Karol Majewski, MD^{1,2}

Paulina Żelechowska, MD¹

Ewa Brzezińska-Błaszczyk, MD, PhD¹

¹Department of Experimental Immunology,
Medical University of Lodz

² Pulmonary Disease Hospital, Lodz

Circulating cathelicidin LL-37 in adult patients with pulmonary infectious diseases

Abstract

Purpose: The antimicrobial peptide cathelicidin LL-37 plays a role in the immune response in the course of lung infections; however, the exact role of LL-37 in defense mechanisms against bacteria within the respiratory tract is has not been precisely described. The aim of our study was to evaluate LL-37 concentrations in the serum of pulmonary tuberculosis (TB) patients, patients with pneumonia caused by Gram-positive and Gram-negative bacteria and to compare them with those of healthy subjects.

Methods: Thirty TB patients, 30 patients with pneumonia caused by Gram-positive bacteria, 30 patients with pneumonia caused by Gram-negative bacteria, and 30 healthy control subjects were enrolled in the study. Serum LL-37 concentration was measured using an enzyme-linked immunosorbent assay (ELISA).

Results: The mean ($\pm$ SEM) LL-37 concentration in patients with TB (13.94 ± 5.13 ng/mL) was significantly higher than that in patients with Gram-positive bacteria-induced pneumonia (7.87 ± 4.58 ng/mL, $P=0.00077$), in patients with Gram-negative bacteria-induced pneumonia (10.27 ± 3.60 ng/mL, $P=0.00730$), and in control healthy subjects (1.75 ± 0.71 ng/mL, $P=0.00004$).

Conclusion: Our data suggest that cathelicidin LL-37 is an important element of host defense in the course of bacterial diseases within the respiratory tract, particularly when the infection is caused by an intracellular pathogen.

Manuscript submitted 16th September, 2016

Manuscript accepted 22nd November, 2016

Clin Invest Med 2017; 40 (1): E34-E39.

Correspondence to:

Ewa Brzezińska-Błaszczyk, Professor

Department of Experimental Immunology, Medical University of Lodz

92-213 Lodz, Pomorska 251, Poland

E-mail: ewab@csk.umed.lodz.pl

The primary function of the immune system is to protect the host against various pathogens, including bacteria, viruses, and fungi. This system acts through mechanisms of innate immunity, involving different humoral factors (complement proteins, acute phase proteins, and some cytokines) and cells (neutrophils, monocytes, macrophages, NK cells, dendritic cells, and mast cells) as well as *via* mechanisms of adaptive immunity, in which an important role is played by B cells, different populations of T cells, and antibodies. More and more data indicate that some small amphipathic molecules called antimicrobial peptides (AMPs), (i.e., cathelicidins and defensins,) take part in both immune activation and regulation, and thus are involved in host defense against infections [1,2].

The only known member of the cathelicidin family expressed in humans is LL-37 (leucine-leucine-37). This peptide is expressed constitutively or is produced in response to different infectious or inflammatory stimuli in various cells, including neutrophils, monocytes, macrophages, mast cells, NK cells, B and T cells, adipocytes, keratinocytes, and mucosal epithelial cells. Cathelicidin LL-37 directly kills pathogens directly by disrupting their membranes, and it neutralizes the activity of bacterial endotoxins. In addition, there is increasing evidence that it exerts proinflammatory effects [3,4], as it acts as a chemoattractant for mast cells, neutrophils, monocytes, and T cells; it also activates inflammatory cells and modulates Toll-like receptors (TLRs) responses and proinflammatory cytokine release [1,5,6,7]. In addition, LL-37 stimulates the production of various chemokines and increases the expression of some chemokine receptors [3,4]. LL-37 has an ability to induce the expression of anti-apoptotic protein Bcl-xl expression and inhibit caspase-3 activity. This results in the suppression of neutrophil apoptosis [8], which extends the life-span of neutrophils and increases phagocytosis. Recently, it was shown that cathelicidin LL-37 up-regulates the autophagy-related gene expression in macrophages and induces autophagosome formation to promote killing of intracellular bacteria [9]. There is also information that LL-37 can influence the course of adaptive immune response [2].

Cathelicidin LL-37 is generated by airway epithelial cells and immune cells that are resident in the respiratory tract; therefore, it contributes to the barrier function of intact respiratory epithelia and plays a role in lung immunity [10,11]. This peptide is also known to play a role also in immune response in the course of lung infection [12]; however, the exact role of LL-37 in the mechanisms of defense against bacteria within the respiratory tract is not precisely described. There is little data regarding this issue and existing data are

unclear. The aim of our study was to evaluate circulating levels of LL-37 in patients with pulmonary infectious diseases caused by different pathogens (intracellular bacterium *Mycobacterium tuberculosis* (pulmonary tuberculosis (PTB)), Gram-positive or -negative bacteria species (pneumonia)) and to compare them with those of healthy subjects.

Methods

Study population

A total of 120 patients were enrolled in this study. All subjects included in the study were informed about the aims and methods of the study, and provided their written informed consent for participation. The study protocol was approved by the Bioethics Committee of the Medical University of Lodz. Adult patients with pneumonia symptoms or with suspected PTB were selected from the patients of the Lung Diseases Hospital in Lodz. All patients were subjected to an initial evaluation that included a clinical examination, radiological findings, basic laboratory blood tests including ALAT, AspAT, total bilirubin, urea, creatinine, glucose, sodium, potassium, C-reactive protein (CRP), white blood cell (WBC) count and microbiology standard laboratory methods. TB was diagnosed in accordance with guidelines of the World Health Organization regarding standard TB cases. Diagnosis of TB was based on tuberculin skin test (TST) positivity, history of contact with an active TB case, acid-fast bacilli and *M. tuberculosis* culture positivity. Criteria for exclusion from the study were as follows: taking antibiotics for last three months; taking immunosuppressive or antihistamine agents; chronic inflammatory diseases; systemic diseases; immunological disorders (AIDS, allergy); and, cancer.

Patients were divided into three clinical groups, according to the infectious agent and each of the groups consisted of 30 subjects. The groups were as follows: (I) subjects infected with *M. tuberculosis*; (II) subjects with pneumonia caused by Gram-positive bacteria (*Staphylococcus aureus* n=26, *Streptococcus pneumoniae* n=4); and, (III) subjects with pneumonia caused by Gram-negative bacteria (*Haemophilus influenzae* n=9, *Escherichia coli* n=12, *Enterobacter cloacae* n=3, *Klebsiella pneumoniae* n=3, *Klebsiella oxytoca* n=3). The control healthy subjects (IV) (n=30) had no history of TB and had not been hospitalized within the preceeding three months.

Measurement of serum LL-37 levels

Blood samples were obtained from all the patients before the commencement of the treatment and from the control subjects. Serum samples were prepared after centrifugation at

FIGURE 1. Serum LL-37 levels in four studied groups.

TABLE 1. Demographic and clinical characteristics of the study population

Group	I	II	III	IV
N	30	30	30	30
Age (years)	53±3	61±3	71±2	47±3
Gender (M/F)	21/9	19/11	19/11	18/12
AspAT (U/L)	27.17±.54	23.77±2.89	20.20±1.34	19.73±1.01
ALAT (U/L)	19.57±2.67	21.70±1.88	19.10±1.96	21.83±2.23
Total bilirubin (mg/dL)	0.58±0.04	0.54±0.06	0.39±0.04	0.42±0.04
Urea (mg/dL)	24.17±1.85	33.87±2.49	32.61±2.87	26.80±1.22
Creatinine (mg/dL)	0.62±0.02	0.90±0.04	0.85±0.04	0.76±0.02
Glucose (mg/dL)	102.93±3.15	106.80±5.45	97.40±3.29	82.80±1.88
Sodium (mmol/L)	134.03±0.77	137.20±0.76	138.50±0.47	136.2±0.82
Potassium (mmol/L)	4.38±0.16	4.38±0.11	4.45±0.10	4.59±0.12
WBC (×10 ⁹ /L)	9.08±0.50	9.01±0.46	9.35±0.62	6.04±0.20
CRP (mg/L)	42.97±6.65	58.43±13.49	59.00±14.02	1.77±0.23

AspAT=aspartate transaminase; ALAT=alanine transaminase; WBC=white blood cell count; CRP=C-reactive protein

3500 rpm for 10 min and then immediately stored at -80°C until analysis. Serum LL-37 levels were measured in all subjects with the application of an enzyme-linked immunosorbent assay (ELISA) kit (MyBIOsource, San Diego, USA) (the sensitivity of this assay was 0.938 ng/mL), according to the instruction provided in the manual. All the samples were compared to the standard curve. Values below the detection threshold were considered as zero, and extrapolated values were subject to a statistical analysis.

Statistical analysis

The statistical analysis was performed using Statistica 12.5 (Statsoft Inc., USA). Simple descriptive statistics (means, standard error of the means) were generated for LL-37, WBC and CRP variables. Normality of distribution was tested with the Shapiro-Wilk test. Serum concentration of LL-37 and CRP as well as WBC count were compared with the use of the Mann-Whitney U test. The relationships between the serum concentrations of LL-37 and CRP in all groups were expressed as Spearman's correlation coefficients. $P < 0.05$ was considered statistically significant.

Results

The demographic and clinical characteristics of the study subjects are given in Table 1. In group I, the mean ($\pm$ SEM) age was 53 ± 3 years and the male/female ratio was 21/9 women. In the group II the mean age was 61 ± 3 years and the male/female ratio was 19/11. In group III the mean age was 71 ± 2 years and the male/female ratio was 19/11. In control group the mean age was 47 ± 3 years and the male/female ratio was 18/12. Age and gender distributions were not statistically different between the groups. The levels of basic laboratory parameters, including AspAT, ALAT, total bilirubin, urea, creatinine, glucose, sodium and potassium in all groups were within normal ranges.

The mean ($\pm$ SEM) WBC counts were significantly higher in all studied groups, as compared with healthy controls (I *vs* IV - $P = 0.0000001$; II *vs* IV - $P = 0.0000001$; III *vs* IV - $P = 0.000001$). Similarly, levels of CRP, an important inflammatory marker in TB patients, and in groups II and III were significantly higher than in healthy controls (I *vs* IV - $P = 0.0000001$; II *vs* IV - $P = 0.000001$; III *vs* IV - $P = 0.000001$). Significantly higher levels of WBC and CRP in the patients with tuberculosis and pneumonia compared to the control group confirmed the ongoing infection.

The serum levels of LL-37 in groups I, II and III ranged from 0.35 to 118.19 ng/mL , from 0.04 to 107.92 ng/mL , and

from 0.1 to 86.12 ng/mL , respectively (Figure 1). In healthy subjects LL-37 concentration in serum varied greatly and ranged from 0.24 to 21.1 ng/mL . The mean ($\pm$ SEM) serum LL-37 levels in groups I, II and III were $13.94 \pm 5.13\text{ ng/mL}$, $7.87 \pm 4.58\text{ ng/mL}$ and $10.27 \pm 3.60\text{ ng/mL}$, respectively. The mean concentration of LL-37 in healthy individuals was $1.75 \pm 0.71\text{ ng/mL}$. There were no significant differences in mean serum LL-37 levels between groups II and IV ($P = 0.65434$), between groups III and IV ($P = 0.32803$) and between groups II and III ($P = 0.93568$); however, the mean LL-37 concentration in group I was significantly higher than that in groups II ($P = 0.00077$), III ($P = 0.00730$) and IV ($P = 0.00004$). Additionally, Spearman's correlation tests revealed no statistically significant correlations between LL-37 and CRP serum levels ($P > 0.05$).

Discussion

It is obvious that the respiratory tract is particularly exposed to constant contact with different particulates and pathogens entering *via* inhalation and through nasal and oral mucosal surfaces; thus, the mechanisms of innate and adaptive immunity within the respiratory system are particularly important. Increasingly, cathelicidin LL-37, as well as other antimicrobial peptides such as defensins, have been shown to play a significant role during immune response within the respiratory system and is strongly involved in pathomechanism of many respiratory diseases [10,11,12]. Increased levels of LL-37 in bronchoalveolar lavage (BAL) fluid of patients with cystic fibrosis were noted. Moreover, cathelicidin concentrations were correlated with pulmonary inflammation and, thereby, disease severity [13]. In turn, circulating LL-37 levels in patients with interstitial lung disease connected with systemic sclerosis were remarkably lower than in both patients with systemic sclerosis without interstitial lung disease and in healthy subjects; consequently, it was suggested that lower LL-37 levels may be associated with the development of interstitial lung disease [14]. It was also noticed that serum cathelicidin LL-37 levels in children with post-infectious bronchiolitis obliterans were higher as compared with the control group [15]. Likewise, bronchiolitis obliterans syndrome in lung transplant recipients was associated with elevated levels of LL-37 in BAL [16]. Considering the proinflammatory effects of LL-37, it can be assumed that significantly higher levels of LL-37 may exacerbate inflammatory processes during bronchiolitis.

Cathelicidin LL-37 has a broad spectrum of antimicrobial activities against various microorganisms [3,4]; thus, it can play an important role in respiratory infectious diseases as well.

Data relating to a role of this peptide in the course of infectious diseases within the respiratory tract are still scarce and ambiguous. It was shown that circulating levels of LL-37 are down-regulated during septic shock [17]. Conversely, neonates with congenital pneumonia demonstrated significantly higher serum levels of cathelicidin LL-37 [18] and newborn infants with pulmonary infections had elevated LL-37 concentrations in tracheal aspirates, as compared with healthy subjects [19]. Cakir *et al.* [20] observed significantly higher LL-37 levels in BAL of children with pulmonary tuberculosis in comparison with healthy children. Zhan and Jiang [21] observed elevated serum levels of this peptide in adult patients with pulmonary tuberculosis in comparison with healthy controls.

The aim of this study was to analyze serum levels of cathelicidin LL-37 in adults with pneumonia caused by Gram-positive bacteria or Gram-negative bacteria, as well as in adult patients with active pulmonary tuberculosis. This is the first study comparing circulating levels of LL-37 in patients with pulmonary infection caused by different pathogens. The main finding is that pulmonary bacterial infections were associated with considerably elevated concentrations of LL-37 in serum. Interestingly, in comparison to healthy subjects and patients with pneumonia caused by Gram-positive and Gram-negative bacteria, patients with tuberculosis demonstrated statistically significant higher LL-37 levels. We also noted that serum levels of LL-37 in both patient groups with pneumonia were higher than the levels observed in the control group; however, the differences were not statistically significant.

Our observations are consistent with view that LL-37 plays a key role in defense against bacterial pathogens and that elevated expression of LL-37 enhances the protection against infective threats [19,20,21,22,23]. Furthermore, low baseline plasma levels of the antimicrobial peptide hCAP18, the precursor of LL-37, in patients undergoing hemodialysis has been found to be associated with an increased risk of death attributable to infection [24]. Likewise, Ong *et al* [25] indicated that decreased expression of antimicrobial peptides in the skin may account for susceptibility of patients with atopic dermatitis to skin infection with *Staphylococcus aureus*. Our findings, showing that serum level of LL-37 was significantly higher in patients infected with *M. tuberculosis* than in subjects with pneumonia reinforce the idea that this peptide is a key molecule for the regulation of tuberculosis during the primary infection [26] and could serve as a marker of increased immune activation in a chronic infectious process such as tuberculosis [27].

In conclusion, our results suggest that cathelicidin LL-37 is an important element of a host's defense against bacterial diseases within the respiratory tract, particularly when the infection is caused by an intracellular pathogen.

Financial support

The Medical University of Lodz
(grant No. 502-03/6-164-01/502-64-083).

References

1. Choi KY, Chow LN, Mookherjee N. Cationic host defence peptides: multifaceted role in immune modulation and inflammation. *J Innate Immun* 2012;4:361-370.
2. Auvynet C, Rosenstein Y. Multifunctional host defense peptides: Antimicrobial peptides, the small yet big players in innate and adaptive immunity. *FEBS J* 2009;276:6497-6508.
3. Vandamme D, Landuyt B, Luyten W, Schoofs L. A comprehensive summary of LL-37, the factotum human cathelicidin peptide. *Cell Immunol* 2012;280:22-35.
4. Kahlenberg JM, Kaplan MJ. Little peptide, big effects: the role of LL-37 in inflammation and autoimmune diseases. *J Immunol* 2013;191:4895-4901.
5. Tjabringa GS, Ninaber DK, Drijfhout JW, Rabe KF, Hiemstra PS. Human cathelicidin LL-37 is a chemoattractant for eosinophils and neutrophils that acts via formyl-peptide receptors. *Int Arch Allergy Immunol* 2006;140:103-112.
6. Bąbolewska E, Brzezińska-Błaszczyk E. Human-derived cathelicidin LL-37 directly activates mast cells to proinflammatory mediator synthesis and migratory response. *Cell Immunol* 2015;293:67-73.
7. De Yang, Chen Q, Schmidt AP, Anderson GM, Wang JM, Wooters J, Oppenheim JJ, Chertov O. LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPR1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells. *J Exp Med* 2000; 92:1069-1074.
8. Nagaoka I, Tamura H, Hirata M. An antimicrobial cathelicidin peptide, human CAP18/LL-37, suppresses neutrophil apoptosis via the activation of formyl-peptide receptor-like 1 and P2X7. *J Immunol* 2006;176:3044-3052.
9. Rekha RS, Rao Muvva SS, Wan M, Raqib R, Bergman P, Brighenti S, Gudmundsson GH, Agerberth B. Phenylbutyrate induces LL-37-dependent autophagy and intracellular killing of *Mycobacterium tuberculosis* in human macrophages. *Autophagy* 2015;11:1688-1699.
10. Doss M, White MR, Tecle T, Hartshorn KL. Human defensins and LL-37 in mucosal immunity. *J Leukoc Biol* 2010;87:79-92.
11. Tecle T, Tripathi S, Hartshorn KL. Defensins and cathelicidins in lung immunity. *Innate Immun* 2010;16:151-159.

12. Tjabrinka G, Rabe KF, Hiemstra PS. The human cathelicidin LL-37: a multifunctional peptide involved in infection and inflammation in the lung. *Pulm Pharmacol Ther* 2005;18:321-327.
13. Chen CI, Schaller-Bals S, Paul KP, Wahn U, Bals R. β -defensins and LL-37 in bronchoalveolar lavage fluid of patients with cystic fibrosis. *J Cyst Fibros* 2004;3:45-50.
14. Hizal M, Bruni C, Romano E, Mazzotta C, Guiducci S, Bellando Randone S, Blagojevic J, Lepri G, Tufan A, Matucci Cerinic M. Decrease of LL-37 in systemic sclerosis: a new marker for interstitial lung disease? *Clin Rheumatol* 2015;34:795-798.
15. Gedik AH, Cakir E, Gokdemir Y, Uyan ZS, Kocyigit A, Torun E, Karadag B, Ersu R, Karakoc F. Cathelicidin (LL-37) and human β 2-defensin levels of children with post-infectious bronchiolitis obliterans. *Clin Respir J* 2015;doi: 10.1111/crj.12331.
16. Anderson RL, Hiemstra PS, Ward C, Forrest IA, Murphy D, Proud D, Lordan J, Corris PA, Fisher AJ. Antimicrobial peptides in lung transplant recipients with bronchiolitis obliterans syndrome. *Eur Respir J* 2008;32:670-677.
17. Jeng L, Yamshchikov AV, Judd SE, Blumberg HM, Martin GS, Ziegler TR, Tangpricha V. Alterations in vitamin D status and anti-microbial peptide levels in patients in the intensive care unit with sepsis. *J Transl Med* 2009;7:28.
18. Gad GI, Abushady NM, Fathi MS, Elsaadany W. Diagnostic value of anti-microbial peptide, cathelicidin in congenital pneumonia. *J Matern Fetal Neonatal Med* 2015;28:2197-2200.
19. Schaller-Bals S, Schulze A, Bals R. Increased levels of antimicrobial peptides in tracheal aspirates of newborn infants during infection. *Am J Respir Crit Care Med* 2002;165:992-995.
20. Cakir E, Torun E, Gedik AH, Umutoglu T, Aktas EC, Topuz U, Deniz G. Cathelicidin and human β -defensin 2 in bronchoalveolar lavage fluid of children with pulmonary tuberculosis. *Int J Tuberc Lung Dis* 2014;18:671-675.
21. Zhan Y, Jiang L. Status of vitamin D, antimicrobial peptide cathelicidin and T helper-associated cytokines in patients with diabetes mellitus and pulmonary tuberculosis. *Exp Ther Med* 2015;9:11-16.
22. Gonzalez-Curiel I, Castañeda-Delgado J, Lopez-Lopez N, Araujo Z, Hernandez-Pando R, Gandara-Jasso B, Macias-Segura N, Enciso-Moreno A, Rivas-Santiago B. Differential expression of antimicrobial peptides in active and latent tuberculosis and its relationship with diabetes mellitus. *Hum Immunol* 2011;72:656-662.
23. Visser DH, Solomons RS, Ronacher K, van Well GT, Heymans MW, Walzl G, Chegou NN, Schoeman JF, van Furth AM. Host immune response to tuberculous meningitis. *Clin Infect Dis* 2015;60:177-187.
24. Gombart AF, Bhan I, Borregaard N, Tamez H, Camargo CA Jr, Koeffler HP, Thadhani R. Low plasma level of cathelicidin antimicrobial peptide (hCAP18) predicts increased infectious disease mortality in patients undergoing hemodialysis. *Clin Infect Dis* 2009;48:418-424.
25. Ong PY, Ohtake T, Brandt C, Strickland I, Boguniewicz M, Ganz T, Gallo RL, Leung DY. Endogenous antimicrobial peptides and skin infections in atopic dermatitis. *N Engl J Med* 2002;347:1151-1160.
26. Rivas-Santiago B, Serrano CJ, Enciso-Moreno JA. Susceptibility to infectious diseases based on antimicrobial peptide production. *Infect Immun* 2009;77:4690-4696.
27. Yamshchikov AV, Kurbatova EV, Kumari M, Blumberg HM, Ziegler TR, Ray SM, Tangpricha V. Vitamin D status and antimicrobial peptide cathelicidin (LL-37) concentrations in patients with active pulmonary tuberculosis. *Am J Clin Nutr* 2010;92:603-611.