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Reduction of Beta-Lactam Antimicrobial Activity in *Staphylococcus aureus* Abscesses by Neutrophil Alteration of Penicillin-Binding Protein 2

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ABSTRACT

We previously demonstrated that brief nonkilling neutrophil exposure diminishes the binding affinity of *S. aureus* penicillin-binding protein (PBP) 2. We sought to investigate further the role of the neutrophil in the alteration of antimicrobial activity and its interaction with PBP-2 by studying the activity of cefotaxime, which highly binds to PBP 2, and cephalexin, which minimally binds to PBP 2. Using *S. aureus*, cultured *in vitro* in sterile-filtered normal and neutrophil depleted abscess fluid, we sought to demonstrate an *in vivo* significance of the neutrophil effect upon the activity of antimicrobials that target PBP-2 by studying the same antimicrobials in an experimental *S. aureus* abscess. Rats were implanted with perforated tissue cages and infected with *S. aureus*; some rats were neutrophil depleted by mechlorethamine. Abscess fluids from normal and neutropenic abscesses were harvested, pooled, sterile-filtered and stored for the time-kill studies. Treatment studies were performed by administering either 300 μg/kg/d cefotaxime or cephalexin for 7 days in other rats with 24 hour-old tissue-cage *S. aureus* abscesses. In time-kill studies, cefotaxime was highly active against stationary phase *S. aureus* in MHB and in neutropenic abscess fluid, but less active in the non-neutropenic ab- scess fluid ($p < 0.05$ compared to neutropenic abscess fluid). Cephalexin was equally active in neutropenic and non- noneutropenic abscess fluids, and more active than cefotaxime in the abscess model after 7 days of therapy (2.1 ± 1.7 $\log_{10}$ kill, $p = 0.029$ vs. 0.81 ± 2.5, p = NS). These data suggest that neutrophil exposure, which diminishes *S. aureus* PBP-2 binding affinity [or total quantity], also adversely affects the antimicrobial activity of cefotaxime, which binds to PBP-2, as compared to cephalexin. Altered PBP targets from neutrophil exposure may be a mechanism of antimicrobial resistance within abscesses.

Keywords: Neutrophils; Penicillin-Binding Proteins; *S. aureus*; Abscesses

1. Introduction

The treatment of abscesses caused by *Staphylococcus aureus* usually necessitates drainage and antimicrobials in the setting of an abscess usually only have limited activity. However some abscesses respond to antimicrobial therapy without drainage, and the mechanisms by which antimicrobial activity is diminished in a suppurative environment are not fully understood, but are not likely related to inadequate antimicrobial concentrations [1]. We have found that the activities of antimicrobials within polymorphonuclear leukocytes (PMNs) correlated with *in vivo* efficacy in the treatment of experimental *S. aureus* abscesses [2]. The PMN may act a sanctuary since we have previously found that killing by PMNs, but not phagocytosis of staphylococci is inhibited systemically by the presence of an abscess, and may be further inhibited in the abscess milieu [3]. We have further shown that cefazolin is more effective in abscesses in which PMN influx was inhibited [4].

A significant virulence mechanism of *S. aureus* is its ability to survive within the PMN [5]. During the time of intracellular survival, global changes in *S. aureus* gene expression occur, including changes in the expression of penicillin-binding proteins (PBPs) genes [6]. PBPs are the targets of beta-lactam activity which inhibits transpeptidase activity of PBPs by forming a covalent penicilloyl-enzyme complex that blocks the normal transpeptidation reaction and results in bacterial death [7]. We have demonstrated that beta-lactam binding affinity to *S. aureus* PBP-2 is significantly decreased by brief non-killing PMN exposure, and postulated that this may be associated with diminished antimicrobial activity within an

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abscess milieu [4]. The diminished binding affinity observed may be secondary to decreased PBP-2 production or alteration of the target for beta-lactam binding [7,8].

Cefotaxime a third generation cephalosporin and cephalexin a first generation cephalosporin, both have similar *in vitro* activity against *S. aureus* and similar pharmacokinetics, including serum half-life and protein binding. However, the PBP targets of the two agents differ. Cefotaxime binds selectively to PBP 1 and 2, and cephalexin binds to PBP 1 and 3, each at its respective minimum inhibitory concentration [9,10]. We sought to demonstrate that the detrimental role of the neutrophil in the alteration of antimicrobial activity is due to its effects on PBP-2 by studying the activity of cefotaxime, which highly binds to PBP 2, and cephalexin, which only minimally binds to PBP 2, against *S. aureus in vitro* in Mueller-Hinton broth (MHB), sterile filtered abscess fluid, and sterile filtered neutrophil depleted abscess fluid. We further sought to demonstrate an *in vivo* significance of the effect on neutrophils upon the activity of antimicrobials that target PBP-2 by studying the same antimicrobials in an experimental *S. aureus* abscess. We postulated that if the diminished beta-lactam binding affinity of *S. aureus* PBP 2 by PMNs has the potential for clinical significance, that cefotaxime, which binds PBP 2, would have diminished activity compared to cephalexin *in vitro* in sterile filtered abscess fluid and in the *in vivo* experimental abscess model, but not *in vitro* in MHB or in sterile filtered PMN depleted abscess fluid.

2. Materials and Methods

2.1. *Ex-Vivo* Studies, Neutropenic and Normal Abscess Fluid Preparation

Utilizing sterile techniques, ten rats were implanted with perforated tissue cages (gas sterilized table tennis balls with 300 1.5 mm diameter holes, 1 per rat) and allowed to encapsulate intra-abdominally for 6 weeks [2]. One day before infection, six rats were administered 0.5 ml mechlorethamine HCl [Sigma-Aldrich, St. Louis, MO] 1 mg/ml in saline i.v. to deplete PMN and precursor cells. This agent—acts against bone marrow precursors of PMN giving a more complete and longer term depletion than anti-rat PMN antibody which we have used in the past. 24 hr following PMN depletion in the six rats, all ten capsules were inoculated with 1×10^7 cfu of a *Staphylococcus aureus* isolated initially isolated from a bacteremic patient, and used in previous studies [2-4]. Three mL abscess fluid was removed by syringe aspiration from each infected capsule daily until at day 7 when as much fluid as possible was harvested at necropsy. The abscess fluids were sonicated at 90 watts for 15 seconds, sterile-filtered (0.2 μM syringe filters) and stored at −70˚C until pooled as "normal" or "neutropenic" abscess fluid and used as growth medium in time-kill trials with antibiotics as described below. All animal experiments were approved by the University of Missouri-Kansas City animal care and use committee and were conducted in accordance with guidelines by the Association for Assessment and Accreditation of Laboratory Animal Care.

2.2. Time Kill of *S. aureus* in Infection Exudates and Broth

Three growth media were used: sterile-filtered neutronpenic abscess fluid, sterile-filtered normal abscess fluid or MHB. The MIC and MBC of the *Staphylococcus aureus* strain were determined by the method of Taylor, *et al*. [11] Stationary phase *Staphylococcus aureus* 1×10^7/mL and 100 μL containing the minimal bactericidal concentration (MBC) of cephalexin or cefotaxime (or no-antibiotic saline control) were added, and the tubes were incubated at 37˚C. Aliquots were removed for dilution plating on blood agar at 0, 3, 6 and 24 hr. *S. aureus* colonies growing on the plates were counted following 24 hr incubation at 37˚C and bacterial kill at each time point was determined. Each drug time kill was repeated at least 3 times.

2.3. *In Vivo* Experiments, Abscess Model

15 SD male rats, 450+ gm weight, were implanted intra-abdominally with perforated tissue cages as described above. Twenty-four hours following infection, animals were administered cefotaxime (n = 8) or cephalexin (n = 7) at 300 mg/kg/day subcutaneously. Doses were given 1/3 in a.m. and 2/3 p.m. based on the experimentally determined MIC and MBC of the drugs for the infective isolate of *S. aureus* and historic beta-lactam peak and trough data in rats. Abscesses were sampled on days 1, 3, 5, and 7 after infection immediately before the a.m. antibiotic dose. Abscess fluid was sonicated at 90 watts for 15 seconds which liberated phagocytized *S. aureus*, dilution plated and bacterial colonies were counted as described above. Aliquots of abscess fluid were individually filtered and frozen for antibiotic assay.

2.4. Bioassay of Abscess Fluid for Antibiotics

Bioassay procedures followed published methods [12]. Briefly, duplicate 20 μL samples of each abscess fluid, which had been filtered at 0.2 μM before storage at −70˚C, were evaluated for zones of inhibition on *Bacillus subtilis* pour plates after 24 hr growth. Antibiotic levels were determined by comparing abscess fluid inhibition zones to regression curves of inhibition zones prepared with drug standards run concurrently.

2.5. Neutrophil Effect on *S. aureus* PBP 2

The six lanes on Western blot of *S. aureus* PBPs represent duplicate pairs of *S. aureus* cell walls from organisms exposed or not to neutrophils. *S. aureus* cell wall protein was isolated from one culture of *S. aureus*, divided then incubated or not with neutrophils. The PBPs were tagged with unlabeled drug (10 × MIC, cefazolin in this case), followed by electrophoretic separation and detection of the PBP-bound drugs with an anti-beta lactam antibody, appropriate secondary and Western blot development.

2.6. Statistical Analysis

Data were expressed as mean ± SD and analyses were performed using Statistica (Statsoft, Tulsa OK, USA). Comparisons of two groups utilized Student's t-test. For statistical analysis in the *in vivo* experiment, end of treatment results were compared to before treatment data. Multiple comparisons used analysis of variance (ANOVA) and the Tukey post-test. Data with a p value of 0.05 or less were considered significantly different, all p values were two-tailed.

3. Results

3.1. *In Vitro* and *Ex Vivo* Experiments

We sought to determine if the presence of PMNs, which diminishes beta-lactam PBP-2 binding affinity in *S. aureus*, adversely effect the activity of cefotaxime, which binds to PBP-2, compared to cephalexin, which binds to more to PBP 1 and 3 and only minimally to PBP-2. Bacterial killing by cefotaxime and cephalexin were compared in time-kill studies utilizing *Clinical and Laboratory Standards Institute* culture in the sterile-filtered neutropenic and non-neutropenic abscess fluids. The MIC and MBC respectively for cefotaxime was 0.5 and 1.0 μg/ml, and for cephalexin was 1.0 and 2.0 μg/ml. The concentration of antimicrobial used in the time-kill studies was 1.0 μg/ml cefotaxime and 2.0 μg/ml cephalexin. The $\log_{10}$ *S. aureus* counts following growth in the two media, with or without cefotaxime or cephalexin are shown at 3, 6, and 24 hr in **Figures 1** and **2**. Cefotaxime, but not cephalexin, was more active in the neutropenic abscess fluid than in the non-neutropenic abscess fluid. Time kill experiments were also performed in MHB. Both cefotaxime and cephalexin killed *S. aureus* in MHB significantly better than no antibiotic (p = 0.001, ANOVA and post-test, data not shown), and cefotaxime was more effective than cephalexin when measured after 24-hours incubation (**Figures 1**, **2**). **Figure 3** is a Western blot demonstrating the effect of neutrophil incubation on *S. aureus* PBPs.

PBP 2 is virtually eliminated in all neutrophil-incubated *S. aureus* cell walls. This occurs regardless of the drug used to label the *S. aureus* preparation.

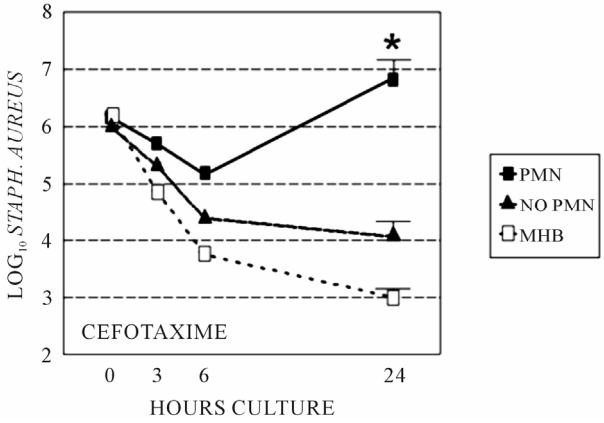

Figure 1. Time kill assays: Stationary phase *S. aureus* kill- ing by cefotaxime, grown in sterile filtered neutropenic ("no PMN") or normal ("PMN") abscess fluids. Killing by cefotaxime in Mueller Hinton broth (MHB) is shown for comparison. *At 24 h, cefotaxime showed greater killing in neutropenic abscess fluid compared to normal abscess fluid, p < 0.05.

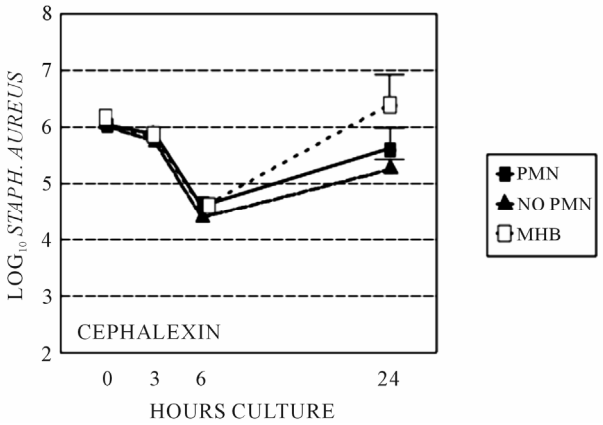

Figure 2. Time kill assays: Stationary phase *S. aureus* killing by cephalexin grown in sterile filtered neutropenic ("no PMN") or normal ("PMN") abscess fluids. Killing by cephalexin in MHB is also shown for comparison. Killing is not significantly different between growth media.

3.2. *In Vivo* Experiments

To confirm the diminished activity of the PBP-2 binding drug cefotaxime compared to the non-PBP-2 binding drug cephalexin in the presence of PMN containing abscess fluid we studied each antimicrobial agent in a tissue-cage *S. aureus* abscess model, shown in **Figure 4**.

Over the 7 day treatment time, cephalexin at 300 mg/kg/d in divided doses exhibited a 2.1 ± 1.7 $\log_{10}$ drop in bacterial cfu/ml from day 0, (p = 0.029, Student's t-test). Treatment with cefotaxime for 7 days exhibited only a 0.81 ± 2.5 $\log_{10}$ drop in bacterial cfu/ml from day 0 (p =

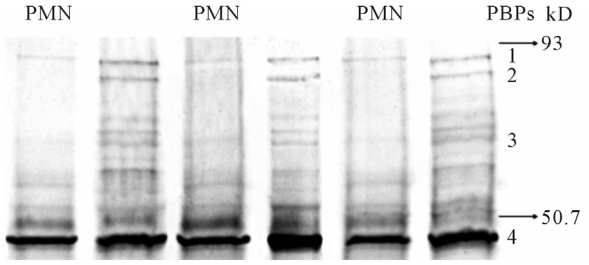

Figure 3. Western blot of the 4 main cell wall penicillin binding proteins (PBPs) of *S. aureus*, incubated or not incubated with neutrophils (PMN). The six lanes on Western blot of *S. aureus* PBPs represent duplicate pairs of *S. aureus* cell walls from organisms exposed or not to PMN. *S. aureus* cell wall protein was isolated from one culture of *S. aureus*, divided then incubated or not with neutrophils. The PBPs were incubated with unlabeled drug (10 × MIC), followed by electrophoretic separation and detection of the PBP-bound drugs with an anti-beta lactam antibody, appropriate secondary and Western blot development. PBP 2 is virtually eliminated in all neutrophil-incubated *S. aureus* cell walls, regardless of the drug used to label the *S. aureus* preparation.

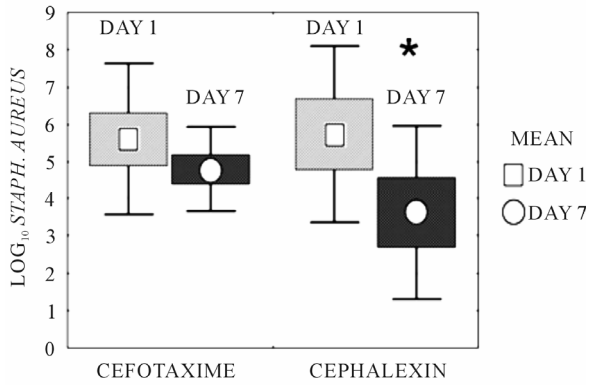

Figure 4. *S. aureus* concentration in abscesses following *in vivo* treatment with cefotaxime or cephalexin for seven days. "Days" are the number of days of systemic antimicrobial administration received by the animal from which abscess fluid was harvested. Bacterial counts in the abscess fluid were determined by dilution plating. Boxes represent ± standard error. Bars represent ± standard deviation. Mean is identified. Cefotaxime exhibited a 0.81 ± 2.5 $\log_{10}$ drop in bacterial cfu/ml over days 0 through 7 (p = NS). *Cephalexin exhibited a 2.1 ± 1.7 $\log_{10}$ drop in bacterial cfu/ml over days 0 to 7, (p = 0.029, Student's t-test).

NS), confirming the *ex vivo* experiments. Trough abscess fluid cefotaxime concentration, measured 15 hours after the afternoon dose at day 7 was 1.56 ± 0.99 μg/ml. Trough abscess fluid cephalexin concentration, measured 15 hours after the afternoon dose at day 7 was 1.45 ± 1.1 μg/ml. Both values exceeded the MIC of the test organism.

4. Discussion

The novelty of these data is through demonstration by several methods, *in vivo*, *ex vivo* and by a new detection process *in vitro*, that the activity of beta-lactams is diminished by PMNs, and that the loss of activity of beta-lactams in an abscess milieu correlates with the PMN induced alteration of PBP 2. The neutrophil, although vital for the initial control of pyogenic infections, may provide a sanctuary by which *S. aureus* survive [5]. The PMN may then induce substantial changes in the in gene regulation in *S. aureus.* Genes encoding proteins that moderate oxidative stress, virulence, and those involved in metabolism and capsule synthesis are generally upregulated, but the effects vary and some PBP genes are down-regulated after exposure for 30 minutes, and upregulated after exposure for 180 minutes [6]. In our previous experiments utilizing biotinylated ampicillin, beta-lactam binding to PBP 2 but not PBP 3 was significantly diminished after PMN exposure [4]. Utilizing a differing methodology by labeling with an anti-beta lactam monoclonal antibody [13], the reduction of beta-lactam binding to PBP-2 after PMN exposure was again demonstrated, and shown in **Figure 4**.

We sought to determine if cefotaxime, a third generation cephalosporin that primarily binds to PBP 1 and 2, has diminished activity in an abscess environment compared to the first generation cephalosporin cephalexin, which primarily binds to PBP 1 and 3, as would be predicted if PMN exposure alters the synthesis of PBP 2 or alters the ability of beta-lactams to bind to the PBP 2 target. Others have noted that strains of *S. aureus*, such as CDC-6, which has an altered PBP-2, is more resistant to cefotaxime as compared to antimicrobials that preferentially bind PBP-3 [14]. Similarly SC 12,700 is an organism with an altered PBP-3 and is more resistant to cephalexin, but is susceptible to cefotaxime [9]. In time-kill curves against stationary phase *S. aureus*, cefotaxime was highly active when tested in MHB, and was more active than cephalexin when measured at 24 h. In abscess fluid that was obtained from neutropenic animals, cefotaxime remained highly active in killing stationary phase *S. aureus* as compared to cephalexin. However in abscess fluid obtained from non-neutropenic animals, cefotaxime had diminished activity in killing *S. aureus*, while cephalexin maintained activity. In the non-neutropenic abscess animal model, cephalexin was more active than cefotaxime after seven days of treatment.

In summary, cephalexin was more active against *S. aureus* exposed to PMNs or PMN products, while cefotaxime was more active against *S. aureus* in the absence of exposure to PMNs. A plausible explanation for these findings is that beta-lactam binding to PBP 2, which is an important target for the activity of cefotaxime but not cephalexin, is altered secondary to PMN exposure, which

results in loss of activity of cefotaxime but not cephalexin. The present data suggest that the effect observed *in vivo* was secondary to the neutrophil.

Previous studies have suggested that the stress of increasing concentrations of antimicrobial may produce selection of altered PBPs with resultant loss of antim- icrobial activity [7]. We and others [15] have found that PMN exposure also effects beta-lactam binding to PBPs. The *in vivo* significance of the altered beta-lactam binding to PBP has not been previously demonstrated. PMN induced alteration of antimicrobial binding proteins with resultant diminished antimicrobial activity defines a novel mechanism of *in vivo* antimicrobial resistance. Antimicrobial resistance to beta-lactams in stationary phase *Streptococcus pyogenes* has also been postulated to be secondary to altered PBP production [16]. The mechanisms by which bacterial persist in abscesses despite the presence of antimicrobials at concentrations above the MIC likely remain multifactorial, however included in the potential mechanisms could be alteration of targets of antibacterials by the PMN.

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