Staphylococcal leukocidin LukE/LukD modulates respiratory burst activity of phagocytes in common carp (*Cyprinus carpio* L.)

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Abstract

Leukocidins are novel leukolytic bicomponent toxins composed of S and F class protein subunits. They are one of major virulence factors produced by various strains of Staphylococcus aureus. Leukocidin subunits bind to phagocytes sequentionally (S prior to F) forming transmembrane pores. The purpose of our study was to determine the in vitro influence of staphylococcal leukocidin LukE/LukD on respiratory burst activity (RBA) of phagocytic cells (neutrophils and macrophages) isolated from head kidney of common carp (Cyprinus carpio L.). The results showed that the highest concentrations of the complete leukocidin LukE/LukD and its LukE subunit (5000-25000 µg/ml) significantly reduced RBA of head kidney phagocytes. Slight stimulation of RBA was observed at the lowest concentrations of the complete leukocidin LukE/LukD used in the experiment (0.32-1.6 µg/ml).

Key words: *fish immunity, respiratory burst activity, staphylococcal leukocidin.*

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Introduction

Staphylococcus aureus bacteria produce many virulence factors including exotoxins that enhance bacterial pathogenicity [1]. Leukocidin LukE/LukD belongs to pore-forming bicomponent exotoxins consisting of two non-associated protein subunits of S and F class [2, 3].

The toxin subunits attach to the leukocyte cell membrane sequentionally [4]. The S part of the toxin binds to a specific unknown receptor present in the membrane and after the conformational change of the S subunit-cell membrane complex free receptors appear enabling the F subunit to attach to the complex. The components form hexameric or octameric ring-shaped transmembrane pore selective to cations [5]. Reduced cell membrane barrier leads to imbalanced ion influx and efflux, metabolic instability of leukocytes, subsequent release of the inflammatory mediators and finally cell lysis [6]. Leukocidins are cytotoxic to mammalian polymorphonuclear cells, monocytes, macrophages and promyelotic leukemia cell line HL-60 [3, 4, 7] but very little is known on the influence of these toxins on the immune system of lower vertebrates such as fish. Neutrophils and macrophages are important in piscine innate immunity functioning as phagocytes, antigen presenting cells, and effector cells in cell-mediated immunity. Many phagocytic activities in vertebrates are modulated by environmental or pathogenic stressors [8]. Phagocytes possess the ability to form bactericidal oxidative radicals in the process known as respiratory burst and this phenomenon has been widely used as a biomarker in immunotoxicology. The purpose of our study was to determine the effect of leukocidin LukE/LukD on respiratory burst activity (RBA) of phagocytes isolated from head kidney of common carp (*Cyprinus carpio* L.).

Material and methods

Bacterial toxin

Leukocidin LukE/LukD produced by the Newman strain of *S. aureus* was kindly provided by Dr. G. Prevost

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Table 1. Percentage of viable phagocytes isolated from head kidney of common carp (*Cyprinus carpio* L.) after 30 min *in vitro* incubation of different concentrations of leukocidin LukD, LukE, LukE+LukD (mean ±SD, * – p<0.05, ** – p<0.01, n=30). Viability of the control phagocytes was 95±5

Concentration of leukocidin (ng/ml)	Component LukD	Component LukE	Complete leukocidin LukE+LukD
25000	85±2*	40±3**	5±4**
5000	82±3*	51±1**	13±3**
1000	83±1*	67±5**	48±3**
200	82±5*	72±3**	68±1**
40	81±4*	78±3**	72±2**
8	86±2*	80±2*	80±4*
1.6	84±1*	83±5*	84±4*
0.32	89±5	92±4	93±2

of Strasbourg, France. All components were purified as described previously by Prevost et al. [9].

Three sets of leukocidin subunits were used in the study:
- Subunit LukE (the S class – 32.2 kDa, purity of 93% determined by SDS-PAGE and fast protein liquid chromatography FPLC),
- Subunit LukD (the F class – 34.3 kDa, purity of 90% estimated by SDS-PAGE and fast protein liquid chromatography FPLC),
- Complete leukocidin LukE+LukD.

The sets of leukocidin subunits were added to the phagocyte suspension at concentrations: 25000; 5000; 1000; 200; 40; 8; 1.6; 0.32 ng/ml of medium RPMI-1640 (Sigma, USA).

Animals

30 healthy fish weighing 100-150 g were maintained in 10 containers with 20 liters of dechlorinated, filtered and aerated water at a temperature of 22°C and fed with commercially-pelleted dry diet (Sorga, Lublin, Poland). The animals were treated in accordance with the opinion and approval the Local Committee of Ethics (approval number 491/2004). Fish were anaesthetized with Propiscin (Institute of Pharmaceutical Industry, Warsaw, Poland) at a concentration of 2 ml/l. The head kidney (hematopoietic organ) was isolated asseptically from all the fish, pooled and squeezed through the cell dissociation sieve (Sigma, USA) into heparinized (10 IU/ml) RPMI 1640 (Sigma, USA) medium. Phagocytes (about 85% macrophages and about 15% neutrophils) were separated by centrifugation of the head kidney isolate at 2000 g for 30 min at 4°C using the Gradisol G (Polfa-Kutno, Poland) gradient.

Cell viability assay

Trypan blue exclusion assay was used to determine the influence of leukocidin LukE/LukD and its subunits at different concentrations on phagocyte viability. The cells isolated from the head kidney were suspended in RPMI and incubated in a 96-well microplate 30 min at 22°C with appropriate concentations of the toxin. After incubation, 20 µl of cell suspension ($0.5x10^6$ cells/ml) from each experimental sample was stained with 20 µl of 0.1% trypan blue and left 5 min at room temperature. The cells were counted in a Bürker chamber using a light microscope. The non-viable cells were stained purpure-violet and viable phagocytes remained unstained. Percentage of viable cells was calculated from the means of triplicates as: Cell viability (%) = number of unstained viable cells/total number of stained and unstained cells x 100%.

Respiratory burst activity (RBA)

Phagocytes were resuspended in RPMI 1640 medium supplemented with 10% of FCS (Foetal Calf Serum, Gibco-BRL, England) at a concentration of $3x10^6$ cells/ml of RPMI 1640 medium. Viability of the cells evaluated by supravital staining with 0.1% w/v trypan blue (1:1 mixture of cell suspension and trypan blue solution) was about 95%. Respiratory burst activity of phagocytes was assessed by a colorimetric method described by Rook et al. [10].

A 96-well culture micoplate (Nunc, Denmark) was used for incubation of 100 µl of phagocytic cell suspension in RPMI 1640 medium. The adjusted concentrations of S subunit (LukE) were added to the cell suspension. After 30 min of microplate incubation at 22°C, the same concentrations of F subunit (LukD) were added to the wells containing mixture of phagocyte suspension/S subunit. Separate microplate wells with phagocyte suspension only and the wells without leukocidin subunits were used as the control samples. The microplate was incubated for 30 min at 22°C. After the incubation 100 µl of 0.2% nitro blue tetrazolium (NBT, Sigma, USA) solution in 0.2 M phosphate buffer at pH 7.2 and 1 µl of PMA (phorbol myristate acetate, Sigma, USA) at a concentration of 1 mg/ml of methanol were added to each microplate well. After 30 min of incubation at 22°C, the supernatant was removed from each well. The layer of adherent phagocytes was washed with absolute ethanol, three times in 70% ethanol and left to dry at a room temperature. The amount of extracted reduced NBT after incubation with 2 M KOH and DMSO (dimethylsulfoxide, Sigma, USA) was measured at 620 nm in a microplate reader (MRX 3, Dynatech, Chantilly, USA). All the samples were tested in triplicate and the mean value served as the result.

The data were analysed statistically by 1-way ANOVA test. Post hoc Duncan test was used in order to describe mean differences between the tested groups. Statistical evaluation of the data was performed using computer program Statistica 5.0. For all calculations $p<0.05$ was

assumed as significant. Means and standard deviations are presented in the figure 1.

Results

Leukocytic properties of the complete leukocidin LukE/LukD and its LukE subunit was demonstrated by highly statistically significant, a dose-dependent suppression of phagocyte viability at concentrations of 25000-1000 ng/ml after 30 min incubation in comparison to the control group (table 1). The modulatory influence of leukocidin LukE/LukD on respiratory burst activity of phagocytic cells isolated from head kidney was observed. The complete leukocidin LukE/LukD at concentrations of 200-25000 ng/ml and single LukE subunit at concentrations of 1000-25000 ng/ml induced statistically significant inhibition of RBA (figure 1). Slight but statistically significant stimulatory effect of the complete toxin on RBA was observed at subcytolytic concentrations of 0.32 and 1.6 ng/ml. On the other hand, single LukD subunit at all concentrations used in the experiment did not evoke changes of phagocytic activity of phagocytes.

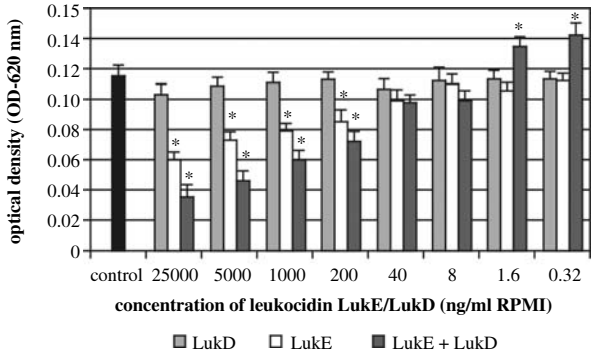

Fig. 1. *In vitro* effects of different concentrations of leukocidin LukD, LukE, LukE+LukD on respiratory burst activity (RBA) of phagocytic cells isolated from head kidney of common carp (*Cyprinus carpio* L.) (mean ±SD, $p<0.05$, n=30, * – statistically significant differences)

Discussion

The *in vitro* studies have demonstrated that staphylococcal leukocidin LukE/LukD, a newly described staphylococcal cytotoxin possesses the modulatory influence on RBA of phagocytes isolated from head kidney of common carp. Suppressive effects evoked by the highest concentrations of the complete toxin LukE/LukD and its subunit LukE on respiratory burst activity of phagocytes were probably associated with significant lysis of cells and, as a consequence, very small number of viable cells exhibiting RBA. Low, subcytolytic concentrations of the complete toxin LukE/LukD induced slight but statistically significant stimulation of phagocytic RBA. This effect could be induced by transmembrane pore-induced influx and accumulation of calcium ions inside the cell leading to enzymatic stimulation resulting in augmented phagocytic activity and/or increased production of phagocytosis-enhancing cytokines such as tumour necrosis factor (TNF) – alpha or macrophage--activating factor (MAF). Separate LukE subunit of leukocidin supressed the viability and RBA of phagocytes at the highest concentrations suggesting that not only pore formation induced by the two subunits (S+F) is the cause of the cytotoxic effect and immunosuppression. At present it is not possible to explain the mechanisms of this phenomenon since very little is known on all metabolic changes in the cells sensitive to LukE subunit. Single LukD component did not induce supression of phagocyte viability or RBA indicating that no specific receptors to LukD are not present in the cell membrane of carp phagocytes or the receptors and binding exist but LukD subunit may not affect RBA. Contamination caused by other leukolytic factors present in the leukocidin LukE and LukD samples should be excluded since the purity of the sample was estimated to be as high as 93% and 90% for LukE and LukD respectively. The immunomodulatory properties of staphylococcal leukocidin confirm findings from the previous experiments on mammals. Leukocidin LukE/LukD was described to modulate RBA of phagocytic cells isolated from dogs [11] and rabbits [12]. Similarily, high concentrations of the complete toxin inhibited the phagocytic activity but the lowest concentrations augmented the phagocytic activity of PMN and MN cells isolated from blood of these mammalian species.

The results obtained in our studies suggest the susceptibility of fish phagocytic cells to leukocidin LukE/LukD. There is a need for further *in vitro* and *in vivo* comparative studies to understand the mechanisms of toxic action of leukocidins and enrich our knowledge on the influence of other staphylococcal leukotoxins such as haemolysins and alpha-toxin on different immunocompetent cells isolated from various fish species.

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