

Modulation by pentobarbital of neutrophil responses to inhaled *E. coli* endotoxin in sheep: role of lung epithelium

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Modulation by Pentobarbital of neutrophil responses to inhaled E. coli endotoxin in sheep: role of lung epithelium. B.T. Peterson, E.J. Miller, D.E. Griffith, R. Rowjee, P. McWaters. ©ERS Journals Ltd 2000.

ABSTRACT: Neutrophils (PMNs) are implicated in the pathogenesis of acute respiratory distress syndrome (ARDS). The role of the epithelium in the modulation of PMN migration within the lungs was examined.

Epithelial integrity and PMN concentrations in the lung air spaces and lymph were measured in sheep anaesthetized with either halothane (1–2.5%) or intravenous pentobarbital ($12 \pm 4 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$). Ventilation with an aerosol containing 25 mg *Escherichia Coli* endotoxin (lipopolysaccharide; LPS) effected neutrophil recruitment to the air spaces. Lymphatic clearance of aerosolized ^{99m}Tc-DTPA provided an index of epithelial integrity.

Three hours after the deposition of LPS, the lung lining fluid of sheep anaesthetized with halothane (n=7) had $4.9 \pm 3.2 \times 10^6 \text{ PMN} \cdot \text{mL}^{-1}$, but the lung lymph had almost no PMNs (3±8%). Sheep anaesthetized with pentobarbital (n=6) had fewer PMNs in the air spaces ($2.4 \pm 1.2 \times 10^6 \cdot \text{mL}^{-1}$) and more PMNs in the lung lymph (30±20%). Control sheep (n=5) that received no LPS had almost no PMNs in the airspaces or lung lymph, regardless of the anaesthesia. Three additional sheep that remained awake after receiving LPS also had no PMNs in the lung lymph. The PMN fraction in the lung lymph correlated well with the extra-alveolar epithelial permeability measured by lymphatic clearance of aerosolized diethylenetriamine penta-acetic acid ($r=0.81$, $p<0.001$).

Aerosolized lipopolysaccharide recruits neutrophils into the lungs of sheep, but they appear to remain in the airspaces unless extra-alveolar permeability is increased by agents such as pentobarbital.

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Neutrophils (PMNs) are often implicated in the pathogenesis of the acute respiratory distress syndrome (ARDS) because of their appearance in the lungs during the early stages of the development of ARDS, their presence near the sites of injury to the lung endothelium and epithelium, and their documented ability to inflict injury *via* release of enzymes and other reactive compounds [1]. Therefore, knowledge about the site of entry of PMNs into the lungs, their routes of movement within the lungs, and whether or not they degranulate may be helpful in the design of methods for preventing or treating ARDS.

Downey *et al.* [2] used histological techniques to show that neutrophils enter the airspaces of the lungs of rabbits at the alveolar level in response to complement fragment C5a. Lung lavage and histological techniques were used to show that the neutrophils, that enter the lungs of rabbits in response to intravenous staphylococcal enterotoxin A, migrate up the airways to create a gradient in the PMN concentrations between the airways and the alveolar regions within 3–18 h [3]. However, we could not determine in that rabbit model whether the PMNs left the airspaces and entered the lung interstitium.

To determine the route of migration of PMNs within the lungs, and to test the hypothesis that epithelial integrity

modulates PMN egress from the air spaces, the lung lymphatic of anaesthetized sheep was cannulated and PMNs recruited into the lungs by ventilating the sheep with an aerosol of *E. coli* endotoxin (lipopolysaccharide; LPS). Kindt *et al.* [4] showed that this model resulted in the appearance of PMNs first in the airspaces and then in the lung lymph. It was reasoned that the addition of an assessment of extra-alveolar epithelial integrity to the model of Kindt *et al.* [4] would provide information about the role of the epithelium in PMN migration from the air spaces and into the lung interstitial spaces drained by the lymphatics. It has been shown that an increase in permeability of the lung epithelium adjacent to regions of the lungs drained by the lymphatics (*i.e.* extra-alveolar) causes the concentration of aerosolized ^{99m}Tc-DTPA in the lymph, to exceed that in the plasma by 30% [5].

However, the present study was unable to reproduce the findings of PMNs in the lymph described by Kindt *et al.* [4] when the standard anaesthetized sheep preparation that included halothane anaesthesia was used. However, when pentobarbital anaesthesia was used, as Kindt *et al.* [4] had used, PMNs were found in the lung lymph as they described. This differential effect of the two modes of anaesthesia provided a unique opportunity to investigate

the role of the lung epithelium in PMN migration from the air spaces into the lung tissue. The present hypothesis was that the appearance of PMNs in the lung lymph in response to aerosolized LPS is modulated by the lung epithelium whose permeability is affected by the type of anaesthesia used during exposure to LPS.

Methods

Surgical preparation of sheep

A total of 21 sheep, each weighing 35 ± 8 kg were first anaesthetized with 500–1,000 mg pentothal sodium (Abbott, N. Chicago, IL, USA), a short acting anaesthetic given intravenously to allow intubation. The sheep were then ventilated with 50% oxygen at a tidal volume of 500 mL and at a rate to keep arterial carbon dioxide tension (P_{a,CO_2}) 35–40 mmHg. In 18 of the sheep, after intubation and cannulation of the jugular vein, anaesthesia was maintained by either ventilating with 1–2.5% halothane (Halocarbon Labs, River Edge, NJ, USA) or by injecting sodium pentobarbital (Abbott, N. Chicago, IL, USA) intravenously as needed to maintain a surgical plane of anaesthesia (average pentobarbital dose = 12 ± 4 mg·kg⁻¹·h⁻¹). Catheters were installed in the carotid artery and in the efferent duct of the mediastinal lymph node *via* a right thoracotomy as described elsewhere [5]. The tail of the lymph node was ligated to minimize contamination from nonpulmonary sources [6].

Anaesthetized protocol

Each sheep was placed in the prone position following surgery. Arterial blood pressure and airway pressures were continuously recorded on a Sormedics four-channel strip chart recorder (Sormedics, Anaheim, CA, USA) and arterial blood and lung lymph samples were collected every 30 min. Following a 30 min baseline period, the sheep were ventilated for 30 min with an aerosol of saline (control) or 25 mg *E. Coli* endotoxin (0127:B8, Sigma Chemical, St. Louis, MO, USA) diluted in 10 mL of saline and delivered with a Hudson jet nebulizer (Hudson RCI, Temecula, CA, USA) in series with the inspiratory line of the breathing circuit. One hour later the sheep were ventilated with an aerosol of ^{99m}Tc-diethylenetriamine penta-acetic acid (DTPA) to assess changes in extra-alveolar lung epithelial permeability [5]. Three hours after the end of the deposition of LPS, three 60 mL single-cycle lavages (right lower lobe, left lower lobe, and left middle lobe (see below [7]) were performed. Lymph and plasma sample collection continued every 30 min for 1 h after the completion of the lavages. The sheep were then euthanized by increasing the level of anaesthesia and injecting saturated KCl intravenously. All procedures were approved in advance by the Animal Research Committee of the University of Texas Health Center at Tyler.

Unanaesthetized protocol

To determine the response to LPS in sheep that received neither halothane nor pentobarbital, three sheep were

anaesthetized with sodium pentothal and had an endotracheal tube installed but received no surgery. Each sheep was ventilated with 25 mg of LPS aerosol and then allowed to awaken and returned to its cage. Three hours later the sheep were anaesthetized with sodium pentothal and maintained anaesthesia with halothane to allow cannulation of the lung lymphatic and performance of the three lavages.

Single-cycle lavage

Details of this technique have been described previously [7]. Briefly, 60 mL of sterile saline was instilled through a bronchoscope into a segment of the lungs. It was immediately withdrawn *via* a series of five syringes on a manifold. The first syringe (~15 mL) contained the saline that was in the dead space and the upper airways and was not analysed. The next four syringes (~3–5 mL each) contained a mixture of saline and the epithelial lining fluid (ELF) from the lower airways and alveoli and was designated as fractions 1–4. A final sample, consisting of the 1–2 mL of fluid remaining in the bronchoscope, was collected after the bronchoscope was withdrawn from the lungs and was designated fraction 5. The lavage aliquots and the blood samples collected immediately before the first lavage and after the third lavage were centrifuged at 450×g for 5 min and the supernatants were collected. Total protein, albumin, myeloperoxidase, interleukin (IL)-8, and urea concentrations were measured on each cell-free aliquot and plasma sample (see below, [8]). The fraction of neutrophils in each lavage aliquot was determined by resuspending the pellet in saline, cytospinning the resuspended cells (Shannon Cyto centrifuge, Pittsburgh, PA, USA), staining the cells, and counting at least 200 cells per sample. The total number of white cells in each fraction was measured with a Coulter Counter (Hialeah, FL, USA).

The concentrations of cells and solutes (proteins, myeloperoxidase, IL-8) in the epithelial lining fluid (PELF) were calculated from the cell or solute concentrations in each collected fraction (P_{fraction}) and the urea concentrations in that fraction ($[urea]_{\text{fraction}}$) and the plasma ($[urea]_{\text{plasma}}$):

$$PELF = P_{\text{fraction}} [urea]_{\text{plasma}} / [urea]_{\text{fraction}}$$

Standard assays

Total protein, albumin, and urea concentrations were measured using commercially available kits (Sigma Chemical Co, St. Louis, MO, USA). The analysis of the total protein assay was modified to correct for alinearity of the standard curve and the dependence of the standard curve on the albumin fraction of the sample by including standards of varying proportions of albumin as described elsewhere [8].

Myeloperoxidase (MPO) concentrations in the cell free lavage fluid were determined by measuring the change in optical density at 450 nm that occurred by oxidation of tetramethylbenzidine as described by SUZUKI *et al.* [9].

Results are reported in nmol of substrate oxidized·min⁻¹·mL ELF⁻¹.

Assessment of extra-alveolar permeability

Extra-alveolar permeability was assessed by depositing an aerosol of ^{99m}Tc-DTPA (DTPA) in the lungs. DTPA is a small molecule (mw=492) that diffuses out of the air spaces relatively quickly and equilibrates in the water spaces of the body. This results in equal concentrations of DTPA in the lung lymph and plasma within 45 min of deposition into the lungs of healthy sheep [5]. It has been shown that in the presence of acute lung injury due to intravenous infusion of air, the steady-state concentration of aerosolized DTPA in the lung lymph exceeds that in the plasma by 30–50% [5]. Therefore, a steady-state L/P DTPA ratio that is significantly greater than 1.0 has been interpreted as evidence of increased extra-alveolar epithelial permeability [5].

Sheep interleukin-8 assay

The concentration of IL-8 in lavage fluid and lymph was measured using an enzyme-linked immunosorbent assay (ELISA) specific for ovine-IL-8 with slight modifications from the method previously described [10]. Briefly, the monoclonal antibody raised against recombinant ovine IL-8 (anti-rOvIL-8) [11], was diluted in 10 mM phosphate buffered saline (PBS), pH 7.4 and dispensed into Nunc Maxisorp 96 well immunoplates (Fisher Scientific, Pittsburgh, PA, USA). The plates were then incubated at 4°C overnight. After removal of unbound antibody, the wells were washed four times with PBS containing 0.05% (v/v) Tween 20 (PBST). Nonspecific binding sites on the plate were blocked by incubation, for 1 h at room temperature, with PBS containing 1% (w/v) bovine serum albumin (BSA), 5% (w/v) sucrose and 0.05% (v/v) Tween 20. Samples and standards were then added to the wells and incubated for 3 h at room temperature. The plates were then washed four times in PBST before the addition of a 1:2000 dilution of rabbit anti-rOvIL-8 and incubation for 1 h at room temperature. After four further washes, horseradish peroxidase conjugated anti-rabbit immunoglobulins (Dako Corp., Carpinteria, CA, USA) was added and incubated for 1 h at room temperature. After five more washes with PBST, tetramethyl benzidine substrate was added to each well and incubated for 20 min at room temperature. The reaction was stopped with 2M H₂SO₄ and the absorbance was read at 450 nm with background correction at 540 nm. Dilutions of rOvIL-8 in PBST containing 0.1% (w/v) BSA were used to construct standard curves.

Statistical analysis

Unless otherwise noted, statistically significant differences among the groups of sheep were determined by a one-way analysis of variance. Differences between specific groups were determined by the Bonferonni post-hoc test [12]. When the distributions were not normal, the non-parametric Mann Whitney test was used and is so indi-

cated in the data tables. A p-value of <5% was considered statistically significant. All values in the text and tables are reported as mean±SD. All values in the graphs are mean±SEM.

Results

Neutrophil migration

Although all noncontrol sheep received the same dose of aerosolized *E. Coli* endotoxin (LPS), the concentration of PMNs in the lymph and ELF depended upon the anaesthetic used. During pentobarbital anaesthesia, PMNs entered the lung lymphatics at a rate that was only slightly slower than that reported by KINDT *et al.* [4]. The mean percent of PMNs in the lavage fractions collected 3 h after the deposition of the LPS was 59±15% and the total PMN concentration was 2.4±1.2×10⁶ PMN·mL ELF⁻¹ (figs. 1, 2, table 1). In contrast, during halothane anaesthesia, the number of PMNs in the lung lymph was not significantly different from zero until after the lavages (see below), and there were more PMNs in the ELF (fig. 2), even though the fraction of PMN in the ELF was similar in both groups (table 1). It should be noted that the fraction of PMNs found in the lymph of control sheep anaesthetized with halothane was not significantly different from zero, but the PMN fraction in the control sheep anaesthetized with pentobarbital was 10±5% 3 h after the deposition of aerosolized saline (table 2, fig. 1).

The pattern of PMN concentration in the various lavage fractions also differed between the two anaesthetics. During pentobarbital anaesthesia the PMN concentration was similar in all of the fractions (fig. 2). However, with halothane anaesthesia the PMN concentration was lower in the more distal fractions (alveolar region) and higher in the more proximal fractions (airways).

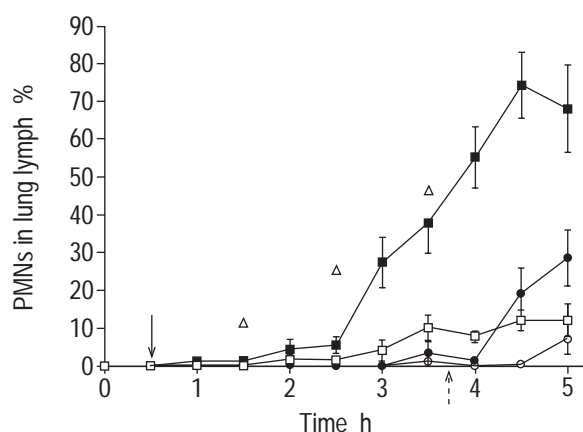


Fig. 1. – Time course of neutrophils (PMN) concentrations in lung lymph for 4.5 h after the beginning of the deposition of aerosolized *Escherichia Coli* endotoxin (lipopolysaccharide; LPS) (indicated by solid arrow) in sheep anaesthetized with either halothane or sodium pentobarbital. PMN concentrations are expressed as a per cent of total leukocytes. The lavages were performed only at the time indicated by the dashed arrow. ■ : pentobarbital and LPS (n=6); ● : halothane and LPS (n=7); □ : pentobarbital control (n=2); ○ : halothane control (n=2); △ : data from KINDT *et al.* [4]. Values are mean±SEM.

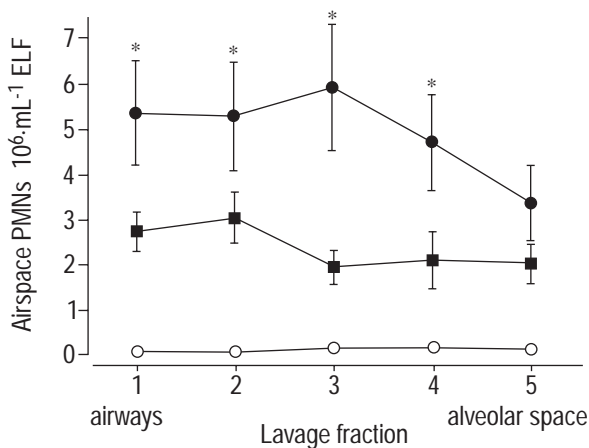


Fig. 2. – The concentration of neutrophils (PMNs) in the epithelial lining fluid (ELF) determined by analysis of the five fractions collected from single cycle lavages performed 3 h after the deposition of lipopolysaccharide (LPS). ■ : pentobarbital and LPS (n=6); ● : halothane and LPS (n=7); ○ : pentobarbital (n=2) and halothane (n=3) control; *: p<0.05 (unpaired t-test). Values are mean±SEM.

Epithelial permeability and neutrophil degranulation

To determine whether there was a change in the epithelial permeability of the extra-alveolar epithelium, each sheep was ventilated with an aerosol of ^{99m}Tc-DTPA and its concentrations in the lymph and plasma were monitored for the next 2 h. A steady-state lymph to plasma DTPA ratio (L/P) >1.0 implies an increase in the permeability of the extra-alveolar epithelium [5]. Steady-state L/P ratio was found to be 1.06±0.06 for all groups of sheep except for those anaesthetized with pentobarbital. These sheep had an L/P ratio of 1.30±0.15 which was significantly greater than that in the other groups (p<0.01) (fig. 3). Figure 4 shows that the fraction of PMN in the lung lymph correlated significantly with the DTPA L/P ratio (r=0.81, p<0.001).

The two anaesthetics also effected different responses to LPS with respect to the amount of total protein, albumin, and MPO in the ELF. During pentobarbital anaesthesia the final albumin and total protein concentrations in the lavage fluid were greater than those measured in the sheep with halothane anaesthesia (p<0.01 by t-test) (table 1). The MPO concentration in the cell-free lavage fluid reflects degranulation of PMNs and was over 20 times greater in

the sheep with pentobarbital anaesthesia versus sheep with halothane anaesthesia (p<0.01 by analysis of variance).

No statistically significant differences were found in the lymph flows or protein concentrations relative to that in the plasma in response to LPS with either anaesthetic or with no anaesthetic during the 3 h after deposition of LPS (table 2).

Interleukin-8 concentrations in epithelial lining fluid and lymph

The concentration of IL-8 in the ELF was very high in response to LPS regardless of the anaesthetic used. Figure 5 shows a strong correlation between the IL-8 concentration and the number of PMNs in the ELF with halothane anaesthesia (r=0.96, p<0.001). However, this correlation was not present when pentobarbital anaesthesia was used. The IL-8 concentration in the lung lymph was significantly elevated only in the sheep that received pentobarbital, but even in these sheep the IL-8 concentration in the ELF was ~80-fold greater than that in the lymph.

Effect of lavage on neutrophils in lung lymph

Figure 1 shows that the three lavages performed 3.5–4 h after the beginning of the deposition of LPS appeared to increase the fraction of PMNs in the lung lymph.

Unanaesthetized sheep

To determine which of the two anaesthetics most closely resembled the response to LPS in unanaesthetized sheep, the effects of LPS in three sheep that received neither pentobarbital nor halothane before, during, or 3 h after receiving LPS were measured. The low concentration of MPO in the ELF and the absence of PMNs in the lung lymph suggest that halothane, rather than pentobarbital anaesthesia, more closely resembles the unanaesthetized response to LPS (tables 1, 2).

Discussion

ARDS is associated with the accumulation of PMNs in the air spaces but the relationship between the increases

Table 1. – Cell and solute concentrations in epithelial lining fluid (ELF)

	Control		Endotoxin aerosol LPS		
	Halothane	Pentobarbital	Halothane	Pentobarbital	No anaesthesia
Sheep n	3	2	7	6	3
Total protein mg·mL ⁻¹	17±3	14±3	11±3	17±3 [#]	8±2
Albumin mg·mL ⁻¹	4.9±0.06	3.8±0.5	3.2±1.4	9.1±4.9 ^{#5}	3.7±0.8
PMN 10 ⁶ ·mL ⁻¹	0.17±0.10	0.02±0.02	4.9±3.2*	2.4±1.2*	3.0±1.4*
PMN %	6±5	1±1	54±17*	59±15**	77±3
MPO nmol substrate oxidized·mL ⁻¹ ·min ⁻¹	0.2±0.1	1.5±1.1	0.2±0.1	3.7±0.7** ⁵	0.6±0.4
IL-8 ng·mL ⁻¹	14±6	14±10	1686±1125*	1904±1368*	NM

Data presented as mean±SD. LPS: lipopolysaccharide; PMN: neutrophils; MPO: myeloperoxidase; IL: interleukin; NM: not measured; *: p<0.05 versus control; **: p<0.01 versus control; #: p<0.01 versus halothane:LPS but only by an unpaired t-test; ⁵: p<0.02 versus halothane by nonparametric analysis.

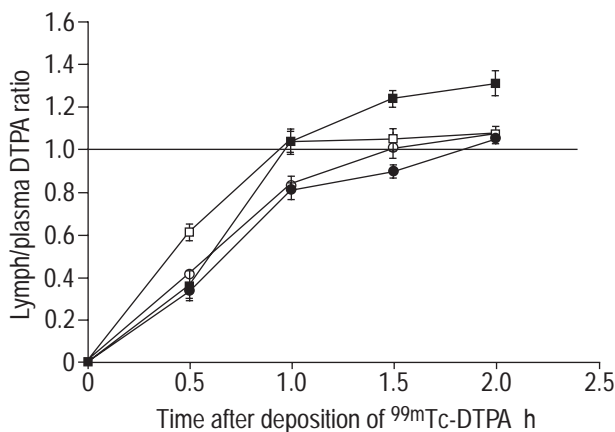


Fig. 3. – The concentration of ^{99m}Tc-diethylenetriamine penta-acetic acid (DTPA) in the lymph relative to that in the plasma for 2 h after its deposition in the lungs of control sheep. □ : pentobarbital control (n=2); ○ : halothane control (n=2); ● : sheep received halothane anaesthesia and lipopolysaccharide (LPS) (n=3); ■ : sheep received pentobarbital anaesthesia and LPS (n=6). The horizontal line represents the equilibration point between lymph and plasma.

in lung endothelial and epithelial permeability and the migration of neutrophils within the lungs is not clear. KINDT *et al.* [4] showed that aerosolized LPS causes neutrophils to enter both the air spaces and lung tissue drained by the lymphatics. Their measurement of chemotactic gradients explained the presence of PMNs in the airways but not the early movement of PMNs into the lung lymph from either the airways or the plasma.

Examination of PMN migration within the lungs led us to consider the effects of anaesthesia. Halothane has been the anaesthesia of choice for sheep even though it has been shown to decrease PMN accumulation in vessels in animals [13, 14]. Pentobarbital is rarely used in sheep, is associated with lung toxicity in rats that receive LPS, and can depress PMN chemotaxis and degranulation [15–17]. The appearance of PMNs in the lung lymph of sheep ventilated with LPS depended upon the anaesthetic used. To determine which of the two responses more closely

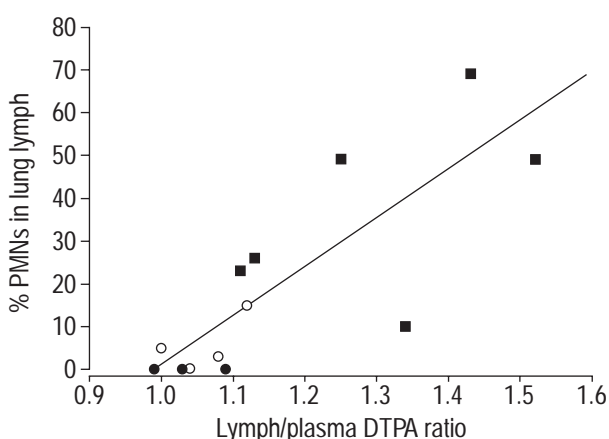


Fig. 4. – The fraction of neutrophils (PMNs) in the last lung lymph samples collected before the lavage compared to the lymph to plasma DTPA concentration ratio for all sheep. Linear regression line is shown ($r=0.81$, $p<0.01$). ■ : pentobarbital and lipopolysaccharide (LPS) (n=6); ● : halothane and LPS (n=3); ○ : pentobarbital (n=2) or halothane (n=2) control.

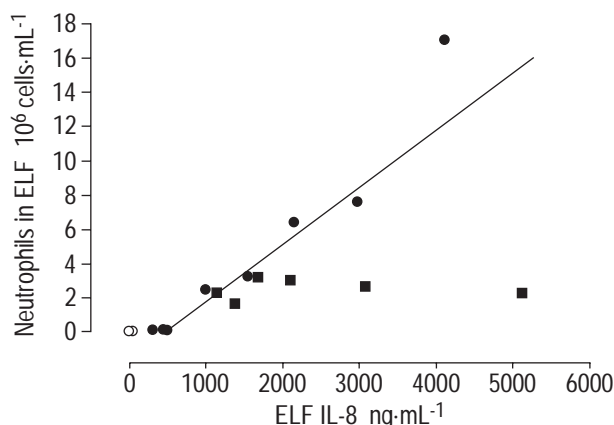


Fig. 5. – Comparison of the concentrations of interleukin (IL)-8 and neutrophils in the epithelium lining fluid (ELF). The two solute concentrations correlated well only in the sheep with halothane anaesthesia ($r=0.96$, $p<0.01$). ■ : pentobarbital and lipopolysaccharide (LPS) (n=6); ● : halothane and LPS (n=7); ○ : pentobarbital (n=2) or halothane (n=3) control.

mimicked the unanaesthetized condition, three sheep that received neither anaesthetic during the 3 h after the deposition of LPS were examined. The strong similarity in the PMN responses seen in these sheep and those that received halothane suggests that the PMN response under halothane anaesthesia is similar to the unanaesthetized response. Therefore, pentobarbital is altering the PMN response.

The normal response to aerosolized LPS was found to involve migration of PMNs from the circulation and into the air spaces, most likely in response to the release of IL-8 in the air spaces. The PMNs do not normally enter the lung lymph in response to aerosolized LPS. The absence of increased lymph flows, increased protein concentrations in the lymph, increased levels of MPO or proteins in the ELF, and the normal DTPA L/P ratio suggests that there are no changes in either the endothelial or epithelial permeabilities in response to aerosolized LPS [18]. However, the discovery of a gradient in the PMN concentration from the lavage fraction 5 (alveoli) to fractions 1–3 (airways) is a new finding in this model of aerosolized LPS although SIMPSON *et al.* [19] also found more PMNs in large airways *versus* alveoli of sheep given intravenous LPS. In an earlier study involving lung histology a similar gradient was found in PMN concentration in rabbits, which was attributed to migration of PMNs from the alveoli to the airways in response to *Staphylococcus enterotoxin A* [3]. The PMN gradient found in the sheep given aerosolized LPS was also probably due to migration rather than entry into the air spaces directly because the DTPA L/P ratio of 1.0 implies that there were no alterations in extra-alveolar epithelial permeability [5].

Compared to the normal response to LPS, the sheep anaesthetized with pentobarbital had fewer PMNs in the ELF, no PMN gradient in the airways, and more PMNs in the lung lymph. They also had more proteins and MPO in the ELF, and an elevated DTPA L/P ratio. The possibility was considered that pentobarbital was increasing vascular endothelial permeability which would allow the PMNs to leave the circulation and enter the lung tissue drained by the lymphatics. However, such an increase in permeability

Table 2. – Cell and solute concentrations in lung lymph[#]

	Control		Endotoxin aerosol LPS		
	Halothane	Pentobarbital	Halothane	Pentobarbital	No anaesthesia
Sheep n	2 [‡]	2	7	6	3
PMN %	2±2	10±5	3±8	30±20** [§]	1±1
IL-8 ng·mL ⁻¹	2±1	10±1	4±2	24±11** [§]	ND
Total protein L/P	0.85±0.06	0.71±0.04	0.63±0.11	0.69±0.07	0.64±0.05
Albumin L/P	0.88±0.08	0.83±0.03	0.62±0.18	0.85±0.10	0.70±0.07
Increase in flow mL·h ⁻¹	0.5±1.4	1.0±1.0	1.1±1.7	0.7±1.5	ND
DTPA L/P	1.06±0.02	1.06±0.06	1.04±0.04 [¶]	1.30±0.15** [§]	ND

Data presented as mean±SD. LPS: lipopolysaccharide; PMN: neutrophils; IL: interleukin; L/P: lymph/plasma ratio; ND: not determined; [#]: values in last lymph sample collected before lavages; [‡]: lymph only available from one sheep; *: p<0.05 versus control; **: p<0.01 versus control; [§]: p<0.02 versus halothane by nonparametric analysis; [¶]: n=3 sheep.

would be expected to increase both the lung lymph flow and the protein concentration of the lung lymph relative to that in the plasma, but this did not occur [20]. The possibility that an undetected increase in endothelial permeability that allowed PMN migration into the lymph cannot specifically be excluded, but this explanation is not consistent with the present data or with the data of KINDT *et al.* [4].

The sheep that received pentobarbital anaesthesia had an increase in lung epithelial permeability as shown by the increase in the concentrations of proteins in the ELF and the increase in the DTPA L/P ratio. This increase in epithelial permeability may have allowed the PMNs in the air spaces to enter the lung tissue drained by the lymphatics, thereby increasing the lymph concentration of PMNs. The extra-alveolar epithelium is the probable site for access into the lymph because lymphatics do not extend down to the level of the alveoli. The movement of PMNs out of the airways would tend to destroy the alveolar to airway gradient in PMN concentration seen in the sheep with the normal response to LPS, which is exactly what was found in the present study (fig. 2). This mechanism would also explain the lack of correlation between the IL-8 and PMN concentrations in the ELF. The IL-8 gradient probably provided the mechanism for PMN migration into the alveoli, but once they arrived in the airways they were carried out of the air spaces by convection of fluid flowing from the airways into the lymphatics as reported by MACKLIN [21]. Furthermore, the correlation between the DTPA L/P ratio and the fraction of PMNs in the lung lymph suggests that the integrity of the extra-alveolar epithelium helped modulate PMN migration into the lymph.

Figure 1 shows that the measurements of PMN fraction in the lymph of sheep anaesthetized with pentobarbital were slightly less than those reported by KINDT *et al.* [4]. This difference can be explained by examining the effect of the lavages on the concentration of PMNs in the lymph. It was found that the PMN concentration in the lymph increased within 30 min after performing the lavages 3 h after deposition of the LPS. This is probably due to convection of PMNs in the excess fluid as it is cleared from the air spaces and into the circulation directly or *via* the lymphatics. In the study by KINDT *et al.* [4] the hourly lavages probably facilitated the movement of PMNs from the air spaces into the lung lymph resulting

in slightly higher values than were measured without the hourly lavages.

The mechanism by which pentobarbital altered the normal response to aerosolized LPS is not known, but it may be related to the findings of increased MPO levels in the ELF of sheep that received pentobarbital. The pentobarbital may have promoted degranulation of PMNs in the airways resulting in the release of several enzymes including elastase and MPO. One or more of these enzymes may have acted directly on the lung epithelium to increase alveolar or extra-alveolar permeability or both [22]. It is concluded that the normal (*i.e.* unanaesthetized) response in sheep given aerosolized LPS is migration of neutrophils into the alveoli by a chemoattractant mechanism that does not cause any significant damage to the endothelium or epithelium. IL-8 may be an important cytokine in the process. After entering the alveoli, the PMNs migrate up the airways resulting in an accumulation of PMNs in the more proximal regions of the lungs. Pentobarbital anaesthesia alters this normal response by increasing epithelial permeability, possibly through a mechanism that includes PMN degranulation. This increase in epithelial permeability allows PMNs to enter the lung tissue drained by the lymphatics. Therefore, the epithelium may be an important barrier for modulating the migration of neutrophils within the lungs.

References

- Baughman RP, Gunther KL, Rashkin MC, Deeton DA, Pattishall EN. Changes in the inflammatory response of the lung during acute respiratory distress syndrome: Prognostic indicators. *Am J Respir Crit Care Med* 1996; 154: 76–81.
- Downey GP, Worthen GS, Henson PM, Hyde DM. Neutrophil sequestration and migration in localized pulmonary inflammation. *Am Rev Respir Dis* 1993; 147: 168–176.
- Peterson BT, Miller EJ, Morris D. Neutrophil influx and migration in rabbit airways in response to staphylococcal enterotoxin-A. *Exp Lung Res* 1999; 25: 41–54.
- Kindt GC, Gadek JE, Weiland JE. Initial recruitment of neutrophils to alveolar structures in acute lung injury. *J Appl Physiol* 1991; 70: 1575–1585.
- Peterson BT, Gray LD. Pulmonary lymphatic clearance of ^{99m}Tc-DTPA from air spaces during lung inflation and lung injury. *J Appl Physiol* 1987; 63: 1136–1141.

6. Roos PJ, Wiener-Kronish JP, Albertine KH, Staub NC. Removal of abdominal sources of caudal mediastinal node lymph in anesthetized sheep. *J Appl Physiol* 1983; 55: 996–1001.
7. Peterson BT, Griffith DE, Tate RW, Clancy SJ. Single-cycle bronchoalveolar lavage to determine solute concentrations in epithelial lining fluid. *Am Rev Respir Dis* 1993; 147: 1216–1222.
8. Peterson BT, Tate RW. Albumin fraction and measurement of total protein concentration. *Am J Physiol* 1993; 264: H1723–H1726.
9. Suzuki K, Ota H, Sasagawa S, Sakatani T, Fujikura T. Assay method for myeloperoxidase in human polymorphonuclear leukocytes. *Anal Biochem* 1983; 132: 345–352.
10. Persson WK, Colditz IG, Flapper P, Seow HF. Leukocyte and cytokine accumulation in the ovine teat and udder during endotoxin-induced inflammation. *Vet Res Comm* 1997; 21: 101–115.
11. Seow HF, Yoshimura R, Wood PR, Colditz IG. Cloning, sequencing, expression and inflammatory activity in skin of ovine interleukin-8. *Immunol Cell Biol* 1994; 72: 398–405.
12. Wallenstein S, Zucker CL, Fleiss JL. Some statistical methods useful in circulation research. *Circulation Res* 1980; 47: 1–9.
13. Miller LS, Morita Y, Rangan U, Kondo S, Clemens MG, Bulkley GB. Suppression of cytokine-induced neutrophil accumulation in rat mesenteric venules *in vivo* by general anesthesia. *Int J Microcirculat: Clin Exp* 1996; 16: 147–154.
14. Kowalski C, Zahler S, Becker BF, *et al.* Halothane, isoflurane, and sevoflurane reduce postischemic adhesions of neutrophils in coronary system. *Anesthesiol* 1997; 86: 188–195.
15. Hoque AM, Marczin HN, Catravas JD, Fuchs LC. Anesthesia with sodium pentobarbital enhances lipopolysaccharide-induced cardiovascular dysfunction in rats. *Shock* 1996; 6: 365–370.
16. Weiss M, Buhl R, Birkhahn A, Mirow N, Schneider M, Wernet P. Do barbiturates and their solutions suppress FMLP-induced neutrophil chemiluminescence? *Eur J Anaesthesiol* 1994; 11: 371–379.
17. Kress HG, Segmuller R. Intravenous anesthetics and human neutrophil granulocyte motility *in vitro*. *Anaesthesist* 1987; 36: 356–361.
18. Wiener-Kronish JP, Albertine KH, Matthay MA. Differential responses of the endothelial and epithelial barriers of the lung in sheep to *Escherichia coli* endotoxin. *J Clin Invest* 1991; 88: 864–875.
19. Simpson JF, Butterfield MJ, Lefferts PL, Dyer EL, Snapper JR, Meyrick B. Role of pulmonary inflammation in altered airway responsiveness in three sheep models of acute lung injury. *Am Rev Respir Dis* 1991; 143: 585–589.
20. Peterson BT. Permeability: theory vs. practice in lung research. *Am J Physiol* 1992; 262: L243–L256.
21. Macklin CC. Pulmonary sumps, dust accumulations, alveolar fluid and lymph vessels. *Acta Anat* 1955; 23: 1–32.
22. Gossage JR, Kuratomi Y, Davidson JM, Lefferts PL, Snapper JR. Neutrophil elastase inhibitors, SC-37698 and SC-39026, reduce endotoxin-induced lung dysfunction in awake sheep. *Am Rev Respir Dis* 1993; 147: 1371–1379.