

Effect of Fat and Casein on Intracellular Killing of *Staphylococcus aureus* by Milk Leukocytes (39856)

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Polymorphonuclear leukocytes (PMN) isolated from milk have reduced *in vitro* phagocytic activity when compared with PMN isolated from blood (1, 2). This reduction in activity has been attributed to competitive interference by fat globules and casein which are present in PMN isolated from milk (3, 4). This observation was further supported by *in vitro* studies showing that milk fat (5) and casein (6) interfered with phagocytosis of *Staphylococcus aureus* (*S. aureus*) by PMN. If a similar relationship between PMN and either fat globules or casein occurs in the mammary gland, this could explain the susceptible nature of the bovine mammary gland to infection by mastitis pathogens (7). The present study was conducted to determine the extent to which milk fat and casein interfere with intracellular killing of phagocytosed *S. aureus*.

Materials and methods. *Cows.* Holstein-Friesian cows at various stages of lactation were tested. All mammary quarters were sampled twice monthly for bacteria, starting 2 months before the experiment. Additional samples were collected for bacteriological study and for microscopic leukocyte counts (8) 2 days before and on the day of the *in vitro* phagocytosis assay. Only quarters free from bacterial infection and having leukocyte counts less than 200,000 cell/ml of milk were used as sources of milk for study.

Polymorphonuclear leukocytes. Two quarters were infused with 100 ml of sterile filtered saline containing 0.1% oyster glycogen immediately after milking. The PMN were isolated (9) and suspended to a concentration of 100×10^6 PMN/ml in phosphate-buffered (0.0132 M, pH 7.4) 0.85% sodium chloride (PBS) at 4°. The cell suspension was kept iced and was used within 15 min. A total of 19 donor cows were used for the collection of PMN.

Staphylococcus aureus. A fresh stock culture of *S. aureus* (strain 305, supplied by

Dr. F. H. S. Newbould, University of Guelph, Guelph, Canada) was prepared once a week by placing a lyophilized culture in 200 ml of brain-heart infusion broth (BHI) and incubating for 18 hr at 37°. The broth (0.1 ml) was streaked on blood agar and incubated for 24 hr at 37°. Forty-eight hours before an intracellular kill assay, a colony from the blood agar plate was transferred to a mannitol salt agar slant and incubated for 24 hr. The resulting colonies were washed from the slant and suspended in 5 ml of BHI. This suspension (0.2 ml) was added to 200 ml of BHI and incubated to an optical density of 0.3979 at 525 nm (4-5 hr), a stage of growth previously determined to be in the logarithmic growth phase. The organisms were centrifuged and resuspended three times in 150-ml volumes of ice-cold PBS and resuspended in PBS to an optical density of 0.6576 [approximately 300×10^6 colony forming units (CFU)/ml]. This suspension was used as the stock culture from which the *S. aureus* suspensions used in the phagocytosis assays were prepared as described below.

Milk sample preparation. Immediately before milking, the mammary gland was washed with disinfectant and dried. After milk "let-down," milk was aseptically collected. A 10-ml aliquot was designated as the whole milk fraction. The rest of the milk was used to prepare the skimmed milk and whey (5), and because sediment results when milk is centrifuged (10), a skimmed milk sample that contained resuspended sediment was also prepared.

Intracellular kill assay. Three milliliters of the stock *S. aureus* suspension was added to 15 ml of PBS (50×10^6 CFU/ml). Two-tenths milliliter (10×10^6 CFU) was then combined with 1 ml of PMN (100×10^6), and either 0.2 or 0.8 ml of whole milk, skimmed milk, skimmed milk plus 10% cream, skimmed milk plus sediment, or

wey was added to siliconized tubes (12 × 77-mm Falcon tube with snap cap, Fisher Scientific Co., Silver Spring, Maryland). Volumes were adjusted to 2 ml with PBS, and the samples were tumbled for 60 min at 37°. Tests were conducted in duplicate. After incubation, one tube received 4 IU of lysostaphin (Schwartz/Mann, Orangeburg, New York) in 0.5 ml of PBS and was tumbled for 60 min at 37° to lyse extracellular and adherent staphylococci (11). Trypsin (0.05%) (Bovine pancreas, type III, Sigma Chemical Co., St. Louis, Missouri), dissolved in Hanks balanced salt solution containing bicarbonate (pH 7.4), was then added (1 ml), and incubation was continued for 30 min at 37° to inactivate the lysostaphin. This tube was used to estimate surviving intracellular *S. aureus*. Ice-cold PBS (1.5 ml) was added to the other tube and was used to estimate surviving extracellular and intracellular *S. aureus*. Surviving extracellular *S. aureus* was obtained by difference and was used as an estimate of phagocytosis.

In an effort to rupture the PMN, all samples were sonicated (Virsonic disrupter, catalog no. 16-850, the Virtis Co., Gardiner, New York) with a Micro-tip at a power setting of 35 for 60 sec. Rupture of PMN was verified by microscopic examination. The samples were diluted in duplicate in 25 ml of sterile PBS and sonicated for 20 sec with the standard 0.75 in. probe at a power setting of 60 to disperse the *S. aureus*. Duplicate trypticase soy agar pour plates were made from each of the diluted samples. The plates were counted after 18 hr of incubation at 37°.

The effect of varying the *S. aureus* to PMN ratio on intracellular and extracellular survival of *S. aureus* was tested by adding 0.8, 0.2, 0.08, and 0.04 of diluted *S. aureus* (6.7 ml of stock and 1.3 ml of PBS) (approximately 250 × 10⁶ CFU/ml), 1 ml of PMN (100 × 10⁶), and 0.2 ml of either whole milk, skimmed milk, whey, or serum. Volumes were adjusted to 2 ml with PBS. These dilutions provided *S. aureus*:PMN ratios of 2:1, 1:2, 1:5, and 1:10, respectively.

Controls. In an effort to test the direct effects of the studied mammary fluids on growth of the *S. aureus*, 0.2 ml of *S. aureus* (approximately 10 × 10⁶ CFU) was added

to 0.2 ml of either whole milk, skimmed milk, skimmed milk plus 10% cream, or whey in a final volume of 2 ml with PBS and incubated for 60 min at 37°.

In an effort to determine whether or not substances released from PMN during phagocytosis were bactericidal, PMN were incubated with yeast cells (12), the mixture was centrifuged, and *S. aureus* was incubated in the supernatant for 60 min. *Staphylococcus aureus* was also incubated with lysed PMN (sonication, 30 sec, standard probe) and with intact PMN in tubes that were not tumbled. Smears were prepared from the non-tumbled sample and stained with Wrights stain. The first 100 PMN were observed microscopically for the presence of phagocytosed or adherent *S. aureus*. All samples were sonicated, diluted in PBS, and plated.

Statistical analysis. Colony forming units were converted to log, and an analysis of variance was made on the transformed data. Duncan's multiple range test was used to determine significant differences among the treatment means.

Results and discussion. In Tables I and II data pertinent to establishing the validity of the intraleukocytic kill assay are summa-

TABLE I. EFFECT OF STUDIED MATERIAL ON GROWTH OF *Staphylococcus aureus* IN THE ABSENCE OF PMN.^a

Studied material ^b	Total colony forming units	
	Arithmetic mean (×10 ⁶)	Log mean ^c
Skimmed milk (0-min control)	7.9	6.87 ^a
Whole milk	11.6	7.05 ^e
Skimmed milk	11.6	7.05 ^e
Skimmed milk plus cream (20 μl)	11.8	7.06 ^e
Whey	11.1	7.03 ^e
Standard error of treatment mean		±0.01
Significance level among means		<i>p</i> < 0.01

^a Represents the mean from four determinations run in duplicate.

^b Studied material (200 μl) was in a total volume of 2 ml with phosphate-buffered saline and was incubated for 60 min at 37°.

^c Means in the column with no superscript letter in common differ from each other (*p* < 0.05).

TABLE II. EFFECT OF PMN AND PMN PRODUCTS ON GROWTH OF *Staphylococcus aureus*.^a

Treatment ^b	Total colony forming units	
	Arithmetic mean ($\times 10^6$)	Log mean ^c
0-hr Control (no PMN)	10.03	7.00 ^j
1-hr Control (no PMN)	11.36	7.05 ⁱ
Intact PMN	0.41	5.60 ^f
Intact PMN (without tumbling) ^d	6.76	6.82 ^g
PMN supernatant ^e	11.70	7.07 ⁱ
Lysed PMN	14.01	7.15 ^h
Standard error of treatment mean		± 0.01
Significance level among means		$p < 0.01$

^a Represents the mean from three determinations run in duplicate.

^b Incubation mixture contained 200 μ l of skimmed milk in a total volume of 2 ml and was incubated for 60 min at 37°.

^c Means in a column with no superscript letter in common differ from each other ($p < 0.05$).

^d Microscopic counts of the first 100 PMN indicated that on the average 9% of the PMN contained either phagocytosed or adherent *S. aureus*.

^e Obtained after phagocytosis of killed yeast (10×10^6).

rized. The average number of *S. aureus* increased 46% ($p < 0.01$) after incubation for 60 min at 37° in the studied materials (Table I). There was no significant difference ($p > 0.05$) in the growth of *S. aureus* in any of the studied materials.

The influence of the PMN on growth of *S. aureus* is shown in Table II. When *S. aureus* was incubated with either lysed PMN, PMN supernatant, or in the skimmed milk medium alone, growth of the *S. aureus* increased significantly ($p < 0.01$) when results were compared with those for the 0-hr control. There was no significant difference ($p > 0.05$) between skimmed milk and PMN supernatant. This finding suggests that during phagocytosis of yeast, the PMN did not release bactericidal substances in concentrations sufficient to slow the growth of the *S. aureus* used in this experiment. However, this test did not take into account the possible release of a labile bactericidal agent by PMN which may have been destroyed during the 60-min incubation period. Growth of *S. aureus* was significantly

greater ($p < 0.01$) during incubation with lysed PMN than with any other incubation medium. Incubation of *S. aureus* with PMN without tumbling significantly decreased ($p < 0.01$) numbers of *S. aureus*, but this method was significantly less ($p < 0.01$) effective in reducing the total number of surviving *S. aureus* than when the samples were tumbled. Microscopic examination of the first 100 PMN obtained from the stationary samples indicated that 9% of the PMN contained either phagocytosed or adherent *S. aureus*.

The effect of altering the *S. aureus* to PMN ratio on phagocytosis and intracellular killing of *S. aureus* is shown in Table III. The data indicate that as the ratio of bacteria to PMN decreases, both phagocytosis and intracellular kill of *S. aureus* by PMN was increased. A *S. aureus* to PMN ratio of 2:1 resulted in the greatest number of *S. aureus* left unphagocytosed and the greatest number of surviving intracellular *S. aureus*. Moreover, the numbers of surviving *S. aureus* were greater when whole milk rather than skimmed milk, whey, or serum was used as the incubation medium. These data suggest that during the initial establishment of infection in the mammary gland by *S. aureus*, where *S. aureus* may be expected to outnumber PMN, the PMN cannot effectively remove and destroy the organisms.

The effect of incubation media on phagocytosis and intracellular kill of *S. aureus* by milk PMN is shown in Table IV. Phagocytosis was significantly lower ($p < 0.01$) in whole milk and skimmed milk plus cream than in skimmed milk, skimmed milk plus sediment, or whey. These results support previous data (5) that showed the inhibitory effect of milk fat globules on phagocytosis. Phagocytosis of *S. aureus* by PMN incubated in either skimmed milk or skimmed milk plus sediment was the same. This finding suggests that the sediment in milk, which is primarily casein (13) and cellular debris (10), does not interfere with phagocytosis of *S. aureus* by PMN. Phagocytosis in skimmed milk was not different ($p > 0.05$) from that in whey. These data indicate that under the conditions of this experiment, casein did not inhibit phagocytosis.

The inhibitory effect of cream on the in-

TABLE III. EFFECT OF *Staphylococcus aureus*:PMN RATIO ON PHAGOCYTOSIS AND INTRACELLULAR KILLING OF *Staphylococcus aureus*.^a

Studied material ^b	<i>S. aureus</i> :PMN ratio ^c	Total surviving <i>S. aureus</i>					
		Intracellular			Extracellular		
		Arithmetic mean		Log mean	Arithmetic mean		Log mean
		(CFU × 10 ⁶)	(%)	(CFU)	(CFU × 10 ⁶)	(%)	(CFU)
Whole milk	2:1	44.5	22.6	7.30	26.0	17.6	7.14
	1:2	5.9	12.2	6.49	6.7	13.8	6.47
	1:5	1.8	9.4	6.03	3.2	16.6	6.21
	1:10	0.6	7.4	5.66	1.5	15.2	5.85
Skimmed milk	2:1	7.6	3.8	6.81	7.4	3.8	6.78
	1:2	1.4	2.9	6.09	1.3	3.1	6.05
	1:5	0.5	2.7	5.62	0.5	2.7	5.68
	1:10	0.2	2.2	5.25	0.2	2.3	5.29
Whey	2:1	9.0	4.6	6.89	11.1	5.7	7.00
	1:2	1.5	3.0	6.12	2.2	4.5	6.29
	1:5	0.5	2.6	5.68	0.9	4.6	5.87
	1:10	0.2	2.4	5.34	0.3	3.1	5.40
Serum	2:1	7.4	3.8	6.80	12.5	6.4	6.91
	1:2	1.3	4.1	6.06	3.0	6.2	6.30
	1:5	0.4	2.3	5.58	1.2	6.2	5.92
	1:10	0.2	2.3	5.30	0.5	5.4	5.49
Standard error of treatment mean				±0.04		±0.04	
Significance level among means				<i>p</i> < 0.01		<i>p</i> < 0.01	

^a Represents the mean from four determinations run in duplicate.

^b Studied material (200 μl) was in a total volume of 2 ml with phosphate-buffered saline and incubated for 60 min at 37°.

^c Prepared by adding either 0.8, 0.2, 0.08, or 0.04 of a *S. aureus* suspension that contained 237 × 10⁶ colony forming units (CFU)/ml.

TABLE IV. EFFECT OF STUDIED MATERIALS ON BACTERICIDAL PROPERTIES OF MILK PMN.^a

Studied material ^b	Total CFU ^c					
	Intracellular			Extracellular		
	Arithmetic mean		Log mean ^d	Arithmetic mean		Log mean ^d
	(×10 ⁶)	(%)		(×10 ⁶)	(%)	
Whole milk	0.31	3.9	5.38 ^{e, g}	0.68	7.9	5.58 ^e
Skimmed milk	0.19	2.2	5.27 ^f	0.12	1.4	4.93 ^{f, h}
Skimmed milk plus cream (20 μl)	0.33	4.4	5.42 ^e	0.70	7.8	5.40 ^g
Skimmed milk plus sediment	0.20	2.4	5.28 ^{f, g}	0.11	1.2	4.85 ^f
Whey	0.21	2.4	5.30 ^{f, g}	0.17	2.0	5.01 ^h
Standard error of treatment mean			±0.04		±0.04	
Significance level among means			<i>p</i> < 0.01		<i>p</i> < 0.01	

^a Each value represents the mean of 12 determinations run in duplicate.

^b Studied material (20 μl) in a total volume of 2 ml with PBS and incubated for 60 min at 37°.

^c Total number added (0 hr) was 8.83 × 10⁶ colony forming units (CFU) providing a *S. aureus*:PMN ratio of 1:11.

^d Means in a column with no superscript letter in common differ from each other (*p* < 0.05).

tracellular killing of phagocytosed *S. aureus* by PMN was significant (*p* < 0.01) (Table IV). This was attributed to degranulation of both primary (3) and secondary (14) granules during phagocytosis of milk fat globules, leaving fewer granules available for

killing phagocytosed *S. aureus*. However, there was no significant effect of casein on intracellular kill of *S. aureus*.

Increasing the concentration of whole milk in the incubation media from 10 (200 μl) to 40% (800 μl) (Table V) resulted in a

TABLE V. EFFECT OF CONCENTRATION OF WHOLE MILK AND SKIMMED MILK IN INCUBATION MEDIA ON PHAGOCYTOSIS AND INTRACELLULAR KILLING OF *Staphylococcus aureus*.^a

Studied material ^b	Total surviving <i>S. aureus</i> ^c					
	Intracellular			Extracellular		
	Arithmetic Mean		Log mean ^d	Arithmetic mean		Log mean ^d
	(CFU × 10 ⁶)	(%)	(CFU)	(CFU × 10 ⁶)	(%)	(CFU)
Whole milk						
200 μl	0.32	3.2	5.46 ^e	0.45	4.2	5.59 ^e
800 μl	0.50	5.2	5.51 ^e	2.03	21.7	5.97 ^f
Skimmed milk						
200 μl	0.19	1.9	5.24 ^f	0.14	1.5	5.19 ^g
800 μl	0.14	1.4	5.10 ^f	0.35	3.1	5.43 ^h
Standard error of treatment mean			±0.04			±0.04
Significance level among means			$p < 0.01$			$p < 0.01$

^a Represents the mean from eight separate determinations run in duplicate.

^b Studied material was in a total volume of 2 ml with PBS and incubated for 60 min at 37°.

^c Total number added (0 hr) was 10.7×10^6 colony forming units (CFU) providing a *S. aureus*:PMN ratio of 1:9.

^d Means in a column with no superscript letter in common differ from each other ($p < 0.05$).

significant decrease ($p < 0.01$) in phagocytosis. When whole milk constituted 10% of the incubation medium, only 4.2% of the *S. aureus* were left unphagocytosed, but when it constituted 40% of the incubation medium, 21.7% of the *S. aureus* were unphagocytosed. Increasing the concentration of skimmed milk from 10 (200 μl) to 40% (800 μl) resulted in a slight but significant ($p < 0.05$) drop in phagocytosis. There were 1.5 and 3.1% *S. aureus* left unphagocytosed in media containing 10 and 40% skimmed milk, respectively. This finding suggests that at higher concentrations of skimmed milk, casein may be exerting an inhibitory effect on phagocytosis, though not nearly the magnitude as that exerted by the fat globules in whole milk. Russell and Reiter (6) recently showed that casein is ingested by PMN and will inhibit *in vitro* phagocytosis of *S. aureus*.

Intracellular killing was significantly lower ($p < 0.01$) in whole milk than in skimmed milk indicating that milk fat globules reduce intracellular killing of *S. aureus* by PMN. However, increasing the concentration of either whole milk or skimmed milk had no effect ($p > 0.05$) on the bactericidal properties of PMN. The failure to detect a decrease in bactericidal activity with an increasing concentration of whole milk was attributed to the decreased phago-

cytosis of *S. aureus* at the higher concentration of whole milk.

Summary. Suspensions of PMN isolated from milk and *S. aureus* were incubated together at different ratios and with different types and concentrations of mammary fluids. Results showed that greater numbers of *S. aureus* were left unphagocytosed and alive within PMN at the higher *S. aureus*:PMN ratios (2:1) than at the lower ratios. This effect was more noticeable in whole milk than in skimmed milk. Phagocytosis and destruction of *S. aureus* by PMN (ratio 1:10) were reduced ($p < 0.01$) to a greater extent in whole milk or in skimmed milk containing 10% cream than in either skimmed milk or whey. There was no difference between skimmed milk or whey. Increasing the concentration of either whole milk or skimmed milk in the incubation mixture from 10 to 40% resulted in fewer ($p < 0.01$) *S. aureus* phagocytosed but had no effect on intracellular kill. The data show that the milk fat globule is a major deterrent to the phagocytosis and destruction of *S. aureus* by PMN. Casein inhibited phagocytosis, but only when the concentration of skimmed milk in the incubation medium was increased to 40%. Casein did not affect intracellular kill.

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