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Elevated concentrations of defensins in bronchoalveolar lavage fluid in diffuse panbronchiolitis

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ABSTRACT: Human neutrophils contain three isoforms of antimicrobial and cytotoxic peptides in the azurophil granules, which belong to a family of mammalian neutrophil peptides named defensins. Here we investigate the role of these peptides in diffuse panbronchiolitis (DPB).

Defensins (human neutrophil peptide-1,-2 and -3) were measured by radioimmunoassay in bronchoalveolar lavage fluid (BALF) of 30 patients with DPB, 16 patients with idiopathic pulmonary fibrosis (IPF) and 15 healthy adults.

The concentration of defensins was higher in BALF of patients with DPB than in patients with IPF and healthy subjects. DPB and IPF patients also had significantly higher plasma concentrations of defensins than controls. In patients with DPB, BALF concentration of defensins correlated significantly with neutrophil count or BALF concentration of interleukin (IL)-8. Immunohistochemistry of open-lung biopsy specimens from four DPB patients showed localization of defensins in neutrophils and mucinous exudate in the airways, and on the surface of bronchiolar epithelial cells. *In vitro* studies showed an enhanced extracellular release of defensins following stimulation of neutrophils with phorbol myristate acetate, N-formyl-methionyl-leucyl-phenyalamine, and human recombinant IL-8. Treatment of DPB with macrolides for 6 months significantly reduced neutrophil count and concentrations of defensins and IL-8 in BALF.

Our results indicate accumulation of neutrophil-derived defensins in the airway in diffuse panbronchiolitis, and suggest that defensins may be a marker of neutrophil activity in this disease.

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Neutrophils are major components of the host defence system, acting through different oxidative and nonoxidative microbicidal mechanisms. Peptides present in azurophil granules of the neutrophils play an important role in the nonoxygen-dependent killing of phagocytosed bacteria, and are, therefore, called defensins [1, 2]. Defensins are small, cationic, arginine- and cystein-rich peptides present abundantly in neutrophils of humans, rats, rabbits and guinea-pigs [2–5]. Six defensins have been recognized in humans, including four forms that are exclusive to neutrophils [2, 6] and two others found in Paneth cells in the small intestinal crypts [7, 8]. Three human neutrophil peptides (HNPs); HNP-1,-2 and -3, have been identified in human defensins and are involved in the destruction of bacteria, fungi and enveloped viruses. Common to these peptides are 29 amino acid sequences constituting 99% of the HNP molecules in the blood. Another HNP, HNP-4, has only 32% amino acid sequence homology with the other HNPs and shows anticorticotropin activity [6]. Defensins are potentially cytotoxic to tumour cells and lung-derived cells [9–11]. Therefore, they may be involved in the pathogenesis of neutrophil-associated lung diseases. While several studies have examined the role of neutrophil elastase [12, 13], to our knowledge, no study has defined the profile of defensins in the bronchoalveolar lavage fluid (BALF) of patients with lung diseases.

Diffuse panbronchiolitis (DPB) is a clinicopathological entity described by HOMMA *et al.* in 1983 [14]. The disease is characterized by chronic sinopulmonary infection and inflammation. The pathogenesis of DPB has been elucidated in recent years, but several aspects remain obscure. Previous reports have shown a marked increase of neutrophils in BALF of DPB patients, suggesting that accumulation of neutrophils in the airway lumen may play an important role in the pathogenesis of this disease [15–18].

We have established a sensitive radioimmunoassay (RIA) for HNPs 1–3 [19]. Using this assay, we measured in the present study the concentration of defensins in plasma and BALF of patients with DPB to examine their involvement in this disease.

Materials and methods

Study population

We studied control subjects and two groups of patients with chronic neutrophil-associated lung diseases [15–18,

20]. The patient groups consisted of 30 patients with DPB (12 females and 18 males) and 16 patients with idiopathic pulmonary fibrosis (IPF) (six females and 10 males). The control subjects were 15 healthy normal adults (two females and 13 males; mean±SD age 28±1 yrs) who were younger than DPB (age: 47±2 yrs) and IPF patients (age: 59±2 yrs). At the time of the investigation, two DPB patients, one IPF patient and five normal subjects were smokers. The experimental protocol was approved by the Ethics Committee for Human Experimentation of our institution and a signed consent was obtained from all subjects. Bronchoalveolar lavage (BAL) was performed in all subjects participating in the study.

The diagnosis of DPB was based on the new criteria published by the Japanese Ministry of Health and Welfare in 1995 [21]. These include the presence of: 1) productive cough and dyspnoea on exertion; 2) rales and rhonchi on physical examination; 3) diffuse disseminated fine nodular shadows, mainly in the lower lung fields on chest radiograph or computed tomography; 4) two laboratory abnormalities including forced expiratory volume in one second (FEV_1) <70% and arterial oxygen tension (P_{a,O_2}) <10.6 kPa (<80 mmHg); 5) cold haemagglutination >64; and 6) the presence or history of chronic sinusitis. All DPB patients satisfied all the above criteria and the diagnosis was further confirmed histopathologically in 10 of these patients by open-lung biopsy.

Measurement of the concentration of defensins in BALF was also repeated in 23 out of 30 patients with DPB after 6 months of continuous treatment with macrolide antibiotics. Ten of these patients were treated with 600 mg·day^{-1} erythromycin, nine with 150 mg·day^{-1} roxithromycin and four with 200 mg·day^{-1} clarithromycin [22–24]. None received other antibiotics or corticosteroids during the course of the study.

All IPF patients included in this study had dry cough and exertional dyspnoea, and their chest radiographs showed reduced lung volumes and diffuse reticulonodular opacities. In addition, the presence of usual interstitial pneumonia was confirmed histopathologically in these patients by open-lung biopsy. There were no significant differences between patients with DPB and IPF with regard to vital capacity (VC) per cent of predicted value (DPB: 83±4, IPF: 77±5% pred) and P_{a,O_2} (DPB: 9.6±0.2 kPa (72±1.8 mmHg) IPF: 10.4±0.4 kPa (78±3 mmHg)), but a significant difference was present in FEV_1 % pred between DPB and IPF (DPB: 66±2, IPF: 81±3%, p<0.01).

Bronchoalveolar lavage

BAL was performed as described previously [17, 25]. Briefly, after local anaesthesia of the upper airway with 4% lidocaine, a flexible fibreoptic bronchoscope (Olympus BF, type p-20; Olympus Co., Tokyo) was wedged into a subsegmental bronchus of the right middle lobe. An aliquot of 50 mL of sterile saline at body temperature was instilled through the bronchoscope. The fluid was immediately retrieved by gentle suction using a sterile syringe, and the procedure was repeated three times. BALF was passed through two sheets of gauze and centrifuged at 500×g for 10 min at 4°C. After washing twice with phosphate-buffered saline (PBS) without calcium and magnesium (Gibco, Rockville, MD, USA), the cell pellets were suspended in PBS supplemented with 10% heat-inactivated foetal calf serum and counted using a haemocytometer. The aliquot was diluted to a concentration of 2×10^5 cell·mL^{-1}, and a differential count was performed on May-Giemsa stained cytospin preparations (Cytospin 2, Shandon Instruments, Sewickley, PA, USA). The remaining fluid was centrifuged at 500×g for 5 min and the supernatant was stored at -80°C until use. Plasma and serum samples were collected on the same day.

Measurement of defensins

The concentration of defensins in BALF and plasma samples was measured by RIA established by our laboratories [19]. We synthesized full-length HNP-1 using a peptide synthesizer Model 430 (Applied Biosystem, NJ, USA), then purified by reverse-phase high performance liquid chromatography (RP-HPLC). In RP-HPLC, synthetic HNP-1 was eluted at a position identical to that of native HNP-1 isolated from human leucocytes. Synthetic HNP-1 (5 mg) was used for immunizing New Zealand white rabbits by multiple intra- and subcutaneous injections. HNP-1 was radio-iodinated and the ^{125}I-labelled peptide was purified by RP-HPLC on a TSK ODS 120A column (Tosoh Co., Tokyo, Japan). The diluted sample or standard peptide solution (100 µL) was incubated with 100 µL of antiserum diluent (final dilution of 1/21,000) for 24 h. The ^{125}I-labelled HNP-1 solution (16,000 counts per minute (cpm) in 100 µL) was added, and the mixture was incubated again for another 24 h. In the next step, normal rabbit serum and anti-rabbit immunoglobulin (Ig)G goat serum were added and stored for 16 h. Bound and free ligands were separated by centrifugation. All procedures were performed at 4°C and samples were assayed in duplicate. In this assay, minimum detectable level was 22 pg and half-maximum inhibition by the peptide was observed at 130 pg. We used 0.5 µL of plasma and 1–10 µL of BALF to determine the level of defensins. The intra- and interassay coefficients of variation were 3.5 and 8%, respectively, at 50% binding. The antiserum equally recognized HNP-1, -2 and -3 on a molar basis, thus the RIA data were expressed as the sum of HNPs-1–3. The RIA system specifically detected HNPs-1–3 and their precursor proteins, which was confirmed by stimultaneous measurements using RP-HPLC and RIA [26].

Measurement of interleukin-8

The concentration of Interleukin (IL)-8 was measured using the enzyme-linked immunosorbent assay (ELISA) kit (Tore Fuji Bionix, Tokyo, Japan). Briefly, murine antibody to human IL-8 was bound to microtitre plates. The samples (50 µL·well^{-1}) were incubated in the wells with horseradish peroxidase-labelled antibody to IL-8 for 90 min under constant shaking at room temperature. The wells were washed three times with 0.1% Tween 20 and incubated with a buffer solution containing tetramethylbenzidine for 30 min at room temperature. The reaction was stopped by adding 50 µL·well^{-1} of 1.8 N sulphuric acid. The plates were read at 450 nm in an ELISA reader. Serial dilutions of each sample were assayed in duplicate. The detection limit was 3.0 pg·mL^{-1}. We also measured

the albumin concentration in BALF by using the turbidimetric immunoassay kits (Wako Pure Chemical Industries, Osaka, Japan).

Immunohistochemistry of lung tissues

Immunohistochemical study of defensins in the lungs of DPB patients was performed as described previously [27]. Open-lung biopsy specimens were obtained from four DPB and two IPF patients. As a control, we also studied macroscopically uninvolved lung tissue specimens resected at surgery from a 45 yr old man with tuberculosis. The tissue samples were immersed in Zamboni's fixative (2% paraformaldehyde and 0.25% picric acid in 0.1 M PBS adjusted to pH 7.4) or in 10% formaldehyde in PBS and, after dehydration in serial ethanol concentrations, they were embedded in paraffin. The specimens (3 mm thick) were deparaffinized in xylene, rehydrated in serial ethanol solutions, and treated with 0.3% hydrogen peroxide for 30 min to inactivate any endogenous peroxidase. Nonspecific binding was blocked with normal goat serum. Anti-HNPs-1–3 antiserum at a final dilution of 1/10,000 was allowed to react overnight with each preparation at 4°C in a moist chamber. Goat-biotinylated anti-rabbit IgG was used as the second antibody. The samples were stained by the ABC alkaline phosphotase (ABC-AP) method using an ABC-AP kit (DAKO Co. Ltd., Carpinteria, CA, USA). The tissue samples were counterstained with haematoxylin.

In vitro *effects of defensin released from neutrophils*

Heparinized venous blood was collected from five healthy subjects and the erythrocytes were separated with dextran. The neutrophils were separated by Ficoll-Paque density gradient centrifugation and suspended in Hank's balanced salt solution (Gibco, Grand Island, NY, USA) containing 0.25% bovine serum albumin at a cell density of 1×10^6 cells·mL^{-1}. The viability of the cells at that stage was 95% or higher as confirmed by the trypan blue exclusion. Neutrophils were incubated with phorbol myristate acetate (PMA; Sigma, St Louis, MO, USA), N-formyl-methionyl-leucyl-phenylalanine (FMLP; Sigma) and recombinant human endothelial IL-8 (Pepro Tech Inc., NJ, USA) at 37°C for 30 min. After culture, the supernatant fluid was examined for the presence of defensins. In the next step, the neutrophils were treated with 1, 10 or 50 μg·mL^{-1} erythromycin at 37°C for 30 min and then incubated with 50 or 500 ng·mL^{-1} IL-8 at 37°C for 30 min and the supernatant fluid was examined also for the presence of defensins.

Statistical analysis

Data were expressed as mean±SEM. The Mann-Whitney U-test and paired t-test were used for comparison between groups. Correlation between two parameters was examined using the Spearman rank correlation. A p-value less than 0.05 was considered significant.

Results

Plasma and BALF concentrations of defensins

The per cent volume of recovered BALF was significantly larger in healthy volunteers (57.0±2.6%) than in DPB (42.7±2.9%, p<0.01) or IPF patients (43.0±3.5%, p<0.01). The mean number of neutrophils in BALF from patients with DPB ($98.2\pm24.3\times10^4$ cells·mL^{-1}) was significantly higher than in controls ($0.15\pm0.05\times10^4$ cells·mL^{-1}, p<0.01) and patients with IPF ($2.2\pm0.9\times10^4$ cells·mL^{-1}, p<0.01). The percentage of neutrophils in BALF of patients with DPB (69.1±3.5%) was also higher than in controls (0.8±0.2%, p<0.01) and patients with IPF (6.8±2.4%, p<0.01). The number and percentage of neutrophils in IPF were higher than in controls (p<0.01).

The mean concentration of defensins (HNPs-1–3) in BALF of DPB (428.5±68.6 ng·mL^{-1}) was markedly higher than in controls (14.4±3.7 ng·mL^{-1}, p<0.01) and IPF patients (22.2±6.0 ng·mL^{-1}, p<0.01) (fig. 1). The mean ratio of defensins to albumin in BALF of DPB patients (6555.3±1179.4 ng·mg^{-1}) was also higher than in controls (695.9±76.4 ng·mg^{-1}, p<0.01) and IPF patients

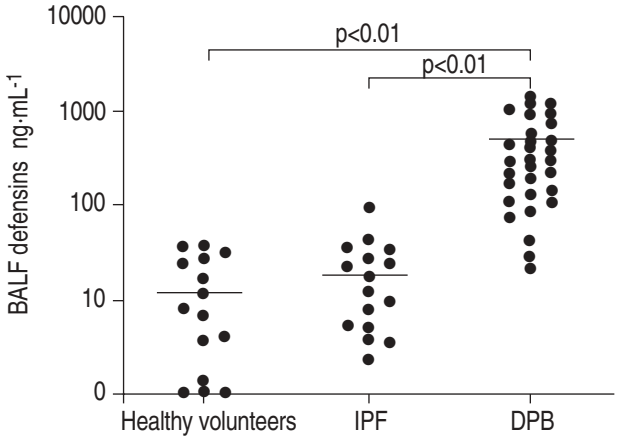

Fig. 1. – Concentration of defensins in bronchoalveolar lavage fluid (BALF) in healthy subjects (n=15), and patients with idiopathic pulmonary fibrosis (IPF; n=16) and diffuse panbronchiolitis (DPB; n=30). Bars represent mean values.

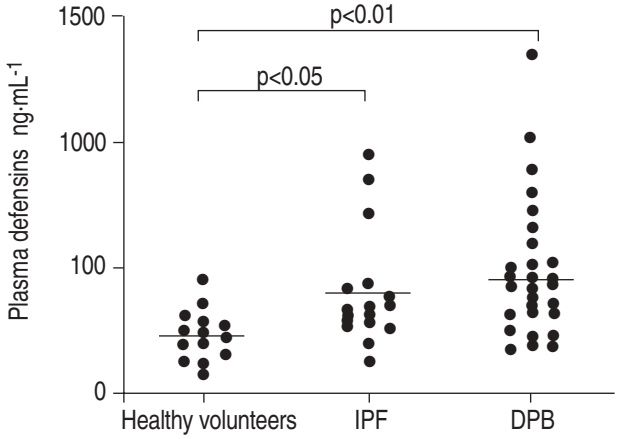

Fig. 2. – Concentration of defensins in plasma of healthy subjects (n=15), and patients with IPF (n=16) and DPB (n=30). Bars represent means. For definitions see legend to figure 1.

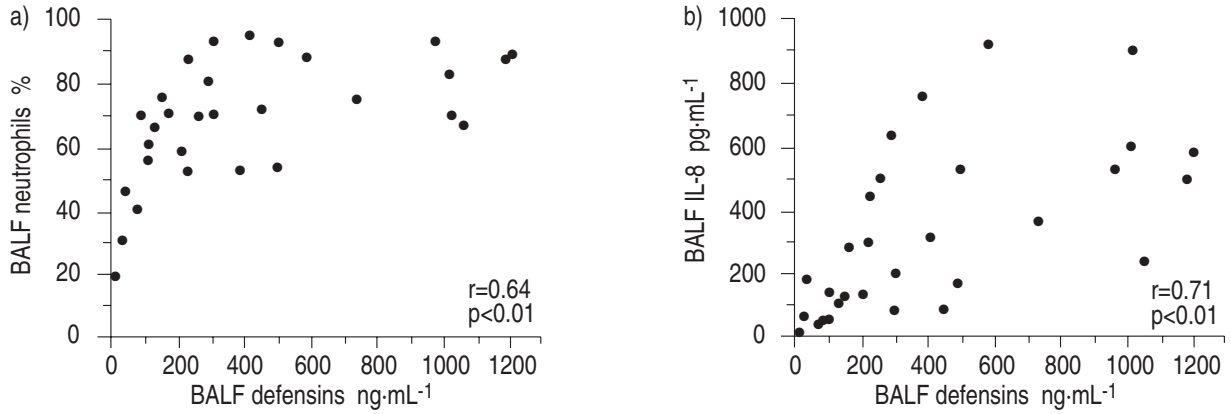

Fig. 3. – Correlation between bronchoalveolar lavage fluid (BALF) concentration of defensins and a) the percentage of BALF neutrophils, and b) BALF concentrations of interleukin (IL)-8 (n=30).

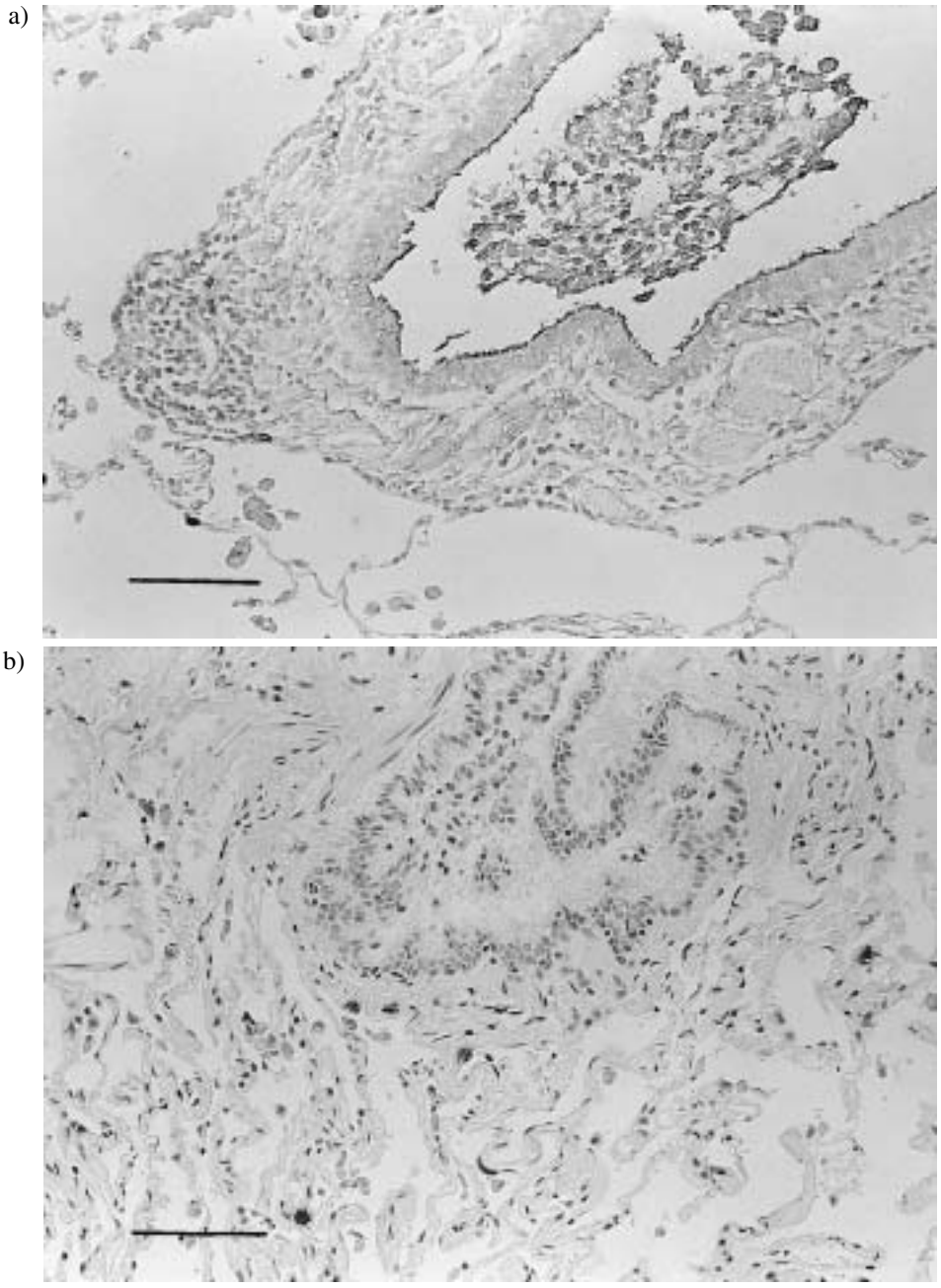

Fig. 4. – a) Immunohistochemical staining of open-lung biopsy specimen obtained from a patient with diffuse panbronchiolitis using anti-human neutrophil peptides (HNPs)-1–3 antiserum. Note the expression of defensins in bronchiolar epithelial cells, and in neutrophils and mucinous exudates in airspaces. Internal scale bar=100 μM. b) In the normal lungs, only neutrophils were stained with anti-HNPs-1–3 antiserum. Internal scale bar=50 μM.

(348.7±76.4 ng·mg^{-1}, P<0.01). The plasma concentration of defensins in DPB (477.7±48.4 ng·mL^{-1}) patients was double that in the control group (233.5±24.7 ng·mL^{-1}, p< 0.01), but not significantly different from that in IPF patients (408.2±57.9 ng·mL^{-1}) (fig. 2). No significant correlation was present between plasma and BALF concentrations of defensins in DPB. The three patients with IPF who had the highest levels of plasma defensins in this group (fig. 2), did not have the highest BALF neutrophil counts (5.5±3.3%) or defensin concentrations (11.1±3.6 ng·mL^{-1}).

The concentration of defensins in BALF correlated significantly with the percentage of neutrophils in BALF of patients with DPB (fig. 3a, r=0.64, p<0.01). The concentration of IL-8 in BALF of patients with DPB (324.4±47.9 pg·mL^{-1}) was markedly higher than in controls (4.7±0.9 pg·mL^{-1}, p<0.01) and patients with IPF (24.6±5.3 pg·mL^{-1}, p<0.01). The BALF concentration of IL-8 in DPB correlated significantly with BALF concentrations of defensins (fig. 3b, r=0.71, p<0.01). No significant relationship was present between plasma or BALF concentrations of defensins and clinical parameters of DPB, such as P_{a,O_2}, VC % pred or FEV1 % pred.

Immunohistochemistry of defensins

Immunohistochemical studies in four patients with DPB using anti-HNPs-1–3 antiserum showed the presence of defensins on the bronchiolar epithelial cells, and in neutrophils and mucinous exudate in airspaces (fig. 4a). No immunoreactivity of defensins was detected in neutrophils or epithelial cells when normal rabbit serum or anti-serum preabsorbed with excessive synthetic HNP-1 was used (data not shown). We also performed immunohistochemical studies using antiserum in samples from two IPF patients and a control. In the control lung tissues, only neutrophils were stained with anti-HNPs-1–3 antiserum (fig. 4b). In IPF patients, neutrophils were stained, but no staining was present in bronchiolar epithelial cells (data not shown). In addition, only partial and weak staining of the basement membrane of bronchioles was present in samples from IPF patients (data not shown).

Effect of neutrophil stimulation on release of defensins

The release of defensins from neutrophils was studied by stimulation with PMA, FMLP or IL-8. In control samples, stimulation of neutrophils with PMA enhanced the release of defensins in a dose-dependent manner (fig. 5). The release of defensins by FMLP- or IL-8-stimulated neutrophils was lower than that by PMA, although a significant increase in defensin release occurred at 500 ng·mL^{-1} of IL-8 and at 10^{-5} M of FMLP (fig. 5b and c). Incubation of neutrophils with erythromycin did not influence IL-8-enhanced defensin release (data not shown).

Effect of macrolides on the concentrations of defensins

Long-term treatment of patients with DPB with macrolides significantly improved lung function tests (VC, pre: 82.4±4.4, post: 91.1±4.4 % pred, p<0.01, FEV1, pre: 66.7±2.3, post: 70.3±2.1% pred, p<0.02) and P_{a,O_2} (pre: 9.7±0.3 kPa (73.3±2.3 mmHg), post: 10.9±0.3 kPa (81.8± 2.1 mmHg), p<0.01). The neutrophil count and their percentage in BALF diminished significantly after treatment (table 1). Treatment also significantly reduced the BALF concentration of defensins and IL-8 (table 1).

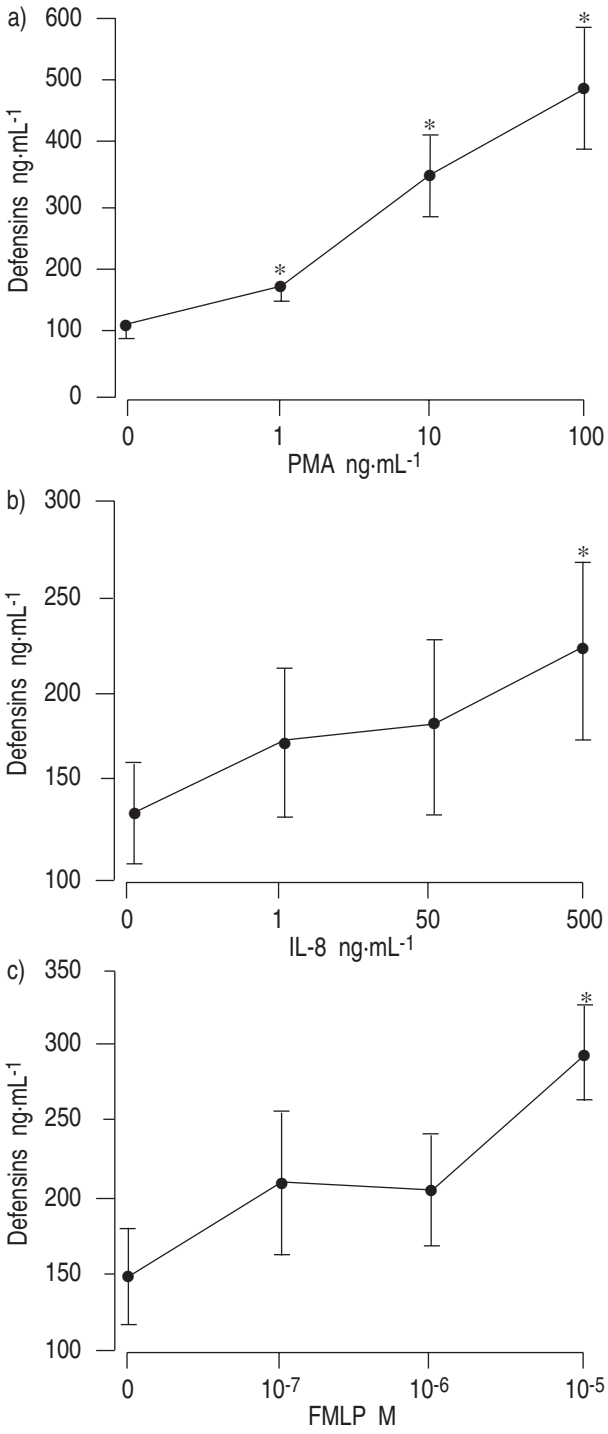

Fig. 5. – Effects of: a) phorbol myristate acetate (PMA); b) interleukin (IL)-8; and c) N-formyl-methionyl-leucyl-phenylaline (FMLP) on extracellular release of defensins from neutrophils. Data are the mean±SEM of duplicate experiments using neutrophils from five different donors. *: p<0.05, significantly higher than control (medium only).

Table 1. – Effect of macrolide treatment on neutrophil count and concentrations of mediators in bronchoalveolar lavage fluid in patients with diffuse panbronchiolitis (n=23)

		Before	After	p-value
Neutrophil	%	65.4±4.2	30.0±6.6	<0.01
	$\times 10^4$ cells·mL^{-1}	93.5±28.6	30.1±13.6	<0.05
Defensins	ng·mL^{-1}	368.4±67.7	79.1±19.1	<0.01
Interleukin-8	pg·mL^{-1}	321.9±60.6	100.0±26.8	<0.01

Values are mean±SD.

Discussion

DPB is characterized histologically by thickening of the walls of the respiratory bronchioles, and infiltration of the walls by lymphocytes, plasma cells and histiocytes [14]. In addition, previous studies have shown a high number of neutrophils and neutrophil-derived elastolytic-like activity in BALF of these patients [15–18], suggesting that accumulation of neutrophils in the airway lumen may be involved in the pathogenesis of DPB through their oxidative and proteolytic products.

The major findings of the present study were that we determined defensin concentrations in BALF as well as in plasma, and showed a markedly high concentration of defensins in BALF of DPB patients compared with normal subjects and IPF. The concentration of defensins in BALF of DPB patients correlated significantly with the percentage of neutrophils present in their BALF. Defensins are restricted to cells of neutrophil lineage [27]. Thus, the high BALF concentration of defensins in DPB must be related to the dense neutrophil infiltration into the airways.

In regard to the plasma concentrations, defensins were higher in patients with DPB and IPF than in control subjects. To our knowledge, there is only one study, by Panyutich *et al*. [28], in which elevated plasma concentrations of defensin were described in patients with sepsis and meningitis. Plasma defensin concentration of healthy subjects in that study was slightly lower (<213 ng·mL^{-1}) than in our control subjects (233.5±24.7 ng·mL^{-1}). This difference may be attributed to different methodologies; an enzyme immunoassay was used in the previous study while we used RIA. Another possible cause for the difference is that our assay detected mature defensins plus their precussor proteins [26].

Defensins are major peptides of human neutrophil granules and have broad microbicidal activity and cytotoxicity to mammalian cells [1, 2, 9–11]. At ~10^{-5} M, they kill a broad spectrum of micro-organisms including gram-positive and gram-negative bacteria, fungi and enveloped viruses *in vitro* [2]. Defensins are released into phagocytic vacuoles from azurophil granules and cause lysis of pathogenic micro-organisms. Defensins also kill human cells since the mechanism employed for killing micro-organisms, the formation of ion channels in cell membranes, is not selective to micro-organisms [29]. The cytotoxic activity of defensins is concentration-dependent. Okrent *et al.* [11] showed that the major cytotoxic activity of neutrophil granule extracts to lung fibroblast and epithelial cells is confined to human defensin-containing fraction, while fractions containing elastase, lysozyme, and other hydrolases were relatively ineffective [11]. Thus, defensins may act as a chemical mediator of tissue destructive events in neutrophil-associated lung diseases, as is the case with oxidants and proteinases in the neutrophil.

The present study identified high concentrations of defensins at the inflammatory sites in DPB. In addition, we also detected a strong immunoreactivity for defensins on the bronchiolar epithelial cells and neutrophils in airspaces of biopsy specimens from DPB patients. In this context, it is possible that under certain circumstances, epithelial cells may synthesize defensins. However, no report has shown the production of the defensins (HNPs-1–3) by human cells other than by neutrophils, and immunoreactivity for defensins was not detected on bronchial epithelial cells of biopsy specimens from IPF. Therefore, we postulate that defensins derived from neutrophils in airspaces of DPB bind to epithelial cells. Thus, these findings suggest that defensins most likely reach their effective cytotoxic concentrations in airway lumen of DPB patients and that the lung injury in DPB may be caused by defensins from neutrophils in the airways.

BALF concentrations of defensins correlated with BALF IL-8 concentrations in patients with DPB. IL-8 is a potent neutrophil chemotactic factor that enhances the transendothelial migration of leucocytes, release of reactive oxygen metabolites and neutrophil degranulation [30–32]. We have demonstrated recently that IL-8 is a major determinant of neutrophil accumulation in airway lumen in DPB [23, 25]. In this regard, Ganz [33] reported previously that defensins are released by human neutrophils in response to PMA. We quantified the extracellular release of defensins after stimulation of human neutrophils with human recombinant IL-8 and found that defensins are released from neutrophils in response to human recombinant IL-8 or FMLP as well as PMA. Considered together, the results of the present study as well as those of other investigators suggest that defensins may participate in neutrophil-mediated extracellular cell lysis or injury and that IL-8 probably contributes to the extracellular release of defensins from neutrophils in DPB. In this regard, the levels of defensins in BALF of DPB patients were not significantly high when neutrophil counts were <50% (fig. 3a). The concentration of defensins in BALF of IPF was similar to that in control subjects in spite of the significant difference in BALF neutrophils. These findings suggest that a significant rise in the concentration of defensins in BALF is critically dependent on a minimum number of neutrophils. While the exact mechanisms of this phenomenon are not understood, it should be noted that a significant increase in defensins occurred only at the highest concentrations of IL-8 in this *in vitro* study while the activity of IL-8 is known to be chemotactic at the lower concentrations [32].

DPB often shows rapid progression, which until recently culminated in respiratory failure due to repetitive respiratory infections. However, since Kudoh *et al.* [21] reported the beneficial effects of long-term treatment with erythromycin, the frequency of these complications has diminished dramatically. Since the description of the efficacy of use of erythromycin in DPB, several researchers have investigated the therapeutic mechanisms of this drug in DPB and suggested that erythromycin acts through its anti-inflammatory properties irrespective of its antimicrobial effect [15–18, 25, 34, 35]. A number of investigators have also recently shown similar clinical benefits using roxithromycin and clarithromycin, two new erythromycin

derivatives [23, 24]. Although the mode of action of macrolides is not fully understood, there is a large body of evidence suggesting that these drugs inhibit intrapulmonary influx of neutrophils [15–18]. A significant reduction of neutrophils and an associated fall in the concentration of neutrophil chemotactic and activating factors, such as IL-8 and IL-1β, in BALF of DPB, have been observed after treatment with these drugs [15–18, 23, 25].

In this study, macrolide therapy reduced defensin concentrations in BALF of DPB patients together with the reduction in neutrophil count and IL-8 concentrations in BALF. While no control group was included in the present study, the specific effect of erythromycin in DPB was confirmed in a large double-blind comparative study by the Research Project Team of the Ministry of Health and Welfare for Specific Disease in Japan [36]. Recent *in vitro* studies have shown an inhibitory effect of macrolides on IL-8 release from bronchial epithelial cells [37] and macrophage-like cell line [38] in response to stimuli. The present study showed, however, that erythromycin did not inhibit the enhanced release of defensins from neutrophils induced by recombinant human IL-8. Together, these results suggest that macrolides may inhibit the production and release of neutrophil chemotactic and activating factors such as IL-8 and IL-1β at inflammatory sites, thus reducing the accumulation of neutrophils into the lungs. These changes may result in a reduction in BALF concentration of defensins in DPB patients.

In conclusion, elevated concentrations of defensins are present in airway lumen of diffuse panbronchiolitis patients and are reduced after treatment with macrolides. These observations suggest that defensins may be a marker of neutrophil activity in this disease.

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