Pseudomonas-induced neutrophil recruitment in the dog airway \textit{in vivo} is mediated in part by IL-8 and inhibited by a leumedin

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ABSTRACT: A bacteria-free supernatant of \textit{Pseudomonas aeruginosa} induces the production of neutrophil chemotactic activity in human bronchial epithelial cells \textit{in vitro} that is due to the potent chemotactic factor, interleukin-8 (IL-8). Because \textit{P. aeruginosa} supernatant itself is not chemotactic, we hypothesized that intratracheal \textit{P. aeruginosa} induces the production of neutrophil chemotactic factors, including IL-8, \textit{in vivo}. Because neutrophils play a key role in cystic fibrosis, inhibition of neutrophil recruitment might be therapeutic.

We studied the effect of \textit{P. aeruginosa} supernatant in the isolated tracheal segment of dogs \textit{in vivo}, and we measured neutrophil chemotactic activity \textit{in vitro} in the tracheal fluid. We also determined the local effect of intratracheal administration of leumedin NPC 15669, an inhibitor of neutrophil recruitment, on IL-8- and \textit{P. aeruginosa}-induced neutrophil accumulation.

\textit{P. aeruginosa} supernatant and IL-8 both caused time-dependent accumulation of neutrophils in the tracheal fluid. Tracheal fluid obtained after \textit{P. aeruginosa} administration had neutrophil chemotactic activity \textit{in vitro} that was significantly inhibited by the IL-8 antibody. Intratracheal NPC 15669 prevented both IL-8- and \textit{P. aeruginosa}-induced accumulation of neutrophils.

We conclude that \textit{P. aeruginosa} supernatant recruits neutrophils into the airway indirectly by inducing the production of chemotactic factors, including IL-8. Our results suggest a potential therapeutic role for leumedins in chronic airway diseases. \textit{Eur Respir J.}, 1994, 7, 1925–1931.

Lung disease in patients with cystic fibrosis is characterized by recurrent and chronic airway infections, frequently with \textit{Pseudomonas aeruginosa} (PA), massive neutrophil infiltration, and mucus hypersecretion [1]. The high concentration of neutrophil-derived deoxyribonucleic acid (DNA) significantly alters the viscoelastic properties of sputum of patients with cystic fibrosis; the large quantities of neutrophil proteases, including elastase, in sputum are incriminated in the pathophysiology of mucus hypersecretion [2–4]. In addition, neutrophils and their products are strongly implicated in the tissue damage which occurs in the airways of these patients [5, 6]. In spite of the marked recruitment of neutrophils, PA is able to evade the host defence mechanisms in the airways of cystic fibrosis patients [6].

Evidence is accumulating that interleukin-8 (IL-8), a potent neutrophil chemotactic factor, plays an important role in neutrophil recruitment in several airway diseases, including cystic fibrosis [7]. Sputum from cystic fibrosis patients contains high concentrations of IL-8 [7]. Recently, we showed that a bacteria-free supernatant of PA that was itself not chemotactic, induced the production of neutrophil chemotactic activity in the culture medium of a human bronchial epithelial cell line that was due to IL-8 [8]. This finding suggested an indirect mechanism for PA-induced neutrophil recruitment; factors released by PA bacteria may be responsible for recruiting neutrophils into the airway \textit{in vivo}. Because this hypothesis is not easily tested \textit{in vivo} in humans, we studied the effect of administering a bacteria-free supernatant of PA on neutrophil recruitment in the isolated canine tracheal segment \textit{in vitro}. We also determined whether the PA supernatant causes neutrophil recruitment directly or indirectly by measuring the neutrophil chemotactic activity \textit{in vitro} in the PA supernatant alone, and in the tracheal fluid removed from the dogs given PA supernatant compared to control dogs.

To determine whether PA induces the production of IL-8-like activity in the dog, we incubated the tracheal fluid obtained after PA administration with a blocking monoclonal antibody to human IL-8.

Because neutrophils and their products play an im-
portant role in the pathophysiology of the airway disease in cystic fibrosis, a drug that inhibits neutrophil recruitment into the airway might prove therapeutically beneficial, especially if such a drug were effective after local administration. One potential inhibitor of neutrophil recruitment into the airways is NPC 15669 (N-[9H-2-(7-dimethylfluorenyl-9-methoxy)carbonyl]-L-leucine), a member of a group of novel low molecular weight anti-inflammatory leucine derivatives known as leumodins [9]. NPC 15669 administered systemically, intraperitoneally or in the gastrointestinal tract has been shown to be an effective inhibitor of neutrophil recruitment in several animal models of inflammation [9–13]. NPC 15669 has been found to inhibit arachidonic acid- and phorbol ester-induced ear oedema in mice [9], to reverse the passive Arthus reaction in rats [12], to prevent leucopenia and mortality in endotoxic shock in mice [10], to inhibit neutrophil recruitment and subsequent epithelial and vascular damage in anesthetic acid colitis in rats [11], and to prevent neutrophil activation and pulmonary damage in the postperfusion syndrome following cardiopulmonary bypass in pigs [14]. However, the effect of local treatment with NPC 15669 on neutrophil recruitment into the airways is unknown. Therefore, we tested the ability of NPC 15669 administered intratracheally to inhibit both IL-8-induced and PA-induced neutrophil recruitment into the trachea.

Methods

In vivo studies

Animal preparation. Four dogs (weighing 14–26 kg) were selected at random from a colony of inbred ragweed-sensitized animals [15]. The experimental protocol was approved by the Committee on Animal Research of the University of California, San Francisco, USA and was in accordance with the published "Guiding Principles in the Care and Use of Animals" of the council of the American Physiological Society. The animals were anaesthetized with sodium pentobarbital (30 mg·kg-1 i.v. initially, and 65 mg·ml-1 i.v. as needed). Intravenous normal saline (50 ml·h-1) was used to prevent dehydration, and a heating pad was used to maintain body temperature at 37°C. The dogs were intubated per os with a modified double-balloon endotracheal tube [16], and ventilated with a constant-volume respirator (Harvard apparatus model 607A, Dover, MA, USA), set to deliver a tidal volume of 10 ml·kg-1 at a frequency of 20 breaths·min-1.

Isolation of tracheal segment. A tracheal segment was isolated and perfused as described previously [16]. Briefly, a standard cuffed endotracheal tube was modified by adding a second balloon, creating an isolated segment 6 cm long. After inflating the two balloons, the isolated segment was perfused through an open system consisting of two small-bore silastic tubes to provide flow of fluid to and from the interballoon segment. A polypropylene

lene 20 ml syringe barrel was used as a reservoir, from which the solution (total volume 20 ml) was pumped to and from the segment by a peristaltic roller pump (Buchler, Fort Lee, NJ, USA; 14 ml·min-1). Sterile Hank’s balanced salt solution (HBSS), supplemented with 100 U·ml-1 penicillin and 100 µg·ml-1 streptomycin to prevent bacterial growth during the experiments, was used in the control experiments. The endotoxin content measured by the limulus amoebocyte lysate test (LAL Pyrotest, Associates of Cape Cod Inc., Cape Cod, MA, USA) was less than 100 pg·ml-1 in HBSS and 13 µg·ml-1 in PA supernatant diluted in HBSS. The test substances (PA supernatant, control bacterial culture medium, IL-8 or NPC 15669) were diluted in HBSS supplemented with the antibiotics and administered into the isolated tracheal segment at the concentrations indicated. All solutions administered into the tracheal segment were isotonic and had a neutral pH.

Experimental protocol. The experiments were conducted in four dogs, which served as their own controls. On separate occasions, each dog was exposed to: 1) control buffer solution of HBSS supplemented with antibiotics (the negative control for IL-8); 2) IL-8 10-8 M (the positive control for neutrophil recruitment); 3) IL-8 10-8 M and NPC 15669 10-5 M (an inhibitor of neutrophil recruitment); 4) trypotcine soy broth dialysate (TSBD) (the culture medium of PA, used as a negative control for PA supernatant); 5) PA supernatant; and 6) PA supernatant and NPC 15669 10-5 M. IL-8 was administered at a concentration of 10-4 M, as this was previously shown to be maximally chemotactic in the isolated dog trachea in vivo [17]. NPC 15669 was administered at a concentration of 10-5 M because the median inhibitory concentration (IC50) for neutrophil infiltration in a mouse model of inflammation after topical administration of arachidonic acid was 14±3 µM (n=6) [9]. To exclude the possibility that the results may have been affected by repeated exposure, two dogs were exposed a second time to HBSS and two other dogs were exposed a second time to PA supernatant. Exposure to the different test solutions occurred at one week intervals. Samples (1 ml) were removed from the reservoir at the beginning of the experiment and at 1 h intervals for 6 h. These samples were replaced with 1 ml of HBSS containing antibiotics, and the results were calculated taking this dilution into account. The samples of tracheal fluid were assessed for cell counts with a haemocytometer. A modified Giemsa stain (Diff-Quik, American Scientific Products, McGaw Park, IL, USA) was performed on the cytospin of cytocentrifuge-concentrated samples. At least 200 cells per sample were examined for differential counts.

Preparation of PA supernatant. PA supernatant was prepared from strain PA103 (kindly provided by J. Wiener-Kronish, UCSF) grown in TSBD (Cell Culture Facility, UCSF) to stationary phase at 32°C for 72 h [18]. This strain was chosen for its toxicity and because it is well-characterized [19]. Supernatant of 2×1010 cultured PA103 bacteria·ml-1 was obtained by centrifugation at 10,000
In vitro studies

Canine neutrophil isolation. Citrate-buffered canine blood was diluted 1:2 with Ca²⁺/Mg²⁺-free HBSS, layered on top of Histopaque-1077 (Sigma, St. Louis, MO, USA), and centrifuged at 400 g for 20 min. Red blood cells were removed from the neutrophil-rich pellet by hypotonic lysis. The neutrophil pellet was washed with phosphate-buffered saline (PBS) enriched with 2% bovine serum albumin (BSA) (Sigma, St. Louis, MO, USA), and resuspended in HBSS with Ca²⁺/Mg²⁺ and 2% BSA to a final concentration of 4×10⁶ cells·ml⁻¹ for the studies of chemotaxis in vitro. The final neutrophil suspension was at least 95% pure, and the cell viability was 98% as determined by trypan blue dye exclusion.

In vitro chemotaxis. In vitro chemotaxis was performed in a 48 well microchemotaxis chamber (Neuroprobe, Cabin John, MD, USA) [20]. The chemoattractant (25 µl) was placed in the lower wells of the chamber in duplicate. Fifty microlitres of the neutrophil suspension was placed in the top wells and allowed to migrate through nitrocellulose filters (Neuroprobe, Cabin John, MD, USA) of 3 µm pore size at 37°C for 25 min. The filters were fixed with 70% methanol, stained with haematoxylin, and cleared with xylene. Migration was assessed according to the leading front method by recording the mean from five fields on duplicate filters [21]. The results are expressed as net migration (mean distance minus random migration). Random migration is defined as the migration towards HBSS alone.

The chemotactic activity of PA supernatant and TSBD at a dilution of 1:80 was assessed. We have previously reported that PA supernatant is not chemotactic at a dilution of 1:1, 1:3, 1:10, 1:30, 1:100, 1:300 or 1:1,000 [8]. To determine whether IL-8-like activity was responsible, in part, for the appearance of neutrophil chemotactic activity 6 h after administration of the PA supernatant, tracheal fluid was incubated with and without F(ab')₂ fragments of a murine monoclonal anti-human IL-8 antibody (A.5.12.14 immunoglobulin 2a (IgG2a); provided by J. Kim, Genentech Inc., South San Francisco, CA, USA). A specific antibody to dog IL-8 was kindly supplied by G. Baker, Genentech, South San Francisco, CA, USA. Human monocyte-derived recombinant IL-8-β interferon, transforming growth factor-β, colony stimulating factor, granulocyte/macrophage colony-stimulating factor, platelet factor IV, β-thyroglobulin, tumour necrosis factor-α, or C5a [7]. Samples were incubated alone or with F(ab')₂ fragments of IL-8 antibody or F(ab')₂ fragments of a control antibody (a monoclonal antibody to gp120, a human immunodeficiency virus (HIV) antigen; provided by B. Fendly, Genentech, South San Francisco, CA, USA). Samples of tracheal fluid obtained from the four dogs 6 h after administration of PA supernatant were incubated with F(ab')₂ fragments (100 µg·ml⁻¹) or with HBSS containing Ca²⁺, Mg²⁺ and 2% BSA for 30 min at 37°C before assessing neutrophil chemotactic activity. The F(ab')₂ fragments of the anti-human IL-8 antibody were previously used in a rabbit model of asbestos-induced pleurisy to demonstrate a role for mesothelial cell-derived IL-8, and were effective in this nonhuman species at a concentration of 100 µg·ml⁻¹ [22].

Chemical reagents and drugs

Sterile HBSS, TSBD, penicillin and streptomycin were obtained from the Tissue Culture Facility, University of California, San Francisco, USA; 0.9% NaCl solution from Travenol laboratories, Deerfield, IL, USA; and sodium pentobarbital from Anthony Products Co., Arcade, CA, USA. Human monocyte-derived recombinant IL-8 was kindly supplied by G. Baker, Genentech, South San Francisco, CA, USA. NPC 15669 was kindly provided by Scios-Nova Inc., Mountain View, CA.

Statistical analysis

The data were analysed using a Macintosh II computer, using the Primer of Biostatistics program (McGraw-Hill Inc., 1988). Data are presented as the mean±SEM and compared using a one-way analysis of variance followed by a two-tailed Student’s t-test with the Bonferroni correction for multiple comparisons. Values of p<0.05 are considered as significant. The effect of the IL-8 antibody on the neutrophil chemotactic activity measured in vitro in the tracheal fluid obtained 6 h after administration of PA was analysed by Student’s paired t-test. The in vitro chemotactic activity in the tracheal fluid obtained at different times after administration of PA supernatant was analysed by multiple linear regression [23].

Results

Recruitment of inflammatory cells in vivo

Effect of interleukin-8 (IL-8). During the first 2 h of each experiment, the fluid removed from the isolated tracheal segment contained few cells, consisting of a mixed
population of macrophages, epithelial cells, neutrophils and, rarely, eosinophils. Six hours after administration of HBSS alone, total cell counts in the tracheal fluid reached a maximum of 82±13 cells·µl⁻¹ (n=4), and consisted of more than 90% neutrophils.

Intratracheal administration of IL-8 10⁻⁸ M caused a time-dependent recruitment of inflammatory cells consisting of more than 92% neutrophils, which became significantly different from HBSS at 3 h (fig. 1a). Intratracheal administration of Pseudomonas aeruginosa (PA) supernatant caused a time-dependent recruitment of inflammatory cells, consisting of more than 90% neutrophils, into the trachea, values which became significantly different from TSBD at 3 h (fig. 1b). Administration of NPC 15669 (10⁻⁵ M) significantly inhibited the recruitment of inflammatory cells induced by administration of PA supernatant (fig. 1b). In the two dogs exposed a second time to PA supernatant and in the other two dogs exposed a second time to HBSS, the numbers of neutrophils recruited into the trachea were similar to the numbers recruited during the first exposure.

Neutrophil chemotactic activity of tracheal fluid samples in vitro

PA supernatant or TSBD alone were not chemotactic for isolated dog neutrophils in vitro at the dilution of 1:80 administered to the dogs (data not shown, [8]). The tracheal fluid removed 6 h after administration of PA supernatant had significantly more neutrophil chemotactic activity than the tracheal fluid removed 6 h after administration of TSBD. When NPC 15669 was administered together with PA supernatant, the appearance of chemotactic activity at 6 h was inhibited (fig. 2). The chemotactic activity induced in the tracheal fluid by administration of PA supernatant was time-dependent (p=0.002) (fig. 3). Thus, the neutrophil chemotactic activity was minimum in the tracheal fluid removed 2 h after administration of PA supernatant (4.4±1.8 µm net migration) and increased at 4 and 6 h (21±7.5 and 29.9±

Fig. 1. – Effect of intratracheal administration of interleukin-8 (IL-8) and Pseudomonas aeruginosa (PA) supernatant on the total number of neutrophils in the tracheal fluid from four dogs. Data are reported as mean±SEM. *: p<0.05 compared to control or inhibited conditions. a) IL-8 10⁻⁸ M (—) caused a time-dependent accumulation of neutrophils compared to control values after administration of Hank’s balanced salt solution (HBSS) (—). This response was significantly inhibited by concomitant administration of NPC 15669 10⁻⁵ M (an inhibitor of neutrophil recruitment) (—). b) PA supernatant (—) caused a time-dependent accumulation of neutrophils compared to control values after administration of trypticone soy broth dialysate (TSBD), the bacterial culture medium alone (—). Concomitant administration of NPC 15669 10⁻⁵ M (—) significantly inhibited PA-induced neutrophil recruitment.

Fig. 2. – Neutrophil chemotactic activity in tracheal fluid obtained 6 h after administration of TSBD (the bacterial culture medium), Pseudomonas aeruginosa (PA) supernatant, and PA supernatant and NPC 15669 given together. Chemotaxis of peripheral blood dog neutrophils was measured toward the different 6 h samples of tracheal fluid in vitro. Data are mean±SEM (n=4). *: indicates p<0.05 compared to trypticone soy broth dialysate (TSBD) or PA supernatant with NPC 15669.
in dogs causes marked neutrophil accumulation in the trachea, and that this occurs by an indirect mechanism. PA supernatant administered into the isolated tracheal segment of dogs in vivo caused time-dependent recruitment of neutrophils. In addition, although the PA supernatant tested alone was not chemotactic for neutrophils in vitro, the tracheal fluid obtained after administration of PA supernatant had neutrophil chemotactic activity: this effect occurred after a delay and increased with time, suggesting that PA supernatant induced the production of chemotactic factors in the airway.

We investigated the possibility that one of these chemotactic factors might be IL-8. There are two reasons to suggest that one of the mediators of neutrophil recruitment could be IL-8. Firstly, a recent study found that PA supernatant stimulated IL-8 production in cultured human bronchial epithelial cells (16-HBE) and in monocytes [8]. In that study, the neutrophil chemotactic activity produced by the 16-HBE cells stimulated with PA supernatant was due primarily to IL-8. Secondly, high concentrations of IL-8 are found in sputum of patients with cystic fibrosis [7]; all the patients were chronically infected with PA, and IL-8 accounted for the majority of neutrophil chemotactic activity in the sputum obtained from these patients. The results from the present study show that production of IL-8 is, at least in part, responsible for the PA-induced accumulation of neutrophils in the trachea. Incubation with the F(ab')2 fragments of the anti-human IL-8 monoclonal antibody in vitro significantly inhibited the neutrophil chemotactic activity in the tracheal fluid samples that appeared 6 h after administration of PA supernatant. Incubation with the control antibody (to gp120) did not affect the in vitro chemotactic activity. These results indicate that PA induces the production of IL-8-like activity in the dog trachea, and that IL-8 is responsible, in large part, for the chemotactic activity in the tracheal fluid at 6 h in vivo.

We then studied the effect of intratracheal administration of NPC 15669, an inhibitor of neutrophil recruitment, on IL-8- and PA-induced neutrophil recruitment. The present study shows that local treatment with NPC 15669 effectively inhibits both IL-8- and PA-induced neutrophil recruitment. Of further interest, we found that NPC 15669 prevented the increase in neutrophil chemotactic activity measured in vitro in the tracheal fluid obtained 6 h after co-administration of this drug and PA. However, this finding may be due to the continued presence of NPC 15669 in the tracheal fluid samples, and a direct effect of NPC 15669 on in vitro chemotaxis of freshly isolated dog neutrophils. In a recent study on antigen-induced recruitment of inflammatory cells into the dog trachea, we found that human neutrophils pre-incubated with NPC 15669 from 10^{-5}–10^{-3} M inhibited chemotaxis in vitro to IL-8 10^{-5} M by 29–34% [24].

To ensure that neither an earlier exposure nor the status of the isolated tracheal segment influenced the neutrophil response to a later exposure, we performed our experiments one week apart to allow for adequate epithelial recovery, and we varied the order in which the

**Discussion**

The present study provides in vivo evidence that intratracheal administration of a culture supernatant of PA...
dogs were exposed to the experimental conditions. In addition, in the two dogs exposed a second time to buffer and in the two other dogs exposed a second time to PA supernatant, the numbers of neutrophils recruited into the trachea during the second exposure were similar to the numbers recruited during the first exposure. Thus, our results were not related to the timing or the order of exposures.

The ability of locally administered NPC 15669 to significantly inhibit IL-8- and PA-induced neutrophil recruitment into the airway in vivo suggests that this leumedin may have a beneficial role in the treatment of chronic airway inflammation where excessive neutrophil recruitment exacerbates and perpetuates the underlying airway disease, as occurs in cystic fibrosis. NPC 15669 has shown to inhibit neutrophil recruitment in a wide range of animal models of inflammation [9–13]. However, the present study is the first to show a beneficial effect with local administration in the airway and to specifically address IL-8-mediated neutrophil recruitment. Previous studies with NPC 15669 have shown that it does not inhibit cyclooxygenase, 5-lipoxygenase, phospholipase A2, phospholipase C or the binding of that it does not inhibit cyclooxygenase, 5-lipoxygenase, phospholipase A2, phospholipase C or the binding of ligands to several receptors, including those for leukotriene B4 (LTB4), platelet-activating factor (PAF), N-formyl-methionyl-leucyl-phenylalanine (FMLP), CsA or interleukin-1 [9]. In addition, leumedins are active in models dependent on recruitment of new leucocytes (Arthus reaction, antigen arthritis, arachidonic acid dermatitis, oxazolone dermatitis, adjuvant arthritis) but not in models independent of recruitment of new leucocytes (carrageenan oedema, anaphylactic bronchoconstriction) [9]. Although NPC 15669 has been shown to prevent adherence of stimulated neutrophils to endothelium and to inhibit the expression of the CD11b and CD18 adhesion molecule subunits on activated neutrophils, it is not an integrin antagonist and probably inhibits and reverses adherence via an intracellular mechanism [25]. Thus, the precise mechanism of action of NPC 15669 has yet to be elucidated. Nonetheless, the present study shows significant inhibition of neutrophil recruitment into the airway mediated by the potent neutrophil chemotactic factor, IL-8.

In summary, administration of PA supernatant into the dog trachea results in a time-dependent accumulation of neutrophils in the trachea and a time-dependent increase in neutrophil chemotactic activity in the tracheal fluid, which is due, in part, to the production of IL-8. NPC 15669 prevents both PA- and IL-8-induced neutrophil recruitment, suggesting a potential therapeutic role for this locally administered leumedin in chronic airway inflammation mediated by an excessive neutrophil response.

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