THE CONTRIBUTION OF A CAPSULE TO SURVIVAL OF STAPHYLOCOCCI WITHIN BOVINE NEUTROPHILS

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SUMMARY. The encapsulated strain M of *Staphylococcus aureus* and the encapsulated strain 76 of *S. simulans* survived incubation with bovine neutrophils in media containing heated bovine serum 0·5% v/v or milk whey 10% v/v. With two unencapsulated strains of *S. aureus*, M12 and M60, < 5% of the original inoculum survived. When the concentrations of bovine serum and milk whey were increased to 2% and 50% respectively, survival of the encapsulated strains was similar to that of the unencapsulated strains. The ability of skimmed milk 50% v/v to opsonise strain M was found in milk from 95% of 62 cows.

The individual values for percentage intracellular survival of strains of *S. aureus* phagocytosed by bovine neutrophils in the presence of bovine serum 2% v/v or milk whey 50% v/v were 0·48, 0·30 and 0·24 for strains M60, M and M12 respectively. Intracellular survival of strain M60 was significantly greater than that of strains M12 and M. The encapsulated coagulase-negative strain 76 (*S. simulans*) was not susceptible to lysis by lysostaphin, and it was not possible to destroy extracellular staphylococci to measure intracellular survival. It is concluded that the capsule of *S. aureus* strain M does not contribute to intracellular survival.

INTRODUCTION

Most staphylococci within neutrophils are killed by the bactericidal reactions of the activated cells (Spitznagel, 1977). Verbrugh *et al.* (1978) reported that c. 88% of neutrophil-associated staphylococci are killed *in vitro* within 20 min with little difference in the killing capacity of neutrophils from 50 human donors. Although there was greater individual variation between cows, Williams *et al.* (1984) found that bovine neutrophils killed, on average, 90% of total staphylococci after incubation for 2 h. However, a small proportion of staphylococci are not killed by phagocytes and it is these persistent organisms that trigger chronic inflammatory reactions. Persistent intracellular staphylococci are a feature of chronic staphylococcal mastitis in cattle (Anderson, 1982).

An antiphagocytic advantage conferred on staphylococci by the presence of a capsule has been demonstrated with *S. aureus* strain M (Melly *et al.*, 1974), the Smith

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diffuse strain of *S. aureus* (Koenig, 1962) and *S. simulans* strain 76 (Anderson and Wilson, 1981). Peterson *et al.* (1978) have shown that the capsule of *S. aureus* strain M interferes with opsonisation by the classical and alternative complement pathways and by heat-stable factors in normal human serum. The capsule masks receptors for complement and immunoglobulin on the cell wall peptidoglycan (Wilkinson *et al.*, 1979) and in this way inhibits ingestion. In the presence of specific anti-capsular antibodies, however, opsonisation and ingestion by human neutrophils occurs (Peterson *et al.*, 1978). It is important to know if a capsule aids the survival of staphylococci once they are within neutrophils.

In this paper we report on the capacity of bovine serum and milk whey to opsonise encapsulated and unencapsulated staphylococci and on the survival of these organisms within bovine neutrophils.

MATERIALS AND METHODS

Neutrophils were obtained from fresh blood by the method of Carlson and Kaneko (1973). After centrifugation, 2.5 ml of packed cells were mixed with 1.5 ml of phosphate buffered saline (PBS) and the red cells lysed by the addition of 10 ml of water. After 30 s, isotonicity was restored by adding 5 ml of saline 2.7% w/v. The neutrophils were washed twice with 25 ml of PBS and the concentration was adjusted to 10^7 cells/ml.

Opsonins. Serum was pooled from 6, 14 and 120 cows to provide three separate batches of serum. Pooled serum was heated for 30 min at 56°C and stored at -20°C. Three separate batches of milk whey were prepared from 3, 9 and 20 cows respectively. The milk was centrifuged at 30 000 *g* for 30 min and the unheated clear whey was frozen at -20°C. Serum and whey were sterilised by membrane filtration (0.45- μ m pore size; Millipore (UK) Ltd, Harrow, Middlesex) before use and dilutions were made in Hanks's Balanced Salts Solution.

Staphylococci. *S. aureus* strain M60 is a coagulase-positive staphylococcus that produces α and β haemolysins on ox-blood agar. Strain M12 is a non-haemolytic strain of *S. aureus*. Strains M60 and M12 are unencapsulated and were originally isolated from cases of bovine mastitis. Strain M (NCTC 10649) is an encapsulated strain of *S. aureus* (Scott, 1969). Strain 76 is an encapsulated coagulase-negative staphylococcus classified as *S. simulans* (Anderson and Wilson, 1981).

Bacteria were grown overnight in 10 ml of Todd-Hewitt Broth, washed twice with physiological saline and adjusted by nephelometry to a concentration of $c. 6 \times 10^6$ cfu/ml of saline. Bacterial counts were determined by the technique of Miles *et al.* (1938). Strain M was incubated on ox-blood agar at 30°C for 18 h because the mucoid colonies tended to coalesce at 37°C. The other strains were incubated at 37°C for 18 h. Extracellular staphylococci were eliminated by treatment with lysostaphin (240 units/mg; Sigma) at a final concentration of 5 μ g/ml for 20 min at 37°C. Lysostaphin was subsequently inactivated by trypsin (Difco) at a final concentration of 1.2 mg/ml, before the mixtures were plated out on blood agar to enable colonies of *S. aureus* to grow from the intracellular survivors (Williams *et al.*, 1985).

Experimental design and analysis. Preliminary tests were performed to establish the sensitivity of the bacterial strains to lysostaphin and opsonins without neutrophils. Suitable working concentrations of these variables with neutrophils were then established. Finally, the susceptibility of the opsonised bacterial strains to neutrophils was measured in five separate assays. The initial number of bacteria (T_0) was recorded together with the total number of surviving bacteria (TS) and the number of intracellular survivors (ICS). Bacterial survival after incubation for 1.5 h with neutrophils and opsonins was expressed as a percentage of T_0 . The number of bacteria phagocytosed was calculated as $T_0 - (TS - ICS)$. Because phagocytosis is a necessary pre-requisite for bacterial destruction, the numbers of intracellular survivors were expressed as percentages of phagocytosed bacteria. The calculations are described in full by Williams *et al.* (1985). Analyses of the data were performed on the logarithms of these values; Student's *t* test was used to analyse the $\log_{10}\%$ TS values of the preliminary experiments. Results of the main experiment were analysed by an analysis of variance mode that allowed for

variations in source of neutrophils, assay and batch of opsonin before fitting bacterial strain, type of opsonin (serum or whey) and their interaction. When significant overall effects were detected, individual means were compared by Student's *t* test.

RESULTS

Strain differences in susceptibility to opsonisation and killing by bovine neutrophils

In the standardised technique used in this laboratory for estimating the bactericidal activity of bovine neutrophils, *S. aureus* strain M60 was opsonised with heated pooled bovine serum 0.5% v/v final concentration (Williams and Bunch, 1981). The final concentrations of neutrophils and bacteria were $3.3 \times 10^6/\text{ml}$ and $1 \times 10^6/\text{ml}$ respectively, and these were incubated for 1.5 h in the presence of opsonin. This concentration of serum or milk whey 10% v/v was opsonic for strains M60 and M12 (mean TS = 4.8%) but failed to opsonise strains M or 76 (mean TS = 100.9%) (Table). When the opsonin concentrations were increased to serum 2% v/v and milk whey 50% v/v, the mean number of survivors of either encapsulated or unencapsulated staphylococci was < 5% (4.9 and 3.8% respectively). These higher concentrations of opsonins failed to kill strains M60, M12 and M in the absence of neutrophils although the numbers of *S. simulans* strain 76 were reduced (table). The percentage TS for *S. simulans* in the absence of neutrophils was significantly lower than for the *S. aureus* strains after treatment with milk whey 10% v/v ($p < 0.01$), serum 2% v/v ($p < 0.001$) and milk whey 50% v/v ($p < 0.001$).

Although the average percentage survival rates for encapsulated and unencapsulated staphylococci were < 5% when the bacteria were incubated with neutrophils and serum 2% v/v or whey 50% v/v, there were several effects of a particular opsonin on different strains and of different opsonins on a given strain. In serum 2% v/v, strain M was less susceptible to phagocytic killing than strain M60 ($p < 0.01$) but the results with strains M12 and 76 were not significantly different from those with strain M ($p > 0.05$). In milk whey 50% v/v, the susceptibilities of strains M, M60 and M12 to phagocytic killing were not significantly different and all three strains were significantly more resistant than strain 76 ($p < 0.05$). There were no differences between the three strains of *S. aureus* in response to type of opsonin, but phagocytic killing of strain 76 was significantly greater in whey 50% v/v than in serum 2% v/v ($p < 0.01$).

The milk whey pooled from three cows opsonised strain M poorly in two assays. In a subsequent assay, two of these three individual wheys failed to opsonise strain M (100 and 140% TS respectively). This deficiency of opsonin for strain M in milk whey was investigated further by testing skimmed-milk samples, at a final concentration of 50% v/v, from 62 cows against strain M with bovine neutrophils. Skimmed milk from three cows (5%) failed to opsonise strain M (120% TS). In the remaining 59 samples, the geometric mean for the survival rates was 9%. When the skimmed-milk samples were heated for 30 min at 56°C, the number of samples that failed to opsonise strain M increased to six (10%).

Strain differences in susceptibility to intracellular killing by bovine neutrophils

In preliminary tests, strains M60, M12 and M of *S. aureus* were killed after

TABLE
The effect of serum and milk whey on survival of four strains of Staphylococcus in the presence or absence of bovine neutrophils

Assay mixture	Source and concentration of opsonin	No of observations	Bacterial survival, after 1.5 h, as a percentage of the inoculum ($\log_{10} \pm \text{SEM}$) for strains			
			M12	M60	M	76
With bovine neutrophils	Serum	0.5% v/v	7.5 (0.88 $\pm$ 0.06)	5.2 (0.72 $\pm$ 0.05)	93.5 (1.97 $\pm$ 0.05)	118.7 (2.08 $\pm$ 0.03)
	Milk whey	10% v/v	4.3 (0.64 $\pm$ 0.09)	3.0 (0.48 $\pm$ 0.08)	120.1 (2.08 $\pm$ 0.14)	77.6 (1.89 $\pm$ 0.07)
	Serum	2% v/v	5.5 (0.74 $\pm$ 0.19)	1.8 (0.24 $\pm$ 0.19)	14.9 (1.17 $\pm$ 0.19)	5.7 (0.76 $\pm$ 0.19)
	Milk whey	50% v/v	6.5 (0.82 $\pm$ 0.19)	3.4 (0.53 $\pm$ 0.19)	6.8 (0.83 $\pm$ 0.19)	1.0 (-0.01 $\pm$ 0.19)
Without bovine neutrophils	Milk whey	10% v/v	91.9 (1.96 $\pm$ 0.03)	88.2 (1.95 $\pm$ 0.03)	114.7 (2.06 $\pm$ 0.05)	62.0 (1.79 $\pm$ 0.04)
	Serum	2% v/v	98.0 (1.99 $\pm$ 0.03)	108.8 (2.04 $\pm$ 0.04)	97.7 (1.99 $\pm$ 0.03)	67.0 (1.83 $\pm$ 0.03)
	Milk whey	50% v/v	148.6 (2.17 $\pm$ 0.06)	191.4 (2.88 $\pm$ 0.04)	117.9 (2.07 $\pm$ 0.05)	24.1 (1.38 $\pm$ 0.05)

incubation for 20 min with lysostaphin 5 µg/ml (<0.01% TS). However, *S. simulans* strain 76 was not killed by lysostaphin concentrations up to 200 µg/ml.

There were no differences between the effects of serum 2% v/v and milk whey 50% v/v on intracellular killing of each of the three strains of *S. aureus*. The percentage intracellular survival ($\pm$ SEM) of phagocytosed bacteria was 0.24 ($\pm$ 0.045) for strain M12 (8 observations), 0.48 ($\pm$ 0.045) for strain M60 (8 observations) and 0.30 ($\pm$ 0.048) for strain M (7 observations). The difference between the intracellular survival of strains M12 and M was not significant, but survival of strain M60 within neutrophils was significantly greater than that of strains M ($p < 0.01$) or M12 ($p < 0.001$). Therefore, although phagocytosis and killing of strain M60 was greater than that of strain M, cells of strain M60 were more resistant to intracellular killing.

DISCUSSION

Investigation of the role of a capsule in intracellular survival of staphylococci was made possible by the finding that bovine serum and whey opsonised the encapsulated strain M. With human serum, Peterson *et al.* (1978) found that ingestion of significant numbers of strain M could not be achieved either by prolonging exposure to the opsonin or by increasing its concentration. In the present study, strain M was similarly resistant to phagocytosis in bovine serum 0.5% v/v or bovine whey 10% v/v, but, when the concentrations were increased to 2% v/v and 50% v/v respectively, phagocytic ingestion was increased to the extent that, on average, <5% of organisms of either encapsulated or non-encapsulated strains remained alive. Control preparations showed that, at these higher concentrations, the serum and whey were not themselves bactericidal but required the participation of neutrophils. The exception was with the coagulase-negative encapsulated *S. simulans* strain that grew significantly less well in the presence of serum or whey than the strains of *S. aureus*.

Because heated serum was opsonic for strain M, it appeared that antibody was predominantly involved. The absence of activity in two whey samples stimulated the examination of skimmed milk from 62 cows. Only 5% failed to opsonise strain M and this percentage increased slightly for heated skimmed milk, indicating the minor participation of complement. Both antibody and complement are normally involved in the opsonisation of unencapsulated staphylococci (Verbrugh *et al.*, 1979) and specific anticapsular antibody is required for the opsonisation of encapsulated staphylococci (Verbrugh *et al.*, 1982). Our results indicate that both antibody and complement are involved in opsonisation of strain M by bovine milk and that this activity is fairly widespread among cattle.

When the relative susceptibility of the three strains of *S. aureus* to killing by neutrophils was examined, strain M was significantly more resistant than one of the other *S. aureus* strains in heated serum 2% v/v, but there was no difference between the strains in unheated milk whey 50% v/v. The presence of small amounts of complement in whey 50% v/v (Mueller *et al.*, 1983) may be sufficient to obliterate any difference in susceptibility to phagocytic killing between encapsulated and unencapsulated strains of *S. aureus*. Consistent with its behaviour in whey in the absence of neutrophils, the coagulase-negative strain 76 was significantly more susceptible to killing in whey in the presence of neutrophils than the *S. aureus* strains.

It was already known that the encapsulated strain M was susceptible to lysis by

lysostaphin (King *et al.*, 1980) and the insensitivity of the encapsulated strain 76 to lysis by lysostaphin (200 µg/ml) was similar to findings with some unencapsulated coagulase-negative staphylococci and with micrococci (Schleifer and Kloos, 1975). Although it was not possible to measure the intracellular survival of strain 76, an earlier study in mice showed that once neutrophils were recruited in response to intramammary inoculation of strain 76, the organisms were rapidly eliminated from the glands (Anderson and Craven, 1984).

Intracellular survival of *S. aureus* was measured after lysis of extracellular staphylococci by lysostaphin (Easmon *et al.*, 1978). Strain M60 had the greatest number of intracellular survivors. Survival was significantly greater than that of strains M12 and M; intracellular survival of strains M12 and M was not significantly different. The experiments indicated that a capsule does not increase intracellular survival of staphylococci.

The importance of this observation is two-fold. First, it helps to reveal the contribution of a capsule to virulence of staphylococci. The cardinal property of encapsulated strains of *S. aureus* is that they resist ingestion by phagocytes and they kill mice (Koenig, 1962; Melly *et al.*, 1974). The identification of a coagulase-negative encapsulated staphylococcus that was resistant to phagocytic ingestion but was not lethal for mice indicated that encapsulation itself was not a lethal determinant (Anderson and Wilson, 1981). The present observation indicates that once encapsulated staphylococci are ingested their survival within neutrophils is not enhanced by the presence of a capsule. This further delimits the function of the capsule as a virulence determinant in staphylococci. Second, many strains of *S. aureus* isolated from cases of bovine mastitis are encapsulated (Opdebeeck and Norcross, 1983) and attempts have been made to immunise cattle against staphylococcal mastitis with capsular antigens (Yoshida *et al.*, 1984). If encapsulated strains are common in clinical infections—and this has been challenged (Anderson, 1984)—the results suggest that anti-capsular antibodies may facilitate ingestion of staphylococci by neutrophils but would have no effect on intracellular persistence of staphylococci, which is the essential feature of staphylococcal mastitis (Anderson, 1982). Furthermore, although higher concentrations of serum and whey were required to opsonise encapsulated staphylococci, both serum and whey were able to support opsonisation at concentrations within physiological limits. Serum passes into milk as soon as inflammation begins in the mammary gland and there appears to be little need to provide additional opsonins in milk by immunisation with capsular antigens.

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