Leukotriene receptor antagonism prevents lung protein leakage and hypoxaemia in a septic cat model

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ABSTRACT: Products of the arachidonic acid cascade have been found to play an important role in the pathophysiology in experimental shock and in ARDS. The effect of cysteinyl-leukotriene (cLT) blockade on the development of respiratory failure during septic shock was examined.

Ventilated cats received an infusion of Escherichia coli bacteria. Pretreatment was given with diethylcarbamazine (DEC), a leukotriene synthetase inhibitor, or a new potent cLT receptor antagonist, ICI 198,615. With a gamma camera, the distributions of plasmatransferrin labelled with indium-113m chloride (113mIn) and erythrocytes radiolabelled with technetium-99m (99mTc) were measured over the lungs. A normalized slope index (NSI) reflecting protein leakage, based on the transferrin extravasation, was calculated.

In the nonseptic control group (n=7) NSI was 4.4±10±0.7×10−4·min−1 (mean±SEM). Unpretreated septict animals (n=7) showed a protein leakage after bacterial infusion, with a NSI of 34±5.5×10−4·min−1. Pretreatment with DEC (n=6) significantly reduced NSI to 16±1.5×10−4·min−1. In the group pretreated with ICI 198,615 (n=8), NSI was 9±1.2×10−4·min−1. Arterial oxygen tension (PaO2) remained at baseline level of 20±1.0 kPa during the experimental period in both the nonseptic control group and the ICI 198,615 pretreated group. In the unpretreated septic group, PaO2 fell progressively from a preseptic value of 21±0.9 to 12±1.5 kPa after 3 h.

Pharmacological blockade of the cLTs could prevent the sepsis-induced development of progressive pulmonary microvascular protein leakage and the correlated fall in PaO2 in the cat.


Materials and methods

Animals

The experiments were performed on 28 cats weighing 2.7–4.1 kg. They were deprived of food 12 h before anaesthesia, but had free access to water.
An intramuscular injection of ketamine (Ketalar, Park-Davis, Barcelona, Spain; 25 mg·kg\(^{-1}\)) was followed by an intravenous injection of chloralose, 50 mg·kg\(^{-1}\) i.v., for deep anaesthesia, repeated with 25 mg·kg\(^{-1}\) after 4 h.

Operative procedures and recordings

After tracheostomy, the cats were connected to a constant volume respirator and ventilated artificially with room air. The tidal volume was set to 15 ml·kg\(^{-1}\) body weight, and the rate was set to 20 breaths·min\(^{-1}\). Dead space was adjusted to keep arterial carbon dioxide tension (\(P_{\text{aCO}_2}\)) slightly below normal.

Inspiratory-expiratory pressure amplitudes in the trachea were recorded through a cannula inserted into the tracheostomy tube. The left femoral artery was cannulated for systemic arterial blood pressure recordings, and a left femoral venous line was obtained for intravenous infusions. Pressures were measured by Statham transducers (Statham Instruments, Hato Rey, PR, USA) and recorded in a Grass polygraph (Grass Instruments, Quincy, MA, USA).

Atropine, 0.5 mg·kg\(^{-1}\), was given intravenously to block parasympathetic influences. A slow intravenous infusion of glucose solution containing bicarbonate (10 mmol NaHCO\(_3\) per 100 ml 5% glucose; 0.1–0.2 ml·min\(^{-1}\)) was started at the induction of anaesthesia and continued throughout the experiments.

Arterial blood gases and pH were measured at intervals throughout the experiments. The blood gas analyzer used was ABL-2 (Radiometer, Copenhagen, Denmark).

Radioactivity measurements and calculations

A gamma scintigraphic double isotope technique was used to measure macromolecular leakage. Erythrocytes (RBC) were radiolabelled with technetium-99m (\(^{99m}\text{Tc}\)) (half-life 6 h; photon energy, 140 keV) and used as a blood pool marker [15]. Plasma transferrin was used as a protein leakage marker, labelled with indium-113m-chloride (\(^{113m}\text{In}\)) (half-life 100 min; photon energy, 392 keV), a binding of high affinity [16]. With a computerized gamma camera, both isotopes could be measured. Areas of interest were subsequently analyzed and evaluated.

The radioactive labelling procedure was as follows (fig. 1): stannous fluoride and sodium methochromat (Amerscan, Amersham International, UK) were injected intravenously. Twenty minutes later, 5 MBq (\(^{99m}\text{Tc}\)) sodium pertechnetate from a sterile generator (Byk-Malinckrodt, Athlone, Ireland), mixed with 5 ml of aspirated blood, were injected. After allowing 10 min for RBC labelling and mixing, the first blood sample for radioactivity measurements was drawn. Transferrin was then radio-labelled in vivo by an intravenous injection of 20 MBq (\(^{113m}\text{In}\)) chloride from a sterile generator (Amersham International, Buckinghamshire, UK). An 0.3 ml blood sample was drawn every 10 min and the radioactivity was measured in a well counter. The labelling degree was estimated by measuring blood samples for \(^{99m}\text{Tc}\) and \(^{113m}\text{In}\) activity before and after centrifugation, with separation of plasma and RBC. It was expressed as the percentage of whole blood \(^{99m}\text{Tc}\) and \(^{113m}\text{In}\) activity detected in RBC and plasma, respectively. The labelling degree ranged between 95–98% for each isotope, both initially and at the end of all experiments. The fraction of unbound tracers could influence the normalized slope index (NSI) values [17]. The free tracer behaviour could be estimated from data of the control animals, as all groups showed the same labelling efficiency [7]. After radiolabelling, the first 20 min showed a rapid accumulation of \(^{113m}\text{In}\) in the lung. This event was followed by a constant weak positive linear NSI, indicating a state of equilibrium of the free tracer fraction, equal in the groups.

The accumulation of radionuclides was measured using a gamma camera (Maxi Camera General Electric). The gamma camera was equipped with a parallel-hole high-energy collimator and connected to a NUD gamma II MTS system. The energy window was 20% around the energy peaks, at 140 and 392 keV, respectively. From the higher energy channel originating from \(^{113m}\text{In}\), there was a scatter into the lower energy channel referred to \(^{99m}\text{Tc}\). In order to correct for this, a factor of scatter was calculated [7]. Every 10 min, the gamma camera image was measured for each isotope for 1 min.

Fig 1. — The experimental protocol. Blood samples were drawn every 10 min to measure isotope activity and haematocrit. DEC: diethyl carbamazine.
The cat was placed in a supine position directly on the collimator of the gamma camera. It was possible to anatomically localize regions of interest from the gamma camera image. The heart contour was easily identified, and lung regions were made semi-circular within the apical and central parts of both lungs and well separated from heart, liver and spleen. All counts were corrected for radioactive decay and for the scatter of high energy photons into the low energy window.

The transferrin accumulation in the lung was assessed by calculating normalized index (NI), defined as the ratio of tagged proteins in the entire lung (plasma and interstitium) to tagged proteins in lung plasma (fig. 2).

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NI(t_i) = \frac{\text{InL}(t_i)}{\text{InB}(t_i)} \times \frac{\text{TcB}(t_i)}{\text{TcL}(t_i)}
\]

where \(t_i\) is the time of injection of \(E.\,\text{coli}\) or NaCl; \(\text{InL}(t_i)\) is the \(^{113}\text{In}\) activity in the measured part of the lung at time \(t_i\) and \(\text{InL}(t_0)\) at time zero; \(\text{InB}(t_i)\) is the \(^{113}\text{In}\) activity in the blood sample at time \(t_i\) and \(\text{InB}(t_0)\) at time zero; \(\text{TcL}(t_i)\) is the \(^{99}\text{Tc}\) activity in the measured part of the lung at time \(t_i\) and \(\text{TcL}(t_0)\) at time zero; \(\text{TcB}(t_i)\) of the \(^{99}\text{Tc}\) activity in the blood sample at time \(t_i\) and \(\text{TcB}(t_0)\) at time zero.

To correct for intravascular isotope content, blood samples drawn from the femoral artery were used. It was assumed that the changes in haematocrit were parallel in the lung vessels and in the systemic circulation. NI measurements started directly after isotope activities were given, and followed every 10 min during the whole experimental period.

This instability upon the bolus infusion of bacteria consists of an initial decrease in cardiac output and systemic blood pressure and a prominent rise in pulmonary arterial blood pressure and tracheal pressure [19]. In parallel to considerable variations in the recordings on isotope distribution, it therefore seems reasonable to exclude this phase when calculating the NSI.

Pulmonary blood volume changes were followed by the ratio of \(\text{TcL}\) and \(\text{TcB}\), normalized at time \(t_0\) in each animal. The assumption was based on \(^{99}\text{Tc}\) as a pure intravascular marker and not corrected for the small isotope fraction unbound to RBC.

**Bacterial preparation**

An *Escherichia coli* bacterial strain (\(E.\,\text{coli}\) 06 K13H1) (WHO designation Su 4344/41) from the WHO Collaborative Centre for Reference and Research on Escherichia (State Serum Institute, Copenhagen, Denmark) was used. For a detailed description, see Schützer *et al.* [19].

**Pharmacological pretreatment**

Diethylcarbamazine (DEC), a 5-lipoxygenase synthetase inhibitor was used in one group, as in previous reports [14]. DEC inhibits both the peptidoleukotrienes \(C_4\) and \(D_4\), as well as, leukotriene \(B_4\) (LTB4) [20].

ICI 198,615, a specific potent cysteinyl leukotriene (\(C_4\) and \(D_4\)) receptor antagonist [10], was used in another group of cats. ICI 198,615 has been found to be more potent and selective than previously available cLT antagonist [21] and in addition has been well tolerated in initial clinical studies [22]. In pilot experiments, a bolus injection of 15 mg·kg⁻¹ ICI 198,615 protected against...
pulmonary protein leakage for 45 min. Due to rapid turnover [21], a continuous infusion of ICI 198,615 (15 mg·kg\(^{-1}\)·h\(^{-1}\)) was needed.

**Experimental procedures**

After the preparation, the cats were allowed to stabilize for 30 min before radiolabelling with \(^{99m}\)Tc and \(^{111}\)In, as described above. Measurements of the distribution of the activity were then performed throughout the experimental period (fig. 1). After radiolabelling, a stabilization period of 1.5 h followed. The *E. coli* bacteria, 10\(^9\) ml\(^{-1}\), were given as an intravenous infusion; 1 ml·kg\(^{-1}\) body weight·min\(^{-1}\) for 2 min, followed by 1 ml·kg\(^{-1}\) body weight·h\(^{-1}\) for the next 3.5 h.

The cats were divided into four groups: 1) Group 1 (n=7) had no pretreatment before i.v. *E. coli* infusion; 2) Group 2 (n=6) was given and intravenous infusion of DEC 10 mg·kg\(^{-1}\) 20 min prior to bacterial infusion [14]. Every 45th min a DEC dose of 2 mg·kg\(^{-1}\) was given until the experiments were ended; 3) Group 3 (n=8) was pretreated with ICI 198,615, 15 mg·kg\(^{-1}\) as an intravenous bolus injection 10–20 min prior to the septic insult, and then a continuous infusion of 15 mg·kg\(^{-1}\) (ICI Pharma on file data); and 4) Group 4 (n=7) served as a control group with no bacteria, but received an infusion of the same solution volume as the other groups with physiological saline.

**Gravimetric lung weight**

At the end of the experiment, the lungs were quickly removed, and the blood was drained. After weighing and homogenization, the lungs were dried in a microwave oven. The extravascular lung water was calculated [23], expressed as gram H\(_2\)O per gram blood-free dry lung.

**Statistical method**

Data are expressed as mean±SEM. When testing statistical significance, first and overall nonparametric analysis of variance by the method according to Kruskal-Wallis (Stat View II, Abacus concepts) was performed on each time point. If there was a treatment dependent variation, differences within each group were tested according to Wilcoxon’s matched-pairs signed-rank test [24]. A p-value ≤0.05 was considered as significant. For the gravimetric data the Mann-Whitney U-test was used.

**Results**

Intravenous infusion of ICI 198,615 had no effects on measured parameters. Slowly administered DEC induced a transient 10% increase in systemic arterial blood pressure, without any effect on the other parameters.

Normalized slope index (NSI), reflecting the protein leakage in the measured parts of the lungs for the whole experimental period, was statistically significantly higher in unpretreated sepsis, 34±3.5×10\(^{-4}\)·min\(^{-1}\), than in the other groups. In the DEC pretreated group, NSI was attenuated to 16±1.5×10\(^{-4}\)·min\(^{-1}\), but still significantly higher than in the saline group. In the ICI 198,615 pretreated animals, the NSI was 9±1.2×10\(^{-4}\)·min\(^{-1}\), which was not statistically significantly different from the nonseptic control group, with a NSI of 4.4±0.7×10\(^{-4}\)·min\(^{-1}\) (fig. 4).

Extravascular lung water (EVLW), reflecting fluid accumulation, was 2.51±0.17 g H\(_2\)O per gram blood-free dry lung tissue in the unpretreated septic group, which was slightly but statistically significantly higher than in the saline group. In the groups pretreated with DEC and ICI 198,615, EVLW was 2.05±0.14 and 2.03±0.15 g H\(_2\)O per gram blood-free dry lung tissue, respectively. These values were not significantly different from EVLW in saline animals with 1.67±0.22 H\(_2\)O per gram blood-free dry lung or from that in the unpretreated septic group.

The pulmonary blood volume did not change significantly during the experiments expressed as the relative volume (ratio TcL to TcB) normated at time 0.

Tracheal pressure amplitude increased instantaneously after bacterial infusion, in animals given no pretreatment, from 4.5±0.3 mmHg during the preseptic control period to a peak at 3 min of 12.3±1.4 mmHg. After 5 min, there was a decline to a stable pressure of approximately 170% of the preseptic value. In the DEC pretreated cats, the initial pressure increase was less pronounced, while ICI 198,615-pretreatment gave a statistically significantly attenuated pressure increase (fig. 5).

The apparent arterial oxygen tension (P\(_{aO2}\)) after the 2 h resting period was 21±0.7 kPa, without significant difference between the groups. In the pretreated septic group, P\(_{aO2}\) fell progressively, reaching 11.8±1.5 kPa after 3 h. DEC pretreatment attenuated this fall to 18.1±1.9 kPa. After pretreatment with ICI 198,615, septic insult had no effect on P\(_{aO2}\) (fig. 6).

P\(_{aO2}\), at 3 h and NSI showed a close linear relation with R=0.86 (fig. 7). The pH was significantly reduced in the untreated and the DEC-pretreated septic groups, concomitant with the P\(_{aO2}\), fall (from 7.34±0.02 and...
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Fig. 5. – Tracheal pressure amplitude before and during experimental period. Data are presented as mean values of ±SEM. *: statistically significant difference between unpretreated septic and ICI 198,615 pretreated animals. - - : sepsis; - - - : diethylcarbamazine; - - - - : ICI 198,615; - - : NaCl.

Fig. 6. – Oxygen pressure (PaO₂) in arterial blood samples. Data are presented as mean±SEM. *: statistically significant difference between the unpretreated septic and the other groups; †: statistically significant difference within each group compared with preseptic values. - - : sepsis; - - - : diethylcarbamazine; - - - - : ICI 198,615; - - - - - : NaCl.

Fig. 7. – The lung protein leakage correlated closely with the impaired oxygenation of the blood. Relationship between NSI and PaO₂ at 3 h. NSI: normalized slope index; PaO₂: arterial oxygen tension.

Systemic arterial blood pressure was about 130 mmHg during the preseptic control period in all series. In the unpretreated septic group there was a rapid fall upon bacterial infusion to 76±7 mmHg, followed by a tendency towards normalization within 3–5 min. During the ensuing experimental period, the cats became progressively hypotensive. In the pretreated group, the blood pressure fall followed the same pattern, without statistically significant difference.

Discussion

This study showed that pharmacological blockade of the cysteinyl-leukotrienes in septic shock could prevent permeability disturbances in the lung, and the ensuing PaO₂ fall.
Experimental [19], as well as clinical [5] studies of pulmonary dysfunction in septic shock are characterized by progressive hypoxia, increase in airway resistance, decrease in lung compliance, and elevation of pulmonary artery pressure and vascular resistance. The disturbed gas exchange could be due to fluid and/or macromolecular leakage into the interstitium and eventually into the alveolar space, ventilation/perfusion mismatch and low mixed venous oxygenation. This study concentrated on leakage as a pathogenic factor in the pulmonary dysfunction.

The present study confirmed an increased vascular permeability with significant protein leakage into the lung tissue, as judged by the gamma scintigraphic measurements, in a feline model of septic shock. Lung protein leakage in experimental septic states has been documented in sheep [6], and also in endotoxin injury in guinea-pig [25], and dog [26], using comparable techniques. To express the transcapillary protein flux accumulation in the lung, the “normalized slope index” (NSI) [18] was adopted. This is a single index to which all previously described permeability equations could be related. Accumulation of plasma proteins in the lung, expressed by indium-113-m tagged transferrin, was calculated [7]. Exclusion of the intravascular transferrin content was possible by labelling erythrocytes by technetium-99m [6]. The intravascular relation of radioactivity for both isotopes in separate blood samples was compared with the measured activity over the lung areas. The extravascular accumulation of transferrin could be calculated, and local blood volume changes could be detected. No significant blood volume changes were registered during septic insult in this or in previous studies [7].

In spite of an increased vascular permeability, gravimetric lung water in a previous report did not implicate fluid accumulation parallel to the permeability changes [7]. However, in the present study EVLW was statistically significantly increased in untreated septic animals compared to nonseptic cats. The pretreated groups with an attenuated permeability showed a parallel decrease in lung water, even though EVLW was not significantly different from either the untreated septic or the saline control animals. In lung injury models, conflicting results have been reported as to whether a fluid accumulation is evident or not. This variability is in line with other septic model studies where, e.g. TURNER et al. [27] found increased lung water in dogs, while BRIGHAM et al. [28] did not in sheep. An increased flux of water into the lungs may be compensated by clearing forces, e.g. lymph flow [29] in several studies. Cardiac failure with elevations in hydrostatic pressures during septic conditions may aggravate the water flux, and may partly explain the variability between different studies.

The lung macromolecular leakage was strongly correlated to the fall in $\text{PaO}_2$, indicating a causal relationship. Another possible contributor to the progressive hypoxaemia is constriction of the small airways. The tracheal pressure reflects both airway resistance and lung compliance [14]. However, in this report, the instantaneous tracheal pressure increase after induction of sepsis was unrelated to the $\text{PaO}_2$ fall. The tracheal pressure remained slightly elevated during the rest of the experiments (after the peak of the first 5 min) but did not correlate to the progressive protein accumulation. Ventilation/perfusion mismatch could possibly contribute to the defective oxygenation, although not specifically measured in this study.

In the present study, the role of LTs for development of vascular permeability changes in the lung and the correlation to the subsequent hypoxia was explored during septic conditions. DEC, a leukotriene synthetase inhibitor on the 5-hydroxyperoxycosatetraenoic acid (5-HPETE) level, was used as in previous reports [14]. However, synthetase inhibition could possibly shunt arachidonic acid metabolism to the cyclo-oxygenase pathway and increase the production of thromboxanes and prostaglandins. Because of these unspecific actions of DEC, a new and highly specific cLT receptor antagonist ICI 198,615 was used for comparison.

Pharmacological blockade of cLT with ICI 198,615 almost abolished the protein leakage and the $\text{PaO}_2$ decrease. In addition, the tracheal pressure increase seen during septic conditions was moderately, but statistically significantly attenuated. Pretreatment with DEC showed a similar but less protective effect on protein leakage, $\text{PaO}_2$ fall and tracheal pressure increase, compared to ICI 198,615. A preserved arterial oxygenation by cLT blockade has also been found in some earlier studies [14], but not in others [30]. In endotoxin shock studies using other leukotriene inhibitors, partial prevention of vascular protein leakage has been demonstrated in rats and dogs by TURNER [27] and BALL et al. [30], whilst the study by COOK and co-workers [31] on rats indicated no preventive effects. The reasons for this discrepancy may be that different LT blockers and/or doses are used, but differences in species could also interfere.

In the groups pretreated with DEC and ICI 198,615, the tracheal pressure was reduced but not abolished, despite a preserved oxygenation. Thus, bronchoconstriction seemed to be of less importance for the hypoxia in septic lung injury. Thromboxanes and prostaglandins might be responsible for this remaining bronchoconstriction as dazmegrel, a thromboxane-A$_2$ inhibitor, partly reduced the tracheal pressure increase after sepsis induction in previous studies [19]. The cellular mechanisms behind the preservation of permeability and blood gases remain to be elucidated.

At present, no rational drug therapy is available in ARDS, and treatment remains symptomatic. Identification of the key humoral mediators in this syndrome is a prerequisite for the development of appropriate therapeutic regimens. The findings in this report of LTC$_3$ - and LTD$_4$-involvement in central ARDS mechanisms, such as permeability and oxygenation, may have therapeutic implications.

We conclude that the cysteinyl-leukotrienes seem to be major mediators of respiratory failure in sepsis-induced lung injury in cats. Changes in lung microvascular sieving properties, with protein leakage and development of hypoxia, were prevented by selective antagonism.

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