Nitrated proteins in bronchoalveolar lavage fluid of patients at risk of ventilator-associated bronchopneumonia

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ABSTRACT: The study was designed to identify markers of oxidative injury, related to the nitric oxide derived cascade, in bronchoalveolar lavage (BAL) fluid from intensive care patients suspected of ventilator-associated pneumonia (VAP) and/or acute respiratory distress syndrome (ARDS).

Thirty-eight patients developing VAP and/or ARDS (VAP/ARDS group) were compared to 20 ventilated patients without VAP/ARDS (control group). Myeloperoxidase (MPO) and elastase, taken as markers of neutrophil activation were measured by enzymatic techniques, and nitrated proteins (NTPs) by an immunological method. The cytotoxicity of the BAL fluid was tested using cultured human epithelial alveolar cells by the release of pre-incorporated ^{51}Cr .

Mean NTP concentration and, MPO and elastase activities were different between the VAP/ARDS and control groups ($p < 0.05$ for NTPs; $p < 0.005$ for MPO; $p < 0.005$ for elastase). NTP concentration correlated with MPO and elastase activity and neutrophil number ($r = 0.93$, 0.91 and 0.87 , respectively), but not to protein concentration and arterial oxygen tension/inspiratory oxygen fraction. The cytotoxicity of BAL correlated with NTP concentration ($r = 0.92$) and MPO activity ($r = 0.89$).

It was concluded that the concentrations of nitrated proteins in bronchoalveolar lavage fluid correlated with the oxidant activity of neutrophils and that, bronchoalveolar lavage fluid cytotoxicity was correlated with the nitrated protein concentration and may be mediated by oxidants.

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In patients with or at risk of acute lung injury or acute respiratory distress syndrome (ARDS), bronchoalveolar lavage (BAL) permits exploration of distal lung regions, which are mainly implicated in the inflammatory reaction and in acute respiratory failure. This bronchoscopy technique allows access to damaged tissues and gives the possibility of early detection of inflammatory mediators that have either accumulated or were produced in the lung. From studies already performed on BAL fluids, it appears that neutrophils constitute the majority of the cell population present in the lungs of ARDS patients, and that products of phagocyte degranulation such as myeloperoxidase (MPO) and elastase (free or complexed to α_1 -proteinase inhibitor) can also be found [1, 2].

These degranulation products argued in favour of activation of phagocytes in the lungs, involving release of reactive oxygen species, especially products derived from the activities of reduced nicotinamide adenine dinucleotide phosphate-oxidase and MPO: superoxide anion, hydrogen peroxide, and hypochlorous acid. Phagocytes also produce nitric oxide. The simultaneous production of $\text{NO}\cdot$ and $\text{O}_2^{\cdot-}$ allows *in situ* generation of peroxynitrite, a potent oxidative species which reacts quickly by incompletely determined mechanisms [3–5]. In the lung, ONOO^- is reported to be responsible for alterations to surfactant of type II alveolar cells [6]. ONOO^- is involved in the oxidation of lipoproteins, nucleic acids, proteins and peptides,

and in the nitration of tyrosine and tyrosyl residues. This has important repercussions for the functions of enzymes and proteins [7, 8]. *In vivo*, direct detection of reactive nitrogen species is difficult, but the presence of nitrotyrosine or nitrated proteins (NTPs) is often presented as a marker of their production, especially that of ONOO^- [9, 10]. *In situ* nitration of proteins has been demonstrated by means of immunofluorescence techniques in lung tissues of patients suffering from acute inflammatory pathologies [11, 12].

The purpose of this study was to detect the presence of NTPs in the BAL fluids of patients suspected of ventilator-associated pneumonia (VAP), and to correlate these NTP concentration with MPO and elastase activity, taken as markers of neutrophil activation and degranulation in the lungs. Another purpose was to demonstrate that BAL fluids could be toxic to alveolar cells, in correlation with BAL fluid NTP concentration and MPO activity.

Materials and methods

Study population

Fifty-eight patients at risk of VAP were included in the study. They were intubated and mechanically ventilated for >72 h, and had a recognized predisposing pathology (table

1). BAL was performed when clinical and radiographic signs suggested VAP. Patients were eligible if they fulfilled the following criteria temperature $\geq 38.5^{\circ}\text{C}$; presence of purulent tracheobronchial secretions, leukocytosis ($\geq 12,000$ cells·mm⁻³) or leukopenia ($< 4,000$ cell·mm⁻³); and new, progressive or persistent (> 24 h) infiltrate on chest radiography [13]. The Acute Physiology and Chronic Health Evaluation II score was obtained on the day of arrival and arterial oxygen tension (P_{a,O_2})/inspiratory oxygen fraction (F_{I,O_2}) on the day of BAL.

Bronchoalveolar lavage protocol and bacteriology

Before BAL was started, all patients were ventilated with 100% oxygen and sedated intravenously with propofol (6–12 mg·kg⁻¹) and atracurium (0.5 mg·kg⁻¹). The site of BAL was chosen according to chest radiographic appearance, into a distal airway area involved by the infiltrate or into a subsegment of the right middle lobe or lingula if the infiltrate was diffuse. Lavage was performed *via* the endotracheal tube as previously described, with four 50-mL aliquots of warm sterile 0.9% sodium chloride [13]. The first aliquot was discarded and the second used for bacteriological studies. The aspirated liquid from the third and the fourth aliquots was strained through sterile gauze and centrifuged for 10 min at 300×g. Total cell counts were determined and leukocyte distribution was analysed (differential counts of 400 cells using May-Grünwald Giemsa counterstaining). The supernatants were frozen at -70°C . On thawing, the total protein concentration was measured in the supernatants.

The determination of bacterial number in the second aliquot of BAL fluid was performed by colony count using standard techniques and expressed in colony-forming units (CFU) per millilitre of fluid. A culture threshold of $\geq 10^4$ CFU·mL⁻¹ was used to support the diagnosis of VAP [14].

Myeloperoxidase activity

The peroxidase activity of MPO was assessed at 25°C by measuring the absorbance increase at 460 nm due to

oxidation of *o*-dianisidine (Sigma-Aldrich, Bornem, Belgium) [15]. BAL fluid (50 μL) was added to 1 mL 50 mM *o*-dianisidine in Sørensen buffer pH 5.5, and the reaction was started by the addition of H₂O₂ (final concentration 0.15 mM). The absorbance increase was monitored for 1 min at room temperature. One unit of activity was defined as the amount of MPO which produced an absorbance increase of 1 optical density unit (ODU)·min⁻¹.

Elastase activity

The proteolytic activity of elastase was assessed by measuring the increase in absorbance at 410 nm, due to proteolysis of a specific substrate for leukocyte elastase (*N*-methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide; Sigma-Aldrich, Bornem, Belgium) [16]. This technique enabled the measurement of free elastase and elastase bound to α_2 -macroglobulin, but not elastase bound to α_1 -proteinase inhibitor [2]. BAL fluid (100 μL) was added to 1 mL substrate (0.65 mM in 0.2 M tris-hydroxymethyl-amino methane, 1 M NaCl, pH 8.5). The absorbance was read after 15 min incubation at 25°C . A standard curve was constructed using porcine pancreas elastase (Calbiochem MerckEuroLab, Leuven, Belgium), 239 U·mg⁻¹protein⁻¹ under the same conditions.

Nitrated protein measurement

The occurrence of NTPs in BAL fluids was quantified using a competitive enzyme-linked immunosorbent assay (ELISA) in microplates precoated with a fixed amount of NTP (nitrated bovine serum albumin (BSA)) (Protein Nitrotyrosine ELISA Kit; TCS Biologicals Ltd, Buckingham, UK). The standard curve was obtained by serial dilution (0.4–100 $\mu\text{g}\cdot\text{mL}^{-1}$) of the standard nitrated BSA. The BAL fluid samples (100 μL) were used directly or after dilution in phosphate-buffered saline. Results were expressed in microgram of nitrated BSA equivalents, and a value of 0.1 $\mu\text{g}\cdot\text{mL}^{-1}$ was taken as the lower limit of detection. The ELISA is specific for free nitrotyrosine and nitrotyrosine in proteins in a sequence-independent manner [17].

Bronchoalveolar lavage fluid cytotoxicity assay

The human epithelial lung adenocarcinoma cell line A549 (American Type Culture Collection, Rockville, MD, USA) was used to test BAL fluid cytotoxicity. This cell line has the characteristics of alveolar type II cells. A549 cells were cultured (at 37°C , 5% CO₂) in minimal essential medium supplemented with 2 mM L-glutamine, 10% heat-inactivated foetal calf serum, penicillin (100 U·mL⁻¹) and streptomycin (100 $\mu\text{g}\cdot\text{mL}^{-1}$) (all from GibcoBRL, Life Technologies, Merelbeke, Belgium). Cells were distributed in 24-well plates at a concentration of 8×10^4 cells·well⁻¹ in 500 μL culture medium. After a 24-h culture period, the cells were labelled overnight with ⁵¹Cr (740 kBq·mL⁻¹) (⁵¹Cr sodium chromate, Amersham Pharmacia Biotech, Benelux, Roosendaal, Netherlands) added to fresh medium. After an overnight incubation of the cells at 37°C with BAL fluid, samples diluted twice in Hanks' balanced salt

Table 1. — Diagnostic distribution on admission, characteristics and clinical parameters of patients

Characteristics and clinical parameters	
Male/female	42/16
age yrs	55.59±16.87 (16–88)
APACHE II score	15.85±5.49 (6–31)
Intubation duration days	19.93±15.49 (1–80)
ICU stay days	29.45±23.99 (4–151)
Mortality rate n (%)	17 (29.3)
Bronchopneumonia number	35
Cause of admission to ICU n	
Cardiovascular surgery	20
Multiple/cerebral trauma	6/4
Neurosurgery/abdominal surgery	3/5
Heart and kidney transplant	5
Haematological malignancies	5
Burns	4
Miscellaneous infections	6

Data are presented as mean±SD (range) (n=58). APACHE: Acute Physiology and Chronic Health Evaluation (on day of admission); ICU: intensive care unit.

Fig. 2. – Correlation between concentration of nitrated proteins (NTPs) and myeloperoxidase (MPO) activity in bronchoalveolar lavage (BAL) fluids ($r=0.93$, $p<0.001$). ODU: optical density units.

51.00±15.00% versus 8.7±1.3% in the VAP/ARDS and control groups respectively ($p=0.01$). Significant correlations were obtained between cytotoxicity and NTP concentration (fig. 3), and between cytotoxicity and MPO activity (fig. 4).

Discussion

The present study demonstrates that the presence of NTPs correlated with the number of neutrophils and the presence of active neutrophil granulocytic enzymes in BAL fluid samples from intensive care unit patients with or at risk of VAP. It was, therefore, plausible that oxidative processes had occurred, leading to the formation of an alveolar milieu which could be cytotoxic to the neighbouring cells and tissues. High levels of nitrotyrosine or NTPs have already been found in tissues and biological fluids from patients with acute lung injury and other diseases involving oxidative damage [10–12, 19]. The nitration of tyrosine residues in proteins and peptides *in vivo* is presented as a footprint of reactive nitrogen species production [10] and more specifically as the marker of ONOO⁻ production [17]. The antibodies used in the pre-

Fig. 3. – Correlations between the cytotoxicity and concentrations of nitrated protein (NTPs) of bronchoalveolar lavage (BAL) fluids ($r=0.92$, $p<0.001$). IC: index of cytotoxicity measured on alveolar type II cells A549.

Fig. 4. – Correlation between the cytotoxicity and myeloperoxidase (MPO) activity of BAL fluids ($r=0.88$, $p<0.001$). IC: index of cytotoxicity measured on alveolar type II cells A549. ODU: optical density units.

sent study for NTP measurement are presented as specific to nitrotyrosine and NTPs [17]. This was confirmed by addition of increased exogenous concentrations of nitrotyrosine that were quantitatively recognized by the antibodies.

ONOO⁻ could thus be involved in the formation of the NTPs we measured in BAL fluids, although the formation of nitrotyrosine *in vivo* is not exclusively indicative of ONOO⁻ formation [10, 20, 21]. It is highly probable that stimulated phagocytes present in alveoli produced NO[·] and O₂^{·-}, which rapidly react together generating ONOO⁻ [4]. The constant rate of this reaction is only limited by the diffusion of the two reagents [3, 5], such that NO[·] can overcome superoxide dismutase for reaction with O₂^{·-}, and that ONOO⁻ will be generated at the sites of active inflammation. ONOO⁻ is unstable; it isomerizes spontaneously into nitrate, but also quickly reacts with biological compounds leading to oxidations by nonradical and free-radical reactions [7, 8, 22–24]. The nitration of amino acid residues seems to be due to NO₂[·] and to be favoured by CO₂ [8, 25].

In the present patients, excessive production of NO[·] in the respiratory tract occurs as a host defence mechanism [26], leading to bacterial protein nitration [27]. However, this could also contribute to lung injury by forming reactive nitrogen species with oxygen and activated oxygen species, especially O₂^{·-}, particularly in alveoli invaded by neutrophils in patients with bronchopneumonia. Under these conditions, there would be *in situ* production of ONOO⁻ correlated with the presence of neutrophils and leading to a nitrating activity that could be favoured by a local increase in CO₂ concentration in poorly ventilated lungs. Additional nitrating agents could be produced in alveoli by the reaction of HOCl (produced by the activity of MPO) with nitrite (the autoxidation product of NO[·]), and by the enzymatic activity of MPO on ONOO⁻ and nitrite [10, 28, 29]. This enzyme was present in its active form in BAL fluid and correlated with NTP concentrations in the present patients.

The BAL fluid MPO activity and NTP concentration correlated with the cytotoxicity of BAL fluids observed on alveolar cells in culture. This cytotoxicity could be explained by the chlorination activity of MPO using H₂O₂

provided by the normal metabolism of the alveolar cells. The HOCl produced by MPO activity could further react with nitrite ions (resulting from the oxidation of NO $\cdot$ produced by alveolar cells) to produce reactive intermediate toxic species. However, underlying this cytotoxicity of BAL fluid, a proteolytic action of elastase, and an effect of nitrotyrosine and NTPs themselves, cannot be excluded. No studies have reported a possible direct toxicity of NTPs, and it remains to be clarified whether NTPs are markers of inflammation or actively contribute to lung cell dysfunction.

It was found that the highest concentrations of NTP in BAL fluid correlated not only with the number of neutrophils but also with the activity of extracellular active MPO and elastase, which indicated that the neutrophils were activated and degranulated. MPO provides evidence of neutrophil degranulation, and its measurement has been used to demonstrate neutrophil activation and infiltration [30, 31]. Active elastase could be produced by degranulating neutrophils, but also by alveolar macrophages, and could be either the free enzyme or the enzyme bound to α_2 -macroglobulin, but not the enzyme bound to α_1 -antiproteinase [2]. However α_1 -antiproteinase and α_2 -macroglobulin are highly sensitive to oxidative damage, which destroys their antiprotease capacity [30, 32].

ONOO $^-$ is an unstable molecule, and the nitrating species that can be produced by the activity of MPO on nitrite or ONOO $^-$ are also unstable (NO $_2\cdot$ or Cl $\cdot$ derived from NO $_2$ Cl) and could therefore not have migrated from the plasma into the alveoli. NTPs, MPO and elastase, if originating from the plasma, would have correlated with the concentrations of albumin and protein in BAL fluid, markers of oedema and capillary leak. However, there was no correlation between NTP concentration and MPO and elastase activity, and the concentration of protein in BAL fluid or the PaO $_2$ /FiO $_2$. It was hypothesized that the presence of high levels of neutrophil enzymes and NTP in BAL fluid was the result of production that occurred in the alveoli: the correlation found between NTP concentration and MPO and elastase activity, and the number of neutrophils sustained this hypothesis. High levels of NTPs in BAL fluid were more frequent in patients with VAP; this could be related to the presence of increased levels of lipopolysaccharide [33], and argued in favour of local activation of phagocytes and production of oxidant species. However, the identification in BAL fluid of NTPs specific to alveoli, such as surfactant proteins, should provide a definitive answer regarding the presence of an intra-alveolar oxidative activity of ONOO $^-$ and related compounds *in vivo*.

From this study, it was concluded that the concentrations of nitrated proteins measured in bronchoalveolar lavage fluids correlated with the oxidant activity of neutrophils, and that the bronchoalveolar lavage cytotoxicity correlated with the nitrated protein concentration and may be mediated by oxidants.

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