ALVEOLAR macrophages have recently been postulated as being involved in the aetiology of adult respiratory distress syndrome (ARDS). To evaluate their role, basal cyclic AMP levels and responsiveness of adenylyl cyclase alveolar macrophages were determined at four intermediate stages of developing respiratory distress in piglets using a protocol with repeated lung lavage. Examination of alveolar cells recovered from the subsequent lavages reveals an influx of granulocytes (neutrophils and eosinophils) within 1 h of two intensive lung lavages. During the developing respiratory distress the basal cyclic AMP-level of alveolar macrophages increases and adenylyl cyclase responsiveness to prostaglandin E₂ (PGE₂) and isoprenaline diminishes. The previously observed impairment of macrophage activity can then be explained at a subcellular level.

Key words: Adenylyl cyclase, Alveolar macrophages, ARDS, cAMP, Granulocyte influx, PGE₂, Phagocytosis

Pulmonary granulocyte influx and impaired alveolar macrophage adenylyl cyclase responsiveness in developing respiratory distress

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Introduction

Adult respiratory distress syndrome (ARDS) is characterized by severe dyspnoea, hypoxaemia, diffuse bilateral pulmonary infiltrates and decreased lung compliance. The induced hypoxaemia is due to increased intrapulmonary shunting.¹ ARDS is related to a damage of the alveolar capillary membrane,² leading to pulmonary oedema, although its pathogenesis is poorly understood.

It was recently suggested that alveolar macrophages (AM) are implicated in the development of ARDS.³ By releasing different chemotactic factors, AM may stimulate neutrophil influx and activity.⁴ Indeed, several groups reported an increase in the number of neutrophils and their proteolytic enzymes in bronchoalveolar lavage fluids (BAL) from patients with ARDS.^{5–9} Thus, activation of polymorphonuclear leukocytes (PMNs) leads to endothelial damage and increased membrane permeability via release of cytotoxic products, lysosomal enzymes and eicosanoids. As a result the alveolar wall is injured and pulmonary oedema develops.

Using a septic shock model to attain an ARDS-like state, Shennib *et al.*³ showed a diminished phagocytic capacity of AM within 1 h of bacterial challenge. In a similar model AM showed enhanced respiratory burst with a concomitant decrease in phagocytic and cytotoxic potency.^{10,11} Surfactant has a protective function in lung defence as it prevents epithelial disruption and

protein leakage into the alveolar space.¹² To investigate the putative involvement of alveolar macrophages in the development of an ARDS-like syndrome, basal cAMP levels and the adenylyl cyclase responsiveness of alveolar macrophages shown to provide appropriate parameters for macrophage activity¹³ were determined. By washing out the surfactant from the lungs of piglets via repeated intensive BAL, respiratory distress rapidly develops as assessed by reduced arterial oxygen tension and pulmonary influx of albumin.^{14,15} In the performance of successive complete lavages, this larger animal model allows alveolar macrophages to be harvested in sufficient number for determining cAMP levels after various incubations.

Materials and Methods

Induction of respiratory distress: Six female outbred Yorkshire piglets (10.1 ± 1.1 kg), were anaesthetized with an intraperitoneal injection of 30 mg/kg sodium pentobarbital and placed in a supine position on a thermocontrolled operation table to maintain body temperature. Anaesthesia was maintained by a continuous infusion of sodium pentobarbital (8.5 mg/kg/h i.v.) Tubocurarine (0.2 mg/kg/h i.v.) was administered to paralyse. Animals were mechanically ventilated under the following conditions: constant tidal volume (P_{aCO₂}) between 38 and 42 mmHg, frequency of 10 per min,

PEEP (positive and expiratory pressure) of 2 cm H₂O, an I:E ratio of 2:3 and F_{IO₂} of 0.6. A polythene single lumen catheter was inserted via the right common carotid artery into the aortic arch for blood sampling.

Finally RDS was induced according to a recently described standardized lavage regimen¹⁴ using the following time schedule: at $t = 0$ min, 35 ml/kg sterile saline was instilled into the lung and re-aspirated. This lavage was immediately followed by a second, using the re-aspirated volume (first complete lavage). Using fresh saline, complete lavage was repeated at $t = 5$ min, $t = 60$ min and $t = 65$ min. The four complete lavages obtained were designated as lavage 1, 2, 3 and 4, respectively. This study was approved by the Institutional Animal Welfare Board.

Purification of macrophages and incubation procedure: The lavage fluids recovered were filtered through surgical gauze to sieve flakes occasionally present in the lavage fluid and centrifuged at $400 \times g$ (4°C) to collect bronchoalveolar cells. The resulting pellet was re-suspended in Gey's Balanced Salt Solution (GBSS) and a Ficoll–Isopaque gradient centrifugation was carried out ($400 \times g$, 4°C, 30 min) to isolate AM. The resulting cell layer was recovered and washed three times. The population obtained contained more than 95% AM viability, tested by Trypan Blue exclusion, always exceeded 95%. Purified AM were re-suspended at a concentration of 5×10^6 per ml and kept on ice until further use.

Cellular differentiation of cells in different lavages (PMNs, lymphocytes and macrophages) was determined according to the May–Grünwald–Giemsa staining of cytofuuge preparations.

Macrophages purified from the different lavage fluids were incubated for 15 min (37°C) with PGE₂ or isoprenaline. IBMX (3-isobutyl-1-methylxanthine) was used in a concentration of 0.4 mM to inhibit phosphodiesterase activity.¹⁶ After incubation, cAMP concentrations were determined using a high-affinity protein-binding method as previously described.¹⁷

Materials: Sodium pentobarbital and tubocurarine chloride were obtained from the Pharmacy Department, Dijkzigt Hospital, Rotterdam, The Netherlands. PGE₂ and isoprenaline were purchased from Sigma (St Louis, USA), IBMX from Janssen Chimica (Beerse, Belgium) and Ficoll–Isopaque from Nycomed (Oslo, Norway). The [³H] cAMP was obtained from Amersham (Amersham, UK).

Statistics: Values were expressed as the mean $\pm$ S.E.M. Differences between the means were determined by the non-parametric Mann–Whitney U test. A p -value < 0.05 was considered significant.

Results

After performing two complete lavages at $t = 0$ and $t = 5$ min respectively, the oxygen tension in arterial blood (P_{aO₂}) decreased from 280 ± 21.5 to 86.8 ± 14.8 mmHg and partly recovered within 60 min to 114 ± 49 mmHg. At this time, lavage 3 was performed and 5 min later lavage 4, resulting in a sharp decrease of P_{aO₂} to 59.2 ± 6.6 mmHg, which persisted for at least 5 h.¹⁴

Present results describe data on the alveolar cells recovered from the four lavages. Table 1 summarizes cytological data on the four subsequent lavage fluids. Cell counts between lavages 1 and 2, and 3 and 4 did not show any differences. However, the first two lavages each contained numbers of pulmonary cells four times higher than lavages 3 and 4. In lavages 3 and 4 cellular viability was slightly but significantly reduced by about 5% compared to the first two lavages.

As the total number of alveolar cells was highest in lavages 1 and 2 and the absolute number of PMNs was the same in all four lavages, the percentage of neutrophils and eosinophils was respectively four and 16 times higher in the latter two lavages, while the percentage of AM had decreased (Fig. 1).

Basal cAMP levels in the macrophage populations were determined for obtaining an indication

Table 1. Lavage recovery^a (% recovery), total cell counts (cell counts), number of cells per ml regained solution (cells/ml) and % cellular viability (viability) of the four subsequent lavage fluids before Ficoll–Isopaque purification (1, 2, 3 and 4). Data expressed as mean values $\pm$ standard deviation from six experiments

	1 (0 min)	2 (5 min)	3 (60 min)	4 (65 min)
Recovery	74.2 ± 2.9	92.7 ± 5.3^b	85.5 ± 4.7^b	85.4 ± 8.9^b
Cell counts (10^{-8})	3.7 ± 2.1	3.7 ± 1.2	0.9 ± 0.4^b	0.8 ± 0.3^b
Cells/ml (10^{-6})	1.0 ± 0.5	0.8 ± 0.3	0.2 ± 0.1^b	0.2 ± 0.1^b
Viability	96.7 ± 1.1	94.7 ± 3.0	91.1 ± 2.5^b	91.4 ± 4.2^b

^arecovery is calculated as % volume regained from volume of instilled saline solution.

^b $p < 0.05$ compared to the value obtained in lavage 1.

FIG. 1. Cytological differentiation of four subsequent complete lavages (1-4). Left vertical axis refers to percentage of neutrophils, eosinophils and lymphocytes (0-10%). Right vertical axis refers to percentage of macrophages (88-96%). * $p < 0.05$ compared to lavage 1.

of the AM's basal cellular activity in the four different lavages.¹³ Figure 2 shows that basal intracellular cAMP-concentrations in AM from lavages 3 and 4 are about 40-60% higher compared to AM from the initial lavage.

FIG. 2. Basal cAMP levels per 10^6 AM purified from four different lavages. ^a $p = 0.07$; * $p < 0.05$ compared to lavage 1.

In addition, adenylyl cyclase responsiveness of AM from the different lavage fluids to the inflammatory mediator, PGE_2 , and the non-selective β -sympathomimetic isoprenaline was determined (Figs 3 and 4). Though both compounds dose-dependently enhanced cAMP levels in AM from all four lavages, the response to both was lower in lavages 3 and 4. Isoprenaline (10^{-4} M) stimulated adenylyl cyclase about 2.5 times less effective in AM from lavages 3 and 4 compared to the AM from the first two lavages. Similar though not as clear differences were observed in the adenylyl cyclase responsiveness to 10^{-5} M PGE_2 .

Discussion

Considering albumin-influx and impairment of hemodynamic values, repeated BALs induced an RDS-like state in piglets within 1 h after the initial two complete lung lavages.¹⁴ Cytological examination of the alveolar cell population in different lavage fluids showed as well an influx of PMNs in the lung within 1 h of the initial two complete lavages. Using a less intensive lavage procedure in monkeys, Kazmierowski *et al.*¹⁸ described PMN influx after two successive BALs. This influx of PMNs may be due to the action of chemotactic factors for PMNs and monocytes, secreted by alveolar macrophages.¹⁹ Release of chemotactic

FIG. 3. Adenylyl cyclase stimulation by increasing doses of PGE₂ in AMs isolated from different lavage fluids (1-4). **p* < 0.05 compared to lavage 1.

factors from AM is involved in various forms of lung injury including ARDS.^{5-7,9} Besides, in different experimental ARDS-models macrophage functions related to phagocytosis, like lysosomal enzyme release and respiratory burst, were shown to be impaired.^{3,10,11}

As such AM functions appear to be intracellularly regulated via second messengers, we investigated whether intracellular cAMP level and adenylyl cyclase responsiveness are affected in the course of developing respiratory distress. Cyclic AMP level in AM from lavages 3 and 4 was considerably

FIG. 4. Adenylyl cyclase stimulation by increasing doses of isoprenaline in AMs isolated from different lavage fluids (1-4). **p* < 0.05 compared to lavage 1.

higher as compared to AM recovered from the first complete lavage. Considering the inverse relationship between intracellular cAMP level and AM functions,¹³ this enhancement indicates a rather rapid impairment (within 1 h of the first two lavages) of AM function. This is in agreement with the previously observed impairment in macrophage activity after induction of respiratory distress.^{3,10,11}

We recently observed in human AM²⁰ that an increase in intracellular cAMP level induces a significant rise in the release of LTB₄ which is known to retain strong chemotactic activity for PMNs. In addition we therefore suggest that the rise in cAMP levels of AM from lavages 3 and 4 is responsible for the observed PMN-influx in lavage 3 and 4 via promoted LTB₄-release from alveolar macrophages.

Most of the surfactant, considered to protect the underlying epithelium and alveolar wall from damaging effects by oxygen and its metabolites,²¹ is removed by the initial two complete lavages. Though surfactant is re-supplied from pneumocyte cells type II, we believe that under the present experimental conditions the alveolar wall and the cells embedded in the lining fluid, including AM, have been exposed to oxygen-toxicity. Considering the albumin influx, the alveolar wall appears to be damaged within 1 h of the initial two complete lavages. Similarly, either re-oxygenation of the poorly protected lung or reactive oxygen intermediates released by recruited PMNs may have contributed to this damage. The impaired receptor-mediated stimulation of adenylyl cyclase (decreased susceptibility to respond to PGE₂ and isoprenaline) in AM isolated from the last two lavages may be a reflection of this damage. Furthermore, β -adrenoceptors contain essential sulphhydryl groups which are sensitive to toxic oxygen species.²² The molecular organization of the receptor types will determine the relative sensitivity to such reactive compounds. This explains why β -adrenoreceptors are more profoundly affected than prostanoid-receptors. In addition lipid peroxidation by oxygen radicals has been reported to impair β -adrenoceptor mediated triggering of the adenylyl cyclase complex.²³ Thus, the impaired adenylyl cyclase responsiveness of the AM rather seems to be the result of the induction of RDS than being one of the triggers of the distress.

In conclusion, induction of respiratory distress by repeated lung lavage is attended by pulmonary PMN-influx and enhanced cAMP levels in AM. The latter observation indicates that phagocytic activity of alveolar macrophages diminishes in the course of the developing distress.

Clinically, AM dysfunction may be an important factor in the injury phase preceding the reparative,

proliferative and fibrotic phase as macrophages are known to secrete a large variety of (pro-) inflammatory mediators.

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