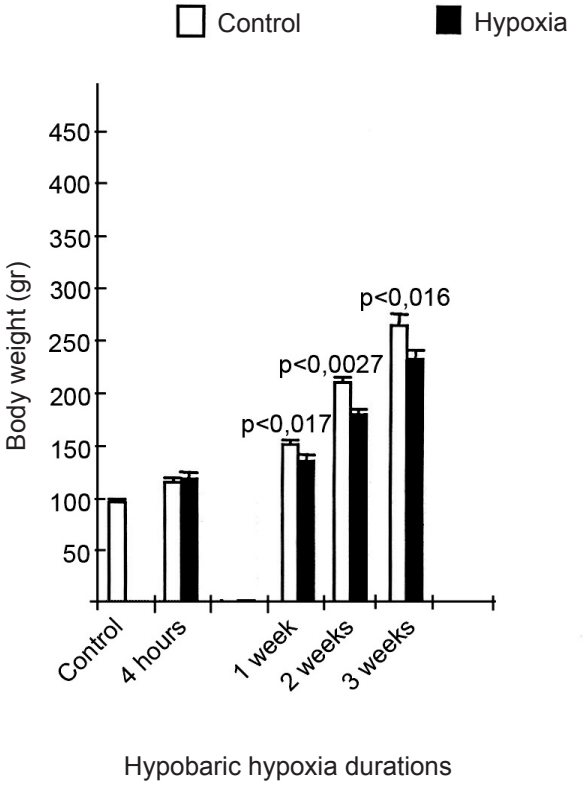

Fig. 1. – Influence of intermittent exposure to hypobaric-hypoxia on body weight (mean ± SEM). Wistar rats were placed in an altitude simulator chamber (see "Materials and Methods") for 4-hour-day sequential exposures, five days a week, for one to three weeks. Barometric pressure and partial O_2 pressure inside the chamber were about 370 mm Hg and 78 mm Hg, respectively. Animals were administered food and water *ad libitum* and were weighted once a week. Hypoxia exposure entailed a significant weight decrease.

INFLUENCE OF HYPOBARIC HYPOXIA ON *P. CARINII* INFECTION

P. carinii growth was assessed at the same intervals in rats exposed to hypobaric hypoxia and in dexamethasone-treated rats housed under normobaric normoxic conditions. As expected, dexamethasone-treated rats developed PcP with parasite rates of 8.8 ± 1.6 10^5 *P. carinii* cysts per animal one-week post-inoculation (p.i.) and 2.2 ± 0.7 10^6 cysts per animal three weeks p.i. (Fig. 2).
Three weeks p.i., neither group-6 control rats nor rats exposed to hypobaric hypoxia conditions had cleared all their parasites from their lungs. At the end of the experiment parasite counts ranged from microscopically undetectable rates to 10^4 cystic forms per animal in either hypobaric-hypoxia exposed (6 ± 3 10^4) or unexposed rats (9 ± 1 10^4). In our work conditions, hypobaric hypoxia condition did not influence therefore significantly the ability of presumably immunocompetent rats to eliminate *Pneumocystis* organisms from their lungs.

EFFECT OF HYPOBARIC HYPOXIA ON RAT PERIPHERAL BLOOD LYMPHOCYTES

Data from rats exposed to hypobaric hypoxia conditions (groups 3-5) were compared with both dexamethasone-treated rats (group 1) and unexposed control animals (group 6) just before *Pneumocystis* inoculation (Fig. 3). In hypoxemic rats, the number of total lymphocytes was unchanged (Fig. 3A). Cell distribution analysis evaluated as percentage of total lymphocytes showed a significant decrease in B lymphocytes (p =

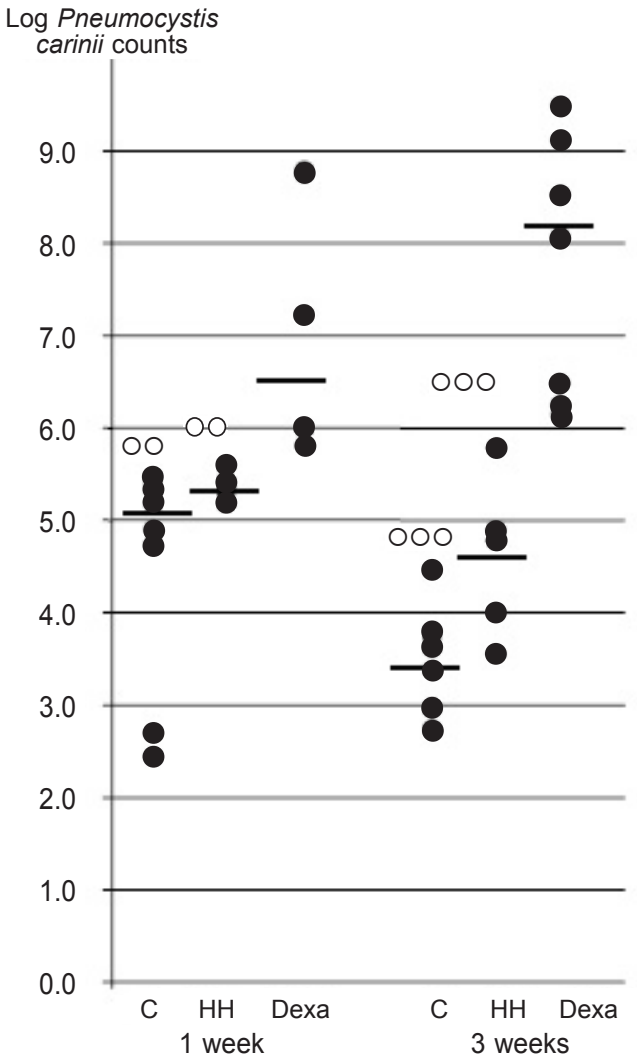

Fig. 2. – Influence of intermittent exposure to hypobaric-hypoxia on *Pneumocystis carinii* rates. Rats were placed in an altitude simulator chamber as indicated in figure 1 caption. The animals were exposed to hypobaric-hypoxia for one week or for three weeks. Each solid circle corresponds to one animal. Horizontal traits represent the average rates. C: control rats endotracheally inoculated with infectious *P. carinii* organisms and maintained in normobaric normoxic conditions; HH: rats endotracheally inoculated with infectious *P. carinii* organisms and exposed to hypobaric-hypoxia conditions; Dexa: dexamethasone-treated rats (see text) endotracheally inoculated with infectious *P. carinii* organisms and maintained in normobaric normoxic conditions. Open circles indicate statistically significant differences: °: $p < 0.05$; °°: $p < 0.005$; °°°: $p < 0.0001$.

0.001, Fig. 2B) and an increase of $CD4^+$ T lymphocytes (Fig. 3C and D).

KRL test was used to evaluate the susceptibility to oxidative stress of lymphocytes from *P. carinii*-infected rats exposed to hypobaric hypoxia compared to those of control rats of group 6 (*P. carinii*-infected and maintained under normobaric normoxic conditions) (Fig. 4). Half slope mean (n = 6) was 53.5 ± 2.0 min for lymphocytes of hypobaric hypoxia exposed rats, and 75.4 ± 5.1 min for lymphocytes of control rats (n = 6), respectively. Peripheral lymphocyte resistance to oxidative cytolysis was thus lowered significantly (17 %) in rats exposed to hypobaric hypoxia.

Effect of hypobaric hypoxia on pulmonary surfactant protein composition

SP-C and SP-D mRNAs were not sensitive to hypobaric hypoxia. In contrast, a 33 %-decrease in SP-B mRNA was recorded after a 4-h-exposure to hypoxia (Fig. 5). However, after 2-3-weeks intermittent exposure to hypoxia, SP-B mRNA tended to return to the level recorded in group-6 control rats. On the contrary, SP-A mRNA decreased regularly and significantly (even compared to 4-h-hypoxic rats) all along the intermittent exposure to hypoxia. Lowest SP-A mRNA amounts were recorded after a two-week exposure to hypobaric hypoxia (Fig. 5).

In order to determine whether decreased amounts of surfactant protein mRNAs were associated with actual protein amount changes, a Western blot analysis of the four surfactant proteins was performed (Fig. 6). Band-densitometry analysis indicated that both hydrophobic protein precursors decreased after 4-h-exposure to hypoxia (55 % for SP-B and 70 % for SP-C). After one to three weeks of 4-h intermittent hypoxia, all the surfactant proteins decreased of almost 45 % (Fig. 6). SP-C protein increased significantly compared to its level after a 4-h hypoxia exposure (Fig. 6).

Intratracheal inoculation of *P. carinii* organisms did not influence surfactant-protein amounts in corticosteroid-untreated rats exposed or not to hypobaric hypoxia (Fig. 7). As expected, corticosteroid administration enhanced the synthesis of pulmonary surfactant components. Consistently, SP-A and SP-B were found to be significantly increased (Fisher *et al.*, 1991) (Fig. 7).

DISCUSSION

The used altitude simulator chamber works in diminishing the barometric pressure from 760 mm Hg (normobaric normoxic conditions) to 370 mm Hg. As in these experiments relative proportions of O_2, CO_2 and N_2 were not changed, it can be deduced that O_2 partial pressure inside the chamber fallen from about 160 mm Hg (at sea level) to about 78 mm Hg. This change has evident physiological impact as biochemical and physiological action of a given gas is depending on the pressure it is exerting.

Several studies showed that alveolar epithelial cells tolerate hypoxia remarkably well and are able to maintain oxidative phosphorylation down to very low oxygen concentrations (Jain & Sznajder, 2005). Little is known however about the consequences of hypoxia on pulmonary surfactant.

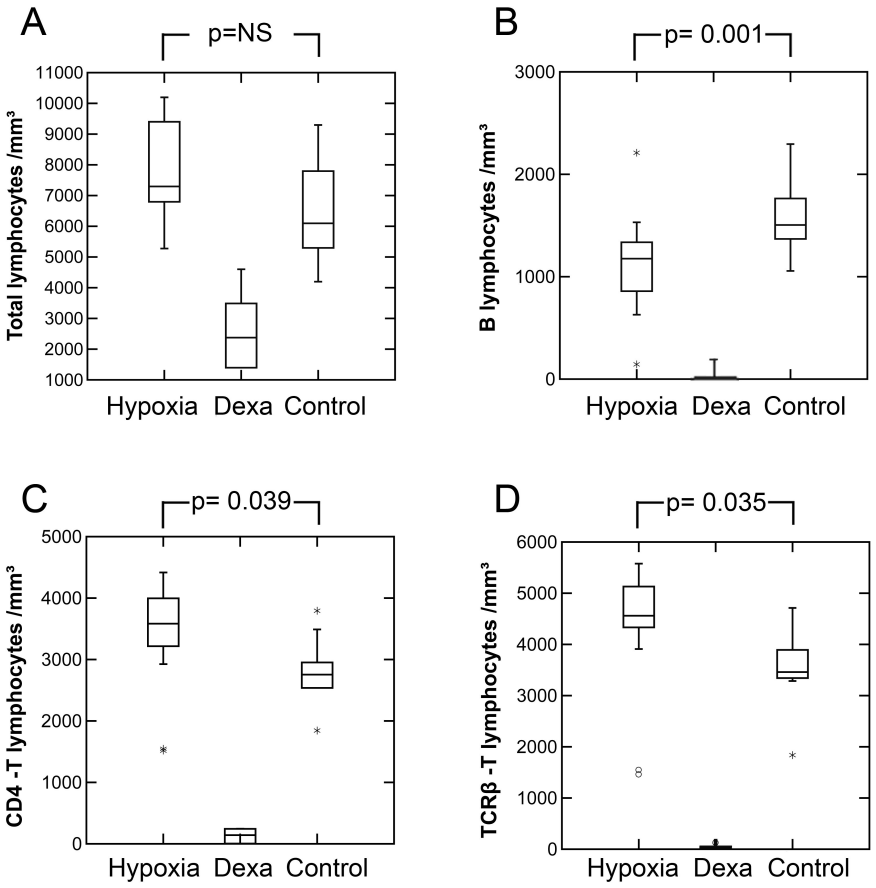

Fig. 3. – Influence of intermittent exposure to hypobaric-hypoxia on peripheral blood lymphocytes assessed by flow cytometry (mean ± SEM). *Pneumocystis-carinii* endotracheally inoculated Wistar rats placed in an altitude simulator chamber as indicated in figure 1 caption. Three groups were compared: animals exposed for three weeks to intermittent hypobaric hypoxia ("Hypoxia"), and dexamethasone-treated ("Dexa") or untreated rats ("Control") maintained in normobaric normoxic conditions. Dexamethasone administration induced both marked decrease of total, B- or T lymphocytes (A-D). In rats of dexamethasone-untreated groups, total lymphocyte counts were apparently not influenced by hypobaric hypoxia (A). In contrast, a significant decrease in B lymphocytes (B) and a small increase of $CD4^+$ T lymphocytes (C and D) were recorded in rats exposed to hypobaric hypoxia.

In previous studies it was reported that hypoxia decreased surfactant protein in preterm infants and in ovine foetuses (Nardo *et al.*, 2005; Gortner *et al.*, 2005). Our study is the first, however, that reports a decrease of all surfactant proteins in adult rats exposed to intermittent hypobaric hypoxia. Thus, in our work conditions, a decrease of the two surfactant hydrophobic proteins was recorded. SP-B-decrease was about 55 % after 4-hour exposure, and 50 % after intermittent long-lasting hypoxia (Fig. 6). The mRNA data suggest that SP-B decreasing could be controlled at a post-transcriptional level. Hydrophobic proteins are critical to life. Especially, decreasing of SP-B has likely a marked impact on the respiratory function (Tokieda *et al.*, 1999; Walther *et al.*, 2002). Interestingly, in the mouse, *Pneumocystis* growth induced a decrease of hydrophobic SP-B and SP-C. This effect could increase surface tension and contribute thus to hypoxemia (Beers *et al.*, 1999; Atochina *et al.*, 2000). With regard to SP-C, the lowest concentration was observed after a 4-h-hypoxia exposure (Fig. 6). In rats exposed to longer hypoxia periods, SP-C levels tended to come near the control level, this trend resulting likely from an adaptive physiological response. As SP-C mRNA level remained stable, SP-C decrease and subsequent increase during hypoxia could result from processes occurring at a post-transcriptional level. SP-C was shown to participate to innate immunity as it can bind lipid A moiety of LPS in a specific and competitive fashion (Augusto *et al.*, 2001, 2002). This interaction prevents the acti-

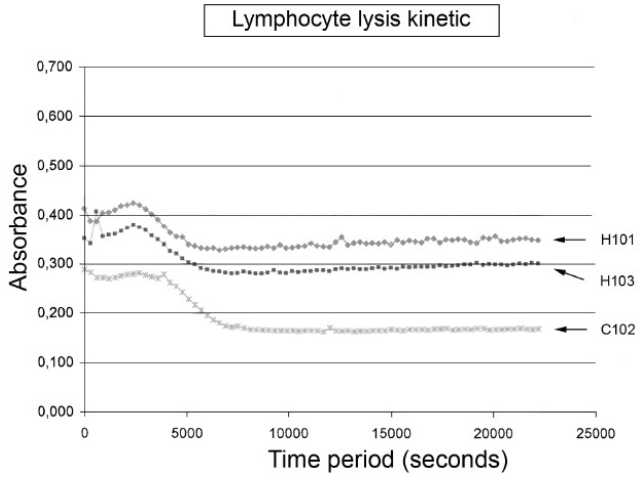

Fig. 4. – Influence of hypobaric-hypoxic conditions (animals placed in an altitude simulator chamber as explained in figure 1 caption) on the susceptibility of rat peripheral blood lymphocytes to oxidative stress (KRL test, see "Materials and Methods"). Control or experimental Wistar rats were endotracheally inoculated with *P. carinii* organisms (see text). Lymphocyte cell lysis was assessed by measuring absorbance. Results of a representative experiment. Lymphocytes of two rats (H101, H103) exposed for four hours to hypobaric hypoxia, and of one control rat (C102) maintained in normobaric normoxic conditions.

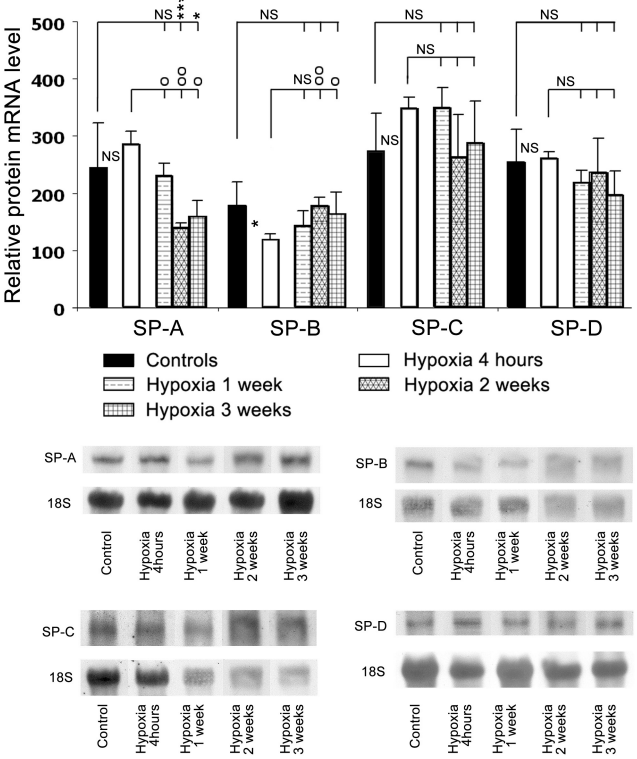

Fig. 5. – Influence of intermittent exposure to hypobaric-hypoxia in an altitude simulator chamber (as indicated in figure 1 caption) on the expression of pulmonary surfactant protein mRNA. Control or experimental rats were endotracheally inoculated with *P. carinii* organisms (see text). Northern hybridization with complementary DNA probes followed by mRNA band quantification (see "Materials and Methods") showed the relative levels of SP-A, SP-B, SP-C and SP-D mRNAs in *P. carinii* endotracheally inoculated rats exposed or unexposed (controls) to intermittent hypobaric hypoxia. The sizes of the transcript were as follows: 1.6 kb (SP-A), 1.5 kb (SP-B), 0.8 kb (SP-C) and 1.3 kb (SP-D), respectively. Significant differences compared respectively to controls (*) or to 4-hour hypoxia rats (°) are shown. * or ° if $p < 0.05$, by ** or °° if $p < 0.005$ and by *** or °°° if $p < 0.0001$ (Student *t* test); NS = no significant.

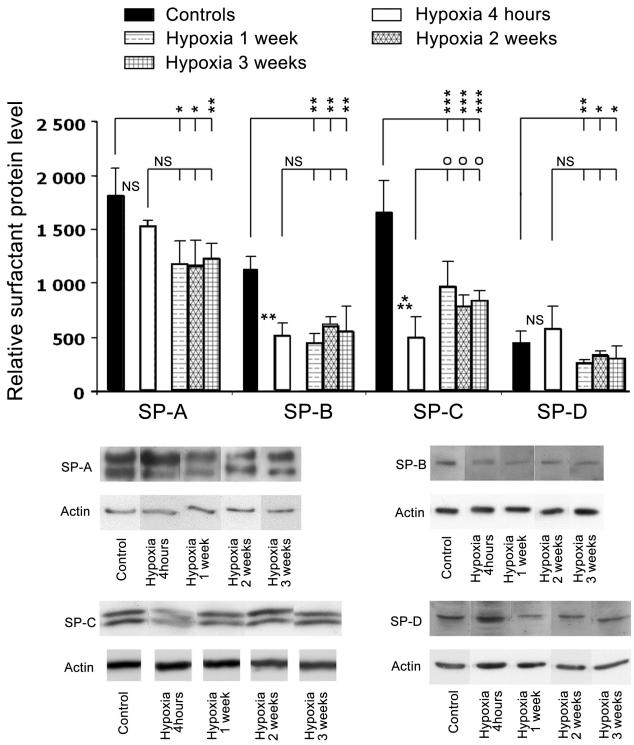

Fig. 6. – Influence of intermittent exposure to hypobaric-hypoxia in an altitude simulator chamber (as indicated in figure 1 caption) on pulmonary surfactant protein production. Control or experimental rats were endotracheally inoculated with *P. carinii* organisms (see text). Anti-SP-A antibody labelled strongly two bands of 32 kDa and 36 kDa glycosylated proteins. Specific antibody against SP-B recognized a 25-kDa pro-protein. Anti-SP-C antibody labelled two specific bands corresponding to 21 kDa pro-protein and 24 kDa palmitoylated pro-protein, respectively. Anti-SP-D antibody recognized one specific band at 43 kDa. Results were normalized after beta-actin hybridization (see text). Significant differences compared respectively to controls (*) or to four hour hypoxia rats (°) are shown by * or ° if $p < 0.05$, by ** or °° if $p < 0.005$ and by *** or °°° if $p < 0.0001$ (Student *t* test); NS = no significant.

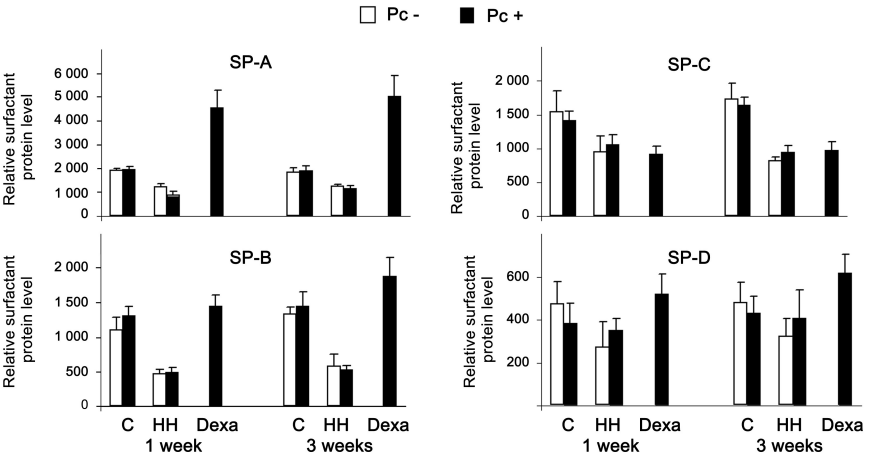

Fig. 7. – Influence of *Pneumocystis carinii* infection on the surfactant protein rates in immunocompetent Wistar rats exposed to hypobaric-hypoxia in an altitude simulator chamber (as indicated in figure 1 caption). *P. carinii*-infected (Pc^+) or uninfected (Pc^-) rats of the control groups (C) were maintained in normobaric normoxic conditions. *P. carinii*-infected or uninfected rats of the group HH were exposed to hypobaric hypoxia conditions. The "Dexa" group is constituted by dexamethasone-treated Wistar rats that were endotracheally infected with *P. carinii* and maintained in normobaric normoxic conditions. Dexa group attested that the used *P. carinii* organisms were infectious. In these experiments, the endotracheal inoculation of *P. carinii* organisms did not influence surfactant-protein amounts in immunocompetent rats exposed or not to hypobaric hypoxia.

vation of macrophage CD14 receptor by LPS and consequently prevents the pro-inflammatory response due to this cytokine and to NO release (Beers *et al*., 2005).

Hypobaric hypoxia conditions induced also a significant decrease in the lung content of SP-A (Figs. 5 and 6) and SP-D (Fig. 6). SP-D protein decrease after intermittent hypobaric hypoxia was about 33 % (compared to control) (Fig. 6) and it was apparently not related to mRNA changes (Fig. 5). Inflammatory changes usually associated to hypoxia (Hirche *et al*., 2004) could affect SP-D concentration as this protein is susceptible to specific degradation by Neutrophil Serine Proteinase. Compared with control animals maintained in normobaric normoxic conditions (Fig. 6), the expression of collectin SP-A, which possesses immuno-regulatory properties and modulates lung inflammatory responses, was also affected by hypobaric hypoxia as reported previously (Facco *et al*., 2005).

Reduction of SP-A after intermittent hypobaric hypoxia could result from the inflammatory process in alveoli. Actually, mediators of the pulmonary inflammatory response could be responsible for surfactant decreasing. Consistently, TNF-α (Tumor necrosis factor-alpha) has been shown to reduce SP-A mRNA and protein level in lung epithelial cells and in NCI-H441 cells (Pryhuber *et al*., 1998; Miakotina *et al*., 2002). Actually, two factors could be involved in the down regulation of surfactant proteins: TNF-α and TGF-β (Transforming growth factor-beta) (Pryhuber *et al*., 1998; Dhainaut *et al*., 2003). In rats, hypoxia induced lung inflammation as early as two hours after exposure, with an over-expression of pro-inflammatory cytokines such as TNF-α (Madjdpour *et al.* 2003). Moreover, TGF-β factor seems also to be increased in hypoxic conditions (Dhainaut *et al*., 2003). Anyway, neither hypoxic conditions, as attested by PaO_2 values (32.4 ± 0.7 mm Hg after 4-hour hypoxia, and 64.8 ± 19.6 mm Hg after intermittent hypoxia exposure, compared to PaO_2 = 99.1 ± 6,8 mm Hg in unexposed group-6 control rats) (Table I), nor pulmonary surfactant protein impairment (Figs 5-7), and potentially associated local immune response alterations, did not affect the ability of non-immunodepressed rats to clear *P. carinii* organisms from their lungs (Fig. 2). Present results suggest therefore that *P. carinii* development depends rather on a deep depression of the immune response than on the alteration of pulmonary surfactant composition. They suggest also that the early perturbation of surfactant properties induced by *Pneumocystis* organisms that we reported previously (Escamilla *et al*., 1992, 1993; Aliouat *et al*., 1998; Prévost *et al*., 1998; Dei-Cas, 2000), is not enough, in absence of immunodepression, to allow the active *Pneumocystis* proliferation associated with PcP.

Most likely, the main finding of the present work was the decrease of lung surfactant proteins (SP-A and SP-D) involved in local defence. Thus, hypobaric-hypoxia conditions, though they were not enough to allow *Pneumocystis* proliferation, weakened nevertheless the lung defence mechanisms. Thus, blood lymphocytes of rats exposed to hypobaric-hypoxia conditions were more susceptible to oxidative stress than those of rats maintained in normobaric normoxic conditions (group 6) (Fig. 4). Paradoxically, surfactant proteins potentially involved in defence mechanisms were found to be increased in dexamethasone-treated rats of group 1 (maintained in normobaric normoxic conditions), which developed PcP. This finding was consistent with previous observations of a drastic increase of SP-A induced by *Pneumocystis* (Atochina *et al*., 2001; Schmidt *et al*., 2006).

Anyway, on the bottom, the basic question of what is the role of SP-A and SP-D in *Pneumocystis* infections remains to be answered. Other experimental models were used to address this question. In a transgenic SP-D-overexpression mouse model, which developed PcP after antibody-induced $CD4^+$-cell depletion and inoculation of *P. murina* Keely *et al.* 2004, it was observed that both *P. murina* infection and lung inflammatory changes were increased (Vuk-Pavlovic *et al*., 2006). With regard with SP-A, in the pulmonary lavage fluid of SP-A deficient mice with corticosteroid-induced PcP were observed higher concentrations of TNF-α, IFN-γ and MIP-2 than in wild-type mice (Linke *et al*., 2005). The authors suggested that SP-A could exert a protective role against *P. murina*. But using the same model no difference in *P. murina* clearance was observed between SP-A deficient and wild-type mice after corticosteroid withdrawal (Linke *et al*., 2006). This observation is consistent with our present observations in rats infected with *P. carinii*.

In fact, *Pneumocystis*-related pulmonary surfactant changes, especially the early changes reported at the beginning of the infection (Escamilla *et al*., 1992, 1993; Aliouat *et al*., 1998) could result from lung inflammatory changes (Grubor *et al*., 2006) that precede the *Pneumocystis* infection. Actually, in corticosteroid-treated hosts or in hosts in which immunodepression resulted from other causes, *e.g.* HIV infection, congenital immunodepression syndromes, or in special situations like spontaneous PcP in rabbits (Allaert *et al*., 1996, 1997; Aliouat *et al*., 1998; Rajagopalan *et al*., 1998), the pulmonary surfactant composition is most likely altered before a noticeable proliferation of *Pneumocystis* organisms.

Both strong increase of SP-A associated with *Pneumocystis* infection (Phelps & Rose, 1991; Atochina *et al*., 2001; Schmidt *et al*., 2006) and the apparent absence of influence of this protein on *Pneumocystis* clearance (Linke *et al*., 2006, and present results) suggest that SP-A might somewhat protect *Pneumocystis* organisms from host defence mechanisms. Although,

alternatively, recent observations suggest that SP-A plays a role in the protection of the host against *Pneumocystis* infection (Atochina *et al*., 2004; Linke *et al*., 2005), in an earlier work Koziel *et al.* (1998) showed that increased levels of SP-A, through binding to the surface of *P. carinii* organisms, may interfere with alveolar macrophage recognition making phagocytosis more difficult. In the same way, though by a different mechanism, Yong *et al.* (2003) showed that SP-D dodecamers mediate optimal aggregation of *P. carinii*, which was shown to be responsible for the impaired phagocytosis of the organisms by alveolar macrophages. SP-D-mediated aggregation of *P. carinii* and SP-A binding to this organism (though available data are contradictory) may represent ways by which it avoids elimination by the host.

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