Review paper

Effects of staphylococcal hemolysins on the immune system of vertebrates

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Abstract

Staphylococcus aureus is pathogenic to animals and humans and produce many virulence factors such as hemolysins which include alpha-, beta-, gamma- and delta-hemolysin. These agents play a very important role in staphylococcal pathogenesis. Hemolysins are cytolytic to a variety of host cells. Toxicity to immune cells makes them a good means for staphylococci to avoid phagocytosis and other forms of immune response. Alpha-hemolysin is a protein toxin released to host environment as a monomer. Oligomerization of the monomers to a heptamer on the surface of the target cell results in a transmembrane pore formation usually leading to metabolic instability and cell lysis. The toxin targets many types of immune cells monocytes, neutrophils and lymphocytes usually causing their death at high concentrations, however at lower concentrations some stimulatory effects on various immune functions were observed. Beta-hemolysin known as an inflammatory inducer is an enzyme with a specific, other than for most of staphylococcal toxins mode of action and its effects depend on sphingomyelinase content in a target cell. Gamma-hemolysins are very unique bicomponent pore-forming toxins consisting of S and F class proteins, released as monomers and forming heterooligomers leading to a cation-selective channel formation. Human monocytes, macrophages and neutrophils are the main cell types susceptible to these bacterial agents. Delta-hemolysin, a heat-stabile, small protein produced by most Staphylococcus aureus strains acts as a cytolysin but also turned out to be a potent polyclonal activator of lymphocytes. Currently, staphylococcal hemolysins are being introduced to many fields of biotechnology.

Key words: *Staphylococcal toxins, hemolysins, immune system.*

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Introduction

Staphylococcus aureus is a Gram-positive bacterium species harmlessly colonizing aproximately 30% of human population. However, under favourable conditions these microorganisms can induce serious diseases such as certain types of skin infections, endocarditis or life-threatening septicemia and pneumonia. Staphylococci produce a number of virulence factors responsible for microbial pathogenesis such as hemolysins belonging to a major group of bacterial toxins known as Pore-Forming-Toxins (PFT). Staphylococcal hemolysins include alpha-, beta-, gamma- and delta-hemolysins. These toxins are important factors in staphylococcal pathogenesis and since they have capability to kill a variety of cell populations, including immune cells, they are very essential factors acting synergistically thus increasing the spread of these bacteria within the host.

Alpha-hemolysin is one of the best characterized pore forming toxin related to other staphylococcal toxins, leukocidin, gamma-hemolysin and beta-toxin from *Clostridium perfringens* and hemolysin II produced by *Bacillus cereus*. This toxin is a protein toxin secreted by bacteria in a water soluble monomeric form of 293 aminoacids (33 kDa) and it undergoes oligomerization into ring-shaped heptamers on the membrane of the target cell forming anion-selective transmembrane pores [1, 2]. The pores allow leakage of ions and small molecules leading to metabolic disorders of the target cell and finally its lysis. The toxin is considered as one of the most important pathogenetic factors in staphylococcal infections. The specific receptor for alpha-

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-hemolysin has not been identified however recent findings suggest that caveolin-1, found on most mammalian cells could play an important role in toxin-target cell interaction since alpha-toxin possesses the caveolin recognition motif [3]. Alpha-toxin induces lysis of variety of cells such as human platelets, erythrocytes, monocytes and endothelial cells and it is known to change functions of different populations of immune cells. There is evidence demonstrating different effects exerted by alpha-toxin on phagocytic cells. Human monocytes turned out to be vunerable even to low concentrations of the toxin (20 ng/ml) [4]. Cytotoxic action of alpha-hemolysin is manifested by depletion of cellular ATP. The *in vivo* and *in vitro* studies delivered some evidence that staphylococcal alpha-hemolysin triggers oversecretion of inflammatory mediators by immune cells enhancing inflammation caused by other bacterial virulence factors. It was demonstrated that the toxin stimulates the secretion of interleukin-1β and tumor necrosis factor-α (TNF-α) from human monocytes [4]. The concentration of interleukin-1β from cultured monocytes exceeds 10 ng/ml in the supernatant 60 min after application of the toxin. Excessive production of interleukin-1β and TNF-α results in tissue injury during inflammation. It was demonstrated in the *in vivo* studies that alpha-hemolysin stimulates interleukin-1 alpha secretion in peritoneally injected mice. A dose of 45 of hemolytic units of alpha-hemolysin also triggers interleukin-6 secretion [5]. Alpha-toxin at low concentrations (3-30 ng/ml) possesses the ability to induce apoptosis in peripheral blood mononuclear cells by mitochondrial pathway associated with the release of TNF-α. Interestingly, blocking the TNF-α receptor with TNF-α antagonists decreased apoptosis of macrophages [6].

Rabbit alveolar macrophages turned out to be susceptible to highly purified staphylococcal alpha-hemolysin. Cell necrosis is usually observed after 4-hour and 8-hour exposure to 1 ug/ml and to 0.1 ug/ml, respectively. Additionally, sublytic concentrations of the toxin significantly reduce the phagocytic activity of these cells [7].

Alpha-hemolysin modulates functioning of polymorphonuclear cells. In the *in vitro* conditions the toxin triggers the arachidonic acid and extracellular Ca^{2+} influx into those cells [8]. Low doses of the toxin (under 10 hemolytic units) enhance phagocytosis and the intracellular killing of neutrophils. Alpha-toxin acts as a potent chemoatractant for polymorphonuclear cells and significantly inceases adherence of human polymorphonuclear cells to rat aortic endothelium after stimulation of the endothelium with the toxin [9, 10]. Alpha-toxin is a factor starting neutrophil-induced cardiac dysfunction in isolated rat heart. The toxin stimulates coronary endothelial expression of intracellular adhesion molecule-1 (ICAM-1) and neutrophil accumulation with subsequent secretion of cysteinyl leukotrienes [11]. The *in vivo* studies performed by Riollet et al., revealed that alpha-hemolysin could be a useful agent in the treatment of staphylococcal mastitis. Immunization of cows with this toxin triggered an early and massive recruitment of neutrophils from blood into the milk compartment in the mammary gland [12].

As early as some decades ago it was demonstrated that partially purified alpha-toxin have mitogenic ability towards rabbit lymphocytes [13]. However, some speculations arose that the toxin isolates used in the studies could be contaminated with other agents that stimulated the blastic transfomation of lymphocytes [14]. Further studies with the purified toxin proved that alpha-hemolysin indeed stimulates human T and non-T lymphocyte proliferation. This property is usually maintained after inactivation of the toxin at 60°C for 10 min but in turn its hemolytic activity is reduced [15].

Alpha-toxin turned out to affect the immunoglobulin screction. Production of antibodies IgM, IgA, IgG was stimulated after treatment of human peripheral blood lymphoctes with alpha-toxin at non-lytic concentrations (1-100 ng/ml). Similar results were obtained with the alpha-toxin toxoid – denaturated form of natural alpha-hemolysin with no hemolytic properties [16].

Staphylococcal alpha-toxin possesses the ability to diminish the opsonic activity of serum for *Staphylococcus aureus*. Levels of the complement units C2, C3 and C9 of the classical pathway were reduced after the exposure [9].

Beta-hemolysin (sphingomyelinase C) is an enzyme with phosphorylase C activity having a different mode of action than the other staphylococcal hemolysins. The toxin requires bivalent Mg^{2+} cations for its biological activity [17]. Beta-hemolysin is more often produced by strains of *Staphylococcus aureus* pathogenic for animals. There is various sensitivity to beta-hemolysin among different animal species. Different susceptibility of vaious cell types is dependent on sphingomyelin content. The toxin decreases viability of sphingomyelin-containing polymorphonuclear cells and lymphocytes. Toxic effects are manifested by morphological changes in polymorphonuclear cells such as ruffled membrane [18] but there are no invaginations as in erythrocytes, so it is possible that leukotoxicity could not be a result of membrane damage. Beta-hemolysin was described as a very potent monocytocidal agent. At a concentration of 0.001 U/ml (5 ng/ml) the toxin kills over 50% human monocytes within 60 minutes. The cells exposed to beta-hemolysin release interleukin-1β, interleukin-6 soluble receptor and soluble C14 receptor to the supernatant. However, beta-toxin at a concentration of 1-5 ug/ml exert no destructive effect on other human immune cells: granulocytes, fibroblasts and lymphocytes [19]. Beta-hemolysin given experimentally induces mild inflammatory changes in the bovine mammary gland. The toxin has no mitogenic influence on human lymphocytes [15], however it is capable to kill such cells during proliferation [20] .

Gamma-hemolysins (Hlg) are apart from leukocidins (Luk) a very unique group of protein toxins composed of two independently secreted proteins of two different classes S (slow-eluting from ion-exchange column) and F (fast-eluting from ion-exchange column), mostly non-toxic when administered separately. Gamma-hemolysins are produced by almost every strain of *Staphylococcus aureus* while leukocidins are released by only 2-3% of strains.

The S class are: HlgA, HlgC, LukE, LukS, LukS-PV and LukS-R. Proteins of F class are: HlgB, LukD, LukF, LukF-R, LukF-PV and LukM. The proteins of each class have similar molecular weight (usually 32000 for S components and 34000 for F components). Appropriate combination of the protein subunit from S and F classes determines the toxic properties of the complex. Theoretically, there are 36 possible combinations of the S and F subunits, but naturally, a single strain of *Staphylococcus aureus* produces two, three or five of these component proteins. The two types of S and F subunits are necessary for the toxic action [22]. After sequentional binding of gamma-hemolysin subunit (S prior to F) to a receptor linked to a divalent cation selective channel or to the channel itself, the channel is opened. Afterwards, the toxin monomers insert into the membrane and oligomerize to a hexamer (3S:3F) or octamer (4S:4F) but not to heptamer as in the case of alpha-hemolysin. Recent findings suggest that gamma-hemolysin components HlgA, HlgC, HlgB are able to form mixed pores containing all three subunits. As a result of transmembrane pore formation a metabolic instability and subsequent lysis of leukocytes occurs. The toxic action of bicomponent leukocidin and gamma-hemolysin subunit combinations was studied in human granulocytes. The higher inflammatory mediator release was induced by toxins LukS-PV/LukF-PV, LukS-PVL/HlgB. The toxins HlgC/LukF-PVL, HlgC/HlgB were less active but the least potent were combinations HlgA/LukF-PVL and HlgA/HlgB [22]. Toxins HlgA/HlgB and HlgC/HlgB are also able to induce permeabilization in model membranes [23].

Delta-toxin is a small (26 amino acids, 3kDa of molecular weight), heat stabile protein produced by 97% of *Staphylococcus aureus* strains. The inactive form of the toxin – protoxin is 45 amino acids in length. Delta-hemolysin is an alpha-helix acting as a surfactant destructing the cell membrane [24]. The toxin is one of the most potent staphylococcal toxins affecting a wide range of cells and organelles, it is able to bind and exert its toxic effects on immune cells. The toxin induces a rapid influx of Ca^{2+} and stimulates oxygen radicals production in human granulocytes [25]. In addition, release of lysozyme and beta-glucuronidase from the cells occurs but only after exposure to higher concentrations of the toxin (15 ug/ml) [26]. A stronger response to bacterial lipopolysaccharide and increased TNF-alpha production was observed when human neutrophils were incubated with delta-hemolysin [25].

Similarily to alpha-toxin, delta-hemolysin and its toxoid act as a medium-strength polyclonal activator of human lymphocytes. Moreover, it was demonstrated that production of IgM, IgA and IgG antibodies is stimulated after incubation of immunoglobulin-producing lymphocytes with this hemolysin at concentrations of 1-100 ng/ml [16]. In the *in vivo* studies itwas shown that rabbits and guinea pigs immunized with delta-toxin produce specific immunoglobulins IgG which however are unable to neutralize the toxin's hemolytic activity [27].

Some efforts are being undertaken to exploit properties of staphylococcal hemolysins in biotechnology. Promising attempts to use of hemolysins' toxoids – inactivated toxin inducing immune response in the injected animals, are well documented. These toxins could serve as a useful carrier to deliver immunotherapeutic agents directly into the cell and structurally modified mutants of such toxins could be a good immunostimmulatory agent. The ability of staphylococcal hemolysins to form pores may be useful to control growth of different cell populations. The level of toxicity might be controlled by setting a proper combination of gamma-hemolysin subunits, for instance. Such innovations could be helpful in improving therapies of certain types of cancer.

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