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Antimicrobial/anti-biofilm activity of expired blood platelets and their released products*

Aktywność przeciwbakteryjna/przeciwbiofilmowa „przeterminowanych" płytek krwi i produktów ich degranulacji

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Introduction: Although platelets are not part of the classical immune system, they have many features that indicate their role in the anti-infective host defense. They come into interactions with microorganisms, which results in co-aggregation and co-adhesion or destruction of the microbes due to the action of antimicrobial peptides released from platelets.
The aim of this study was to evaluate the killing effect of platelets against planktonic and biofilm cultures of *Staphylococcus aureus* and to test their synergy with antibiotics.

Materials and Methods: *S. aureus* ATCC 29213; platelet rich plasma (1-3 days post shelf life). Evaluation of bactericidal activity of platelets or their lysates against planktonic cultures of *S. aureus* – CFU calculation after 4- and 24-hour co-incubation. Assessment of *S. aureus* biofilm viability under the influence of platelets – Live/Dead® BacLight™ Bacterial Viability Kit. Determination of minimum inhibitory concentrations (MICs) (oxacillin, vancomycin, linezolid) and estimation of the synergistic action of antibiotics and platelet lysates – a gradient-diffusion test strip.

Results: Microbicidal activity of "expired" platelets and their lysates has been shown as a significant reduction in the population of staphylococci in their planktonic cultures by 56-87% and a decrease in metabolic activity of biofilm formation by 7-38%. These activities were enhanced after activation with ADP. Platelet lysates showed a synergistic effect with β-lactam antibiotic (oxacillin) and glycopeptide (vancomycin) but not with oxazolidinone (linezolid).

Conclusions and Discussion: In summary, platelets even after the medical expiry date are still a good source of antimicrobial low molecular weight proteins (PMPs). Testing of bacterial resistance to PMPs may be advisable as a predictive indicator of susceptibility to treatment of infections such as infective endocarditis and other local infections of biofilm nature.

Keywords: **blood platelets • platelet microbicidal peptides • *S. aureus* • biofilm • infective endocarditis**

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Introduction

Although blood platelets (thrombocytes) are not considered as part of the classical immune system, they have many features indicating their important role in the anti-infective defense of the host. These cells express receptors for the Fc fragment of immunoglobulin, complement component C3a/C5a, various chemokines and Toll-like receptors (TLRs). They have an ability to generate reactive oxygen (ROS) and nitrogen (NOS) species, and their intracellular granules are rich in peptides with microbicidal activity [2,3,7,11,14,16,18]. The platelets undergo rapid stimulation by thrombin, ADP (adenosine diphosphate), collagen, and other mediators released into body fluids as a result of infection or exposed on the surface of the damaged tissues. Activated platelets interact mainly with the vascular endothelial cells; they stimulate the recruitment and facilitate the adhesion of neutrophils and other leukocytes to the affected tissue, acting as a specific connection between hemostasis, infection, inflammation and immunity processes [12,15,17]. They can also participate in direct contact (co-adhesion) with microorganisms, resulting in the creation of stable cell-to-cell aggregates (sequestration of bacteria) or pathogen destruction (phagocytosis/killing). Findings from several investigations aimed at elucidating this antimicrobial role of platelets revealed the presence of platelet microbicidal peptides (PMPs) and other released factors, which possess potential antimicrobial properties. It has been agreed that PMP activity may have positive importance in the pathogenesis of various types of infection and the resistance to their action is relevant to the outcome of the particular disease [2,4,5,7]. Special attention has been paid to the participation of PMPs in pathogenesis of such diseases as sepsis, infective endocarditis (IE) and other chronic localized infections. It has been proved that these types of infections are very often complicated by formation of biofilms / aggregates of bacteria or fungi. Besides IE and sepsis, examples of such pathologies are non-healing diabetic foot ulcers, cancer-related ulcers, burn-associated wounds, surgical site infections and many others [1,2,8,9,17]. It has been reported that bacteria recovered from patients with, for example, IE are consistently resistant to PMP-induced killing, whereas similar species of bacteria isolated from other types of infections are susceptible [2,8]. For many years scientific interest of our team has been focused on the analysis of drug resistance and virulence factors of *Staphylococcus aureus* – bacteria taking part in the pathogenesis of the above-mentioned types of infections (common endovascular pathogen). It is noteworthy that staphylococci are able to bind fibrinogen/fibrin, possess procoagulant activity, easily aggregate and interact with platelets. Moreover, their activity leads to both clot formation (mainly based on direct surface protein interactions and coagulase activity) and fibrinolysis (due to staphylokinase properties). These two processes could be relevant to the course of IE, making staphylococci one of its most important etiological agents [2,6,8,10,19]. A novel approach to alternative therapies of such infections, presented in this study, involves characterization of the antimicrobial role of platelets toward *S. aureus*. An additional question which we want to ask is whether we can expect synergy between platelet-derived antimicrobial products and antibiotics routinely used for therapy of infective endocarditis and other infections. Knowledge regarding the role of platelets in the development and severity of various disorders besides thrombosis continues to emerge, particularly in the field of inflammation and the immune response to infection [5,7,13,16].

The aim of this study was to evaluate the microbicidal activity of platelets and their products against *S. aureus* in suspension (planktonic) and sessile (biofilm) cultures. Expired platelet concentrates (platelet-rich plasma product in the bag) were used in this study. We asked the question if 6-8-day-old platelets themselves or their lysates prepared by freezing can still be microbicidally effective and may become a source of alternative or auxiliary products to antibiotics.

Material and Methods

The test organisms.

The reference strain of *Staphylococcus aureus* ATCC 29213 grown for 24 h at 37°C on Müller-Hinton agar (MHA) or tryptic-soy broth (TSB) supplemented with 0.25% D-(+)-glucose (TSBGlc) was used.

Platelets, platelet-derived products.

The expired platelet concentrates were obtained from the Regional Blood Center in Lodz. It was platelet-rich plasma (PRP) from 5 donors, within 1-3 days after the expiry date. Total platelet count in the bag was equal to 3×10^{11} and contained <1% of leukocytes. Cells taken from the bag were centrifuged (10 min, 2000 g), then washed twice in Tyrode buffer (Sigma, USA) and suspended in the culture medium RPMI-1640 (CytoGen, Poland) with a density of 10^8/ml. Platelets were counted by the photometric method according to Walkowiak et al. [15]. The platelet suspension was left without stimulation or was stimulated with ADP (adenosine diphosphate, Sigma, USA) at a final concentration of 20 mM, 15 min, 37°C. Thus, in the experiments platelets were used as: a) a suspension of unstimulated cells, b) a suspension of platelets after ADP stimulation, c) and d) as cell lysates prepared, respectively, from these two types of platelets (in a density equivalent to those used in point a/b), then they were frozen and thawed three times. Prepared lysates were kept frozen

(-80°C) until testing, but no longer than 1 month.

Microbicidal activity of platelet-derived products against *S. aureus* planktonic cultures

In time-kill studies platelets or their lysates were used against S. aureus ATCC 29213 strain at the inoculum of 3 × 10^7 CFU/ml in RPMI-1640. Platelets (or quantitatively equivalent lysate) were mixed with bacteria in the proportion of 3:1 in a 24-well culture microplate. At predetermined time points (0, 4, and 24 h of incubation at 37°C), 100 µl samples were serially diluted in sterile normal saline. Then, a 100 µl aliquot of each dilution was plated onto an MHA plate for CFU counting, after further incubation at 37°C for 24 h. The results were reported as the mean CFU ± S.D. of all four replicates and the percentage of growth inhibition was calculated (compared to the control).

S. aureus biofilm eradication under the influence of platelet-derived products.

The suspension of *S. aureus* ATCC 29213 prepared from fresh overnight culture in TSBGlc was added to the wells of a 96-well polystyrene plate (Nunc, Denmark) and cultured for 24 h at 37°C. Biofilms were carefully washed and undiluted platelet lysates were applied (100 µl/well). After subsequent 24-h co-incubation, the degree of biofilm survival (%) was assessed using a LIVE/DEAD® BacLight™ Bacterial Viability Kit (Molecular Probes, USA), as recommended by the manufacturer. Results are presented as the percentage of the formed biofilm, calculated from mean fluorescence values (measured at 485/535 nm for green Syto9 and at 485/620 nm for red PI) ± S.D. in comparison to control biofilm considered as 100% grow rate. The assay was done in quadruplicate to obtain the statistical average of the result.

Determination of antibiotics' MICs and assessment of their synergistic activity with platelet lysates.

The prepared inocula of *S. aureus* ATCC 29213 (density of 0.5 according to McFarland scale) were lawned evenly by a sterile cotton swab on the surface of the A) control MHA or B) MHA containing blood platelet lysate (with the ratio 1:1). Antibiotic gradient strips – MIC Test Strip (LiofilChem, Italy) containing oxacillin, vancomycin or linezolid (the range of gradient concentrations for all antibiotics was 0.002-32 mg/l) – were used. MHA plates with overlayered strips were then incubated at 37°C for 24 h and the inhibition zones were measured. Differences in MIC values obtained on the control and test plates were recorded (end-points were determined according to the manufacturer's instructions).

Results and Discussion

The aim of the present study was to provide answers to two important questions: whether blood platelets, when they are medically useless (shelf life – 5 days), possess antibacterial activity and whether they could be a source of products which can enhance antibiotic activity. Indeed, our results confirm these two assumptions by showing that the biocidal activity of such platelets, as well as their products, is significant against *S. aureus* planktonic and biofilm cultures. The source of thrombocytes was platelet rich plasma (PRP) obtained from the Regional Blood Center in Lodz. The cells were used at a density of 10^8/ml, which corresponds to the average content of platelets in 1 ml of whole blood. It was shown that platelets, even after the expired time for transfusion (total time 6-8 days of life), maintain significant microbicidal activity. Direct contact with the platelet suspension limited the number of *S. aureus*, assessed after 4-hour co-incubation as CFU, by 85-87%. Quantitatively equivalent platelet lysates were also active, since those obtained from nonactivated cells were able to limit the multiplication of the tested bacteria by 56.70 ± 9.54%, and when platelets prior to lysis were activated with ADP, the effect increased to 72.70 ± 5.45% of inhibition. After extending the time of co-incubation of platelets or their products (lysates) with staphylococci, the observed results were weaker (data not shown). It was due to the multiplication of bacteria in inoculum which were not destroyed during the first 4 hours of co-incubation. As shown in Table 1, both platelets and their lysates were more effective after activation with ADP. It can be assumed that platelet activation with thrombin, instead of ADP used by us, could have given a much better response with respect to the release of antimicrobial products. However, due to the expected strong platelet aggregation and clot formation caused by thrombin, we decided to use ADP.

Table 1. The influence of platelets and released products on *Staphylococcus aureus* ATCC 29213 planktonic culture growth and biofilm eradication.

Platelets/lysates	*S. aureus* CFU x 10^7 ± SD (inhibition of planktonic growth [%] ± SD)	Inhibition of biofilm survival ± SD [%]
Unstimulated cells	11.51 ± 1.98 (87.86 ± 2.09)	7.53 ± 9.84
Stimulated cells	13.63 ± 1.9 (85.63 ± 2.00)	32.94 ± 15.36
Lysate of unstimulated cells	41.06 ± 9.04 (56.70 ± 9.54)	28.68 ±14.75
Lysate of stimulated cells	25.89 ± 5.17 (72.70 ± 5.45)	38.24 ± 1.64
Control	94.83 ± 18.54 (0)	0

Although ADP is regarded as a weak agonist of circulating blood platelets, it is an important mediator of platelet activation induced by other stimulators (thrombin, collagen), which promote ADP release from intraplatelet storage pools, such as dense granules, where it is present in high concentrations. Additionally, ADP acts synergisti-

cally to all other platelet agonists, even faint ones, such as serotonin, adrenaline or chemokines [2,3,12,21]. In our hands, platelet activation with ADP, although not able to deliver a spectacular effect, has revealed that ADP is an equally potent agonist to the occurrence of microbicidal/anti-biofilm action. In the present study the impact of certain variables, such as pH, was not included, although we are aware that in this way we could have obtained a more reliable result. It has been reported that platelet-derived peptides exerted greater activities and border spectra under slightly acidic conditions [2,18,19]. Thus, it will be interesting to continue the research on the isolation of proteins with microbicidal activity, the assessment of their stability and the impact of physical and chemical conditions on their activity.

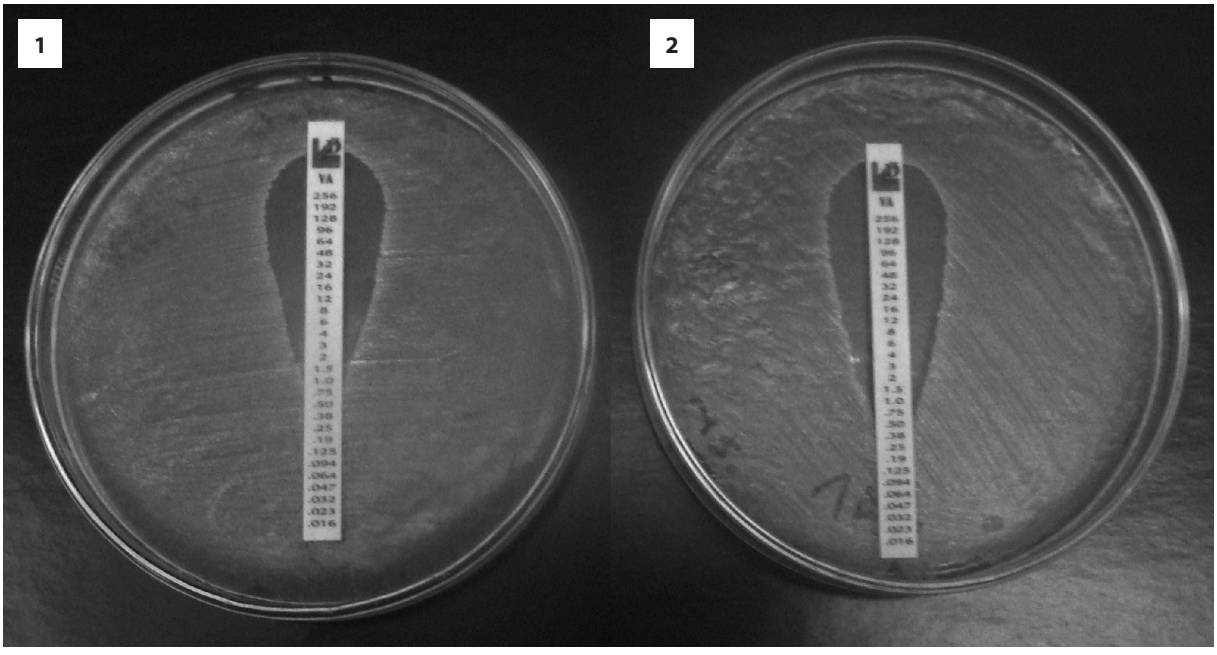

Fig. 1. Synergistic effect of vancomycin and ADP-activated platelet lysate against *S. aureus* ATCC 29213 evaluated by gradient-diffusion strip test as MIC value. A – control plate; B – medium with platelet lysate (1:1 dilution).

In the present study we made another important and unique observation concerning the activity of platelets and their products against *S. aureus* biofilm. Viability of the sessile biomass was measured by differential staining using a LIVE/DEAD® BacLight™ Bacterial Viability Kit, compared to the control biofilm (Table 1). ADP-activated platelets and their lysates applied onto already formed biofilm reduced its viability by approximately 32-38%, assessed after a further 24 h of incubation. Given the known extremely high resistance of biofilm, even partial destruction of the biofilm by platelet-derived products should be regarded as encouraging. Many *in vitro* studies and clinical observations indicate that complete eradication of biofilm-embedded bacteria by chemotherapy is hard to achieve [1,8,10]. Therefore, it is important to search for compounds that could increase the susceptibility of biofilms to conventional therapeutic agents. It is hoped that antimicrobial peptides from blood platelets could be such prospective compounds as alternative or supplementary to classical pharmacological products to combat infections. We observed that platelet lysate used even in a sub-inhibitory concentration (diluted 1:1 in agar diffusion test), enhanced *S. aureus* susceptibility to β-lactam antibiotic (oxacillin) and to glycopeptide antibiotic (vancomycin), but not to oxazolidinone (linezolid). A typical view of the test in which a synergistic effect of platelet lysate and antibiotics has been examined is shown in Fig. 1.

Both antibiotics which in our study showed synergism with platelet-derived lysates are known to have a bactericidal effect, by interfering with cell wall synthesis. It can be suggested that in the presence of oxacillin or vancomycin the uptake of platelet-derived peptides is facilitated, and in turn, due to a combined action, MICs of antibiotics were lowered. MIC of oxacillin assessed on a control plate was 0.25 mg/l but on agar containing platelet lysate it was 0.19 mg/l. Similarly, MIC of vancomycin dropped from 1.0 mg/l (control) to 0.5 mg/l (test plate). This is consistent with earlier reports concerning synergy of platelet-derived microbicidal proteins, isolated from fresh thrombocytes, with antibiotics such as nafcillin, ampicillin and vancomycin [2,8,9,10,20]. The similar activity of a product coming from outdated platelets, presented in this study, can be considered interesting. The observed effect of increased sensitivity of bacteria to antibiotics due to platelet proteins has another, more important effect *in vivo*: the possibility of reduced adhesion of bacteria to platelet aggregates. Such an initial observation was reported many years ago by Yeaman *et al.* [19,20]. In summary, the group of results presented here suggests that platelets may enhance the effects of antibiotics used against *S. aureus*. Of course, it is possible that certain antimicrobial peptides also fa-

cilitate the host defense functions of other endogenous mechanisms of innate or adaptive immunity.

In the light of our results it is clear that platelets significantly contribute to antimicrobial host defense and potentially enhance the antimicrobial mechanisms of distinct classes of conventional anti-staphylococcal agents. The activity of platelets/released antimicrobial products towards planktonic and biofilm *S. aureus* cultures observed in this study are relevant, although the achieved ranges of inhibition were not significant. However, it can be suggested that after further processing, the expired platelet concentrate will have a potential medical application as a source of prophylactic or therapeutic modern biodrugs. In view of the rising prevalence of multi-resistant staphylococci such combined therapy may be very useful in future [8, 9, 21]. Here are a few data supporting the submission of the presented opportunities for discussion: in Poland there are 21 Regional Centers for blood donation, and 2 Departmental Centers under the supervision of the National Blood Center. Taking into account the diversity of platelet-rich products prepared in each regional blood center and their short time of suitability for transfusion (platelets are discarded after five days of storage at 22°C), they seem to be a very rich and inexhaustible source of natural biocidal peptides.

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