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Decreased neutrophil bactericidal activity during phagocytosis of a slime-producing *Staphylococcus aureus* strain

Belén BARRIO, Frédéric VANGROENWEGHE, Hilde DOSOGNE,
Christian BURVENICH*

Ghent University, Faculty of Veterinary Medicine, Department of Physiology,
Biochemistry and Biometrics, Salisburylaan 133, 9820 Merelbeke, Belgium

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Abstract – Phagocytosis and intracellular killing by bovine polymorphonuclear leukocytes (PMN) are important host defence mechanisms against mastitis caused by *Staphylococcus aureus*. We compared the phagocytosis and overall killing of a non slime-producing (NSP) *S. aureus* and its slime-producing (SP) variant by blood PMN, using an in vitro bacteriological assay. Seven clinically healthy Holstein-Friesian dairy cows in mid-lactation stage were used for this purpose. The percentages of overall killing for the NSP and SP variant were $34 \pm 3\%$ and $21 \pm 4\%$ ($P < 0.05$) and the corresponding percentages of phagocytosis were $40 \pm 4\%$ and $31 \pm 4\%$, respectively. A significant positive correlation ($r = 0.79$; $P < 0.001$) was found between phagocytosis and overall killing. These results suggest that the presence of slime was responsible for a decreased phagocytic ingestion and overall killing.

bovine blood neutrophil / *Staphylococcus aureus* / slime / bacteriological assay

Résumé – Diminution de l'activité bactéricide des neutrophiles d'une souche de *Staphylococcus aureus* productrice de « slime » au cours de la phagocytose. La phagocytose et la destruction intracellulaire sont deux importants mécanismes de défense des leucocytes polymorphonucléaires (PMN) contre la mammites provoquée par *Staphylococcus aureus* chez les bovins. Nous avons comparé la destruction intra- et extracellulaire par les PMN du sang pour un *S. aureus* non-producteur de « slime » (NSP) et son variant producteur de « slime » (SP), en utilisant une analyse bactériologique in vitro. Sept vaches saines en moyenne lactation ont été utilisées à cette fin. Les pourcentages de destruction totale pour le NSP et SP étaient de $34 \pm 3\%$ et $21 \pm 4\%$ ($P < 0.05$) et les pourcentages de phagocytose étaient de $40 \pm 4\%$ et $31 \pm 4\%$, respectivement. Une corrélation significativement positive ($r = 0.79$; $P < 0.001$) entre la phagocytose et la destruction totale était observé. Ces résultats suggèrent que la présence de « slime » pourrait être responsable d'une ingestion phagocytaire et d'une destruction totale diminuées.

leucocyte bovin sanguin / *Staphylococcus aureus* / slime / analyse bactériologique

* Correspondence and reprints
Tel.: (32) 9 2647321; fax: (32) 9 2647499; e-mail: christian.burvenich@rug.ac.be

1. INTRODUCTION

Mastitis is a serious problem worldwide in the dairy industry. *Staphylococcus aureus* is one of the most virulent organisms involved in bovine mastitis [15]. To establish intramammary infection, *S. aureus* has to overcome several host defence mechanisms (phagocytosis, recruitment of polymorphonuclear neutrophils (PMN), etc.). Polymorphonuclear neutrophils play a crucial role in the elimination of invading bacteria [6, 10] and their phagocytic and bactericidal functions are important host defence mechanisms. Bacteria possess factors that protect them from host reactions. At least two factors interfere with phagocytosis by immune cells and contribute to the virulence of the bacterium. One is the production of an extracellular polysaccharide layer (exopolysaccharide or slime) by *S. aureus*. This is an important aspect of virulence [4, 9, 22] as strains of *S. aureus* with exopolysaccharide production are hardly phagocytosed in vitro [13, 14, 20, 27]. Second, capsule polysaccharides enhance virulence of the organism by impairing complement- and antibody-mediated opsonisation [13, 27, 28]. It has been postulated that the slime layer has certain functional similarities with the constitutive capsule, both masking the cell wall antigens [7, 29]. Furthermore, *S. aureus* strains isolated from clinical cases of bovine mastitis, have been shown to produce slime under in vivo conditions rather than true capsules [24]. These studies might suggest the implication of slime as a virulence factor during bovine *S. aureus* mastitis. To our best knowledge, however, the influence of slime production on overall killing has never been assessed. In the present study, phagocytosis and overall killing of the slime-producing and non slime-producing *S. aureus* variant by isolated bovine PMN are evaluated.

2. MATERIALS AND METHODS

2.1. Experimental animals and blood sampling

Seven clinically healthy dairy cows of the Holstein-Friesian breed in mid-lactation (159 ± 1 days post-partum) from the Ghent University dairy herd (Biocentrum Agri-Vet, Melle, Belgium) were selected. Freshly drawn peripheral blood (80 mL) was aseptically collected from the external jugular vein of each cow by venipuncture in evacuated tubes (Laboratoire EGA, Nogent-le-Roi, France) containing 125 I.U. heparin as anticoagulant.

2.2. Isolation of polymorphonuclear leukocytes

Polymorphonuclear leukocytes were isolated according to the method of Mottola et al. [17]. The cell pellet was washed with Ca^{2+} and Mg^{2+} -free Hank's balanced salt solution (HBSS; GIBCO Life Technologies, Paisley, Scotland) and the final cell pellet was resuspended in 0.5 mL HBSS containing Ca^{2+} and Mg^{2+} supplemented with 0.1% bovine serum albumin (BSA; Sigma Chemicals, St. Louis, MO, USA) and 25 mM HEPES buffer (Sigma). After isolation, the cells were counted (Coulter Counter Z2, Coulter Electronics Ltd., Luton, UK). Viability of PMN was determined after isolation by trypan blue dye exclusion. Differential cell counts were performed on smears stained with Haemacolor[®] (Merck diagnostica, Darmstadt, Germany). On average, 93% of the isolated cells were PMN with viabilities of 98%. After cell counting and differentiation, cell suspensions were adjusted to a final concentration of 5×10^6 viable PMN per mL for the bacteriological assay.

2.3. Preparation of bacteria

Non slime-producing (NSP) *S. aureus* Newbould 305 [18] and its slime-producing (SP) variant [7] were grown overnight (37 °C) in brain heart infusion (BHI; Difco Laboratories, Detroit, MI, USA) and tryptic soy broth (TSB; Difco), respectively. Slime production by staphylococci has been shown to be enhanced in vitro by static culture in TSB [1, 8]. Bacteria were washed twice with Ca²⁺ and Mg²⁺-free-HBSS (1 000 × g, 10 min) and finally resuspended at 7 × 10⁷ colony-forming units per mL (cfu·mL⁻¹). Bacterial concentrations were quantified using measurement of absorbance at 450 nm (0.32 corresponds to 1 × 10⁸ cfu·mL⁻¹) with a Titertek Multiskan PLUS spectrophotometer (Labsystems, Helsinki, Finland). For opsonisation, bacteria were incubated (20 min, 37 °C) with 10% pooled bovine complement-inactivated serum (30 min, 56 °C). Bacteria were washed and resuspended in HBSS containing Ca²⁺ and Mg²⁺ supplemented with 0.1% BSA and 25 mM HEPES at a concentration of 5 × 10⁷ cfu·mL⁻¹. Slime production was estimated from the morphology of the colonies [11] on Congo red agar (CRA) plates. Rough appearance was characteristic of the SP variant (Fig. 1a) whereas the NSP variant colonies were typically smooth in their appearance (Fig. 1b).

2.4. Bacteriological assay

A bacteriological test was performed according to Barta [2] with some modifications. The assay was run in duplicate in Eppendorf® tubes (Netheler-Hinz GmbH, Hamburg, Germany) in a final volume of 1 mL with the following composition: 100 µL preopsonised NSP or its SP *S. aureus* variant (5 × 10⁷ cfu·mL⁻¹), 500 µL viable PMN (5 × 10⁶ cells·mL⁻¹) and 400 µL pooled bovine serum diluted to a final concentration (v/v) of 5% (complement-inactivated serum, 56 °C, 30 min).

Control samples (C0, C60; expressed as cfu·mL⁻¹) contained bacteria, HBSS and serum without PMN. The bacteria to neutrophil ratio was 2:1. The tubes were rotated end-over-end at 37 °C for 60 min. Twenty-five microliter samples were taken from the mixture assay (Ma0, Ma60; expressed as cfu·mL⁻¹) at 0 and 60 min, diluted into distilled water and kept at 0 °C for 3 h in order to disrupt the PMN. Extracellular (Ec; expressed as cfu·mL⁻¹) bacteria were separated from the neutrophils by centrifugation (100 × g, 1 min, 4 °C). A 25 µL sample of the supernatant from Ma60 was taken and diluted as for the mixture assay samples. Ten-fold dilutions of C0, C60, Ec, Ma0 and Ma60 were performed and the 2 last dilutions were spread onto Columbia sheep blood agar (Biokar Diagnostics, Beauvois, France). After overnight incubation at 37 °C, colony counts were related to the original bacterial suspension. The results from the bactericidal assay were expressed as the percentages of killed (% killing) and phagocytosed (% phagocytosis) bacteria. The percentages were calculated using the following formulas:

$$\text{Bacterial growth } G = \frac{C60 (\text{cfu} \cdot \text{mL}^{-1})}{C0 (\text{cfu} \cdot \text{mL}^{-1})}$$

$$\% \text{ killing} = 100 - \left\{ \left[\frac{\text{Ma60} (\text{cfu} \cdot \text{mL}^{-1}) / G}{\text{Ma0} (\text{cfu} \cdot \text{mL}^{-1})} \right] \times 100 \right\}$$

% phagocytosis =

$$\left\{ \left[\text{Ma0} (\text{cfu} \cdot \text{mL}^{-1}) - \left(\frac{\text{EC} (\text{cfu} \cdot \text{mL}^{-1})}{G} \right) \right] / \text{Ma0} (\text{cfu} \cdot \text{mL}^{-1}) \right\} \times 100$$

with C0: control sample at 0 min, C60: control sample at 60 min, Ma0: mixture assay sample at 0 min, Ma60: mixture assay sample at 60 min, Ec: mixture assay sample supernatant after centrifugation (extracellular).

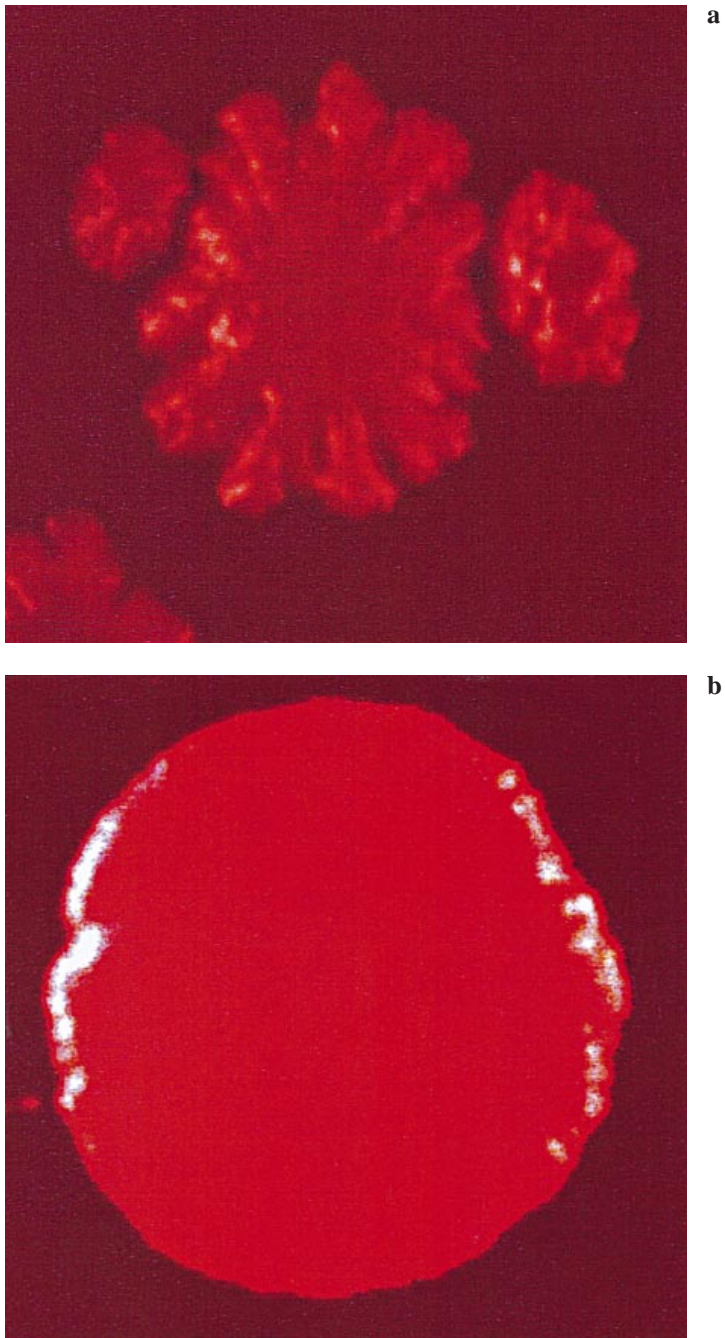


Figure 1. Slime-producing *S. aureus* colony (a) and non slime-producing *S. aureus* colony (b) on Congo Red agar*.

* This figure is available in colour at www.edpsciences.org

Table I. Killing and phagocytosis of non slime-producing (NSP) and slime-producing (SP) variants of *Staphylococcus aureus* Newbould 305 by bovine blood polymorphonuclear leukocytes. The results are presented as mean percentage \pm standard error of the mean obtained from bacteriological assay with PMN of 7 cows. The asterisk indicates the significant difference of the mean ($P < 0.05$).

	NSP <i>S. aureus</i>	SP <i>S. aureus</i>
% Phagocytosis	40 \pm 4	31 \pm 4
% Overall killing*	34 \pm 3	21 \pm 4

2.5. Statistical analysis

Data were analysed with Statistix® (Analytical Software, Tallahassee, FL, USA) and are expressed as means \pm standard error of the mean. The significance ($P < 0.05$) of the difference of means was tested by the Stu-

dent *t*-test and the correlation coefficient, *r*, was calculated.

3. RESULTS

The percentage of overall killing was significantly lower ($P < 0.05$) in the SP than in the NSP *S. aureus* variant (21 \pm 4% and 34 \pm 3%, respectively). The percentage of phagocytosis was 40 \pm 4% and 31 \pm 4% for the NSP and SP *S. aureus* variants, respectively (not significantly different) (Tab. I). A significant positive correlation ($r = 0.79$; $P < 0.001$) was observed between phagocytosis and overall killing (Fig. 2).

4. DISCUSSION

In the present study, an uptake of 40% of NSP *S. aureus* by PMN was detected.

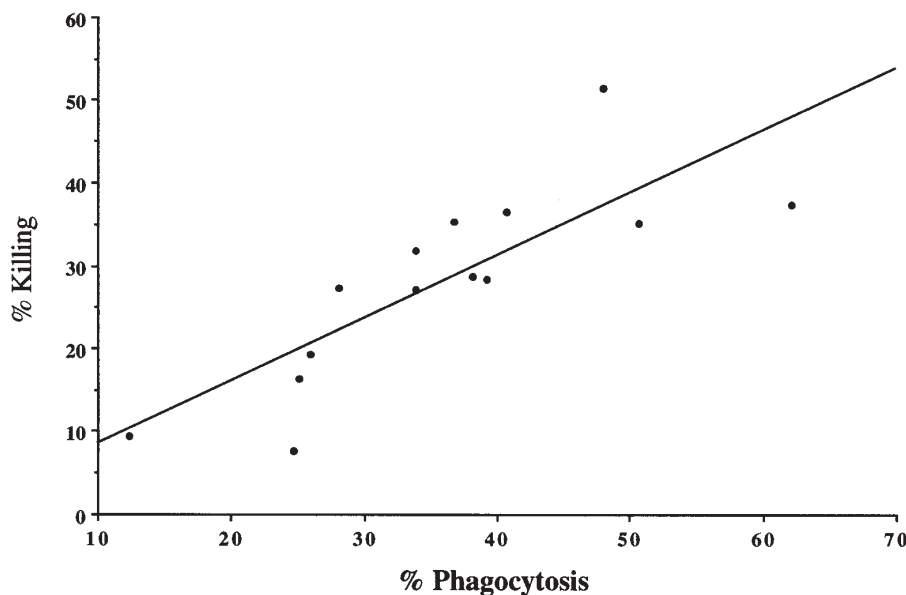


Figure 2. The correlation between phagocytosis and overall killing of NSP and SP *Staphylococcus aureus* strain Newbould 305 by bovine polymorphonuclear leukocytes ($r = 0.79$, $P < 0.001$). Data are from 7 independent experiments performed with PMN of different cows. Each experiment included the SP strain and its NSP variant.

This was lower than data observed in other studies, where zymosan-activated serum was used [26]. The difference of these results clearly indicates the importance of the complement for the phagocytosis of *S. aureus*. This is in agreement with data from the literature [5, 25]. As normal bovine milk is known to exert an inhibitory effect on the hemolytic and bactericidal activity of complement [21, 23], only heat-stable serum factors were used as opsonins in this study.

As expected, phagocytosis of SP *S. aureus* was lower than the NSP variant. This was in agreement with data from the literature [13, 14, 20, 27]. A reduction in the killing of coagulase-negative *Staphylococci* by human PMN was similarly found in the presence of slime [16, 19]. Under our experimental conditions, the decrease in phagocytosis amounted to 38%. Overall killing was decreased to a similar extent and amounted to 22%. From these results, it can be tentatively concluded that the decrease in overall killing was directly related to the decrease in phagocytosis, and that the mechanism of the SP variant of *S. aureus* to evade phagocytosis by PMN was probably attributed to the presence of slime. Indeed, a significant positive correlation was found between overall killing and phagocytosis.

Several mechanisms can be postulated to explain the decrease in overall killing of SP *S. aureus*. In a study performed by Johnson et al. [12], an alteration of superoxide generation, degranulation and phagocytosis was observed in the presence of slime. Efficient destruction of *S. aureus* by bovine PMN appears to depend on oxidative burst and release of bactericidal granule contents [10]. In a study performed by Johnson et al. [12], it was observed that lactoferrin binding to *S. aureus* was decreased by the presence of slime.

Our findings contribute to the understanding of the importance of the virulence mechanisms of SP *S. aureus* in bovine mastitis. Indeed, in vivo studies have revealed that SP variants can reverse to the NSP state

within the mammary gland after experimental infection [3]. The SP *S. aureus* strain had a higher colonisation capacity, which could imply that slime would be an important colonisation factor.

In conclusion, our study confirms the impaired phagocytosis of SP *S. aureus* but also suggests an inhibitory influence of slime layer on the killing of this bacteria by bovine neutrophils. This could be one aspect in the persistence of *S. aureus* intramammary infection during subclinical mastitis.

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