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Effect of iNOS inhibitor LNMMA along with antibiotics Chloramphenicol or Ofloxacin in murine peritoneal macrophages regulates *S.aureus* infection as well as inflammation: An in vitro study

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ABSTRACT

Death due to sepsis by *S. aureus* is rapidly increasing because of their potent weaponries against macrophage mediated killing. Macrophages serve as intracellular reservoirs of *S. aureus*. Although significant resources have been invested during the last decade in new treatments for sepsis, only antibiotic therapy has failed to improve outcomes. Moreover the host pathogen interaction resulted in host cell death triggering inflammation. So, successful therapy requires amalgamation of therapies to delineate pathogen along with providing protection to host cell. With this idea, LNMMA, the iNOS inhibitor is used along with antibiotics Ofloxacin or Chloramphenicol on *S. aureus* infected mouse peritoneal macrophage. ROS like H_2O_2, O_2^- production has been measured. NO inhibition by iNOS inhibitor and antioxidant levels has been analysed. COX2, TLR2 and iNOS expression along with proinflammatory cytokine level was studied. It was found that the use of iNOS inhibitor LNMMA along with antibiotics not only enhances bacterial clearance but also decreases proinflammatory responses in *Staphylococcus aureus* infected macrophages. Inhibition of TLR2 as well as COX2 has also been found in combined treatment groups. The use of iNOS inhibitor LNMMA plus Ofloxacin or Chloramphenicol pretreatment enhanced bacterial clearance by increasing ROS. Decreases in NO protect the cell from harmful peroxynitril as well as inflammatory damage by changes in iNOS, COX2 activity along with reduced proinflammatory cytokines like TNFα, IFNγ, IL1-β etc. Changes in antioxidant level has been found. This in-vitro realm of augmented bacterial clearance and regulated inflammation may be considered as a novel and important therapeutic intervention.

1. Introduction

Staphylococcus aureus, an adaptable pathogen, versatile in nature and varying in severity of infection affecting skin, soft tissue, respiratory system, bone joints and endovascular tissues [1]. The magnitude of alarming increase in *S. aureus* infection leading to high morbidity and mortality rates is mainly due to emergence of multiple antibiotic resistance [2]. Methicillin resistant *S. aureus* are not restricted to any particular geographic area, it is a global health problem [3]. Invasive MRSA conditions are reported to include septic shock, pneumonia,endocarditis, bacteremia and cellulitis [4]. The success of *S. aureus* as a pathogen can be attributable to its vast repository of virulence determinants and its residence inside the macrophage serves as its weaponry to anticipate innate and adaptive response by the host cell.

Macrophage comprises a complex population of cells that display a spectrum of functional states which are determined by the presence of signals such as cytokines. Regardless of the existence of heterogeneous macrophage populations, these cells are all unified through their capacity to combat infection, albeit with variable efficiency, because they are endowed with the innate

List of Abbreviations: CHL, Chloramphenicol; COX-2, Cyclogenase; CPCSEA, Committee for the purpose of control and supervision of experiments on animal; ELISA, Enzyme linked immunosorbent assay; iNOS, Inducible nitric oxide synthase; LNMMA, L-NG-monomethyl Arginine; OFX, Ofloxacin; VRSA/VISA, Vancomycin Intermediate/Resistant *Staphylococcus aureus*; NF-kB, Nuclear factor -kappa beta; ROS, Reactive oxygen species; RPMI, Roswell Park Memorial Institute medium; SDS-PAGE, Sodium dodecyl sulphate polyacrylamide gel electrophoresis; MARCO, Macrophage receptor with collagenous structure.

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ability to ingest particulates, such as bacteria, through phagocytosis. *S. aureus* are rapidly phagocytosed by macrophages mainly relying on non-opsonic receptors MARCO and CD36 [5]. Along with this TLR2 [toll like receptor 2] plays a role as a pattern-recognition receptor in the innate immune response involving secreted proteins against microbial pathogens [6].

TLR which consist of 12 structurally related proteins, each of which basically recognizes a distinct PAMP [pathogens associated molecular patterns] present at either the periphery or the inside of microbes. They represent PRR (pattern-recognition receptor[s]) responsible for the induction of responses that direct the production of various secreted factors and cytokines [7]. There is increasing evidence that TLR2 found on monocytes/macrophages is involved in the detection of *S. aureus* PAMPs via TLR1 or TLR6 heterodimers and acts with other co receptors to mediate phagocytosis of the bacterium [9]. Cascade of events follow after recognition of PRR by PAMPs, triggering the host immune response leading to activation of signaling pathways exemplified by NF-KB, AP1 followed by series of protein covering inflammatory cytokines and other inflammatory mediators to provoke the host immune response [8,9].

The escalation of bacterial resistance to antibiotics has been recognized as a reality since some time, but the frequency of emergence of gaining antibiotic resistance has amplified to such extent, to claim post antibiotic era as future fact [10]. The evolution of VISA and VRSA strains which are not strictly opportunistic, makes them even more dangerous [11]. The emergence of MRSA strains with reduced vancomycin susceptibility [SA-RVS] further reduces treatment options. Factors to be included while choosing antibiotics include enhanced efficacy, low toxicity, lesser potentiality for resistance development, penetration into tissue and low cost [12].

Additionally, it has been demonstrated that the rapid release of abundant peptidoglycans, lipoteichoic acid, and exotoxins from *S. aureus* induces inflammatory response and cytokine release [13].

Therefore, novel therapeutic strategies that are efficient against this pathogen further reduces the deleterious effect to the host cell is an immediate need of the time. The use of LNMMA an iNOS inhibitor along with chloramphenicol or Ofloxacin has been experimented in our in-vitro studies.

The therapeutic effectiveness of an antimicrobial agent may depend in part on the interactions between the drug and phagocytic cells. An optimal antibiotic would kill extracellular organisms, would enter phagocytic cells, have no adverse impact on phagocytic cell function, and eradicate surviving intracellular organisms.

Unfortunately, most antibiotics [especially β-lactam drugs] fail to enter leukocytes efficiently and, therefore, are unable to kill phagocytosed organisms. Conversely, even antibiotics that achieve high cellular levels may not kill antibiotic-sensitive, intraphagocytic bacteria. One potential cause for the failure of some highly concentrated drugs to kill intraphagocytic organisms is antibiotic-induced inhibition of phagocytic cell's antimicrobial activity. Indeed, we found that several antibiotics that are highly concentrated within human polymorphonuclear neutrophilic leukocytes [PMN]; clindamycin, macrolides, and trimethoprim inhibit the stimulated respiratory burst response in these cell.

In spite of the constantly increasing need and the alarming epidemic of multidrug-resistant bacteria, antibiotic drug discovery and development seem to have greatly decelerated in recent years, which has forced clinicians to reintroduce forgotten antibiotics into their practice. Due to the low levels of use of many of the old antibiotic compounds, these have remained active against a large number of currently prevalent bacterial isolates. MRSA clinical isolates have shown susceptibility to chloramphenicol with minimum inhibitory concentrations [MICs] of 8 μg/mL [14]. Thus, clinicians are beginning to reevaluate their use in various patient populations and infections, regardless of the fact that they were previously thought to be less effective and/or more toxic than newer agents. Chloramphenicol is a broad-spectrum bacteriostatic antibiotic drug that stops bacterial growth by inhibiting protein-chain elongation and by inhibiting the peptidyl transferase activity of the bacterial ribosome. Along with its broad-spectrum antibacterial nature, chloramphenicol is also effective against *Enterococcus faecium*, which has led to its being considered for treatment of vancomycin-resistant *Enterococcus*. Because of its excellent blood–brain barrier penetration, chloramphenicol remains the first-choice treatment for staphylococcal brain abscesses [15].

Ofloxacin [OFX] is a fluoroquinolone derivative consisting of racemic mixture, having 50% levofloxacin [the biologically active component] and 50% of its enantiomer dextrofloxacin. This fluroquinolone hinders bacterial multiplication by interacting with type II topoisomerases [i.e. gyrase and topo IV] that are involved in the replication and repair of bacterial DNA. Since Ofloxacin targets type II topoisomerases, and as it is found in most of the bacteria, Ofloxacin can be termed as broad-spectrum antibiotic [16].

Reactive oxygen species [ROS] are critical weapons in the phagocyte arsenal. ROS like superoxide and hydrogen peroxide [H_2O_2] are produced inadvertently in the cytoplasm primarily when oxygen collides with various redox enzymes that have solvent-exposed flavins [17]. Both H_2O_2 and superoxide anion has the potent ability to destroy wide range of microorganisms.

The choice of the antibiotic has been done not only because of their bioavailability or penetration inside the cell but also because of enhanced ROS production by both Chloramphenicol as well as Ofloxacin [18,19]. ROS have a role in cell signaling, including; apoptosis; gene expression; and the activation of cell signaling cascades. ROS can serve as both intra- and intercellular messengers [20].

TLR signaling triggers translocation of the master transcriptional regulator NF-kB [nuclear factor kB] to the nucleus, where it governs the expression of proinflammatory cytokines [for example, tumor necrosis factor alpha [TNF-α, interleukin IL-1 and IL-6] and chemokines, macrophage inflammatory protein-1α.Macrophage-produced cytokines and chemokines provide a crucial bridging point between the innate and the adaptive immune systems, making these cells an excellent target for immunostimulatory strategies [21]. Apart from the antimicrobial effect of antibiotics, they can also be said to be effective immunomodulators, [22], they can reduce the production of pro-inflammatory cytokines like TNFα, IFNγ, IL-6,IL1β etc [23,24].

A number of enzymes can transform ROS into less toxic products. Among the most important of these are catalases, peroxiredoxins, and superoxide dismutases [SODs]. Regulation of balance between oxidants and antioxidants is utmost necessity for maintaining cell functionality [25].

Reactive nitrogen intermediates [RNI], including nitric oxide, are also involved in the antimicrobial activity of activated macrophages. It is an essential mediator of macrophage cytotoxicity against a variety of microorganisms, and tumor cells. Excess production of NO damages tissue and has been implicated in a variety of immunopathological situations including septic shock. NO is formed from the terminal guanidino nitrogen of L-arginine, in a reaction catalyzed by the enzyme NO synthase In theory, O_2- and nitric oxide can combine to form highly reactive peroxynitrite [$ONOO^-$] which is one of the potent free radicals causing oxidative damage and has powerful cytotoxic properties. The decomposition products of peroxynitrite, OH′ and NO; are also tissue-damaging agents, and will further contribute to the damage resulting from the inflammatory response [26]. NO can participate in