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A specific neutrophil elastase inhibitor (ONO-5046·Na) attenuates LPS-induced acute lung inflammation in the hamster

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A specific neutrophil elastase inhibitor (ONO-5046·Na) attenuates LPS-induced acute lung inflammation in the hamster. S. Yasui, A. Nagai, K. Aoshiba, Y. Ozawa, Y. Kakuta, K. Konno. ©ERS Journals Ltd 1995.

ABSTRACT: We have examined the effect of ONO-5046·Na, a synthetic specific inhibitor of neutrophil elastase, on lipopolysaccharide (LPS)-induced acute lung inflammation.

Syrian golden hamsters were injected intraperitoneally with either 300 mg·kg^{-1} of ONO-5046·Na or saline, 30 min before and 1 h after intratracheal administration of 0.1 mg·kg^{-1} LPS. Animals were sacrificed 2 and 24 h later and the wet-to-dry lung weight ratio (W/D) was determined. Bronchoalveolar lavage (BAL) was performed, and tissue sections were examined histologically. The effect of ONO-5046·Na on migration of isolated neutrophils was determined.

W/D was not significantly different at 2 h, but was increased at 24 h in the LPS-treated animals. This increase was attenuated in the LPS-treated animals injected with ONO-5046·Na. Analysis of BAL fluid revealed that both at 2 and 24 h after LPS administration the total cell number and neutrophil number, albumin concentration, and elastase-like activity were significantly lower in the LPS-treated animals injected with ONO-5046·Na than in those given LPS alone. Histological examination of the lungs of the animals treated with LPS alone showed intra-alveolar haemorrhages and inflammatory cell infiltration 24 h after LPS administration, whereas the lungs of the LPS-treated ONO-5046·Na injected animals were only sparsely infiltrated by inflammatory cells, as indicated by the inflammation score.

Although ONO-5046·Na had no effect on neutrophil migration *in vitro*; the present findings suggest that ONO-5046·Na attenuates LPS-induced acute lung inflammation by inhibiting neutrophil migration *in vivo*, and conversely that neutrophil elastase plays a considerable role in the pathogenesis of the acute lung inflammation induced by LPS.

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Adult respiratory distress syndrome (ARDS) is characterized by neutrophil accumulation, increased vascular permeability, increased extravascular lung water and impaired gas exchange [1–3]. Sepsis caused by Gram-negative bacilli is a major cause of this syndrome [4]. Lipopolysaccharide (LPS), an endotoxin of Gram-negative bacilli, is widely used in animal models of acute lung inflammation and pulmonary emphysema [5–11]. In LPS-induced acute lung inflammation, numerous products released by neutrophils, such as a variety of proteolytic enzymes, reactive oxygen species and arachidonic acid metabolites, are thought to explain the development of the inflammation [12]. Thus, neutrophils appear to play a major role in LPS-induced lung inflammation [13–15]. Most previous studies of proteinase involvement in lung inflammation have focused on neutrophil elastase [16–21], since neutrophil elastase is prominent in the BAL fluid of such patients [22, 23]. However, the precise role of neutrophil elastase in the early stage of the acute lung inflammation has not yet been evaluated. In previous studies of specific elastase inhibitors in several models of lung inflammation, endotoxin was found to cause endothelial cell injury and was almost completely inhibited by elastase inhibitors [16–18]. Furthermore, GOSSAGE *et al.* [24] have shown that the synthetic neutrophil elastase inhibitors SC-37698 and SC-39026 reduce endotoxin-induced lung dysfunction in sheep [24]. Eglin C, a potent elastase inhibitor originally isolated from leeches, however, had no effect in an ovine model of endotoxaemia [25].

Recently, a specific neutrophil elastase inhibitor, ONO-5046·Na, was developed. This agent is characterized by having no antiprotease effects, except on neutrophil elastase [26]. The purpose of this study was to determine whether ONO-5046·Na attenuates LPS-induced acute lung inflammation, and if so, how the elastase inhibitor affects inflammatory cell migration and serum albumin leakage into intra-alveolar spaces at different stages of inflammation, 2 and 24 h after LPS instillation.

Materials and methods

Male Syrian golden hamsters weighing 130–150 g were obtained from Charles River Breeding Laboratories (Kanagawa, Japan) and the neutrophil elastase inhibitor ONO-5046·Na (a kind gift of the Ono Pharmaceutical Co. Ltd, Osaka, Japan) were used in this study. The experimental design is shown in figure 1. The animals (n=45) were randomly assigned to three groups (15 animals per group): Group l (LS group: LPS control); Group 2 (LE group: treated with a specific elastase inhibitor); and Group 3 (SS group: saline-treated control). Thirty minutes before and 1 h after administration of LPS (*Escherichia coli*-derived; Sigma Chemical Co., St. Louis, MO, USA), the animals were intraperitoneally injected with either ONO-5046·Na, 300 mg·kg^{-1} body weight (BW), diluted with 1 mL of physiological saline solution (LE group) or the same volume of physiological saline solution (LS group). The animals were anaesthetized by intraperitoneal injection of sodium pentobarbital (30 mg·kg^{-1} BW). A midline incision was made above the sternum, and the trachea was exposed by blunt dissection. A 27-gauge needle was inserted into the trachea above the carina, and LPS, 0.1 mg·kg^{-1} BW, diluted with 0.15 mL of sterile physiological saline was instilled. The SS group was given the same volume of physiological saline both intratracheally and intraperitoneally.

After recovery from the anaesthesia, the animals were returned to their cages and given access to food and water *ad libitum*. Two and 24 h later, five animals in each group were weighed, anaesthetized by intraperitoneal injection of sodium pentobarbital, and exsanguinated by cutting the abdominal aorta. Thoracotomy was performed, and the lungs were examined. After endotracheal intubation, the right lung was ligated at the right main bronchus and surgically removed. The right lung was weighed, dried in a conventional laboratory oven for 48 h, and weighed again, and the wet-to-dry lung weight ratio (W/D) was calculated.

Bronchoalveolar lavage (BAL)

BAL of the left lung was performed *via* the tracheal cannula, using a total of 10 mL (4, 3, 3 mL) of sterile physiological saline solution. The BAL fluid was then filtered through two layers of sterile gauze to remove mucous strands, and the fluid and cellular fractions were separated by centrifugation at 400×g for 10 min at 4°C. The cell pellet was resuspended in Hank's balanced salt solution, the total number of cells was counted with a haemocytometer, and differential counts of 200 cells were made on a Wright-stained smear of cytospin preparations (Shandon Southern Instruments, Sewickley, PA, USA). The albumin concentration of the supernatant was measured by direct spectrophotometric determination according to the method of ROCKLEY [27]. The remaining supernatant was concentrated 10 fold in a Centricon 10 (Amicon Co., Denver, CO, USA) with a threshold molecular weight of 10,000 and frozen at -80°C until assayed for elastase-like activity.

Concentration of ONO-5046 in plasma and BAL fluid

In a separate experiment, 25 animals were used to measure ONO-5046 concentrations. The concentrations of ONO-5046 in plasma and BAL fluid 2, 12 and 29 h after intraperitoneal injection of ONO-5046·Na, 300 mg·kg^{-1}, into nontreated hamsters (n=5 at each time point), and the concentration of ONO-5046 in the BAL fluid of LPS-treated animals 2 and 24 h after ONO-5046·Na injection (n=5 at each time point), were measured at the Minase Research Institute, Ono Pharmaceutical Co. Ltd. BAL was performed using 6 mL of physiological saline (recovery rate 77±2%). Samples of spiked plasma and BAL fluid with an internal standard (ONO-EI-547) were diluted with distilled water and 1 N HCl, and then applied to a Sep-Pak C_{18} short column (Waters Co., Milford, MA, USA). ONO-5046 and the internal standard (ONO-EI-547) were eluted with methanol from the short column, and the methanol solution was applied to a Bond Elut SAX short column (Varian Co., Harbor City, CA, USA). ONO-5046 and the internal standard (ONO-EI-547) were eluted with 0.1 N HCl-CH_3OH (40:60 v/v) solution from the short column, and the compounds in this solution were extracted with ethyl acetate. The organic phase was evaporated to dryness, and the residue was dissolved in 150 μL of the mobile phase and subjected

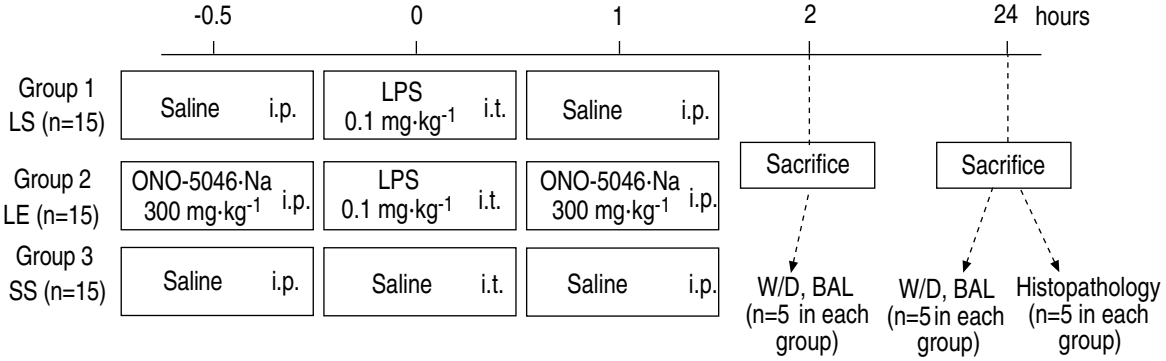

Fig. 1. – The design of the experiment. i.p.: intraperitoneally; i.t.: intratracheally; LS: treated with LPS intratracheally and with saline intraperitoneally; LE: treated with lipopolysaccharide (LPS) intratracheally and with a specific neutrophil elastase inhibitor (ONO-5046·Na) intraperitoneally; SS: treated with saline both intratracheally and intraperitoneally; W/D: wet to dry lung weight ratio; BAL: bronchoalveolar lavage.

to high-performance liquid chromatography (HPLC). A liquid chromatograph system equipped with a pump, an ultra violet (UV) detector and an autosampler (LC-module-1; Waters, Milford, MA, USA) was used. A Chemcosorb 3Dph column (3 μm, 150×4.6 mm internal diameter (ID); Chemco, Osaka. Japan) was used as the analytical column. The aliquot (50 μL) was injected through the auto-sampler onto an analytical column, and the mobile phase composition was changed from 0.02 M KH_2PO_4 (pH 3.8)-CH_3CN (4:1 v/v) to 0.02 M KH_2PO_4 (pH 3.8)-CH_3CN (5:4, v/v) in the linear gradient mode for 40 min. Separation of each sample was completed within 55 min, and quantitation of ONO-5046 in plasma and BAL fluid was carried out based on the calibration lines obtained by the peak area ratio to the internal standard (ONO-EI-547).

Elastase-like activity

The elastase-like activity of the BAL fluid was measured using a synthetic elastase substrate, succinyl-trialanine-nitroanilide [Suc-$(Ala)_3$-NA], according to the method of BIETH *et al*. [28]. Briefly, 0.5 mL of the concentrated supernatant of the BAL fluid was mixed with an equal volume of 0.2 M Tris buffer (pH 8.0), and the mixture was incubated with 20 μL of 0.125 M [Suc-$(Ala)_3$-NA] at 37°C for 30 min. Optical density was read at 410 nm before and after the addition of [Suc-$(Ala)_3$-NA], and any increase in absorbance above background was attributed to elastase-like activity in the sample. Elastase-like activity (nmol of NA released·h^{-1}·0.1 mL^{-1}) in the BAL fluid was determined by referring to a standard curve constructed using known concentrations of porcine pancreatic elastase. To determine whether the elastase activity detected was attributable to metalloproteinase (derived from macrophages) or serine proteinase (derived mainly from neutrophils), enzyme inhibition was performed by incubating BAL fluid samples with either the metalloproteinase inhibitor disodium ethylenediamine tetra-acetate (EDTA) (100 mM) or the serine proteinase inhibitor diisopropyl fluorophosphate (DFP) (100 mM) prior to addition of [Suc-$(Ala)_3$-NA].

Histopathological findings

Histological studies were performed in 15 animals 24 h after intratracheal administration of LPS (LS and LE groups) or physiological saline (SS group) (n=5 in each group). The hamsters were sacrificed, their lungs were inflated *via* a tracheal cannula with 2.5% glutaraldehyde buffered with sodium cacodylate (pH 7.4) at a constant pressure of 20 cmH_2O for at least 48 h, and the lungs were then used for light microscopic study. Three specimens of pulmonary tissue were obtained from the left lung (medial third, middle third, and lateral third). Paraffin-embedded tissue blocks were cut 3 μm thick, and stained with haematoxylin and eosin. Inflammatory cell infiltration of the lung was assessed, and each lung section was assigned an inflammatory score as follows: 1=rare or occasional inflammatory cells throughout the lung; 2=numbers of inflammatory cells intermediate between 1 and 3; 3=a large number of inflammatory cells throughout the lung. The total inflammation score for each animal was calculated as the mean of the three lung sections. All assessments were made independently by two observers (A.N. and S.Y.) without any knowledge of the procedures to which the hamsters had been subjected.

Assay for neutrophil migration

In a separate experiment, neutrophils were isolated from nontreated hamsters according to the method of TAHAMONT *et al*. [29]. Briefly, fresh blood was drawn from 20 hamsters by venipuncture and immediately supplemented with 1/10 volume EDTA. The leucocyte population was separated from the red cells by sedimentation in 1.5% dextran for 45 min at room temperature. The leucocyte-enriched plasma was then layered over a discontinuous Percoll gradient (80, 70, 62, 55% (vol/vol)) and centrifuged at 400×g for 10 min at 20°C. The polymorphonuclear leucocyte (PMN)-rich layer was collected, washed once in excess hydroxyethylpiperazine ethanesulphonic acid (HEPES) buffer (140 mM NaCl, 10 mM HEPES, 10 mM KCl, 0.1 mM $CaCl_2$, 11.9 mM $NaHCO_3$, 5 mM glucose and 14.5 μM albumin), and resuspended in HEPES at 2×10^6 cells·mL^{-1}. The purity of the isolated neutrophils was greater than 90%, and cell viability exceeded 95% on the basis of the trypan blue dye exclusion criterion. Portions of the PMNs prepared were treated with ONO-5046·Na (3, 30, 300 μg·mL^{-1}) for 30 min at room temperature, and the rest of the cells were made available as a control. Our selection of the concentrations of ONO-5046·Na was based on the finding that 30 μg·mL^{-1} is in the vicinity of the peak level in the peripheral blood of hamsters. Neutrophil migration was determined by the microchamber technique, using a 48-well microchemotaxis chamber (Neuroprobe Inc., Pleasanton, CA, USA) in accordance with the method described by FALK *et al*. [30]. To assess whether ONO-5046·Na affects capacity for migration toward known chemoattractants, 25 μL of 10^{-6} M N-formal-methionyl-leucyl-phenylalanine (fMLP), 0.2 mg·mL^{-1} LPS diluted in HEPES was placed in the lower well of the chemotaxis chamber, and 50 μL of PMN suspension (2×10^6·mL^{-1}) preincubated with ONO-5046·Na or HEPES alone was placed in the upper well. These two wells were separated by a 3 μm pore size polyvinylpyrrolidone (PVP)-free polycarbonate filter (Nucleopore Corp., Pleasanton, CA, USA). The chemotaxis chambers were then incubated at 37°C in air with 5% CO_2 for 30 min. After incubation, the filters were removed, fixed and stained with Diff-Quick stain (Harleco, Gibbstown, NJ, USA). All samples were run in duplicate. The numbers of cells that had migrated across the filters were randomly counted in five oil immersion fields (×1000) and averaged. The mean value of the buffer control (random neutrophil migration) was subtracted from the experimental values, so that all reported data represent net neutrophil migration.

Statistical analysis

Data for each treatment group are shown as mean±SEM. The means for all of the treatment groups were compared by analysis of variance (ANOVA) to test for significance of differences, and this was followed by further analysis using student-Newman-keul's test to assess differences between individual treatment groups. Statistical significance was assumed at a p-value of less than 0.05.

Results

There were no deaths in any of the groups during the 2 and 24 h test periods.

Concentration of ONO-5046 in plasma and BAL fluid

The plasma concentration of ONO-5046 reached 28.8±12.1 μg·mL^{-1} (mean±SEM) at 2 hours, 9.35±1.3 μg·mL^{-1} at 12 h and 1.7±1.5 μg·mL^{-1} at 29 h after intraperitoneal administration of ONO-5046-Na, 300 mg·kg^{-1} (fig. 2). No ONO-5046 was detected in the BAL fluid of non-treated hamsters 2 and 29 h after LPS administration. The concentration of ONO-5046 in the BAL fluid of LPS-treated ONO-5046·Na-injected animals was 0.093±0.013 μg·mL^{-1} at 2 h and 0.013±0.002 μg·mL^{-1} at 24 h after LPS administration.

Findings 2 h after LPS administration

The W/D and the BAL fluid findings are shown in table 1. W/D was the same in all three groups. Albumin concentrations in the LE group were significantly lower than in the LS group. No albumin was detected in BAL fluid (<20 μg·mL^{-1}) in the SS group.

The total cell number, neutrophil number and elastase-like activity in the BAL fluid in the LE group were similar to the SS group but significantly lower than in the LS group. Elastase-like activity was completely abolished by the addition of DFP, but only partially abolished by EDTA.

Findings 24 h after LPS administration

The W/D and BAL fluid findings are shown in table 2. W/D in the LE group was significantly lower than in the LS group, but significantly higher than in the SS group.

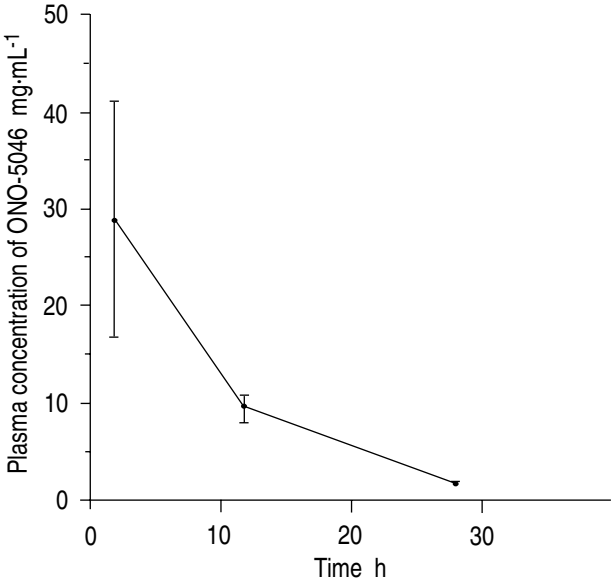

Fig. 2. – Time course of plasma concentration of ONO-5046. ONO-5046·Na, 300 mg·kg^{-1} body weight, was administered into the animals intraperitoneally. Values are presented as mean±SEM.

The BAL fluid albumin concentration was significantly lower in the LE group than in the LS group. No albumin was detected in the BAL fluid (<20 μg·mL^{-1}) of the SS group.

The total cell number and neutrophil number in the BAL fluid in the LE group was significantly lower than in the LS group but significantly higher than in the SS group.

Elastase-like activity in the LE group was significantly lower than in the LS group, but not significantly different from the level of activity in the SS group.

In the animals in the SS group, comparable measurements between 2 and 24 h were not significantly different.

Lung histopathology

Light microscopic examination of the lungs of the LS group 24 h after LPS administration revealed intra-alveolar haemorrhage and a large number of inflammatory cells, especially neutrophils, infiltrating the alveolar spaces (fig. 3a). In contrast to the LS group, there were few inflammatory cells in the lungs of the LE group (fig. 3b). The lungs of the animals in the SS group appeared normal (fig. 3c). The inflammation scores assigned by

Table 1. – Wet to dry (W/D) lung weight ratio and BAL fluid findings at 2 h

	W/D	Albumin concentration μg·mL^{-1}	Total cell number ×10^{5}cells·mL^{-1}	Neutrophil number ×10^{5}cells·mL^{-1}	Elastase-like activity nmolNA·h^{-1}·mL^{-1}
LS group (n=5)	4.57±0.13	127.3±55.4*	22.2±8.1*	19.5±7.8$^{\dagger\#}$	3.30±0.51**
LE group (n=5)	4.41±0.11	22.3±15.1	4.8±1.1	0.94±0.55	0.96±0.30
SS group (n=5)	4.46±0.04	ND	6.2±1.5	1.03±0.58	1.02±0.47

Values are presented as mean±SEM. LS: treated with lipopolysaccharide (LPS) intratracheally and with saline intraperitoneally; LE: treated with LPS intratracheally and with a specific neutrophil elastase inhibitor (ONO-5046·Na) intraperitoneally; SS: treated with saline both intratracheally and intraperitoneally; ND; not detected; BAL: bronchoalveolar lavage; *: significantly ($p<0.05$) different from LE group; **: significantly ($p<0.05$) different from LE and SS groups; †: significantly ($p<0.02$) different from LE group; $^{\#}$: significantly ($p<0.01$) different from SS group.

Table 2. – Wet to dry (W/D) lung weight ratio and BAL fluid findings at 24 h

	W/D	Albumin concentration $\mu g \cdot mL^{-1}$	Total cell number $\times 10^5 \cdot mL^{-1}$	Neutrophil number $\times 10^5 \cdot mL^{-1}$	Elastase-like activity nmol NA$\cdot h^{-1} \cdot mL^{-1}$	Inflammation score
LS group (n=5)	5.68±0.11†	179.2±80.0*	134.0±37.0*#	114.9±30.5†	2.79±0.09†	2.40±0.21†
LE group (n=5)	5.00±0.09#	51.3±14.4	43.3±17.7**	26.1±10.7#	0.40±0.09	1.32±0.16#
SS group (n=5)	4.41±0.14	ND	3.1±1.2	0.31±0.12	0.59±0.05	0.30±0.20

Values are presented as mean±SEM. NA: succinyl-trialanine-nitroanilide. For further abbreviations see legend to table 1. *: significantly ($p<0.05$) different from LE group; **: significantly ($p<0.05$) different from SS group; #: significantly ($p<0.01$) different from SS group; †: significantly ($p<0.01$) different from LE and SS groups.

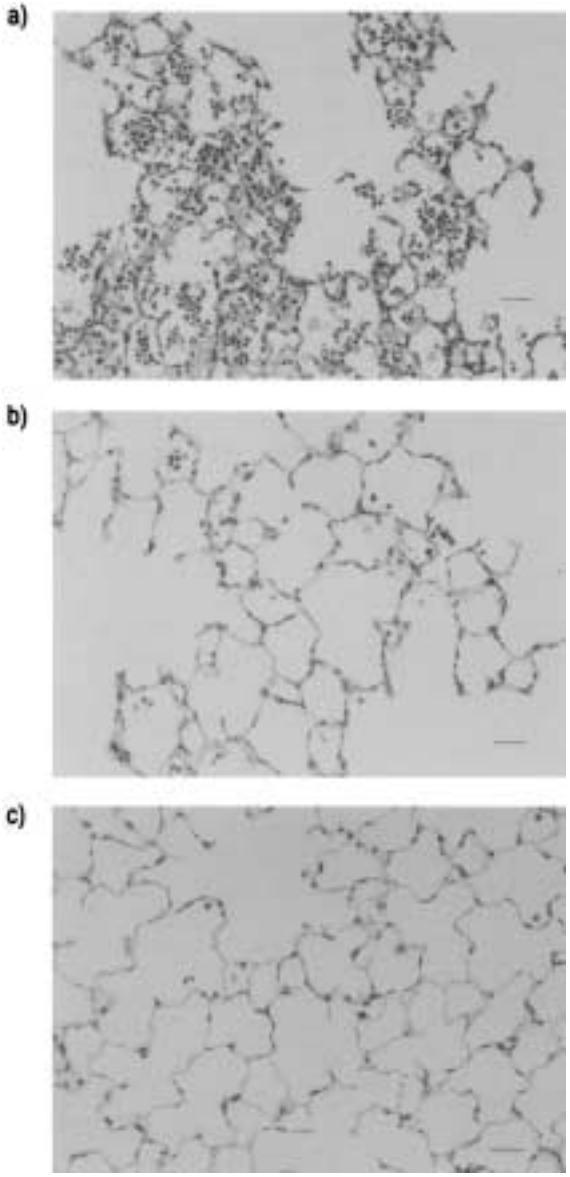

Fig. 3 – a) Light micrograph of the lungs of the animals 24 h after lipopolysaccharide (LPS) treatment alone. Numerous inflammatory cells and haemorrhage are observed in the intra-alveolar space. b) Light micrograph of the lungs of the animals 24 h after LPS treatment and administration of neutrophil elastase inhibitor. Inflammatory cells are less prominent than in the lungs of the LS animals. LS: treated with LPS intratracheally and with saline intraperitoneally. c) Light micrograph of the lungs of the animals 24 h after saline treatment both intratracheally and intraperitoneally. Normal observation of the alveolar region. (Internal scale bars=10 μm).

the two examiners were highly correlated(r=0.91). The inflammation scores assigned by one of them are shown in table 2. The LS group had a significantly higher score than the LE group, whilst the score in the SS group was significantly lower than in the other two groups.

Neutrophil migration

When ONO-5046·Na and 10^{-6} M fMLP were placed in the upper and lower chambers, respectively, of the chemotaxis plate, the number of cells migrating in the absence of ONO-5046·Na was 9.0±0.8 cells·field^{-1} (mean±SEM) *versus* 7.0±1.2, 7.4±1.0 and 6.8±1.1 cells·field^{-1} in the presence of ONO-5046·Na at concentrations of 3, 30 and 300 $\mu g \cdot mL^{-1}$, respectively. There was no correlation between dose and migration. When ONO-5046·Na was placed in the upper chamber of the chemotaxis plate and 0.2 $mg \cdot mL^{-1}$ LPS in the lower chamber, the number of cells migrating in the absence of ONO-5046·Na was 6.3±1.1 cells·field^{-1} (mean±SEM), *versus* 5.9±0.8, 5.4±0.6 and 5.1±0.8 cells·field^{-1} in the presence of ONO-5046·Na at concentrations of 3, 30 and 300 $\mu g \cdot mL^{-1}$, respectively.

Discussion

ONO-5046·Na, sodium N-[2-[4-(2,2-dimethylpropionyloxy) phenylsulphonylamino] benzoyl] aminoacetate tetrahydrate (MW 528.51), is a chemical compound that competitively inhibits human neutrophil elastase as well as leucocyte elastase obtained from rabbits, rats, hamsters and mice [26]. ONO-5046 is inactive against bovine pancreatic trypsin, porcine pancreatic kallikrein, human plasma kallikrein, bovine pancreatic chymotrypsin and human plasma cathepsin G [26], and possesses no antioxidant activity [31].

The histological findings, W/D, albumin concentration, intra-alveolar cell number and elastase-like activity in BAL fluid in the present study showed that a single intratracheal dose of LPS induced acute lung inflammation 2 and 24 h after LPS injection. Increases in neutrophil number and elastase-like activity were observed in the BAL fluid of the LPS-injected animals, but these increases were smaller in the ONO-5046·Na-treated animals.

The present findings in the ONO-5046·Na-treated animals 2 h after LPS instillation are of considerable

interest. An albumin leak was found in these animals, but neutrophils were not increased in BAL fluid. These findings suggest that the serum albumin leak precedes neutrophil migration in LPS-treated animals. This may not be surprising, since the neutrophil sequestration in the pulmonary vasculature produced by LPS administration may first damage endothelial cells, and the serum albumin leak may thus precede neutrophil migration into the intra-alveolar spaces. This notion is supported by previous reports that intra-alveolar oedema was observed beginning 2 h after the LPS injection, whereas intra-alveolar neutrophils were rare even after 4 h [32], and that there may be dissociation between changes in vascular permeability and the transvascular migration of neutrophils in the lung after LPS injection [14].

The present findings, that the LPS-induced increase in elastase-like activity at 24 h was inhibited in ONO-5046· Na-treated animals as early as at 2 h despite an increased neutrophil influx into the intra-alveolar spaces, suggest that endogenous inhibitors, such as α_1-protease inhibitor, may have played some role in inhibiting elastase-like activity at 24 h in those animals. The concentration of ONO-5046 in the BAL fluid in LPS-ONO-5046·Na-treated animals was 0.214±0.031 μM at 2 h, which was higher than the concentration causing 50% inhibition (IC_{50}) value of 0.037±0.004 μM for inhibition of hamster leucocyte elastase [26], and declined to 0.029±0.005 μM at 24 h. Since a high concentration of ONO-5046 was present in the intra-alveolar spaces at 2 h of LPS-ONO-5046·Na-treated animals, it would enhance the anti-elastase screen compared to 24 h. Nevertheless, elastase-like activity was still low at 24 h when ONO-5046 levels were low, suggesting that increased endogenous antielastases had contributed to the result.

Our *in vitro* study showed that ONO-5046·Na had no effect on neutrophil migration. However it is still possible that ONO-5046·Na inhibits neutrophil migration into the intra-alveolar spaces *in vivo*, since it has been reported that elastase is required for neutrophil migration accompanied by degradation of capillary basement membrane collagen [33], and for solubilization of alveolar extracellular matrix [34]. A recent study has shown that secretory leucocyte proteinase inhibitor (SLPI), a highly selective inhibitor of neutrophil elastase, reduces elastase activity in the alveolar wall and the intra-alveolar spaces of LPS-treated animals [9]. The authors suggested that infiltrating neutrophils release elastase, which facilitates migration from capillaries through the interstitium into the intra-alveolar spaces. In our study, it is possible that the plasma concentration of ONO-5046 at 2 h (28.8 $\mu g \cdot mL^{-1}$) may have been high enough to prevent neutrophils from degrading the matrix collagen of basement membrane, thus impairing their migration. This concept may be supported by the findings that continuous intravenous infusion of 1 $mg \cdot kg^{-1}$ ONO-5046·Na-inhibited neutrophil accumulation in the lungs of hamsters after LPS instillation, and the plasma concentration of ONO-5046 in the study was above 25 $\mu g \cdot mL^{-1}$ (personal communication, T. Hagio). Even when the plasma concentration of ONO-5046 decreased at 24 h (near 1.70 $\mu g \cdot mL^{-1}$), the neutrophil influx was inhibited, although not completely. Since a relatively moderate amount of ONO-5046 was present in the intra-alveolar spaces at this stage, neutrophil chemotaxis and solubilization of alveolar extracellular matrix would have been inhibited. Another possible explanation for the inhibitory mechanisms of the neutrophil migration is that the elastase inhibitor reduced the activity of chemotactic factors, such as interleukin-8 (IL-8) [35, 36] and tumour necrosis factor-α (TNF-α) [37] in the lungs of LPS-treated animals. However, further studies will be required to clarify this possibility.

As seen in the present study, a single intratracheal dose of LPS causes a massive influx of neutrophils and results in acute lung inflammation or lung injury [5–7]. Induction of emphysema, on the other hand, has been reported to occur in response to repeated intratracheal instillation of LPS [8–11]. In the LPS-induced model of emphysema, LPS promotes neutrophil influx into the lung, and neutrophil elastase may produce the emphysema [8–11]. The results of the present study suggest that neutrophil elastase is also involved in acute lung inflammation induced by LPS.

In conclusion, ONO-5046·Na, a novel elastase inhibitor specific for neutrophil elastase, clearly reduced LPS-induced acute lung inflammation. The data in our study suggest that the effect of the inhibitor is not only due to simple elastase inhibition within the lung itself, but may well be due to a major effect on cell migration or infiltration, either directly or indirectly. However, the precise mechanism of the inhibitory effects remains uncertain and further experimentation may be necessary to clarify the mechanisms. Although, at the present time, we cannot conclude that the elastase released by neutrophils is a primary causative factor in the acute lung inflammation induced by LPS, the evidence presented here supports the possibility that elastase plays a considerable role in the development of LPS-induced acute lung inflammation.

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