TNO report
PML 1996-B63

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Infecties door gram-negatieve bacteriën zijn vaak dodelijk, ondanks therapie met antibiotica. Algemeen wordt aangenomen dat de vergiftigingsverschijnselen, zoals 'septic shock', veroorzaakt worden door de zogenaamde lipopolysacchariden (LPS; ook wel endotoxinen genoemd), een belangrijke component van het buitenste celmembraan van gram-negatieve bacteriën, waartoe de meeste pathogenen behoren.

Tot nu toe bestaat er geen adequate behandeling van zulke endotoxinebesmetting. Dit is de reden dat deze endotoxinen zijn aangemerkt als Midspectrum Agents en dus van belang voor de Nederlandse Krijgsmacht.

Een kwetsbaar targetorgaan voor endotoxinen is de long. Endotoxinen die per inhalatie in de longen komen, stimuleren de alveolaire macrofagen die meteen het cytokine TNFα (tumor necrosis factor) afgeven. In het hier aangeboden rapport is dit fenomeen heel duidelijk te zien in figuur 5c. TNFα dat gesynthetiseerd en afgegeven wordt door de macrofagen in de long, zorgt ervoor dat er een geweldige infiltratie van neutrofielen (PMNs) vanuit de bloedbaan in de long plaatsvindt. Deze neutrofielen produceren proteases en vrije zuurstofradicalen die ernstige longweefselschade aanrichten waardoor oedeem ontstaat en de ademhaling (zuurstofopname) ernstig in gevaar komt. Ernstige schade wordt ook toegebracht aan het long surfactant systeem waardoor een collapse van de alveoli optreedt en de gasuitwisseling in toenemende mate belemmerd wordt. Dit rapport geeft een beeld van de pathologische gebeurtenissen die in de long plaatsvinden ten gevolge van een endotoxine-besmetting. Het ziektebeeld dat zich manifesteert komt sterk overeen met dat van ARDS ('shocklong').

De in dit rapport gekarakteriseerde longpathologie ten gevolge van een endotoxinebesmetting vormt een uitstekend model waarmee alternatieve behandelingen onderzocht kunnen worden.

In de research groep Chemische Toxicologie van het TNO Prins Maurits Laboratorium, Divisie Toxische Stoffen wordt reeds gewerkt aan de ontwikkeling van antilichamen tegen endotoxinen en er worden antagonisten van TNFα gesynthetiseerd. Deze behandelingsstrategieën kunnen eveneens in het hier beschreven model getest worden.

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1 Introduction

The Adult Respiratory Distress Syndrome (ARDS) is characterized by high-permeability pulmonary oedema containing plasma-derived proteins, decreased lung volumes, decreased compliance, and arterial hypoxaemia. ARDS results from a number of different causes, of which Gram-negative sepsis and endotoxin (lipopolysaccharide, LPS) from bacteria and aspiration are thought to be major causes to the development of this life-threatening syndrome [1, 2].

Although insight into the clinical course of ARDS patients is increasing, the sequence of pathophysiological events remains poorly understood [3, 4, 5]. Currently, it is becoming widely accepted that an inflammatory reaction occurs in the lungs, in which numerous cellular and humoral mechanisms are involved, including macrophages, neutrophils, platelets, coagulation and fibrinolytic systems [6]. Activated alveolar macrophages (AM) may contribute to this inflammatory reaction by the *in vivo* production of reactive oxygen intermediates (ROI) and chemotactic cytokines, of which TNFα seems to be the major one [7, 8, 9]. Some of the damage done by ROI *in vivo* is assumed to be due to hydroxyl radicals ($OH^{\cdot}$) [10, 11] that emerge from the conversion of $O_2^{\cdot}$ and H_2O_2. Not only *in vivo* experiments were in favour of a potential role for TNFα in pulmonary damage [12], but also *in vitro* experiments supported these findings [13, 14]. *In vitro* TNFα was capable of 'priming' PMNs for secondary stimuli [15, 16, 17]. In addition to the central role of ROI and TNFα, there is clear support for the involvement of a multimediator network that leads to lung tissue injury [18]. During the development of ARDS, the generation of various mediators contribute to the development of severe lung damage. For example, damage to the surfactant system significantly contributes to the lung dysfunction associated with ARDS [19, 20, 21].

Since ARDS may develop as a complication of a variety of disorders, and so far an effective treatment is rudimentary, a wide variety of animal models has been developed. Based on the assumption that sepsis as a consequence of exposure to LPS is one of the main factors contributing to the development of ARDS, models based on challenge of animals with bacterial endotoxin (LPS) were developed [22, 23, 24, 25, 26]. Bacterial LPS is a potent activator of the immune system which induces local or systemic inflammation [27, 28]. Intratracheal aerosolization of LPS in the rat revealed functional, cytological and histological changes in the lungs, comparable to those observed in ARDS patients [26].

The present study extends existing evidence that intratracheal aerosolization of LPS in the rat, may serve as a very relevant model to study human ARDS [26].

2 Material and methods

2.1 Animals

Male albino Wistar rats (180-200 g) obtained from Charles River (Germany) were used in this study. The animals were kept at a regular 12 h light /dark cycle, at a temperature of 22 ± 2 °C. Food and water were given ad libitum.

2.2 Chemicals and solvents

All chemicals were obtained from Sigma (Bornem, Belgium), unless stated otherwise. LPS from Samonella Enteritidis, lyophilized powder, was also obtained from Sigma, L6761, Lotno. 55 F4013. Endotoxin-free Dulbecco's phosphate buffered saline, PBS, at pH 7.4 was used to dissolve LPS for intratracheal aerosolization. Water was demineralized and further purified in a Milli-Q UF plus water purification system (Millipore, Etten-Leur, The Netherlands).

2.3 Administration of LPS and dose-response curve

Under brief halothane anaesthesia each rat was placed on a specially constructed PVC 'bed' at an angle of 60° with the horizontal plane with its head supported by hooking its incisors over an elastic band. This position facilitated intubation with the aerosolizer guided by a fibre optic light source. PBS (vehicle) or LPS solution (0.5 ml) was instilled using a miniature nebulizer (Penn-Century, Philadelphia, PA, USA). The nebulizer consists of a 1 ml syringe in combination with a miniaturized nozzle mounted on the tip of a 5.5 cm stainless steel tube (diameter 1 mm), as described by Wheeldon et al. [26]. This nebulizer allows dispersion of a small volume (0.5 ml) of liquid into the lungs without the necessity of a large volume of air to act as a vehicle, which could damage the small rat lung. The LPS solution was dispersed in the trachea just before the bifurcation. In this way a dose-response curve of the effect of LPS on respiration was constructed after administration of 9, 11, 13, 15, 20, 25 or 40 mg/kg of LPS dissolved in PBS (4 rats per dose). After LPS administration, each animal was placed in a whole-body plethysmograph to measure respiratory frequency (RF). These relatively high dosages of LPS were needed to obtain a manifest clinical picture of ARDS in 24 h. The guiding principle was the deterioration of respiration, i.e. the gradual increase in RF and decrease in tidal volume (Tv). Animals receiving ≥ 20 mg/kg should be ventilated artificially on the second day after LPS challenge. Wheeldon et al. [26] used 7 mg/kg of LPS from Salmonella Enteritidis to induce ARDS in rats. To investigate the course of events following LPS challenge, an LPS dose of 16 mg/kg was used (see below).

2.4 Experimental design

Three groups each containing 42 animals were used in this study: (1) sham-treated animals, i.e. intubation with the aerosolizer without fluid (LPS or PBS) dispersion; (2) animals receiving PBS buffer only; (3) animals receiving LPS (16 mg/kg) dissolved in PBS. Each animal was then put in a whole-body plethysmograph to measure respiratory frequency. After set periods of time (1, 2, 3, 5, 10, 16 and 24 h), groups of 6 animals per time point were sacrificed to determine lung wet weight and to carry out lung lavage (see point 6).

2.5 Assessment of respiratory function

a The equipment to measure the respiratory frequency (RF) consisted of (i) a stand-alone computer with an analogue-to-digital converter card type DAS8/PGA and (ii) a PVC tube with a diameter of 110 cm and a length of 300 cm containing the animal. This tube could be closed on one side and connected to a pneumotachograph (Gould Godart type 17212, with a flow transducer (Fleisch: 1 mm H_2O = 5.9 ml/sec)) on the opposite side. Through the tube passed a flow of 1 l/min. The capacity of the flowmeter (Econorm Comp. Hoekloos, The Netherlands) was 1-15 l/min. The breathing pattern of the animal was superimposed on the constant flow of 1 l/min, which was considered as the base-line. A microprocessor (type 386) collects 512 data points (pressure differences) from the D/A converter at 3 sec intervals. A timer started to run when the first data point was registered and terminated when 512 points had been collected. Each time the sinusoid respiratory signal crossed the x-axis, the programme registered a change from positive to negative and vice versa. In this way the respiratory frequency could be calculated. The integrated area under the curve of the respiratory sinus served as a measure for tidal volume (Tv). Frequency multiplied by tidal volume yielded the respiratory minute volume (RMV).

b PaO_2 and $PaCO_2$ were measured under artificial ventilation. Separate groups of anaesthetized and artificially ventilated LPS (16 mg/kg) treated (n=6) or control (naif) rats (n=6) were used. These values were determined 24 h following LPS administration. Under halothane anaesthesia, a catheter (containing 5000 IU of heparine/ml saline) was placed in the left carotid artery. Before tracheotomy, the animals received an injection of pentobarbitone (60 mg/kg/ml i.p.). The trachea was cannulated with a tube which was secured by ligature. Six animals at the same time were connected to a distributor unit that in turn was connected to a Servo Ventilator (900 C, Siemens-Elema, Solna, Sweden). The animals were ventilated simultaneously at a respiratory rate of 30 breaths/min, a fraction of inspired oxygen (F_iO_2) of 1.0, an inspiration ratio of 1:2 and a peak inspiratory pressure (PIP) of 28 cm H_2O which included a positive end-expiratory pressure (PEEP) of 8 cm H_2O. Blood gas analysis was

performed with a blood gas analyser (Radiometer Copenhagen ABL 500). At the start of the experiment, blood was taken from the arterial catheter to determine pretreatment values of the animals under the described ventilatory settings.

2.6 Lung wet weight and bronchoalveolar lavage (BAL)

To determine lung wet weight, animals were sacrificed and the thorax was opened. The trachea was separated from thymus and oesophagus and cut just below the larynx. The lungs still connected to the heart were dissected and weighed. Then bronchoalveolar lavage was carried out using 5 subsequent volumes of 8 ml of PBS buffer. After centrifugation of the volumes of BAL-fluid, two samples of 100 µl were taken from the supernatant of the first lavage volume of 8 ml to assess TNFα. After mixing the 5 volumes of lavage fluid, a sample of 300 µl was taken for cell counting and assessment of other parameters (see below). The rest of the BAL fluid was stored at -20 °C for analysis of surfactant parameters. Lung tissue was stored at -70 °C for analysis of glutathione (GSH).
Following lavage, the lung lobes were dissected from the trachea. To determine lung wet weight, the total weight of heart and trachea were subtracted from total weight of trachea plus lungs plus heart before performing the lavage. Lung wet weight is expressed as a percentage of whole body weight (% LWW/BW).

2.7 Determinations in BAL

2.7.1 Cellular contents

Cellular constituents were determined by cytocentrifuge preparations. The slide was air-dried for 24 h before it was stained with May-Grunwald and Giemsa (Merck, Darmstadt, Germany). A hundred cells were counted and the percentage alveolar macrophages (AM), polymorphonuclear neutrophils (PMN), other leukocytes and erythrocytes was calculated.

2.7.2 Lactate dehydrogenase (LDH) and total protein

LDH in BAL fluid was assessed according to Bergmeyer et al. [29]. Protein in BAL fluid was determined using the method of Bradford [30] with BSA as a standard.

2.7.3 TNFα assessment

Rat TNFα levels in BAL fluid and plasma were determined by means of a commercially available immunoassay kit specific for rat TNFα (ImmunoSource, Zoersel-Hall, Belgium). The detection limit of this kit was 1 U corresponding with 50 pg TNFα protein. TNFα was determined exactly according to the manufacturer's instructions. To determine its biological activity, TNFα was also measured

by bio-assay. In this assay, 1U of TNFα is defined as the amount of TNFα that has a cytopathologic effect on 50% of WEHI-1640 cells. This colorimetric method is based on the capacity of mitochondrial enzymes of viable cells to convert tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium (MTT) into the blue-coloured formazan [31].

2.7.4 Detection of $OH^{\cdot}$ radicals

Detection of $OH^{\cdot}$ radicals was performed according to the method of Sloot and Gramsbergen [32]. In brief, 2 h before sacrificing the animals, salicylate was injected (300 mg/kg/ml i.p.) for trapping $OH^{\cdot}$ radicals by forming mainly 2,3- and 2,5-dihydroxybenzoic acid (2,3-DHBA and 2,5-DHBA, respectively. Salicylic acid and its 2 hydroxylated products were measured by HPLC using UV and EC detection. [33, 34]. Moreover, a 2 h infusion (1 ml/h from a concentration of 25 mM) of tertiary butyl hydroperoxyde (TBH) (25 mM; 1 ml/h, during 2 h), a compound producing $OH^{\cdot}$ radicals *in vivo*, resulted in a marked increase in the ratio of 2,3-DHBA/salicylate (data not shown). In the present study, $OH^{\cdot}$ radicals were determined 0, 2 and 3 h after LPS administration. These relatively early time points were chosen because the start of the production of oxygen radicals is expected to be early in the development of ARDS.

2.7.5 Isolation and quantification of SP-A

SP-A was isolated from BAL fluid as described by Haagsman et al. [35]. Aliquots of the purified protein were stored in 5 mM HEPES, pH 7.4, at -20 °C. The concentration of SP-A was measured using a sandwich enzyme-linked immunosorbent assay as described by Oosterlaken-Dijksterhuis et al. [36]. Purified rat SP-A was used for the standard curves. Surfactant phospholipids were measured using a standard method to assess phosphorus. Antibodies were prepared as described by Oosterlaken-Dijksterhuis et al. [36]. SP-A levels in BAL were related to the total volume of BAL fluid (40 ml).

2.8 Determinations in lung tissue

2.8.1 Histology

Lungs dissected from rats 24 h following administration of LPS (20 mg/kg) were used for histologic examination. The trachea was cannulated and the lungs dissected free from the thoracic wall. Lung fixation was performed by intratracheal instillation of 10% formalin under pressure (30 cm H_2O). All tissues were processed using standard histologic methods, sectioned at 3 μm, and stained either with haematoxylin and eosin or with Martius scarlet blue, a specific stain for fibrin [37].

Homogeneous distribution of LPS was evaluated by 'blind' assessment of the degree to which each of the 5 lung lobes were infiltrated by PMNs 24 h after intratracheal dispersion of LPS. This was considered to reflect the extent of the

inflammatory process throughout the lungs. For that purpose, a 5 points scale was used, each point being equal to approximately 20% of the maximal PMN density found in any lobe. The mean scores per lobe calculated for 8 control and 8 LPS-treated animals were calculated.

2.8.2 Measurement of glutathione in lung tissue

The assay is based on the reduction of 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) into 2-nitro-5-mercaptobenzoic acid by free -SH groups. The method used has been described by Polhuijs et al. [38], consisting of two parts: (1) assessment of the amount of reduced non-protein thiols (NPSH); (2) assessment of the total amount of glutathione (reduced (GSH) and oxidized glutathione (GSSG). In tissues containing a high concentration of GSH (liver, kidney and lung), the contribution of other non-protein thiols than GSH can be neglected so that the concentration of NPSH represents the concentration of GSH. The amount of oxidized GSH was calculated from the amount of total glutathione and the amount of reduced NPSH.

2.9 X-rays

Chest X-rays were made of anaesthetized rats using a Philips type ZN20 Mammadiagnostic-4 equipment (exposure time 0.4 sec, 30 mA).

2.10 Statistics

All data are presented as means ± SEM. For homogeneity of variance, the Bartlett's test was used. If the variances were homogeneous, a one-way ANOVA was performed. For intergroup comparisons, the Newman-Keuls method was used. If they were not homogeneous, the Kruskal-Wallis test was performed to analyse the data. Differences between the means were considered to be statistically significant if $p < 0.05$.

3 Results

In general, animals intratracheally exposed to LPS (9-40 mg/kg) displayed symptoms of illness 24 h after exposure in that their fur had raised, and they hardly ate and drank, and breathed in a fast and superficial way.

Distribution of PMNs over the lungs and dose-response effect

A semi-quantification of the density of the inflammatory process throughout the various lobes of the lungs, revealed a remarkable homogeneous distribution of the infiltration of PMNs 24 h after LPS (20 mg/kg) administration (Figure 1a). There were no significant differences between the scores in the different lobes. This indicates a homogeneous distribution of LPS-induced inflammation over the various lobes of the lungs.

Already at the lowest dose (9 mg/kg) tested, LPS induced a significant rise in respiratory frequency (RF) 24 h after administration compared to PBS-treated animals (Figure 1b). At the higher doses of LPS (15, 20, 25 mg/kg), no great changes in RF occurred, although approximately 50% of the animals died on the subsequent days due to respiratory distress and hypoxaemia.

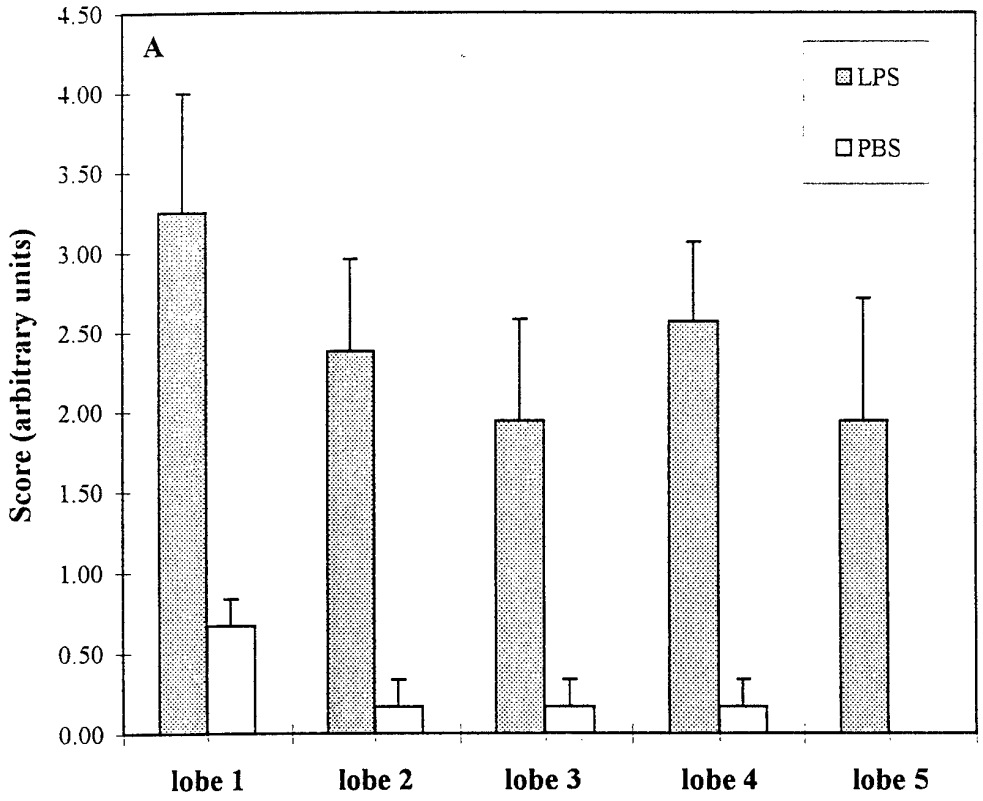

Figure 1a: Semi-quantification of the density of the PMN infiltration representing the extent of the inflammatory process throughout the lobes of the lungs 24 h after LPS (20 mg/kg) according to a 5 points scale. Expressed is the mean ± SEM score per lobe per 6 animals.

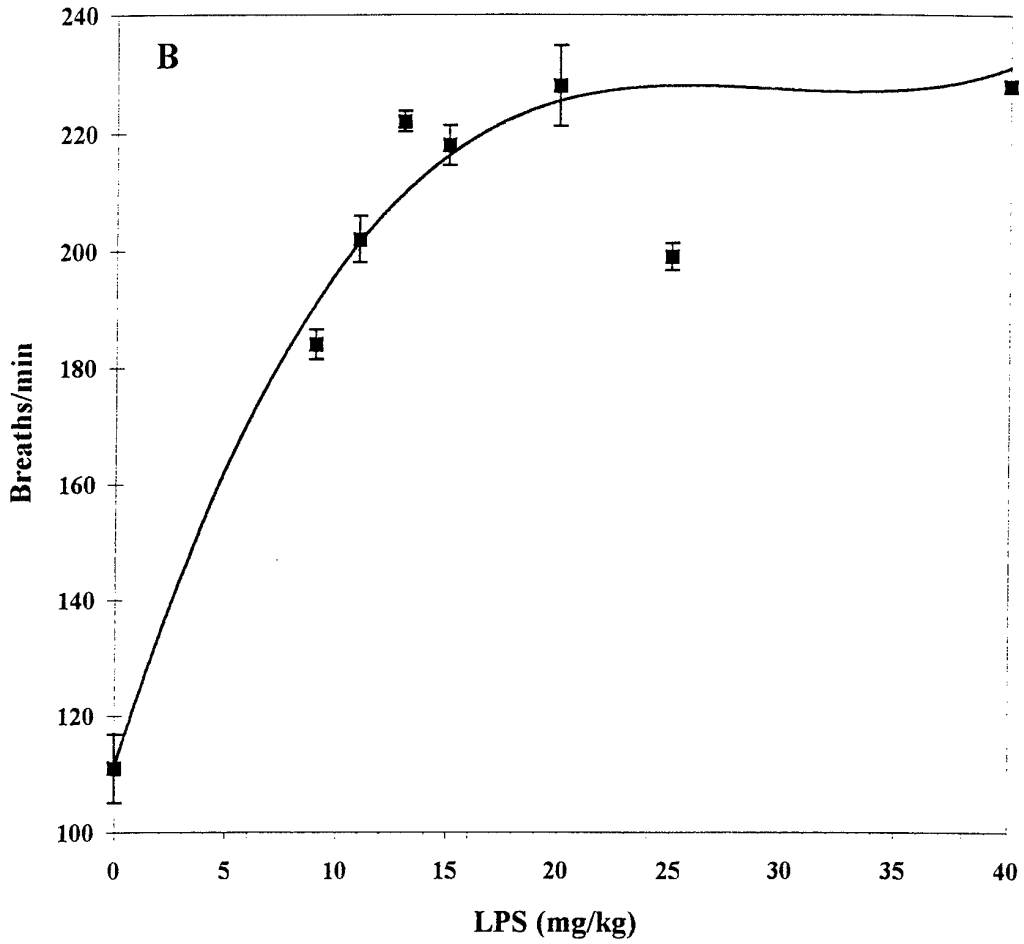

Figure 1b: Dose-response curve of LPS on respiratory frequency (breaths/min), measured 24 h after intratracheal aerosolization (n=4 animals per dose).

Development of ARDS

3.1 Changes observed in RF, Tv and RMV

A time course study following administration of 16 mg/kg revealed a gradual increase in the respiratory frequency (Figure 2a), a considerable decrease in tidal volume (Tv) and an increased respiratory minute volume (RMV) (Figure 2b). All these values differed significantly from control values 7 h after LPS.

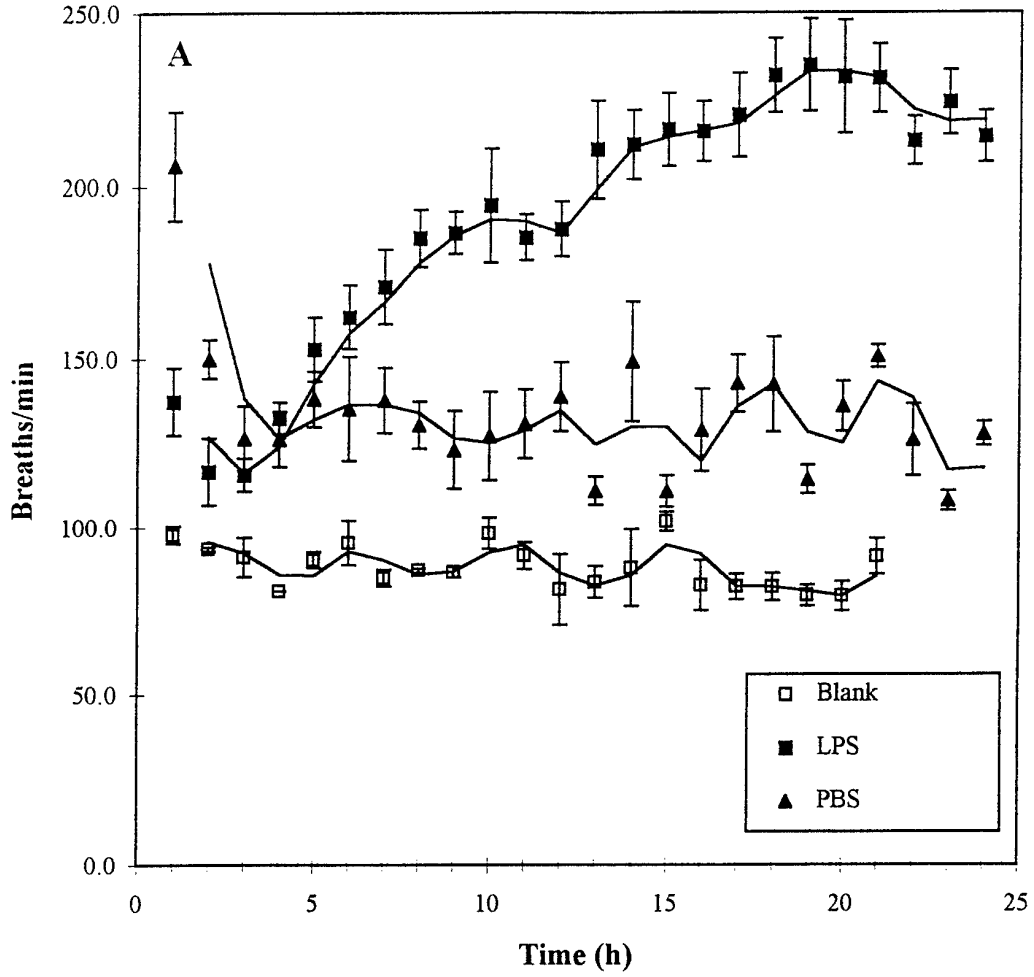

Figure 2a: Respiratory frequency (breaths/min) of rats (n=6) after intratracheal aerosolization of LPS (16 mg/kg), PBS at t=0, or unexposed (blank).

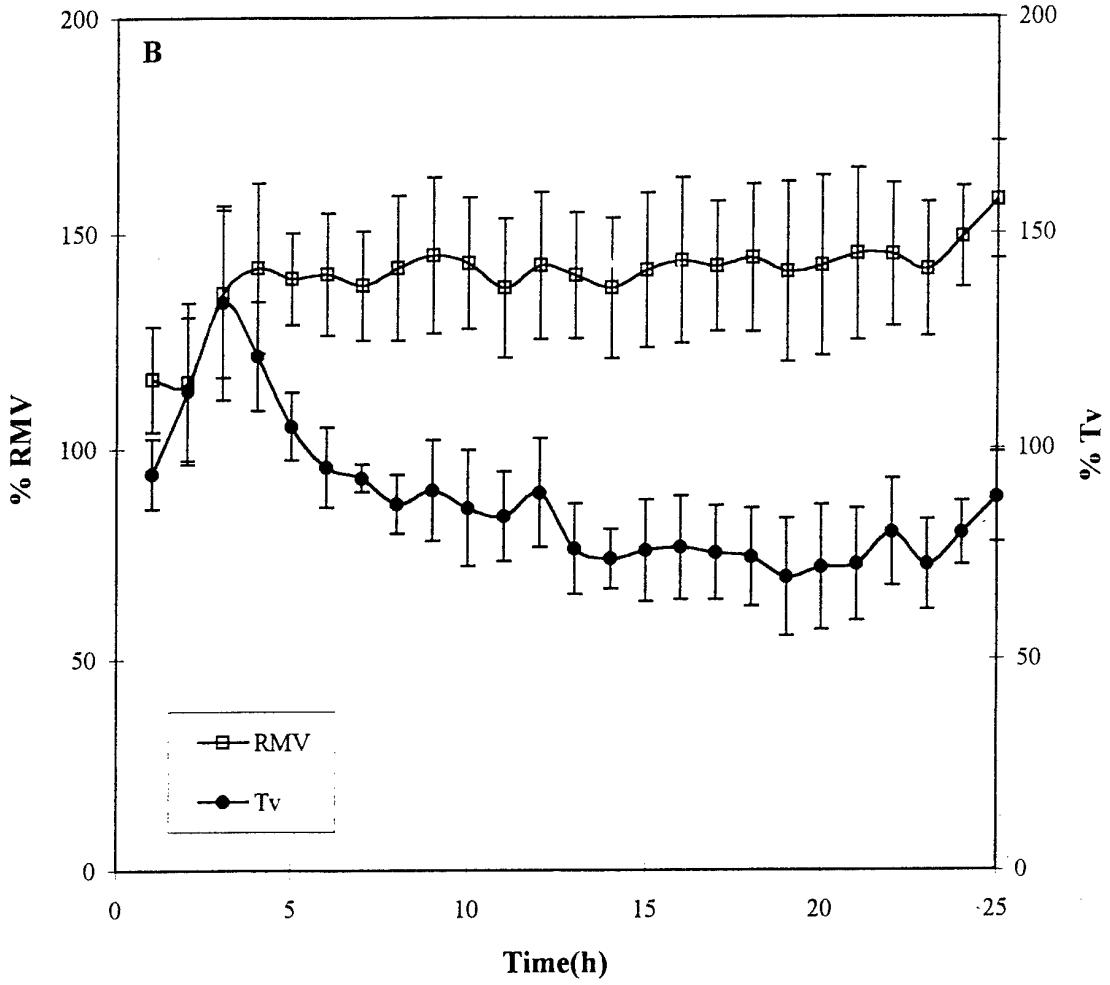

Figure 2b: Tidal volume (Tv) and Respiratory Minute Volume (RMV) after LPS. The latter values are not shown for the blank.

3.2 Changes in PaO_2, $PaCO_2$, HCT and Hb

Whereas control animals demonstrated a mean PaO_2 value of approximately 500 ± 15 mm Hg and a $PaCO_2$ of about 36 ± 6 mm Hg under the ventilatory conditions used, the LPS challenged animals had a mean PaO_2 value of approximately 100 ± 16 mm Hg and a $PaCO_2$ of 53 ± 10 mm Hg (not shown). Haemoglobin (Hb) and haematocrit (HCT) were significantly enhanced 24 h after LPS (2.5% and 5% from control values, respectively, not shown).

3.3 Changes in oedema parameters (LWW, protein, LDH in BAL)

Lung wet weight demonstrated a 3-fold increase at 5 h after LPS ($p < 0.05$), a clear dip at 10 h ($p < 0.05$), and a subsequent 2-fold final increase (Figure 3). BAL fluid contained an increased amount of protein; at 10h after LPS it had reached a plateau at which the amount was 16-fold higher than before LPS administration. LDH, only measured 24 h after LPS, was increased significantly (35-fold, compared to control values. Already 3 hrs after LPS challenge, significant changes could be observed in these parameters. To reduce the number of figures, these values of BAL fluid proteins and LDH were not shown.

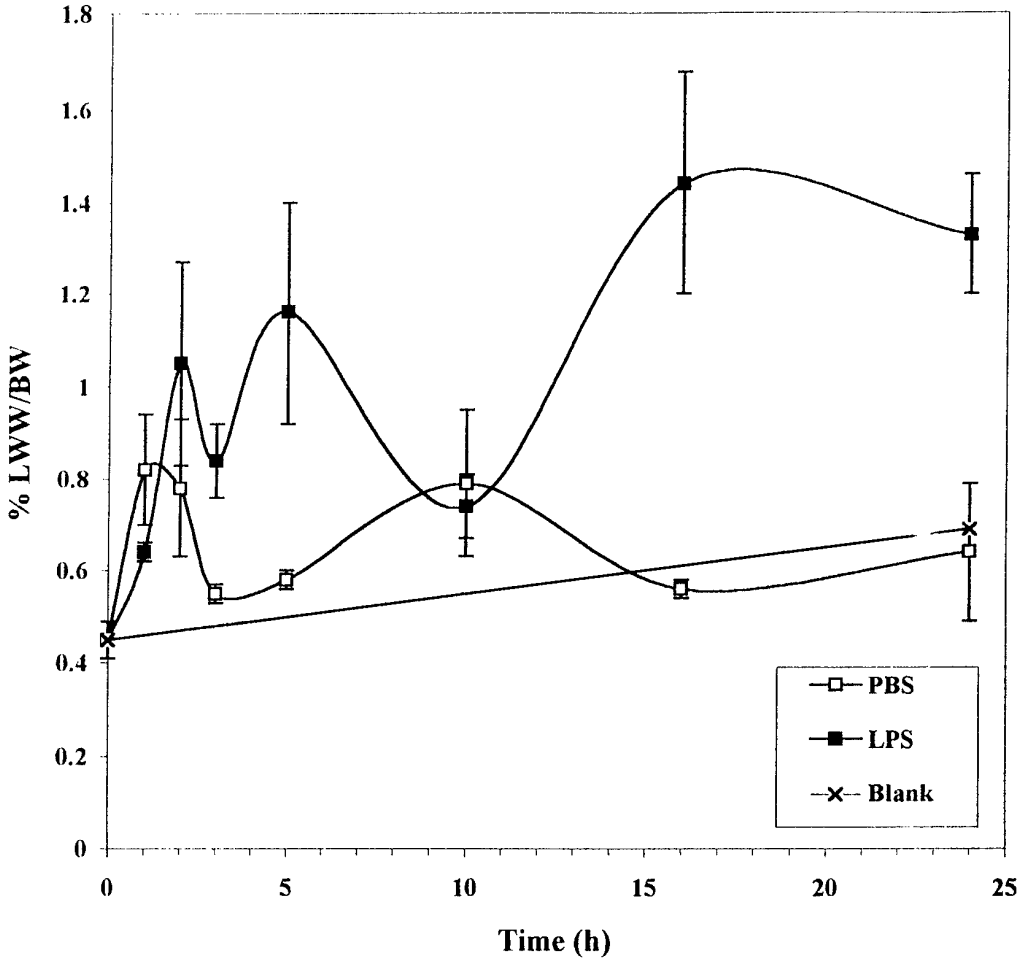

Figure 3: Lung wet weight (% LWW/BW after LPS (16 mg/kg), PBS or sham (blank) exposure (n=6 animals per group).

3.4 Changes in cellular constituents in BAL and lung tissue

The numbers of PMNs, erythrocytes and alveolar macrophages (AM) increased in time after administration of LPS (Figure 4a) compared to control values (Figure 4b). The number of erythrocytes was already significantly ($p< 0.05$) increased 1h after LPS (0.5×10^6 cells) and increased 32-fold 5 h after LPS. The number of PMNs increased gradually in time: it was significantly ($p< 0.05$) increased at 5h and reached a plateau-like level 10 h after LPS (4×10^6 cells). The number of AMs hardly increased after LPS administration. Also epithelial cells were present in BAL fluid from LPS-challenged rats, but they were not counted. In the PBS challenged animals, there was only a slow and temporary insignificant increase of PMNs at 10 h.

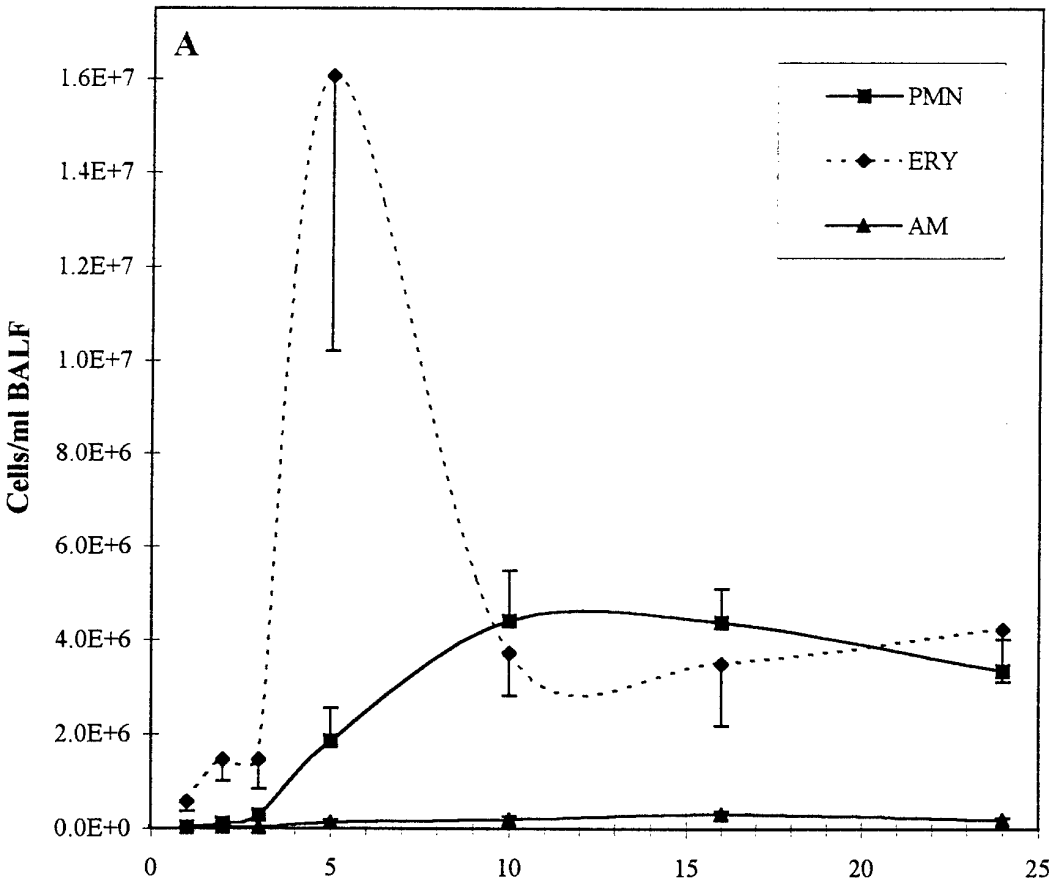

Figure 4a: Counts of alveolar macrophages (AM), PMNs and erythrocytes (ERY) in BAL fluid after LPS (16 mg/kg) challenge.

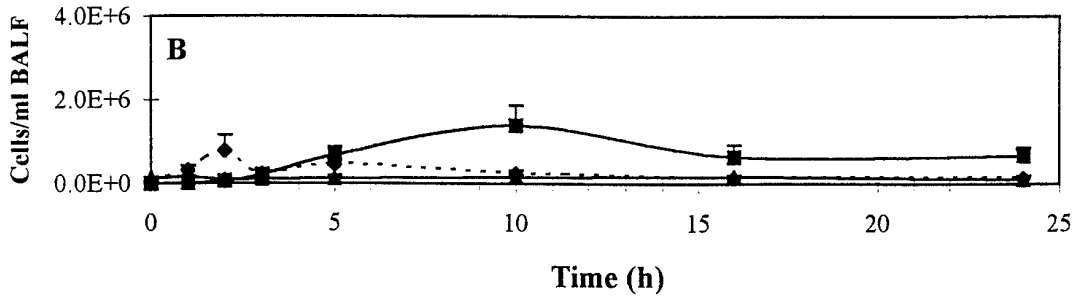

Figure 4b: After PBS challenge at t=0.

3.5 Changes in ROI, GSH and TNFα

There was a significant ($p < 0.05$) decrease in the amount of $OH^{\cdot}$ radicals in BAL fluid 2 h and 3 h after LPS administration (Figure 5a); Figure 5b shows that the amount of oxidized GSH (GSSG) in lung homogenates 24 h after LPS decreased significantly ($p < 0.05$), whereas the total amount of glutathione (GSH and GSSG) remained unaltered. As a consequence, the GSH/GSSG ratio largely increased. A time course study of the amount of TNFα measured in BAL fluid showed a maximal increase (500 U/ml corresponding to 25.000 pg/ml) of this cytokine about 5 h after LPS challenge (Figure 5c). In plasma, the amount of TNFα was below 3 U/ml (corresponding to 150 pg/ml) plasma at each time point. Assessment of the biological activity of TNFα showed that approximately 50% of the total amount of TNFα determined by ELISA at each time point was biologically active.

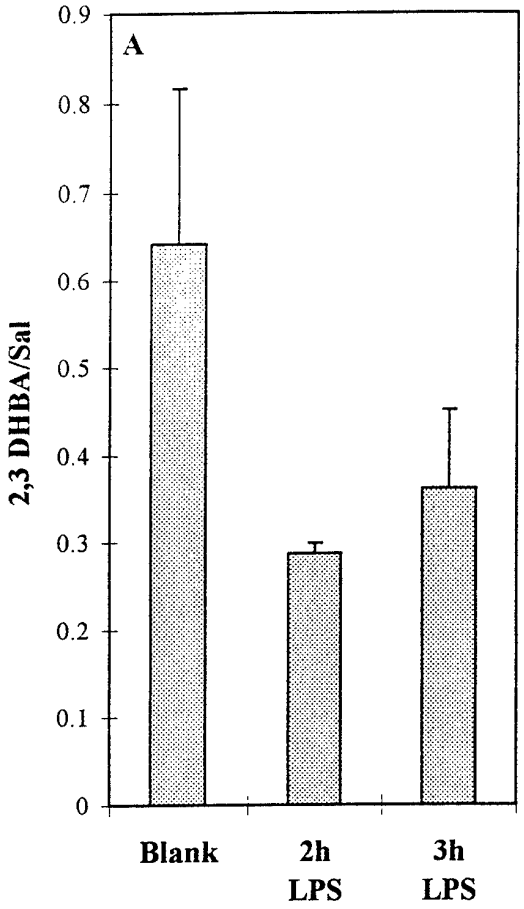

Figure 5a: Amount of OH· radicals in BAL fluid before (control) and 2 h and 3 h after LPS (16 mg/kg).

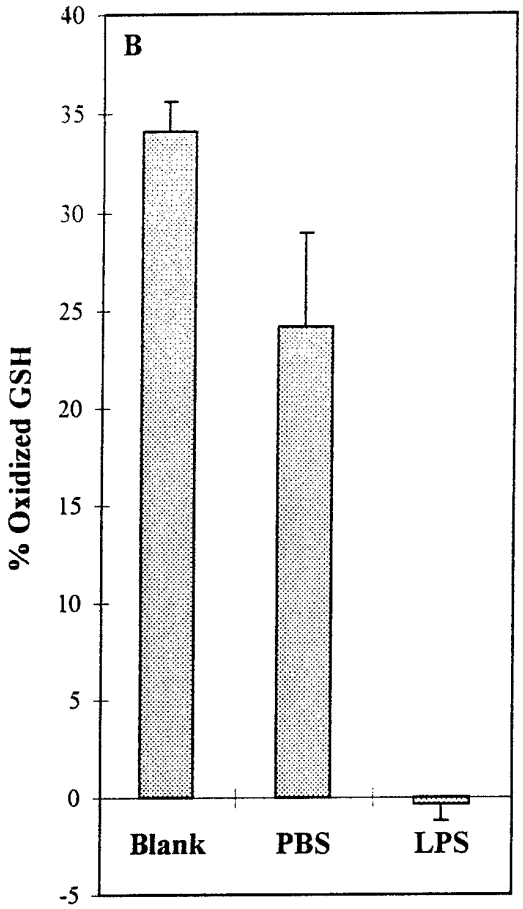

Figure 5b: Amount of oxidized GSH in lung homogenates 24 h after LPS (16 mg/kg), PBS or sham (blank) challenge.

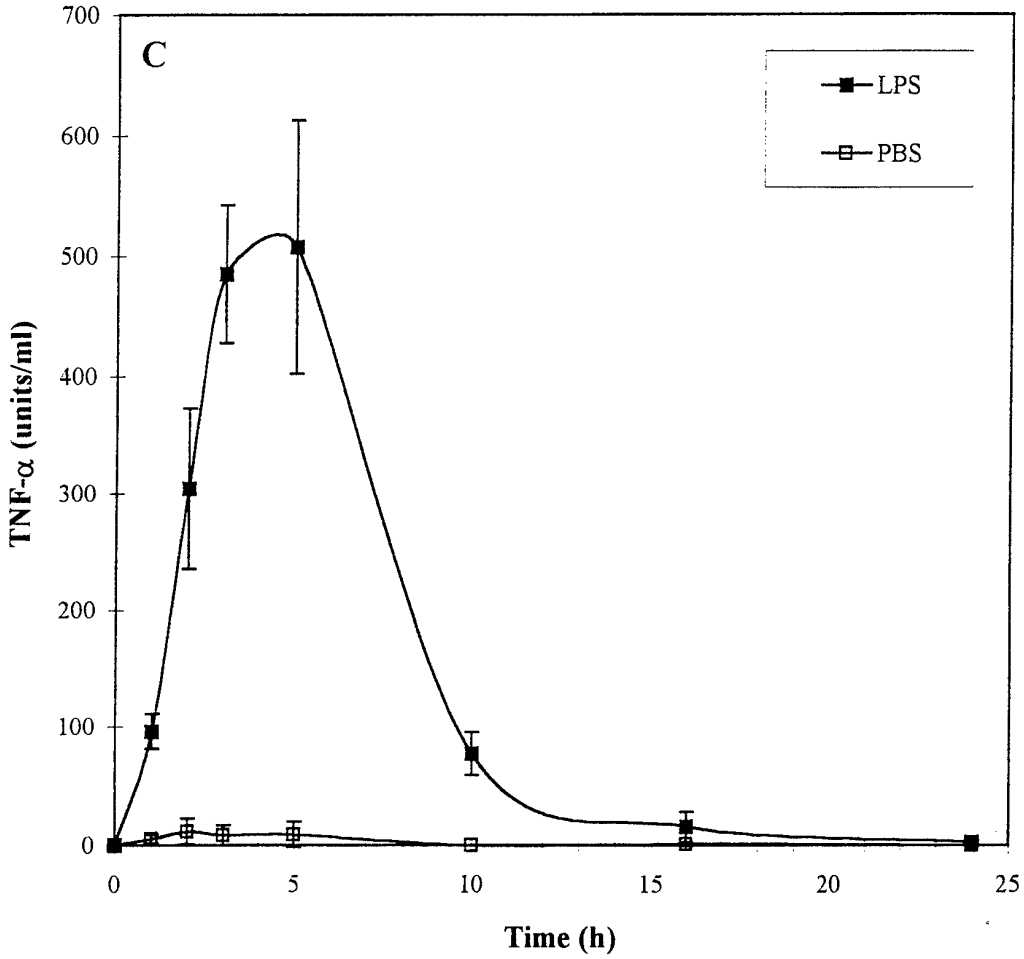

Figure 5c: TNFα determined in BAL fluid after LPS (16 mg/kg), or PBS challenge.

3.6 Changes in the surfactant system

Figure 6 shows a rapid two-fold significant ($p < 0.05$) increase of SP-A in lavage within the first hour, followed by a gradual increase until 10 h after LPS (16 mg/kg) and attained control values 16 h after LPS challenge. There was a short-lasting increase of SP-A in BAL fluid of control animals.

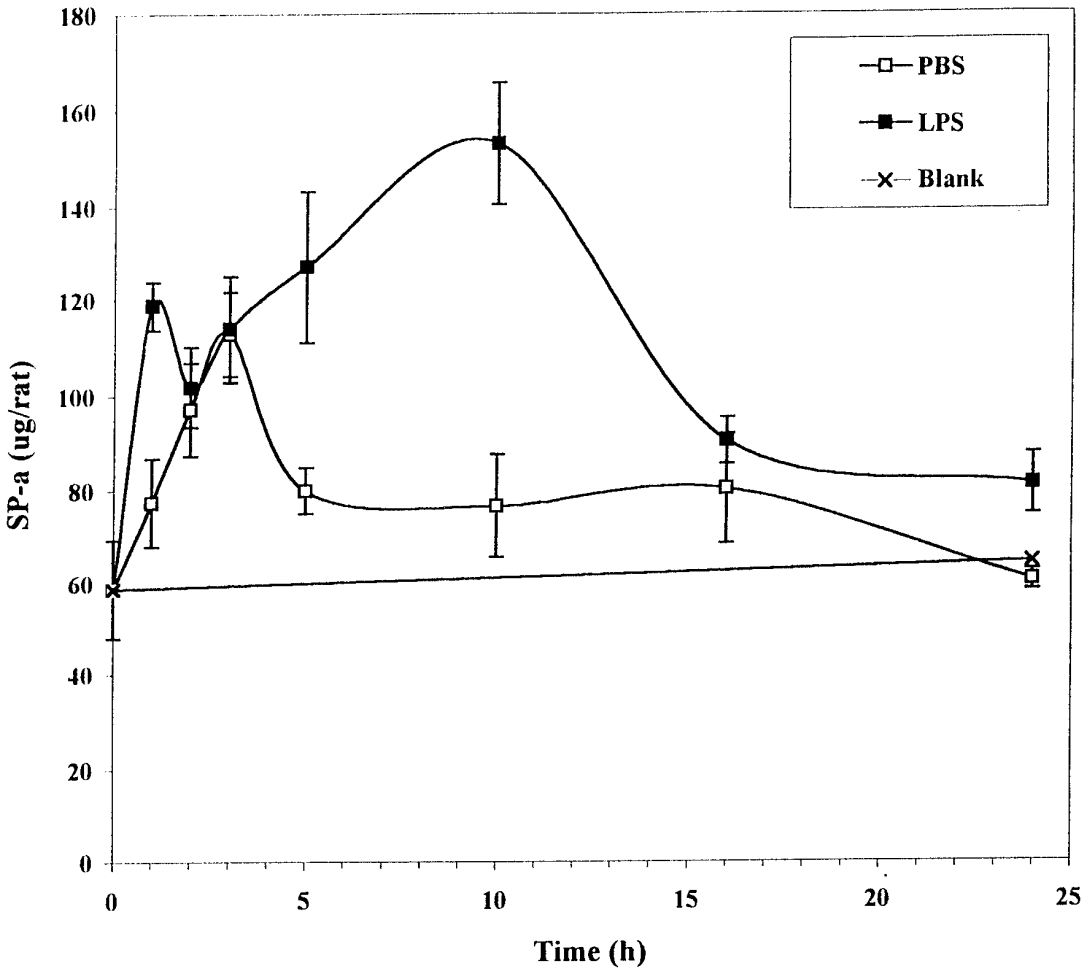

Figure 6: Amount of SP-A in BAL fluid of animals (n=6) following exposure (at t=0) to LPS (16 mg/kg), PBS or unexposed (blank).

3.7 Histopathology and X-ray

The histopathology of ARDS was characterized 24 h after LPS (not shown). As in BAL fluid, there were extensive PMN infiltrates in the alveolar space and in the interstitial tissue. Irregular areas of consolidation were present as well as a diffuse thickening of the alveolar wall and loss of the alveolar-capillary wall. Thorax X-rays demonstrated a marked alveolar consolidation in all quadrants of the lungs compared to control (not shown).

4 Discussion

The present data demonstrate that intratracheal aerosolization of LPS in the rat results in the development of an acute (within 24 h after LPS) and sturdy neutrophilic alveolitis that closely resembles that of human ARDS. Several points should however be addressed.

Comparison with other models of ARDS

In the past, several other models have been proposed to mimick ARDS [20]. From a pathogenetic point of view some arguments may be put forward why the model presented here is more relevant than previous ones and could therefore be used in future studies dealing with the pathogenesis and therapy of ARDS.

- Statistically 70% of clinical ARDS pictures appear to be associated with sepsis (40%) and aspiration of gastric contents (30%) [43]; the latter probably also reflecting infection. Based on the assumption that sepsis as a consequence of exposure to the natural pathogen LPS is one of the main factors contributing to the development of ARDS, a model based on challenge of animals with bacterial endotoxin (LPS) should be preferred.
- In ARDS patients, inflammatory reactions occur in the lungs in which alveolar macrophages, neutrophils, platelets and several mediators, such as TNFα, proteases, arachidonic acid metabolites and perhaps oxygen radicals are involved, which all together are thought to be responsible for injury to the alveolar-capillary membrane [39]. It is unlikely that these complex and sequential events will be mimicked in animal models of ARDS which are not based on LPS challenge[20], albeit that the lung injury obtained, often seem to be physiologically and morphologically similar to changes seen in ARDS patients.
- In our model, the lungs were directly exposed to LPS opposed to i.v. administration of LPS in some other models. However, i.v. administration of endotoxin does neither consistently cause pulmonary alveolar oedema, nor alveolar epithelial injury [40]. Since endotoxin has been measured in blood of ARDS patients [41], it suggests that direct exposure of the lungs to LPS or bacteria had caused ARDS. Inhalation of LPS-bearing aerosol has been suggested to contribute largely to lung inflammatory reactions to inhaled organic dusts in humans [42, 43]. Since alveolar oedema is consistently present in our model, it suggests that ARDS induced by intratracheal LPS-challenge is most suitable.

The relevance of the rat model presented here for human ARDS

In our rat model, the development of ARDS could be diagnosed on the basis of a lung injury score developed for human ARDS by Murray et al. [44] (see Table 1). This injury score includes the extent of X-ray densities, the degree of hypoxaemia, the reduction in compliance and the level of PEEP. With exception of the compliance (not measured), all measured parameters indicated severe ARDS.

Table 1: Lung injury score by Murray et al. (1988). Indicated are the scores found in our animal model.

		Value	Score
1 X-ray	No alveolar consolidation		0
	Consolidation 1 quadrant		1
	2 quadrant		2
	3 quadrant		**3**
	4 quadrant		**4**
2 Hypoxeamia	PaO_2 (when ventilated)	> 300	0
		225-299	1
		175-224	2
		100-174	**3**
		< 100	**4**
3 Compliance	(when ventilated)	≥ 80 ml/cm H_2O	0
		60-79	1
		40-59	2
		20-39	3
		≤ 19	4 (see text)
4 PEEP	(when ventilated)	< 5 cm H_2O	0
		6-8	1
		9-11	2
		12-14	3
		>15	**4**
	Sum of scores/ no. of parameters = 0 : no injury		
	0.1-2.5 : mild to moderate		
	> 2.5 : severe injury (**ARDS**)		

Although we did not measure the compliance, it can easily be calculated by dividing the tidal volume (Tv) by the plateau airway pressure minus the PEEP. A normal value for Tv in rats is 1.5 ml. The mean Tv of the rats 24 h after LPS administration was 75% of the control value, i.e. 1.12 ml (Figure 2b). The mean plateau airway pressure 24 h after LPS was 28 cm H_2O (PIP=28 cm H_2O; PEEP=8 cm H_2O), i.e. the lungs required much higher than normal airway pressures to inflate them to normal Tv. Thus, the mean compliance is 1.12/20 = 0.056 ml/cm H_2O. Extrapolated to human values (Tv is about 350 x higher) this would correspond to 19 ml/cm H_2O (compare Table 1). Taken together, all clinical lung injury scores as suggested by Murray et al. are present in our rat model, supporting its relevance.

Moreover, other features of human ARDS appeared also to be present in our animal model: (1) oedema formation, (2) generation of ROI and TNFα (3) alterations in the amount of SP-A in BAL fluid.

4.1 Oedema formation

Oedema emerged in the first hours following exposure to LPS, indicated by an increased lung wet weight. This coincided with a strong increase in protein content in BAL fluid together with large numbers of PMNs and erythrocytes (indicating endothelial and epithelial damage).
In the early phase of respiratory failure from acute lung injury, ARDS patients also develop severe alveolar oedema accompanied by large numbers of inflammatory cells, primarily neutrophils, in the alveoli and interstitium of the lung [45]. Initially, the oedema fluid has a high concentration of protein (75-95% of plasma protein concentration), which is characteristic of an increased-permeability oedema [46]. Ultrastructural studies of the lungs from patients who died in the first 24 h after developing ARDS showed evidence of lung endothelial and considerable epithelial injury, presumably leading to capillary leakage and pulmonary oedema [47].

4.2 Generation of ROI and TNFα

Although it was assumed that LPS would stimulate alveolar macrophages to generate massive amounts of ROIs, the amounts of oxidized GSH and $OH^{\cdot}$ radicals were lower in BAL fluid of animals challenged with LPS than in those not challenged. This suggests that the naturally present antioxidant systems in the lung were able to cope with the production of oxygen radicals. An explanation for this may be that an increased glutathione redox cycle may be induced by hypoxia and protects the lungs against oxidants. This phenomenon was previously reported by White et al. [48] who demonstrated that pre-exposure of rats to hypoxia increased survival and reduced the GSH/GSSG ratio in animals subsequently exposed to continuous hyperoxia.

In the rat model described, here TNFα emerged in BAL fluid during the first hours following exposure to LPS (peak at 5 hrs). The generation of TNFα preceded the huge infiltration of PMNs, suggesting generation of TNFα by alveolar macrophages in the early development of ARDS. In ARDS patients, high levels of TNFα have been found in BAL fluid [9, 49]. Moreover, Van Nhieu et al. [50] reported that alveolar macrophages derived from ARDS patients produced TNFα. Thus, from a pathogenetic point of view, these data indicate that alveolar macrophages are triggered by LPS to generate and release TNFα. Upon release of TNFα, endothelial cells are activated to express adhesion molecules to allow neutrophil adhesion and transmigration [51]. Infiltrated PMNs may then be triggered by TNFα to release proteases and ROIs which could damage the surfactant system. In our rat model, an increase of mobilized PMNs was always associated with a deterioration of lung function.
Although their role is not yet fully elucidated, infiltrated PMNs certainly contribute to the further development of ARDS [52, 53]. Indications are:

- the vast majority of BAL cells recovered from ARDS patients are PMNs [54];
- the number of PMNs in the lungs reflects the severity of ARDS. At least infiltrated PMNs will be activated by TNFα [12, 15, 16, 17].

Another remarkable finding concerning TNFα was that the amount of this cytokine was high in BAL fluid, whereas that in plasma remained very low. Ghofrani et al. [55] demonstrated in buffer-perfused rabbit lungs that although intravenous or alveolar administration of LPS induced large amounts of TNFα release in both compartments, TNFα was found to be largely confined to the compartment in which the LPS challenge had occurred. This observation is in line with data from Boujoukos et al. [56], who did not detect an increase in the TNFα mRNA expression in BAL cells originating from human volunteers undergoing intravenous endotoxin challenge. These findings together support the hypothesis that human ARDS is induced by intra-alveolar exposure to LPS, and this again suggests that an ARDS model induced in a similar way is most suitable.

4.3 Alterations in the amount of SP-A in BAL fluid

It was found that SP-A levels in BAL fluid doubled within 1 h after LPS challenge. This suggests that SP-A play a role in the immediate inflammatory response and may represent an early diagnostic marker for ARDS.

An interesting finding in the present study was that at about 10 hours after LPS challenge, two events occurred:

- SP-A in BAL fluid was maximal (Figure 6), whereas;
- this coincided with a dip in the amount of oedema (Figure 3). This may reflect an important property of SP-A: the protection of surfactant against inactivation by serum protein. This effect of SP-A has been observed both *in vitro* [57] and *in vivo* [58].

TNFα demonstrated a maximal concentration at t=5 h after LPS (Figure 5c). When TNFα was down at t=10 h, SP-A was maximal. This might reflect that TNFα had triggered the release of SP-A. It has been reported that TNFα affects SP-A production [59, 60] and vice versa [61]. The ultimate decrease in SP-A in the BAL fluid between t=15 h and t=24 h (Figure 6) may point to a shortage in SP-A, resulting in a large increase in oedema (Figure 3). This finding indicates that administration of SP-A containing exogenous surfactant could be very promising in the treatment of ARDS.

In conclusion, intratracheal aerosolization of LPS in the rat resulted in the diagnosis ARDS within 24 h. The development of typical signs of human ARDS appeared to resemble those found in this model. As all present parameters can be measured *in vivo* at several time points in the sequence of pathogenic events, this model is useful to test specific therapeutic interventions for this complex syndrome.

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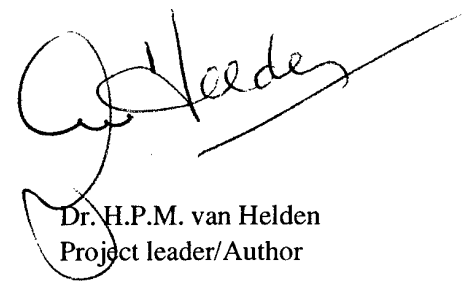

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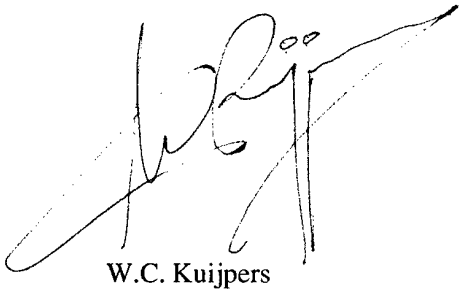

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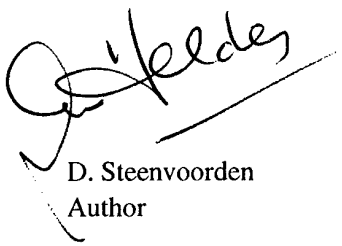

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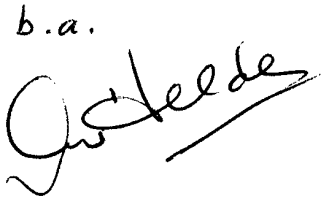

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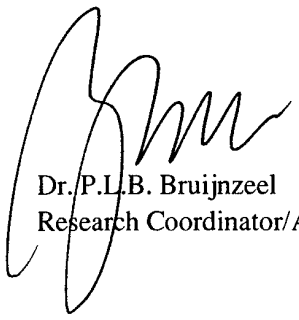

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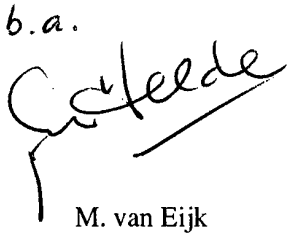

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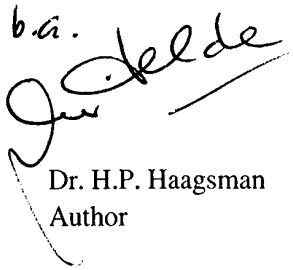

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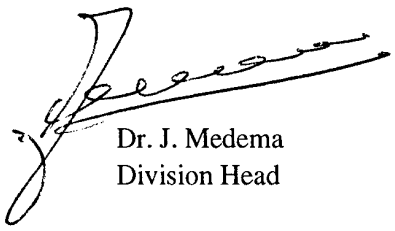

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Intratracheal aerosolization of endotoxin (LPS) in the rat: a comprehensive animal model to study adult respiratory distress syndrome (ARDS)

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14. SUPPLEMENTARY NOTES

The classification designation Ongerubriceerd is equivalent to Unclassified.

15. ABSTRACT (MAXIMUM 200 WORDS (1044 BYTE))

The aim of the study was to extend existing evidence that intratracheal aerosolization of LPS may serve as a very relevant model to study ARDS.
We investigated the sequence of pathogenic events reflected by changes in levels of tumor necrosis factor alpha (TNFα), surfactant-associated protein A (SP-A) in BAL fluid, in addition to cell count, oedema formation and respiratory function. Within 24 h following intratracheal aerosolization of LPS in the rat, ARDS could be diagnosed according to the lung injury score for patients. This score includes the extent of the inflammatory density on chest X-rays, the severity of hypoxaemia, the decline in lung compliance, and the level of PEEP. In addition, other typical features of human ARDS appeared to be present in our model: (1) increased microvascular permeability reflected by oedema, elevated levels of protein and of LDH, and increased numbers of PMNs in BAL fluid; (2) high levels of TNFα in BAL fluid preceding the appearance of PMNs; (3) changes in breathing pattern and a gradual development of respiratory failure with decreased compliance. SP-A levels in BAL fluid doubled within one hour after LPS administration suggesting that this collectin may play a role in the immediate inflammatory response.
Taken together, the findings presented here suggest that intratracheal LPS administration mimicks the clinical development of ARDS very closely.

16. DESCRIPTORS	DESCRIPTORS
ARDS	TNFα
Animal model	Hydroxyl radical
Intratracheal LPS aerosolization	Surfactant protein A
Lung injury score	Respiratory failure

17a. SECURITY CLASSIFICATION (OF REPORT)	17b. SECURITY CLASSIFICATION (OF PAGE)	17c. SECURITY CLASSIFICATION (OF ABSTRACT)
Ongerubriceerd	Ongerubriceerd	Ongerubriceerd

18. DISTRIBUTION AVAILABILITY STATEMENT	17d. SECURITY CLASSIFICATION (OF TITLES)
Unlimited Distribution	Ongerubriceerd

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