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Effect of Phagocytosed Fat and Casein on the Intraphagosomal pH in Bovine Polymorphonuclear Leukocytes[1,2] (41431)

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Abstract. Polymorphonuclear leukocytes (PMN) were isolated from bovine blood or milk and incubated with heat-killed yeast stained with pH indicator dyes. Observation of changes in the color of the phagocytosed yeast allowed a determination of the temporal and maximal depression in the pH of the newly formed phagosomes. Results indicated that the maximal depression of pH was to approximately 5.0 for PMN isolated from both sources, although cells from milk produced fewer ($P < 0.01$) phagosomes reaching pH 5.0 than cells from blood. The effect of casein or milk fat globules on the temporal depression in pH of phagosomes was determined by preincubating PMN from blood with isolated preparations of casein and milk fat globules. Results indicated that the prior ingestion of fat globules by PMN inhibited the subsequent depression of pH within phagosomes compared with untreated control cells ($P < 0.05$). Ingestion of casein micelles, however, had no effect ($P > 0.05$). The results are discussed with reference to previous reports that PMN in milk have reduced phagocytic ability and bactericidal properties compared with PMN in the systemic circulation.

Polymorphonuclear leukocytes (PMN) isolated from milk are deficient in their phagocytic (1–3) and bactericidal properties (4) when compared to PMN isolated from blood. The PMN in milk are known to ingest milk fat globules and casein micelles (4–6) and this is thought to contribute to their reduced phagocytic and bactericidal activity.

Phagocytosis of material by PMN leads to the formation of phagosomes in which the pH drops rapidly (7–9). This depression in pH ensures that enzymes from azurophil granules with low pH optima are maximally active during the intracellular digestion and killing of phagocytosed material. It is therefore possible that in PMN isolated from milk the internalized fat and casein interferes with the formation and number of acid phagosomes. The present study was therefore conducted to compare the temporal and maximal depression of pH within phagosomes of PMN isolated from blood and milk and to determine if ingestion of milk fat globules or casein micelles interferes with this depression.

Materials and Methods. *Source of PMN.* Mastitis-free Holstein–Friesian cows were used as a source of PMN. Immediately after milking, two mammary quarters were infused with 100 ml of 0.1% oyster glycogen in sterile 0.85% saline (10). Milk was collected from these quarters 16 hr later and immediately placed on ice. Blood was also collected at this time from the subcutaneous abdominal vein of the same cow into 40-ml heparinized (10 IU/ml) evacuated tubes and iced immediately. The PMN in milk were isolated according to the method of Paape *et al.* (10) and PMN in blood by sedimentation in dextran (11). The

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concentration of isolated PMN was adjusted to 50 × 10^6/ml.

Preparation of yeast. On the day before an experiment, 5 mg of Baker's yeast (*Saccharomyces cerevisiae*, ICN Pharmaceuticals, Cleveland, Ohio) were heat killed in 1 ml of modified Krebs–Ringer phosphate (KRP) buffer (12), pH 7.4, at 100° for 60 min. The solution was cooled to room temperature and the pH adjusted to 7.4, using 0.1 *M* K_2HPO_4. The killed yeast were stained in solution by adding 3.5 mg of indicator dye per milliliter. After 2 hr, the solution was centrifuged and the yeast cells washed once with KRP. The pH was then adjusted to 7.4 and the yeast cells resuspended to the original concentration. Under these conditions all yeast were stained.

Assay of pH within the phagocytic vacuole. Four pH indicators were chosen to monitor the pH change within the phagosome. Neutral red (NR) (Fisher Scientific Co., N-129), bromocresol purple (BCP) (Eastman Kodak Co., EKC 6266), bromocresol green (BCG) (Fisher Scientific Co., B-90), and bromophenol blue (BPB) (Eastman Kodak Co., EKC 6137) were used to stain the yeast. The indicators BCP, BCG, and BPB were all sodium salts. Aliquots of yeast (100 μl at 1.5 × 10^9 cells/ml), stained with one of the indicators, were incubated separately with 0.5 ml PMN isolated from blood or milk and 200 μl of 10% normal bovine serum (NBS) diluted with 0.0132 *M* phosphate-buffered saline (PBS) pH 7.4. The yeast to PMN ratio was 6:1 and the total volume was adjusted to 1.3 ml with PBS. All vials, each containing serum, yeast stained with one of the four indicators and PMN isolated from blood or milk of the same cow, were incubated together at 37° with shaking (80 oscillations/min, 3 cm transverse). After 20 min the shaking was stopped to prevent disruption of PMN.

Each vial (seven for each indicator) was then examined by bright-field microscopy by two persons at time intervals up to 240 min. At each observation time, one drop of the incubation mixture was placed on a glass microscope slide and covered with a cover slip. Duplicate slides were read by two separate individuals. The number of yeast staining the acid color (red for neutral red, yellow for bromocresol purple, etc.) within the first 100 PMN observed on each slide was recorded. After making the wet mounts, 12 mg of disodium ethylenediaminetetraacetate (EDTA) was added to the vials to stop phagocytosis, after which the vials were reincubated for 15 min with shaking (13). Duplicate dry smears were then made and later stained with Wright's stain. The total number of yeast contained in the first 100 PMN from these smears was determined in duplicate and recorded; and the percentage of ingested yeast that stained acid at each incubation time was then determined.

Preparation of PMN containing casein micelles. On the day of an experiment, PMN were isolated from venous blood (11) and suspended in PBS. The PMN were divided into two equal pools; one was incubated in an equal volume of PBS with 10% NBS, while the other was incubated in an equal volume of PBS with 10% NBS containing isolated casein micelles (30 mg protein/ml). The casein was isolated from milk by centrifuging whole milk at 750*g* at 4° for 30 min. The cream layer and pellet were discarded and the supernatant was removed and recentrifuged at 35,000*g* under the same conditions. The thin layer of cream was discarded and the supernatant was removed and centrifuged at 100,000*g* for 60 min at 4°. The casein pellet was resuspended in PBS and the protein concentration was adjusted to 90 mg/ml (15). This preparation was frozen in 330-μl aliquots in 5 ml siliconized capped plastic falcon tubes. On the day of an experiment, casein was thawed, leukocytes and NBS were added, and the volume was adjusted to 1 ml with PBS. All tubes (those with casein and controls) were then incubated for 60 min at 37° with tumbling (six inversions/min) for the first 20 min. At the end of this period, stained yeast were added to obtain a yeast to PMN ratio of 3:1 and the volume was adjusted to 1.3 ml with PBS. The depression of pH within the phagosome was determined as described above.

Preparation of PMN containing fat globules. On the day of an experiment, PMN were isolated from venous blood (11) and suspended in PBS. The PMN were di-

vided into two equal pools. One was incubated in an equal volume of PBS with 10% NBS; the other was incubated in an equal volume of a mixture of 10% NBS and cream. Both pools were incubated in 5-ml siliconized glass centrifuge tubes at 37° for 30 min with tumbling (six inversions/min). The NBS–cream mixture was prepared each time a determination was made. Cream was prepared from milk collected the day before the experiment. The milk was placed in a separatory funnel and allowed to stand at 4° overnight. On the day of the experiment 2.2 ml of the upper portion of cream was removed and mixed with 7.8 ml of NBS. This mixture was then incubated at 37° with shaking (80 oscillations/min, 3 cm transverse) for 30 min to prevent coalescence of fat globules. At the end of this incubation, the pH was adjusted to 7.3 with 0.1 *N* NaOH. One pool of isolated PMN was then incubated in this mixture and the other in PBS.

At the end of the incubation both pools were centrifuged at 2300*g* in 5 ml glass tubes for 10 min at 4°. The supernatant was then removed and the PMN pellet was resuspended in PBS containing stained yeast at a yeast to PMN ratio of 6:1 in a total volume of 1.3 ml.

To determine the extent of phagocytosis of fat globules by PMN an aliquot of each pool was removed prior to centrifugation and diluted 100:1 with PBS. Duplicate slides were made from each using a Shandon Elliot SCA 0030 Cytospin (14) and later stained with Wright's stain. From these smears the percentage of cells containing fat was determined by counting the number of fat globules contained within the first 100 PMN.

Results. The maximal depression of pH within the phagosomes of PMN isolated either from blood or milk was to pH 5.0–5.5. The BCG-stained and BPB-stained yeast did not change color, indicating that the pH was not depressed further than 5.0. The percentage of phagosomes reaching pH 6.5 increased up to at least 240 min (Table I). Because phagocytosis was complete at 60 min (data not shown), PMN were apparently actively depressing the pH to approximately 6.5 within earlier formed phagosomes for at least 3 hr after formation of the phagosome. The depression to pH 6.5 in PMN isolated from blood occurred at a similar rate as the depression to pH 5.0 up to approximately 90 min of incubation (40.2 and 27.9% of the phagosomes reaching pH 6.5 and 5.0, respectively), and then occurred at a slower rate up to 240 min (83.1 and 52.0% of the phagosomes reaching pH 6.5 and 5.0, respectively). After 240 min of incubation PMN from blood contained 2.7

TABLE I. COMPARISON OF TEMPORAL DEPRESSION OF INTRAPHAGOSOMAL pH FOR PMN ISOLATED FROM BLOOD AND MILK[a]

Incubation time (min)	Ingested yeast which turned red (pH ≃ 6.5)		Ingested yeast which turned yellow (pH ≃ 5.0)	
	Blood*	Milk	Blood*	Milk
30	9.2[b]	5.0	9.4	3.7
40	10.2	7.2		
60	15.0	5.6	27.1	6.8
90	40.2	20.8	27.9	18.4
120	61.7	35.0	38.9	27.6
180	65.1	28.1	52.0	33.0
240	83.1	30.6	52.0	27.1
Significance level	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$
Standard error of treatment mean	±1.24	±1.24	±6.4	±6.4

[a] Each value represents the mean of six cows determined in duplicate.
[b] Results are percentages.
* Blood significantly greater than milk, $P < 0.01$.

TABLE II. THE EFFECT OF INGESTED CASEIN ON THE PERCENTAGE OF INGESTED NEUTRAL RED-STAINED YEAST WHICH TURNED RED (pH ≃ 6.5) AND BROMOCRESOL PURPLE-STAINED YEAST WHICH TURNED YELLOW (pH ≃ 5.0)[a]

	Ingested yeast which turned red (pH ≃ 6.5)		Ingested yeast which turned yellow (pH ≃ 5.0)	
Incubation time (min)	Control PMN*	PMN containing casein[b]	Control PMN*	PMN containing casein[b]
60	10.3[c]	8.9	7.8	7.3
120	16.6	14.9	13.3	12.3
180	23.8	23.9	21.2	17.5
240	31.2	30.1	26.2	27.2
Significance level	$P < 0.05$	$P < 0.01$	$P < 0.05$	$P < 0.05$
Standard error of treatment mean	±4.75	±4.75	±6.53	±6.53

[a] Each value represents the mean of five cows determined in duplicate.
[b] On the average, 76.5% of the PMN had 3.2 casein-containing phagosomes.
[c] Results are percentages.
* Control PMN not significantly different ($P > 0.05$) than PMN containing casein.

times more phagocytic vacuoles that reached pH 6.5 and 2 times more that reached pH 5.0 than did PMN from milk ($P < 0.01$).

Ingestion of casein by PMN had no effect on the depression of pH in incubations up to 240 min (Table II). On the average, 76.5% of the PMN incubated with casein contained 3.2 casein-containing phagosomes. Reduction of intraphagosomal pH to 6.5 or 5.0 occurred at the same rate and in a similar number of phagosomes whether casein was present or not ($P > 0.05$).

In contrast to the ingestion of casein, ingestion of fat inhibited the subsequent depression of intraphagosomal pH (Table III). After incubation with fat globules, on the average, 72% of the PMN isolated from blood contained 1.6 fat globules per cell and reduced the pH to 6.5 in a smaller ($P < 0.05$) percentage of phagosomes than control PMN isolated from blood at all incubation times examined. PMN containing fat also reduced the pH to 5.0 in a smaller ($P < 0.05$) percentage of phagosomes after 60

TABLE III. THE EFFECT OF INGESTED FAT GLOBULES ON THE PERCENTAGE OF INGESTED NEUTRAL RED-STAINED YEAST WHICH TURNED RED (pH ≃ 6.5) AND BROMOCRESOL PURPLE-STAINED YEAST WHICH TURNED YELLOW (pH ≃ 5.0)[a]

	Ingested yeast which turned red (pH ≃ 6.5)		Ingested yeast which turned yellow (pH ≃ 5.0)	
Incubation time (min)	Control PMN*	PMN containing fat globules	Control PMN*	PMN containing fat globules
60	38.1[b]	11.7	24.0	8.5
120	54.4	24.6	25.4	22.6
180	60.4	30.4	33.8	28.1
240	65.7	37.4	39.7	22.5
Significance level	$P < 0.05$	$P < 0.05$	NS	$P < 0.05$
Standard error of treatment mean	±5.7	±5.8	±6.3	±4.5

[a] Each value represents the mean from 10 cows determined in duplicate.
[b] Results are percentages.
* Control PMN significantly greater ($P < 0.05$) than PMN containing fat.

and 240 min of incubation. However, the differences after 120 and 180 min of incubation were not significant.

Discussion. The results of this study show that the maximal depression in pH within phagocytic vacuoles of bovine PMN is to 5.0–5.5. This compares with a pH of 6.0–6.5 for the human (8), 3.5–4.5 for the mouse, rat, and rabbit (7), and 4.7–5.2 for the guinea pig (9). Reasons for these differences are not known.

The effect of the phagocytosis of fat globules on the depression of intraphagosomal pH in PMN isolated from blood indicated that ingestion of fat interfered with pH depression. However, phagocytosis of casein did not inhibit the depression of pH. Thus, the phagocytosis of fat globules by PMN in milk may largely be responsible for the deficiency in production of acid phagosomes, and for their reduced bactericidal properties when compared with PMN from blood.

The mechanism by which fat but not casein interferes with the intraphagosomal depression of pH is not clear. Cytochemical evidence has been reported (4) that indicates peroxidase-positive material in phagosomes containing only fat. Therefore, azurophil granules may be migrating to and fusing with phagosomes that contain fat, thereby reducing the concentration of pH depressing substances that reach phagosomes containing yeast. Also, it has been reported (16) that PMN in milk have reduced glycogen levels compared to PMN in blood. Thus, PMN in milk may not have sufficient energy reserves to lower the pH in all phagosomes formed. There are few data to indicate whether or not either of these hypotheses is correct.

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1. Jain NC, Lasmanis J. Phagocytosis of serum-resistant and serum-sensitive coliform bacteria (*Klebsiella*) by bovine neutrophils from blood and mastitic milk. Amer J Vet Res 39:425–427, 1978.
2. Kent GM, Newbould FHS. Phagocytosis and related phenomena in polymorphonuclear leukocytes from cow's milk. Canad J Comp Med 33:213–219, 1969.
3. Russell MW, Reiter B. Phagocytic deficiency of bovine milk leukocytes: An effect of casein. J Reticuloendothel Soc 18:1–13, 1975.
4. Paape MJ, Wergin WP. The leukocyte as a defense mechanism. J Amer Vet Med Assoc 170:1214–1223, 1977.
5. Paape MJ, Guidry AJ, Kirk ST, Bolt DJ. Measurement of phagocytosis of ^{32}P-labeled *Staphylococcus aureus* by bovine leukocytes: Lysostaphin digestion and inhibitory effect of cream. Amer J Vet Res 36:1737–1743, 1975.
6. Russell MW, Brooker BE, Reiter B. Electron microscopic observations of the interaction of casein micelles and milk fat globules with bovine polymorphonuclear leucocytes during the phagocytosis of staphylococci in milk. J Comp Pathol 87: 43–51, 1977.
7. Jensen MS, Bainton DF. Temporal changes in pH within the phagocytic vacuole of the polymorphonuclear neutrophilic leukocyte. J Cell Biol 56:379–388, 1973.
8. Mandell GL. Intraphagosomal pH of human polymorphonuclear neutrophils. Proc Soc Exp Biol Med 134:447–449, 1970.
9. Sprick MG. Phagocytosis of M. tuberculosis and M. smegmatis stained with indicator dyes. Amer Rev Tuberc Pulm Dis 74:552–565, 1956.
10. Paape MJ, Pearson RE, Wergin WP, Guidry AJ. Enhancement of chemotactic response of polymorphonuclear leukocytes into the mammary gland and isolation from milk. J Dairy Sci 60:53–62, 1977.
11. Hahn G, Tolle A. Zur bedeutung der bakteriziden aktivität boviner blut-und milchgranulozyten gegenüber B-streptokokken für die pathogenese der mastitis. Milchwissenschaft 35:466–469, 1980.
12. DeLuca HF, Cohen PP. Methods for preparation and study of tissues and enzymes. In: Umbreit WW, Burris RH, Stauffer JE, eds. Manometric Techniques. Minneapolis, Burgess, 4th ed, p132, 1964.
13. Guidry AJ, Paape MJ, Miller RH. In vitro procedure for measuring phagocytosis of blood neutrophils. Amer J Vet Res 35:705–709, 1974.
14. Dulin AM, Paape MJ, Weinland BT. Use of the cytospin centrifuge in the performance of differential milk somatic cell counts. J Dairy Sci 65:200–205, 1982.
15. Gornall AG, Bardawill CJ, David MM. Determination of serum proteins by means of the biuret reaction. J Biol Chem 177:751–766, 1949.
16. Naidu TG, Newbould FHS. Glycogen in leukocytes from bovine blood and milk. Canad J Comp Med 37:47–55, 1973.

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