IN VITRO EFFECT OF STAPHYLOCOCCAL LEUKOCIDINS (LUKE, LUKD) ON THE PROLIFERATIVE RESPONSES OF BLOOD LYMPHOCYTES IN DOG (CANIS FAMILIARIS)

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The influence of Staphylococcus aureus leukocidin E/D on mitogen stimulated proliferation of lymphocytes in dogs was studied in vitro. The immunosuppressive effect of the leukocidin on proliferative response of the lymphocytes was demonstrated.

Key words: dogs, Staphylococcus aureus, leukocidins, lymphocytes.

Staphylococci are ubiquitous bacteria existing in all ecosystems and some species are pathogens (26). Staphylococcus aureus induces infectious diseases in rabbits, dogs, calves, and is one of the most frequently isolated bacteria in hospitals (4). Different S. aureus strains produce various virulence factors including bicomponent synergohymenotropic toxins (SHT): gamma-haemolysins (Hlg) and leukocidins (Luk) (5, 8, 16, 21, 25). These toxins consist of two separately secreted and non-associated protein subunits of S class (slow-eluting from the ion exchange column) and F class (fast-eluting from the ion exchange column). Six proteins of S class and 6 of F class were found in different S. aureus strains. The S class proteins are: HlgA, HlgC, LukE, LukS, LukS-PV, LukS-R. Proteins of the F class include HlgB, LukD, LukF, LukF-R, LukF-PV, LukM.
The proteins have similar molecular weight (about 32 kDa for S subunit and 34 kDa for F subunit) (7, 18). Staphylococcus intermedius – a species belonging to bacterial flora of nasal cavity in dogs also produces bicomponent toxin proteins of S class (LukS-I) and F class (LukF-I) (20). These organisms are not found very frequently in humans even having a constant contact with their pets (14, 27).

Leukocidal toxins are able to reduce the number of immunocompetent cells and as a consequence, inhibit the process of phagocytosis. Bicomponent synergohymenotropic toxins of S. aureus act synergistically and lyse polymorphonuclear (PMN) and mononuclear (MN) cells in humans and rabbits (2, 6, 11). Moreover, gamma-haemolysins are also known to cause destruction of erythrocytes of many mammalian species (3).

The knowledge on mechanisms of cytolytic action of leukocidins is not complete yet. The cytotoxic action of leukocidins includes the interaction of two toxin components S and F with the leukocyte cell membrane. The leukocidal toxins induce disturbances in basic physiological processes of the immunocompetent cells. The biological effect of the toxins is a result of direct binding of S and subsequently F protein components to the cell membrane (10). This process induces formation of transmembrane pores in leukocytes and macrophages (17). Secondary reactions are associated with the increase of intracellular calcium cations concentration (Ca\(^{2+}\)) and inflammatory mediators release (1, 9, 10, 23).

Leukocidins are produced by various S. aureus strains inducing furunculosis, dermonecrosis, necrotic pneumonia, cellulitis, osteomyelitis and abscesses in animals and humans (4, 12). Frequent isolation of these strains suggests that secreted leukocidins could play an important role as a virulence factor in cutaneous infections.

Very little is known about the influence of staphylococcal leukocidins on lymphocytes of different animal species. Leukocidin E/D is a newly found bicomponent synergohymenotropic cytotoxin produced by Newman strain of S. aureus (7). The purpose of the study was to determine the in vitro effect of leukocidin E/D on lymphocyte proliferation stimulated by mitogens in dogs.

**Material and Methods**

**Animals.** The study was carried out using 20 healthy dogs (Canis familiaris) weighing 25-40 kg, aged 2-5 years. All the animals lived in a refuge for homeless dogs, remained under veterinary control and were diagnosed as healthy based on a clinical examination. Peripheral blood was obtained by venous puncture by Vacutainer system to the heparinized tubes (Vacutainer set – Vacuette Greiner Labortechnik; 50 IU/ml of heparin). The blood taken from each animal was used for the preparation of the control and experimental samples.

**Leukocidal toxins.** Two components of leukocidin: LukE and LukD from the S. aureus Newman strain were obtained from Dr G. Prevost from Strasbourg, France. All the components were purified from culture supernatants of respective staphylococcal strains as described previously by Prevost et al. (21). The structural analysis revealed that the molecular weight of purified fractions ranged from 31 to 35 kDa. All the fractions were dissolved in 50 mM Na-phosphate buffer with 150 mM NaCl at pH 6.5-8.5. The stock solutions of the components contained 0.58-0.82 mg of purified protein per ml. The fractions were stored at –40°C before the use for the experimental study.

In this study, the following leukocidins were used: Leukocidins LukE (purity of 93%) (the S class – 32.2 kDa) + LukD (purity of 90%) (the F class – 34.3 kDa). The
following concentrations were used: 25 000, 5 000, 1 000, 200 and 40 ng/ml of RPMI-1640 medium (Sigma Chemicals, USA).

**Experimental design and assay procedure.** Lymphocytes were isolated from blood by centrifugation at 2 000 g for 30 min at 4°C on the Lymphoprep gradient (Nycoderm Pharma, USA), washed three times in PBS (phosphate buffered saline) and resuspended in RPMI 1640 medium (Sigma, USA) supplemented with 10% of FCS (Foetal Calf Serum, Gibco-BRL, England) at a concentration of 1 x 10^6 cells/ml of medium. Viability of the cells was evaluated by supravital staining with 0.1% w/v trypan blue (1:1 mixture of cell suspension and trypan blue solution).

The proliferative response of the blood lymphocytes stimulated by mitogen concanavalin A (ConA, Sigma, USA) or lipopolysaccharide (LPS from Serratia marcescens, Sigma, USA) was determined by MTT assay described by Mosmann (15) and used in dogs by Wagner et al. (28) and Siwicki and Mizak (22). MTT [3-(4,5-Dimethyl thiazol-2-yl) 2,5-diphenyl-tetrazolium bromide] (Sigma, USA) was dissolved in PBS at concentration of 5 mg/ml. A 96-well culture plate (Costar, USA) was used for incubation of 100 μl of blood lymphocytes in RPMI 1640 medium containing 10% FCS, 2 mM L-glutamine, 0.02 mM 2-mercaptoethanol, 1% hepes buffer and penicillin/streptomycin (100U/100 μg/ml). The toxin subunits were added sequentially. The prepared concentrations of subunit S (LukE) were added to the cell suspension. After 30 min of microplate incubation at 37°C, the same concentrations of subunit F (LukD) were added to the wells containing mixture of lymphocyte suspension/subunit S. Separate microplate wells with cell suspension only and without leukocidin subunits were used as the control samples. The microplate was incubated for 30 min at 37°C. Mitogens: 10 μl ConA (5 μg/ml RPMI) or 20 μl LPS (20 μg/ml RPMI) were added for stimulation of T or B lymphocyte proliferation, respectively. After 72 h incubation at 37°C with 5% carbon dioxide atmosphere (Asab Incubator, Sweden), 50 μl of MTT solution were added into each well and microplates were incubated for 4h at 37°C. After incubation, the plates were centrifuged (1400 g, 15°C, 5 min). Supernatants were removed and 100 μl of DMSO (Sigma, USA) were added into each well and incubated for 15 min at room temperature. After incubation, the solubilized reduced MTT was measured colorimetrically at 620 nm in a plate microreader (MRX 3 Dynatech). All the samples were tested in triplicate and the mean value served as the result.

The results from three sets of experiments were pooled. The mean values and standard deviations from pooled experiments were used for comparison with the control and experimental groups. Statistical significance was evaluated with the use of the Statgraphics 2.1 Win and Statistica 5.77 software (analysis of variance, comparison of regression lines, Wilcoxon’s twin pair analysis). For all calculations P ≤ 0.05 was assumed as significant.

**Results**

The proliferative response of T lymphocytes stimulated by ConA was reduced by LukE (molecular weight - 32.2 kDa) + LukD (molecular weight - 34.3 kDa) at the concentrations of 25 000 ng/ml and 5 000 ng/ml of medium in comparison to the control. (Fig. 1) The proliferative response of blood B lymphocytes stimulated by LPS was also diminished by LukE + LukD toxin at the highest concentrations of 25 000 ng/ml and 5 000 ng/ml of medium in comparison to the control (Fig. 2). The cell viability was 5% for
T cells and 4% for B cells at 25 000 ng/ml. At the concentration of 5 000 ng/ml the lymphocyte viability was 40% for T cells and 30% for B cells. We have also observed statistically significant optical density reduction for the T and B cells at the subcytolytic concentrations of 1 000 ng/ml (50% of T and 55 of B living cells) and 200 ng/ml (55% of T and 55% of B living cells) of the leukotoxin. A slight but statistically significant suppressive effect on the proliferative response of blood T and B lymphocytes was noted at the concentration of 40 ng/ml of LukE + LukD (75% of T and 60% of B living cells).

Fig. 1. Influence of different concentrations of leukocidins (LukE + LukD) on the proliferative response of blood T lymphocytes stimulated by ConA (mean ± SD, n=20, P ≤ 0.05, * statistical significance in comparison to the control).

Fig. 2. Influence of different concentrations of leukocidins (LukE + LukD) on the proliferative response of blood B lymphocytes stimulated by LPS (mean ± SD, n=20, P ≤ 0.05, * statistical significance in comparison to the control).
Discussion

There are few data on the in vitro influence of leukocidins on selected metabolic pathways of lymphocyte proliferation. The results of our in vitro studies have shown immunosuppressive effect of leukocidin E/D on proliferative response of blood lymphocytes in dogs. The immunosuppressive effect was probably a result of nearly complete (25 000 ng/ml) or partial (5 000 ng/ml) lysis of blood lymphocytes induced by leukocidin what was documented by cell viability results (25 000 ng/ml – 5%-T cells; 4%-B cells) We can conclude that pore formation in the cell membrane of dog lymphocytes probably caused enzymatic imbalance and disturbed ion regulation. Such metabolic disorders could induce lymphocyte lysis and as a consequence, diminished T and B cell proliferation. We need more in vitro studies on different animal species to explain the mechanism of cytotoxic action of various staphylococcal leukocidins.

Up to now, very few data can be found on the effects of various staphylococcal leukocidins including leukocidin E/D on mammalian lymphocytes. Pfannenberg et al. (19) showed that leukocidin produced by bovine P83 strain of S. aureus caused destruction of 25% of bovine lymphocytes in vitro. The cells, however, did not demonstrate morphological changes during contrast - phase observation. Loeffler et al. (13) using $^{51}$Cr release assay also described a slight but statistically significant cytotoxic influence of leukocidin produced by P83 S. aureus strain on bovine lymphocytes. The reviewed results suggest suppressive effect of leukocidins and seem to be in agreement with the data obtained in the performed in vitro studies.

In vivo studies have also shown the reduction of total number of lymphocytes in blood. Szmigielski et al. (24) reported that staphylococcal leukocidin given intravenously to rabbits decreased total number of lymphocytes in the peripheral blood.

Our in vitro studies revealed the susceptibility of dog lymphocytes to cytolytic influence of staphylococcal leukocidin E/D. There is a need for further in vitro and in vivo studies on the effects of other leukocidins that have not been tested using different animal species. The results would improve our knowledge on the role of staphylococcal cytotoxin in pathogenesis of certain diseases and would possibly help to elaborate effective methods for curing staphylococcal diseases and administering the selected drugs to the cells by using transmembrane pores.

References