INTRODUCTION

Polymorphonuclear leukocytes (neutrophils; PMNs) are important effector cells in the immune system that play an essential role in a host’s defence against microbial pathogens. Neutrophils use various biocidal mechanisms to kill bacteria including phagocytosis, the generation of reactive oxygen species, the production of enzymes and toxin production (proteinase, DNase, lipase/ phospholipase, Panton-Valelind leucokinidin), and reduces opsonin-dependent phagocytosis by protein A (Watkins et al., 2012).

Bacterial cells exist not only as planktonic cells but also as bacterial layers located on surfaces and associated with the extracellular matrix. Such biofilms improve bacterial survival in the external environment and during human infection (Donlan & Costerton, 2002). Formation of S. aureus biofilms may increase staphylococcal resistance to antibiotics and to attacks from specific and non-specific immune effectors (Donlan & Costerton, 2002). Biofilms provide features of virulence to staphylococci, including antiphagocytic factors (Archer et al., 2011). However despite this, human neutrophils provide an innate immune defence against staphylococcal biofilms. Human leukocytes are able to attack S. aureus biofilms and penetrate them. The ability of leukocytes to destroy S. aureus biofilms has been clearly illustrated, and leukocyte activation mechanisms identified (Leid et al., 2002). Günther et al. showed that the potency of PMNs for direct phagocytosis of bacterial biofilm depends on its maturation stage (Günther et al., 2009). The adhesion of PMNs to staphylococcal biofilms was independent of IgG- and C3-opsonization of biofilms, although the intensity of the reactive oxygen species production could increase after opsonization (Stroh et al., 2011). S. aureus biofilms, opsonized with normal human serum, were destroyed by PMNs during phagocytosis accompanied by elastase and lactoferrin release. Lactoferrin alone inhibited S. aureus biofilm formation (Meyle et al., 2010). The same authors observed the phenomenon of DNA release during the death of PMNs. This process explains the origin of insoluble DNA complexes that appear after neutrophil cell death, generating the basis for NET (Lögers et al., 2009). The death of PMNs is also accompanied by release of another substrate (membrane lipids) that is not capable of forming the true aqueous solutions. Indeed, the vesicular exocytosis was observed simultaneously with the formation of neutrophil extracellular traps (NET) (Pilszcz et al., 2010). The authors of this study considered the vesicle budding as a secondary mechanism of NET formation. However, the

Abbreviations: DLLS, dynamic laser light scattering; FAL, factor of absorption of light; HBSS, Hanks’ balanced salt solution; NET, neutrophil extracellular trap; PMN, polymorphonuclear leukocyte (neutrophil); PTA, phosphotungstic acid; c.f.u., colony forming unit.

Staphylococcus aureus, a major opportunistic pathogen, is a leading cause of biofilm-related infections in clinical practice. Staphylococcal biofilms are highly resistant to antibacterial medicines and immune effector cells. The main result of our work is the discovery of nano-vesicles in the supernatant of the human neutrophil–S. aureus biofilm system. We also found that phospholipase C treatment causes complete destruction of these vesicles. While the addition of proteinase K led to a partial structural disorganization of the vesicles, DNase treatment did not influence the vesicle structure. These observations allowed us to conclude that phospholipids and proteins play a structure-forming role in the formation of these nano-vesicles. The vesicles demonstrated anti-biofilm activities when tested against Staphylococcus epidermidis (strains 178M and 328/5) biofilms, but were ineffective for S. aureus (strains 5983/2, 5663 and 18A) biofilms.
independent evolution of PMN lipids during the contact with PMN–biofilm was not studied in this work. Thus, the objective of this study was to investigate the structural properties and the antibacterial and antibiofilm activities of nano-sized membrane vesicles, which are formed in the system of human PMNs and S. aureus biofilm.

METHODS

Bacterial strains, media and biofilm formation. S. aureus strain 5983/2 was used for the study. The strain was isolated from an infected wound surface and was identified by morphology and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) with a MALDI Biotyper according to the manufacturer’s recommendations (Bruker Daltonik). The bacterial cells were cultured in tryptophic soy broth (TSB; Becton Dickinson) overnight at 37 °C and diluted (under a spectrophotometric control of cell density of approximately 10^6 c.f.u. ml⁻¹) in TSB with 1 % glucose. For biofilm formation, staphylococcal suspension (4 ml) was inoculated into Petri dishes (35 mm, Corning 430165). Biofilms were grown as a static culture for 48 h at 37 °C.

Isolation of PMNs. Peripheral venous blood was obtained from healthy volunteers followed by the addition of 10 U ml⁻¹ heparin as an anticoagulant. PMNs were isolated by density-gradient centrifugation on Histopaque (Sigma-Aldrich), which yielded a 90–95 % pure PMN population (Brinkmann et al., 2010). The PMNs were suspended (2 x 10^6 in 1 ml) in Hanks’ balanced salt solution (HBSS) with 0.5 % BSA and used within 30 min.

PMN–biofilm interaction and obtaining vesicle-containing supernatant. S. aureus biofilms (48-hour-old) were washed gently twice in Petri dishes with HBSS. PMN suspension (4 ml) was placed on the biofilm, and the specimens incubated for 45 min at 37 °C. Then the supernatant was collected and centrifuged for 30 min at 7500 g at 4 °C. After centrifugation, 1 ml aliquots from the supernatant were taken and filtered through a sterile syringe filter (0.2 μm, polyethersulfone (PES) membrane; Corning) to remove residual bacteria and cellular debris. Formation of biofilms and their destruction during interaction with PMNs were observed at both the macroscopic and microscopic level. At the macroscopic level, biofilm formation and PMN-dependent biofilm destruction were visually controlled by fixing the sample with ice-cold 3 % (w/v) formaldehyde (pH 7.2–7.4) and stained with 1 % crystal violet. At the microscopic level, the interactions between PMNs and biofilms were investigated by laser scanning (confocal) microscopy using the laser scanning microscope 710 Meta (Carl Zeiss Microscopy). After 45 min of incubation with PMNs, the biofilms were stained by using a LIVE/DEAD Viability/Cytotoxicity kit (Invitrogen) according to the manufacturer’s recommendations. For controls, we used Petri dishes with: (1) biofilm with no PMNs; (2) live PMNs (4 ml suspension) with no biofilm; (3) biofilm with heat-inactivated PMNs (4 ml suspension) (Hatch et al., 1978).

Treatment of supernatant with enzymes. To study the chemical origin of vesicular structures, the supernatant was treated with phospholipase C, type 1 (P7633; Sigma), proteinase K (0706; Amresco) and DNase I (EN0521; Fermentas). Phospholipase C was dissolved in 0.005 M Tris/HCl buffer with 0.25 M sucrose and 1 mM CaCl₂ (pH 7.4) at a concentration of 100 units ml⁻¹. The phospholipase C solutions were added to the vesicle suspension at a ratio of 1:9 up to the volume of 1 ml, and the reaction mixtures were incubated at 37 °C for 10 min. DNase was dissolved in 10 mM Tris/HCl (pH 8.0) containing 2 mM MgCl₂ at a final concentration of 1000 μg ml⁻¹. Proteinase K was dissolved in 100 mM Tris/HCl (pH 7.5) at a final concentration of 1000 μg ml⁻¹. These enzymes were added to the vesicle suspension with a 1:9 ratio, up to the volume of 1 ml, and the reaction mixtures were incubated at 37 °C (DNase for 1 h and proteinase K for 2 h). In the control samples (enzyme-untreated vesicles), enzyme-free matching salt solutions were added to the vesicles.

Transmission electron microscopy. Samples were fixed by addition of an equal volume of ice-cold 5 % glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.4) followed by 5 min of exposure. A drop of 30 μl of the fixed sample solution was placed on a pioloform resin film (SPI Supplies) on a slot grid (SPI Supplies) for 3 min, and then stained by phosphotungstic acid (PTA). Ten microlitres of 2 % PTA (previously neutralized to pH 7.4 with 2 M NaOH) aqueous solution was dropped onto the pioloform resin surface. After 1 min, the excess staining agent was removed with filter paper, and the grid was dried at 25 °C for 10 min before electron microscopy observation. Samples were examined with a Morgagni 268D electron microscope (FEI).

Vesicle size analysis. Vesicle size was measured in suspension via an estimation, using laser light, of the intensity fluctuation rate scattered by vesicles (dynamic laser light scattering, or DLLS) with a Beckman Coulter Submicron Particle Size Analyser (model N5 Analazer; Beckman Coulter). The sample (0.5 ml of centrifuged and filtered supernatant) was mixed with an equal volume of buffered isotonic NaCl (pH 7.2), placed into a cuvette and analysed according to the manufacturer’s recommendations.

Evaluation of antibacterial activity of the vesicles. An evaluation of antibacterial activity of the vesicles was studied using planktonic bacteria from clinical isolates of S. aureus (strains 5983/2, 5663/2, 18A) and Staphylococcus epidermidis (strains 178M, 328/5). Overnight staphylococcal cultures on BBL Mueller–Hinton broth (Becton Dickinson) were washed with HBSS three times by centrifugation and diluted under a spectrophotometric control of cell density of approximately 10^6 c.f.u. ml⁻¹ in BBL Mueller–Hinton Broth. An aliquot of bacterial suspension (0.5 ml) was mixed with an equal volume of vesicles standardized by DLS; samples were incubated for 60 min at 37 °C. After pipette mixing, an aliquot (0.02 ml) was taken from each sample and placed onto trypticase soy agar in Petri dishes (90 mm). The cultures were incubated for 24 h at 37 °C and the number of c.f.u. was then evaluated. In the control samples, the supernatant purified from vesicles by centrifugation (100 000 g, for 60 min at 4 °C) was used.

Evaluation of antibiofilm activity of the vesicles. An evaluation of vesicle antibiofilm activity was studied using 48-hour-old biofilms formed by the clinical isolates of S. aureus (strains 5983/2, 5663/2, 18A) and S. epidermidis (strains 178M, 328/5). Biofilms were cultured previously in Petri dishes containing BBL Mueller–Hinton Broth (Becton Dickinson) with 1 % glucose, as described above. An aliquot of vesicle-containing suspension (1.5 ml) was placed onto the biofilm surface, incubated for 60 min at 37 °C, washed twice with HBSS, dried, fixed with 95 % ethanol, stained with 1 % crystal violet, and rinsed with distilled water. The dye was eluted by using 95 % ethanol (4 ml) and the ethanol coloration was measured by spectrophotometry (590 nm). The factor of absorption of light (FAL) in the experimental samples was expressed as a percentage of FAL in the control samples. The supernatant purified from vesicles by centrifugation (100 000 g, for 60 min at 4 °C) was used in the control samples.
Statistical Analysis. Each data point was examined in triplicate in four independent experiments. Data were analysed using Statistica v 7.1 (StatSoft) and the software of the equipment used (submicron particle size analyser model N5, Beckman Coulter; and 268D electron microscope, Morgagni).

RESULTS

Confirmation of PMN–biofilm interaction

After 45 min of incubation with PMNs, the biofilms were completely destroyed. After contact with PMNs, the biofilms had less intensive staining by crystal violet compared to the biofilms in control samples without PMNs (Fig. 1a). Confocal microscopy confirmed that PMNs were attached to the biofilm and penetrated into it. More than 90% (91.3 ± 4.1%) of PMNs fixed to the biofilm which remained had signs of damage. The intensive red fluorescence of PMNs stained by ethidium homodimer-1 (component ‘B’ of LIVE/DEAD Viability/ Cytotoxicity kit) and the absence of green fluorescence with calcein AM (component ‘A’ of LIVE/DEAD Viability/ Cytotoxicity kit) indicated PMN cell membrane damage (Fig. 1c). PMNs without biofilms (control) were intact and viable. These cells were stained by calcein AM but not by ethidium homodimer-1 (Fig. 1b).

Vesicles in the supernatant from PMN–biofilm system

The centrifuged and filtered supernatant (after 45 min of incubation with PMNs) had regular structures that were well contrasted by PTA. These structures were of roundish shape and resembled vesicles (Fig. 2). The vesicles varied in size within the 20–60 nm range (33.4 ± 6.8 nm) and had sharp contours. Few vesicles were attached to each other. In the control samples (biofilm with live or heat-inactivated PMNs and suspension of live PMNs without biofilms), the vesicular structures were not detected.

An analysis of supernatant by DLLS showed similar results (Fig. 3). The particle size, as estimated by DLLS, varied from 15.7 to 121.4 nm (the average was 41.7 ± 14.6 nm). Nano-sized particles were not present in control samples (data not shown).

Changes of vesicles after enzyme treatment

The treatment of samples with DNase did not change the vesicles (Fig. 4a). The samples exposed to proteinase K had formations (of 15.0–34.1 nm in diameter) which were visible on electron microscopy images (Fig. 4b). These structures could not be characterized as vesicles but had some of the features of ordered structures; they had a roundish shape and were not in contact with each other, and their contours were not sharp. Pretreatment of samples with phospholipase C induced the complete disappearance of the vesicles (Fig. 4c). The control samples (incubation of vesicles without enzymes) contained vesicles without changes in shape or size (Fig. 4d).
Antibacterial activity of the vesicles
The results of the antibacterial activity of the vesicles for staphylococci (S. aureus strains 5983/2, 5663 and 18A; S. epidermidis strains 178M and 328/5) are summarized in Table 1. The viability of bacteria did not change after treatment with vesicles, and the number of c.f.u. was about 100 per Petri dish in experiments and in control tests (P>0.05).

Antibiofilm activity of the vesicles against S. epidermidis biofilms
The results of the study of vesicle activity against staphylococcal biofilms (S. aureus strains 5983/2, 5663 and 18A; S. epidermidis strains 178M and 328/5) are summarized in Table 2. Co-incubation of vesicles with S. aureus biofilms (60 min) did not reduce biofilm staining (in all cases P>0.05). On the contrary, the dye colour of biofilms formed by S. epidermidis was less intensive after treatment with vesicles. The FAL for S. epidermidis strain 178M was reduced by 16 % and for S. epidermidis strain 328/5, by 57 %. These data indicate a significant destruction of S. epidermidis biofilms when using the vesicle treatment.

Discussion
In the present study, we observed the PMN-dependent destruction of S. aureus biofilms at the macroscopic and microscopic levels. PMNs died during interaction with the staphylococcal biofilms. These phenomena were not unexpected. The death of PMNs in contact with staphylococcal biofilms and the PMN-dependent destruction of staphylococcal biofilms were described previously (Günther et al., 2009; Meyle et al., 2010). In fact, our novel finding is the release of nano-sized vesicles into the supernatant of the PMN–S. aureus biofilm system. The presence of vesicle structures is common for many biological systems. They are known as membrane microparticles, microvesicles, large dense core vesicles, exosomes, ectosomes, synaptic vesicles, etc. (Hugel et al., 2005; Sadallah et al., 2011). In our experiments, nano-sized vesicles of 20–60 nm in diameter appeared in the system of PMN and S. aureus biofilm. These vesicles were not observed in control samples, including the supernatant of staphylococcal biofilms with live or heat-inactivated PMNs, and the supernatant from PMN suspensions without biofilms. Therefore, the vesicles appeared only in systems where active interaction between PMNs and biofilm staphylococci took place.

Apparently, the important question is the origin of these vesicles. Theoretically, there are three ways of vesicle formation: firstly, nano-vesicles may originate from PMN membranes; secondly, their origin might be connected with staphylococcal membranes; and thirdly, these vesicles may result from a complex of PMNs and staphylococcal derivatives. Human PMNs are responsible for the generation of membrane nano-sized products of 50–300 nm in diameter, which are known as secretory vesicles and ectosomes (Borregaard et al., 1995; Hess et al., 1999; Gasser et al., 2003). The vesicles from S. aureus range in diameter from 20 to 130 nm and consist of 90–143 proteins, which have been examined with a proteomics approach (Lee et al., 2009; Gurung et al., 2011).

We support the third version of the nano-vesicle origin. We believe that these vesicles are produced in conjunction with PMNs and S. aureus. Vesicle formation may result from intimate contact between PMNs and staphylococci. The phagocyte–staphylococcus interaction observed in our experiments with confocal microscopy could be an example of such intimate contact. The fast (45 min) loss of PMN viability was confirmed by staining with vital dye, and could result in enzyme release. Rapid destruction of the membrane derivatives by PMNs and staphylococcal enzymes might create conditions for self-assembly of phospholipid–protein complexes into vesicular structures.

The results of our experiments with enzymes allowed us to identify the biochemical components which are important
for the vesicle structure. The complete destruction of vesicles by phospholipase C demonstrates that phospholipids represent a key component of the nano-vesicular structure. In theory, the vesicles might be formed not only from phospholipids but also from proteins and nucleic acids. Our experiments with proteinase K clearly show the disorganization of vesicles as a result of such treatment. It points towards the presence of proteins in the vesicle composition.

Table 1. The viability of planktonic staphylococci under the influence of vesicles from the PMN–S. aureus biofilm system

<table>
<thead>
<tr>
<th>Strain of Staphylococcus</th>
<th>Number of c.f.u. (per Petri dish)</th>
<th>Significance of differences</th>
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<tr>
<td></td>
<td>Staphylococci treated with vesicles</td>
<td>Staphylococci not treated with vesicles (control)</td>
</tr>
<tr>
<td>S. aureus 5983/2</td>
<td>105.5 ± 7.5</td>
<td>96.8 ± 5.0</td>
</tr>
<tr>
<td>S. aureus 5663</td>
<td>105.5 ± 5.4</td>
<td>100.5 ± 4.7</td>
</tr>
<tr>
<td>S. aureus 18A</td>
<td>111.0 ± 9.9</td>
<td>107.0 ± 5.9</td>
</tr>
<tr>
<td>S. epidermidis 178M</td>
<td>94.3 ± 3.9</td>
<td>103.8 ± 8.4</td>
</tr>
<tr>
<td>S. epidermidis 328/5</td>
<td>99.5 ± 7.4</td>
<td>109.6 ± 5.9</td>
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Fig. 4. Electron micrograph of vesicles after enzyme treatment. (a) Vesicle structures after DNase treatment. (b) Transformation of vesicles after proteinase K treatment; nanoparticles in the micrograph are not vesicles but preserve some features of organization. (c) Complete disappearance of the vesicle structures after phospholipase C treatment. (d) Vesicles in the control samples (without enzyme treatment). Transmission electronic microscopy ×140 000. Bar, 0.2 µm. Staining with phosphotungstic acid. Diameter (nm) of vesicles (a, d) and nanoparticles (b) is shown by black arrows.
and highlights the contribution of these proteins to the maintenance of the vesicle structure. Besides, it is already known that vesicles of PMN origin and vesicles of staphylococcal origin are composed not only of phospholipids but also of a considerable amount of protein (Gasser et al., 2003; Lee et al., 2009; Gurung et al., 2011). Thus, our results do not contradict the well-known published data but rather support them. DNase treatment did not have any effect on the nano-vesicles suggesting that DNA is not the structure-forming component of the vesicle architecture.

Other questions concern the biological role of the nano-vesicles. The phenomenon of vesicles budding from PMNs reacting with S. aureus is already known and reported in the literature (Pilszcz et al., 2010). The authors considered the vesicles filled with nuclear DNA and suggested that the vesicles were, in fact, the products of nuclear membrane deterioration. However, the authors analyzed the process of vesicle formation in the context of NET formation, and not as an independent phenomenon of its own significance. We believe that the vesicle formation has significance which can be justified in many ways. The vesicles showed antibiofilm activity against S. epidermidis biofilms; breakdown of these was not absolute but still significant. This indicates that the vesicles are biologically active and can play an independent role in host–bacteria interactions. The antibiofilm effect of vesicles can possibly be explained by the presence of functionally active enzymes of PMN and/or staphylococcal origin trapped in their structure. Many substances (including biologically active substances) are packaged in the PMN and S. aureus vesicles, rendering them as potential antimicrobial and pro-inflammatory agents (Hess et al., 1999; Gasser et al., 2003). Such components might be harmful for the obligatory biofilm attributes, that is, bacterial cells and extracellular matrix. Resistance of S. aureus biofilm to the vesicles can be attributed to the special properties of a biofilm matrix (Otto, 2008; Jabbouri & Sadovskaya, 2010) and to S. aureus phospholipase activity, which can directly destroy the vesicular structures.

Finally, we think it is too early for strong conclusions regarding the clinical relevance of nano-vesicles due to their antibiofilm effect. Nevertheless, our understanding of mechanisms of vesicle-mediated breakdown of bacterial biofilms can be crucial for the development of antibiofilm pharmacological agents in the future.

In conclusion, the vesicles of the PMN–S. aureus biofilm system are structures with enigmatic roles in the evolution of biofilm-based infections. A detailed study of their biological activity and composition could be an important step in future research.

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