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., 2000; Gigliotti et al., 2003; Chabé et al., 2004). Thus, though immunocompetent hosts are usually able to radically eliminate the parasites from their lungs (Septkowitz 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

Summary:

It has been suggested that patients with pulmonary surfactant impairment are more susceptible to Pneumocystis infection than healthy controls. Owing to 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-immunocomprimé.

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-immunocomprimés infectés avec Pneumocystis carinii par voie endotrachéale, 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 facteurs 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-immunocomprimés. 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.

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as a dynamic reservoir to Pneumocystis species (Deicas, 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-spraying (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 (Kuchandani 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 & Tokhtayev (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; Deicas 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

Weeked 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-Gas 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 (Anonymous, 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 experiments.

**Assessing respiratory function**

Breath frequency was measured in wide-awake unrestrained rats before and after hypobaric-hypoxia exposure using a plethysmograph, Datanalyset and Iox$^\text{®}$ 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 (PaO$_2$, PaCO$_2$, 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-TCR$\alpha$-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$^\circ$ 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. carinii detection and quantification (see below). A second part was immediately snap-frozen (in liquid nitrogen) and stored in deep freezer (~ 80$^\circ$ 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 H2O, 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 GAA GAC AAG TCA CAG A-1115; (b) for SP-B (462 bp), 280-AAG TTC CTG GAA CAA and 856-TGC AGG GTT TTA CAG ACC-833; (c) for SP-C (377 bp), 178-ATG GAA TGT GA and 742-CCC AGC AGT GCA TCT; (d) for SP-D (498 bp), 639-GGC CTT CAC ATG AGT CAG-199 and 1137-CTA CAC ACC TAC-557 bp), 299-CCT GGA GGA CGT GGA GAC AAG-320 and 856-TGC GAA GAC AAG 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) or rabbit (Promega, France) horseradish peroxidase (HRP) conjugated was used as secondary antibody. For detection, we used Chemiluminescence Luminol Reagent according to manufacturer instructions. For detection step, 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).

RESULTS

Characterizing the experimental model

At 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 PaO2 of 32.4 mm Hg after a 4-hour exposure. The high PaCO2 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).
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 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 =

<table>
<thead>
<tr>
<th>Data</th>
<th>Control (n = 6)</th>
<th>4-h exposure to hypobaric hypoxia (n = 6)</th>
<th>1-week exposure to hypobaric hypoxia (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breath/min^b^</td>
<td>111.5 ± 6.2</td>
<td>144.2 ± 4.9^a^</td>
<td>146.2 ± 17.8^a^</td>
</tr>
<tr>
<td>pH</td>
<td>7.35 ± 0.1</td>
<td>7.35 ± 0.004</td>
<td>7.33 ± 0.1</td>
</tr>
<tr>
<td>PaO₂ (mm Hg)^c^</td>
<td>99.2 ± 6.8</td>
<td>32.4 ± 0.7^a</td>
<td>64.8 ± 19.6</td>
</tr>
<tr>
<td>PaCO₂ (mm Hg)</td>
<td>46.9 ± 5.1</td>
<td>46.6 ± 1.05</td>
<td>43.7 ± 2.5</td>
</tr>
<tr>
<td>SaO₂ (%)</td>
<td>93.8 ± 1.6</td>
<td>38.0 ± 7.5^a</td>
<td>76.4 ± 20.0</td>
</tr>
<tr>
<td>Hb (g/dL)</td>
<td>13.3 ± 1.0</td>
<td>15.5 ± 0.5</td>
<td>15.3 ± 0.2</td>
</tr>
<tr>
<td>HCO₃⁻ (mM/L)</td>
<td>25.5 ± 1.5</td>
<td>24.9 ± 0.4</td>
<td>22.4 ± 1.6</td>
</tr>
<tr>
<td>P. carinii cysts (× 10^6)^d</td>
<td>0.20 ± 0.1</td>
<td>ND</td>
<td>0.30 ± 0.2</td>
</tr>
</tbody>
</table>

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 post-inoculation (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.
^e Hemoglobin; PaO₂ and PaCO₂ = Partial O₂ and CO₂ pressures, respectively; SaO₂ = O₂-hemoglobin saturation; HCO₃⁻ = bicarbonate level in blood; ND = not done.
^a Significant difference.

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

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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 O₂ 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.

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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 SURFACANT 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 O₂, CO₂ and N₂ were not changed, it can be deduced that O₂ 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.
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. 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 PaO2 values (32.4 ± 0.7 mm Hg after 4-hour hypoxia, and 64.8 ± 19.6 mm Hg after intermittent hypoxia exposure, compared to PaO2 = 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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REFERENCES


COQUETTE A., VRY B. & VANDERPAS J. Role of vitamin E in the protection of the resident macrophage membrane against oxidative damage. Archives Internationales de Physiologie et de Biochimie, 1986, 94, 529-534.


Prevost M.C., Escamilla R., Aliouat E.M., Cere N., Coudert P. & Dei-Cas E. Pneumocystosis pathophysiology. FEMS Immunology and Medical Microbiology, 1998, 22, 123-126.


Sing A., Roggenkamp A., Autenrieth I.B. & Heesemann J. Pneumocystis carinii carriage in immunocompetent patients with primary pulmonary disorders as detected by single or nested PCR. Journal of Clinical Microbiology, 1999a, 37, 3409-3410.


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