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Ability of *Staphylococcus aureus* Coagulase Genotypes To Resist Neutrophil Bactericidal Activity and Phagocytosis

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This study investigated the functional capabilities of neutrophils against different *Staphylococcus aureus* genotypes isolated from cows with mastitis. Six strains of *S. aureus* were chosen for use in the study, two with a common genotype, two with an intermediate genotype, and two with a rare genotype. The interaction between bacteria and neutrophils was measured by phagocytosis and bactericidal effect. The average percent killing of bacteria was lowest (40.0%) with strains belonging to the most common genotype, medium (50%) with strains belonging to the intermediate type, and highest (64.2%) with strains belonging to the rare type ($P \leq 0.001$). Statistically significant differences ($P \leq 0.001$) in the numbers of phagocytized bacteria were also found between the most prevalent type (6.27 bacteria per cell) and the other two types (intermediate type, 9.26/cell; rare type, 10.5/cell). These findings suggest that one of the reasons for the variation in prevalence of different genotypes of *S. aureus* in the mammary gland is due to the superior ability of some types to resist phagocytosis and/or killing by bovine neutrophils.

Mastitis is an inflammation of the mammary gland which is usually caused by bacterial infection. Mastitis is the most economically important disease to the dairy industry (3). The disease also has human health implications, since it occurs in approximately one-third of all lactating women (23). *Staphylococcus aureus* is recognized as one of the most common mastitis-causing pathogens worldwide and is especially well-known for its tendency to cause chronic infections (28). Like most bacterial species, *S. aureus* can be divided into many subtypes, the identification of which is especially important in order to confirm the epidemiological relationship between isolates. Many subtypes of *S. aureus* have been isolated from cows with mastitis, but the general results of all investigations have been that one to a few subtypes predominate (5, 14, 16). These results suggest that these prevalent types have some characteristics which enable them to evade important host defense mechanisms and establish intramammary infections more successfully than less prevalent types.

The defense of the mammary gland is mediated by several humoral and cellular factors. Studies have shown that the neutrophils are the principal line of defense in the mammary gland once bacteria penetrate the teat end opening (21). The abilities of neutrophils to phagocytize and kill invading bacteria is critically related to the establishment of new intramammary infection. If the neutrophils fail to eliminate the bacteria, an infection will manifest and may eventually become chronic. Therefore, any factor that compromises neutrophil function may have severe consequences regarding susceptibility to mastitis.

Various pathogenic mechanisms may mediate the suppression of neutrophil populations. Previous researchers have shown that *S. aureus* is capable of surviving intracellularly, which may contribute to the chronic nature of *S. aureus* mastitis (19, 20). Furthermore, *S. aureus* produces several substances that have been associated with virulence (2), including the enzyme coagulase (15). Several studies have shown that coagulase-deficient mutants of *S. aureus* lose their virulence (12, 17), but other studies failed to find such an association (13, 22, 27). A direct suppression of neutrophil function by coagulase has been postulated (10), but this effect was not reproducible in a later experiment (4). A possible explanation for these conflicting reports may the experimental use of different subtypes of *S. aureus*, which may vary in the type of coagulase they produce. To date, no studies have compared the different types of coagulase in relation to the virulence of parent organisms.

The purpose of this investigation was to compare the phagocytic and bactericidal activities of bovine neutrophils against distinct *S. aureus* coagulase genotypes found at different prevalences. Variation in neutrophil efficiency against common, intermediate, and rarely found *S. aureus* genotypes is described. The implications of these variations and their relevance to virulence of the bacteria are discussed.

Bacterial strains. Prior to this study, a collection of 187 *S. aureus* strains isolated from cows with mastitis in 187 different herds were screened for their coagulase gene types and restriction fragment length polymorphisms of the gene encoding rRNA (ribotyping) (1). From this collection, six strains of *S. aureus* isolated from cows with subclinical cases of mastitis were chosen for use in the present study. The six strains belonged to coagulase gene types that among the investigated population were found in frequencies of 35% ($n = 2$), 8% ($n = 2$), and 1% ($n = 2$) and to ribotypes that were found in frequencies of 30% ($n = 2$), 7% ($n = 2$), and 1% ($n = 2$), respectively (Table 1). All strains were tested for production of enterotoxins A to D by reverse passive latex agglutination with a commercially available kit (Oxoid, Wesel, Germany). Production of hemolysins was detected on bovine blood agar as described by Skalka et al. (25). Production of staphylokinase was performed on fibrin plates with canine plasma as described by Devriese and Van de Kerckhove (7). Coagulation of bovine plasma was performed by the method of Devriese (6). Detection of the gene for toxic shock syndrome toxin was performed by a PCR technique as described by Johnson et al. (11). No capsules could be observed in India ink after growth in RPMI

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TABLE 1. Prevalences of the different coagulase gene types and ribotypes in *S. aureus* strains by three techniques

Technique(s)	Prevalence (%) of type in strains:		
	A1 and A4	A44 and L9	L42 and 47
Coagulase gene typing	35	8	1
Ribotyping	30	7	1
Coagulase gene typing and ribotyping	28	5	1

1640 medium (Sigma, St. Louis, Mo.) supplemented with 1% L-glutamine and 5% fetal bovine serum (RPMI+).

A bacterial growth curve was made for one strain (A1) to determine the incubation period needed to reach a logarithmic growth phase. All bacterial strains were subsequently grown overnight in RPMI+. Bacterial cells were washed and resuspended in RPMI+, and 10-fold dilutions made in order to measure the bacterial concentrations on blood agar plates. Bacteria were adjusted to a concentration of 10^8 CFU per ml in RPMI+. In order to determine whether there were differences in growth among the six bacterial strains under assay conditions, an initial bacterial concentration of 0.5×10^8 CFU/ml was incubated in RPMI+ for 1 h at 37°C. At the end of this time point, another set of 10-fold dilutions was made to quantify the bacteria and detect any changes in the initial concentration.

Isolation of neutrophils. Eight lactating holstein-friesian dairy cows from the Center for Mastitis Research Herd were used in this study. All experimental animals were free of intramammary infection on the basis of repeated microbiological analyses of quarter foremilk samples. Venous blood samples were collected with 10% (vol/vol) 2× citrate anticoagulant. Blood was centrifuged at 500 × *g* for 35 min, and the plasma and buffy coat were discarded. Neutrophils were isolated from the remaining erythrocytes by adding an equal volume of double-distilled water for 18 s and then by adding a 2× concentration of minimum essential medium (Sigma) to regain the isotonicity of the solution. The remaining cells were washed twice with Hanks balanced salt solution (devoid of calcium and magnesium) and resuspended in RPMI+ to a final concentration of 10^7 cells per ml. The cell isolation procedure yielded eight separate neutrophil populations of more than 90% purity, and viability was more than 99%, as determined by trypan blue exclusion.

Bactericidal assay and phagocytic ratios. Bovine anti-*S. aureus* sera was obtained from three lactating holstein-friesian dairy cows diagnosed with *S. aureus* mastitis. The optimum concentration of pooled bovine anti-*S. aureus* sera required to opsonize the different strains of *S. aureus* was determined by making twofold serial dilutions of serum in phosphate-buffered saline in a 96-well U-bottom plate in 100-μl volumes and adding 100 μl of *S. aureus* (10^6/ml) to each well. The plate was mixed for 5 min and incubated at room temperature overnight. The first dilution that did not agglutinate *S. aureus* was used to opsonize bacteria for the assay.

Bacterial killing by neutrophils was measured by colorimetric determination of formazan production as described previously (26). Briefly, each strain of *S. aureus* in stationary-phase growth was opsonized and incubated in microtiter plates with isolated cells at a ratio of 10 bacteria per neutrophil. Following 1 h of incubation, neutrophils were lysed with saponin (20 mg/ml) and the tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well (100 μg/well). The ability of live *S. aureus* to reduce MTT to purple formazan was quantitated by measuring optical density at 595 nm. Percent killing of *S. aureus* by neutrophils was determined by comparison to a standard formazan curve derived by adding MTT to known concentrations of bacteria. The percentages of bacteria killed were calculated by the following equation: percent killed = $90 \times \{1 - [(\text{OD sample} - \text{OD 90\% dilution})/(\text{OD 0\% dilution} - \text{OD 90\% dilution})]\}$, where OD is optical density.

The abilities of neutrophils to phagocytize the different strains were quantitated by light microscopy. Briefly, neutrophils isolated from one of the eight experimental cows and bacteria of each strain were incubated together in 1.5-ml polypropylene microcentrifuge tubes under assay conditions. Mixtures were pelleted at 12,000 × *g* for 10 s. Pellets were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, postfixed in 1% osmium tetroxide, stained en bloc with aqueous uranyl acetate, dehydrated in a graded series of ethanol, bathed in propylene oxide, and embedded in Jembed 812 epoxy resin (JBS, Dorval, Quebec, Canada). Sections 1.0 μm thick were cut and stained in 0.1% toluidine blue and borax and observed at a magnification of ×300. Phagocytic ratios (neutrophils/bacteria) were determined by enumerating cells and phagocytized bacteria per five individual fields of view within a section. The five resultant values were then averaged to obtain a single ratio for each strain. For each experimental strain, phagocytic ratios were determined twice on cultures embedded on two different days to account for assay variation.

The percentages of bacteria killed or phagocytized for strains within each prevalence group were compared by a paired *t* test for the eight cows or by day of embedment, respectively. The statistical analyses of the strains belonging to different types were carried out by means of the GLM procedures in SAS/STAT 6.03 (24) by the model $Y = \mu + \alpha_i + \beta_j + \varepsilon_{ijk}$, where Y = bacterial killing or phagocytosis, μ = general mean, α_i = effect of the ith cow or day of embedment, β_j = effect of the jth bacterial type, and ε_{ijk} = random variation.

Bacterial growth was measured by inoculating strain A1 in 100 ml of RPMI+, incubating at 37°C, making serial dilutions (0.5 ml) of the culture, and plating each hour for a total of 24 h. The growth study of bacterial strain A1 yielded the following characterization: after incubation in RPMI+ at 37°C, the initial concentration remained constant for approximately 2 h after inoculation. From 2 to 4 h after inoculation, an exponential increase in bacterial concentration occurred. From 4 to 7 h after inoculation, bacterial growth entered a stationary phase, after which the bacterial concentration remained constant for at least 24 h. Under assay conditions, an average increase in bacterial number of 5% (−5 to 15%) for the six strains was observed after 1 h. No differences in bacterial growth among the six different strains were observed.

The average bacterial killing for all strains of *S. aureus* was 51.4%. A significant variation ($P < 0.001$) among the killing ability of neutrophils isolated from the different cows was found. No significant variation in bacterial killing was observed among the strains belonging to the same type. In contrast, the bactericidal capacity of the neutrophils varied significantly ($P < 0.001$) among the three groups of bacteria selected from each level of prevalence. The average percentages of killing were as follows: 40 for the two strains belonging to the most common *S. aureus* type, 50 for the two strains belonging to the intermediate type, and 64.2 for the two strains belonging to the rare type. The mean and standard deviation adjusted for cow effect are shown in Fig. 1.

The average phagocytic ratio for all *S. aureus* strains was 1:8.69. No significant differences were found between neutrophil phagocytosis of strains belonging to the same type ($P \leq$

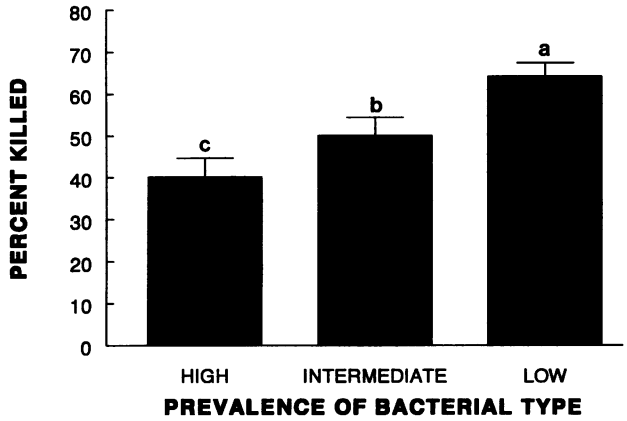

FIG. 1. Neutrophil killing of *S. aureus* types isolated from bovine mastitis at different prevalences. Values shown are averages ± standard errors for neutrophils from eight cows tested with two strains per prevalence type. Mean percentages with different letters are significantly different.

0.5). Conversely, a statistically significant difference was found between the phagocytosis of the most common type and the other two types ($P \leq 0.001$) but not between strains belonging to types of intermediate and low prevalence. As shown in Fig. 2, the average phagocytic ratios were 1:6.27 for strains of the most common type, 1:9.26 for the intermediate type, and 1:10.5 for the rare type.

The coevolution of hosts and their disease-causing pathogens is important to the genetic diversity of both host and pathogen (9, 18). Selection in the pathogen population will favor mechanisms to avoid host defense and to colonize the host. The pathogens that are most efficient in avoiding the host's defense mechanisms will be the most prevalent type found in the microenvironment of interest. This phenomenon is supported by the findings in this investigation in which we studied the abilities of neutrophils to phagocytize and kill different *S. aureus* genotypes found at different prevalences in cases of bovine mastitis. We discovered that the most common genotype of *S. aureus* was also the type against which the

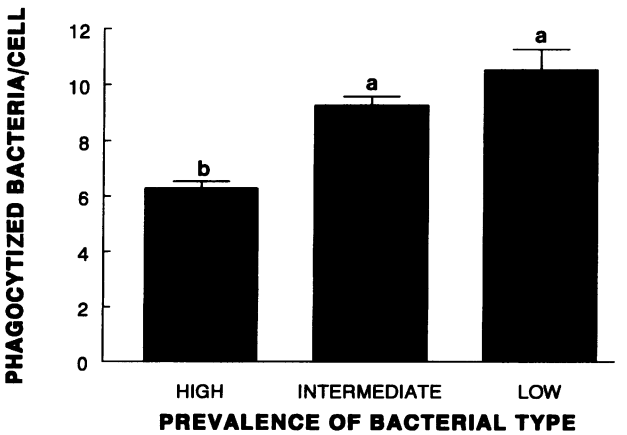

FIG. 2. Phagocytosis of different types of *S. aureus*. Data are expressed as means ± standard errors for two strains per prevalence type in duplicate assays. Means with different letters are significantly different.

neutrophils and thereby the host's initial defense mechanisms were least efficient. In contrast, the neutrophils were highly efficient to the rarely found genotype. These results suggest that types found in high prevalence have unique characteristics which, in contrast to rare types, endow them with the superior abilities to suppress or resist phagocytosis and killing by neutrophils.

Bacterium-host interaction depends on the growth rate of the bacteria, on the production of virulence factors, and on the efficiency of the host's defense. In this study, no differences in growth potential were observed among the strains used; therefore, the disparate killing of different *S. aureus* subtypes may be due to the release of soluble substances by the bacteria or to an inherently increased resistance to the phagocytic or intracellular killing mechanisms of neutrophils. The strains used in this study did not differ in their production of enterotoxins, toxic shock syndrome toxin, staphylokinase or hemolysins; therefore, any effect of soluble substances on killing efficiency in this study must be due to some other unscreened substance or possibly to a variation in the type of coagulase produced by the different coagulase gene types.

Other than the effect of soluble substances, variations in the resistance of different strains to neutrophils might also be explained by differences in bacterial cell surface determinants. Since none of the strains used in this study produced detectable capsules under our particular growing conditions, it appears that a factor other than capsular formation may be of importance for resistance to bacterial killing by neutrophils. For example, neutrophil phagocytosis can be stimulated independently of opsonization as a result of the binding of bacterial cell wall carbohydrates to lectin-like receptors on neutrophil membranes (8). Greater proportions of receptor-compatible carbohydrates on the cell walls of less prevalent strains may be responsible for the increased phagocytosis observed in this experiment. Although uninvestigated at this point, the same or other highly antigenic bacterial cell wall components of strains may make certain strains more likely to elicit antibody formation within the host. Higher antibody titers against these strains may make them more susceptible to opsonization and subsequent phagocytosis by local neutrophil populations. These conditions may increase the chances of initially clearing an infection with such highly antigenic strains and could ultimately reduce their overall prevalence.

The present study investigated the interaction between neutrophils and different *S. aureus* genotypes. On the basis of experimental results, it is likely that variations in resistance to phagocytosis and killing by bovine neutrophils are a major factor in variations in the prevalence of *S. aureus* genotypes in bovine mastitis. The specific mechanism by which this occurs is still unknown but may involve variations in molecular expression on the bacterial cell wall or secretion of virulent substances. Enhancing the neutrophil function specifically to these more prevalent *S. aureus* genotypes, then, could be an effective way to reduce the prevalence of *S. aureus* mastitis.

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