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Effect of Immune Complexes from Mastitic Milk on Blocking of Fc Receptors and Phagocytosis

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Fc receptors on the surface of milk leukocytes from normal glands, bronchial leukocytes, mastocytoma P-815 cells, and murine leukemia L1210 cells were blocked significantly ($P < 0.01$) by cavian and bovine milk collected from inflamed glands (mastitic milk), their wheys, and in vitro-prepared immune complexes composed of the whey from normal milk and serum. Blocking of Fc receptors indicated the presence of immune complexes in the mastitic milk and was detected by inhibition of rosette formation with sensitized erythrocytes or attachment of the aggregated immunoglobulin G. The binding of immune complexes to these cells was also determined by staining with fluorescein isothiocyanate-labeled protein A. As the mastitis subsided, the blocking effect of the mastitic milk also declined markedly. There was no significant difference in blocking capacity between mastitic milk and its whey. The blocking capacity of normal cavian or bovine milk and their wheys was insignificant. Whey from mastitic milk also inhibited phagocytosis of opsonized staphylococci by alveolar macrophages. We suggest that the blocking of Fc receptors on phagocytic cells adversely affects phagocytosis.

Immune complexes (Ic) block Fc receptors (FcRs) for immunoglobulin G (IgG) on macrophages and thereby impair their ability to bind and phagocytize IgG-opsonized particles (6, 7, 14). Blocking by Ic composed of IgG antibody and the corresponding antigen is selective for FcRs and does not affect the complement receptor or other receptors (6). In vivo, Ic impair phagocytosis and clearance of IgG-opsonized erythrocytes. The impairment of phagocytosis is related to the concentration of Ic in the circulation (5, 8).

During inflammation of the mammary gland (mastitis), the percentage of milk leukocytes expressing FcRs is diminished significantly compared with leukocytes from normal mammary glands (20). We suspect that the milk during mastitis contains Ic capable of blocking FcRs on leukocytes. The purpose of this study was to determine the effect of Ic of lacteal origin on the blocking of FcRs on the surface of milk and bronchial leukocytes and on the inhibition of the phagocytic capacity of alveolar macrophages.

MATERIALS AND METHODS

Collection of normal and mastitic milk. Secretion (0.5 to 1.0 ml) from each mammary gland of eight guinea pigs during the first lactation was collected separately at 48 h (5 days postpartum) and 24 h before and at 6, 16, 23, 30, and 54 h after intramammary infusion with 0.5 μg of *S. abortus equi* lipopolysaccharide (LPS) (Difco Laboratories, Detroit, Mich.) per ml in 50 μl of phosphate-buffered saline (PBS). At 6, 16, and 23 h postinfusion, milk secretion was markedly reduced, and consequently, difficulty in collection occurred. Nevertheless, at least 0.5 ml of milk was collected at these sampling periods. Other details of the collection of milk samples have been described previously (20). The LPS was used as a nonspecific irritant to induce mastitis. Sixteen bovine lactating glands (4 months postpartum) were infused with 100 μg of LPS suspended in 5 ml of PBS, and milk samples were collected at 24 h before infusion and at 6, 16, and 23 h postinfusion. The LPS induced the most severe mastitis at 6 h, and then the severity of the inflammation declined. The severity of the mastitis was monitored by the number of total somatic cells in the milk. The secretion collected during inflammation is called mastitic milk. Mammary glands containing milk with more than 5×10^5 cells per ml before infusion (mastitic milk) or with bacteria (detected by inoculation of blood agar plates) were excluded from this investigation.

The milk fat separated by centrifugation ($7{,}000 \times g$ for 5 min) was discarded, and skim milk was used in this study. Whey was prepared from cavian and bovine milk by the addition of 1 N acetic acid to precipitate casein. After centrifugation, the supernatant fluid, whey, was neutralized with 1 N NaOH.

Collection of FcR-bearing cells. Milk leukocytes were separated from the secretions by centrifugation at $5{,}000 \times g$ for 1 min. Approximately 80% of the cells were viable as determined by Trypan blue dye exclusion. All smears were examined microscopically (200 cells per smear) by the same observer.

Bronchial leukocytes were obtained by a tracheobronchial lavage (16) with Eagle minimum essential medium from nonlactating guinea pigs which had not been used for collection of milk. The lavage cells contained 70% alveolar macrophages, 15% lymphocytes, and 15% eosinophils. The yield of bronchial leukocytes was approximately 30×10^6 viable cells per animal. Alveolar macrophages represented a stable population of cells with regard to the yield of the harvested cells, the percentage of FcR-bearing cells, and their phagocytic capacity.

P-815 mastocytoma cells (possess only FcRs for IgG on the surface) (4) were propagated in syngeneic DBA/2 mice (Jackson Laboratory, Bar Harbor, Maine). Each mouse was injected intraperitoneally with 10^6 cells. The mice were sacrificed within 2 weeks, and mastocytoma cells (approximately 10^9 cells per mouse) were harvested. Almost 100% of the cells were masocytoma cells, and 95% were viable. The cells were washed with sterile PBS, and a suspension at a concentration of 10^6 cells per ml was prepared.

Murine lymphocytic leukemia L1210 cells (ATCC CCL 219) were propagated in Fisher medium with 10% horse

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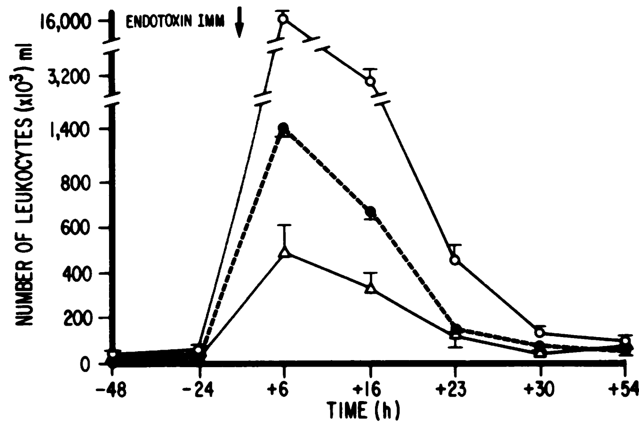

FIG. 1. Mean number ± standard error of PMNs (○), macrophages (●), and lymphocytes (△) in 16 milk samples collected before and after infusion of LPS.

serum and 0.5% gentamicin sulfate at 37°C in an incubator with a 5% CO_2, humidified atmosphere. Cells used for testing for the presence of FcRs were harvested 24 h after fresh medium had been added.

Human lymphoblastic leukemia CCRF-CEM cells (ATTC CCL 119) were propagated in Eagle minimum essential medium with 10% fetal bovine serum. These cells are T lymphocytes (FcR negative).

Detection of antibody to whey components. Serum samples from five females (collected after completion of the third lactation) and five males of comparable age were examined for presence of antibodies to cavian whey from normal milk. The examination was performed by an indirect hemagglutination test (tannic acid) (1) in a microtiter U-shaped plate (Flow Laboratories, Inc., McLean Va.).

Preparation of Ic in vitro. Whey from normal cavian milk was used to form complexes with cavian serum. Sera were collected from blood of normal nonlactating females or males. The inactivated undiluted and diluted (1:2, 1:8, 1:16, 1:32, and 1:64) sera were mixed with equal volumes of whey. The mixtures were incubated in a water bath at 37°C for 40 min and then overnight at 4°C. The preparations were mixed thoroughly after overnight incubation and used for blocking of FcRs.

Preparation of indicator erythrocytes for FcRs. Sheep erythrocytes were washed three times with PBS. A 10% suspension of erythrocytes was mixed with a subagglutinating dose of the IgG fraction of rabbit anti-erythrocyte serum (Cordis Laboratories, Miami, Fla.). This erythrocyte suspension, at a concentration of 2% (vol/vol), was then incubated at 37°C for 1 h; the sensitized erythrocytes (EAs) were subsequently washed three times with PBS and resuspended in PBS to a final concentration of 0.5% (vol/vol) (19).

Blocking of FcRs by mastitic milk and Ic. The presence of Ic in mastitic milk was detected by blocking of FcRs. Blocking of FcRs on the bronchial leukocytes, milk leukocytes, and P-815 mastocytoma cells was determined by an inhibition of rosette formation with EAs. Various dilutions of in vitro-prepared Ic, skim milk, or whey from mastitic milk (150 μl each) were mixed with 20 μl of suspended FcR-bearing cells (5×10^6 cells per ml). The mixture was then incubated at 4°C for 1 h. This mixture was washed in PBS, and 50 μl of EAs was added to the pelleted cells. Subsequently, the preparation was mixed and centrifuged at $500 \times g$ for 5 min. Inactivated serum, PBS, normal skim milk, and normal skim milk whey served as controls in each test. The pelleted cells were incubated at 4°C for 1 h and then gently suspended. The number of rosette-forming cells (three or more EAs per cell) per 100 cells was determined. Smears were subsequently prepared from each suspension and stained, and differential cell counts were performed.

Detection of Ic or aggregated IgG by FITC-labeled protein A or anti-IgG. Attachment of Ic from the mastitic milk to bronchial leukocytes, P-815 cells, L1210 cells (FcR positive), and human leukemia cells (FcR negative) was also detected by fluorescein isothiocyanate (FITC)-labeled protein A (E-Y Laboratories, Inc., San Mateo, Calif.). Protein A binds with a high affinity to the Fc portion of IgG (13). These cells (20 μl), at a concentration of 5×10^6 cells per ml, were incubated at 4°C for 1 h with 150 μl of the whey from cavian normal or mastitic milk. Some of these preparations were washed and incubated at 4°C for 30 min with 50 μl of FITC-labeled protein A. Other preparations of cells were subsequently incubated at 4°C for 1 h with 50 μl of human aggregated IgG. The aggregated IgG was prepared by heating monomeric human IgG (Miles Laboratories, Inc., Kankakee, Ill.) at a concentration of 10 mg/ml in a water bath at 63°C for 20 min and subsequently centrifuging the mixture at $100{,}000 \times g$ for 1 h. The pellet containing aggregated IgG was then dissolved in PBS for use. After the second incubation, the cells were washed and incubated at 4°C for 30 min with 50 μl of the FITC-labeled $F(ab')_2$ fraction of goat anti-human IgG (Pel-Freez Biologicals, Rogers, Ark.). Cells incubated with FITC-labeled protein A or the $F(ab')_2$ fraction of goat anti-human IgG were washed twice in RPMI 1640 medium and then twice in PBS at 4°C. One

TABLE 1. Blocking of FcRs by skim milk and whey from cavian mastitic milk

Sampling periods (h)[a]	% EA rosette formation by[b]:					
	Milk leukocytes with:		Bronchial leukocytes with:		P-815 cells with:	
	Skim milk	Whey	Skim milk	Whey	Skim milk	Whey
−48	43.0 ± 4.4	37.5 ± 2.1	37.8 ± 2.6	39.8 ± 3.9	41.1 ± 4.5	41.0 ± 6.4
−24	ND[c]	ND	38.0 ± 2.9	40.6 ± 2.6	43.9 ± 4.5	41.8 ± 3.7
+6	18.5 ± 3.7	14.3 ± 3.3	23.5 ± 1.7	23.6 ± 1.8	21.0 ± 3.6	22.4 ± 2.1
+16	ND	ND	24.1 ± 1.7	22.6 ± 2.0	18.6 ± 3.6	19.8 ± 1.6
+23	ND	ND	29.4 ± 1.8	30.3 ± 1.2	24.7 ± 3.9	32.0 ± 4.9
+30	ND	ND	33.0 ± 2.0	33.2 ± 1.7	35.4 ± 2.4	38.2 ± 2.6
+54	ND	ND	40.5 ± 0.3	37.0 ± 2.8	47.8 ± 5.0	50.2 ± 3.5
Control (PBS)	41.8 ± 3.0	41.8 ± 3.0	46.1 ± 2.8	46.1 ± 2.8	48.7 ± 3.3	49.2 ± 4.3

[a] Milk samples (16) collected before (−) and after (+) LPS infusion.
[b] Mean percentage ± standard deviation.
[c] ND, Not done.

TABLE 2. Blocking of FcRs by skim milk and whey from bovine mastitic milk

Sampling periods (h)[a]	% EA rosette formation by[b]:				
	Milk leukocytes with whey	Bronchial leukocytes with:		P-815 cells with:	
		Skim milk	Whey	Skim milk	Whey
−24	41.3 ± 1.5	41.3 ± 10.0	42.2 ± 7.0	42.8 ± 3.5	42.4 ± 3.4
+6	21.5 ± 3.5	28.2 ± 5.2	18.4 ± 5.2	22.6 ± 3.4	29.8 ± 6.2
+16	22.0 ± 1.4	29.8 ± 3.8	23.2 ± 4.2	21.2 ± 4.0	33.6 ± 3.5
Control (PBS)	45.0 ± 4.0	56.5 ± 6.9	56.5 ± 6.9	43.7 ± 2.3	43.7 ± 2.3

[a] Milk samples (16) collected before (−) and after (+) LPS infusion.
[b] Mean percentage ± standard deviation.

drop of the suspension was placed on a microscope slide, and 300 cells were observed for staining. Cells incubated with medium and then stained with FITC-labeled protein A or anti-IgG served as an additional control.

Inhibition of phagocytosis by mastitic milk. Phagocytosis by alveolar macrophages was measured in Linbro chambers. A suspension of bronchial leukocytes (50 μl) (5×10^4 cells) was distributed into each chamber, and the cells were allowed to adhere to the glass. After 2 h of incubation, nonadherent cells were removed by gentle washing with Eagle minimum essential medium, and 50 μl of whey from normal (48 and 24 h preinfusion) and mastitic (6, 16, 23, 30, and 54 h postinfusion) milk from guinea pigs was added. The cells were incubated for 45 min and washed with Eagle minimum essential medium, and 50 μl of a suspension (10^7 cells per ml) of heat-killed *Staphylococcus aureus* opsonized with inactivated cavian antistaphylococcal serum was added to each chamber. This strain of *S. aureus* (Smith strain, ATCC 13709) has an antiphagocytic capsule, and phagocytosis is minimal without opsonization. The culture was then centrifuged at 500 × *g* for 5 min and incubated for 30 min. Subsequently, 32 IU of lysostaphin (Sigma Chemical Co., St. Louis, Mo.) was added to each chamber, and the mixture was incubated at 37°C for 1 h to lyse the extracellular staphylococci. The cultures were then washed, fixed in 10% Formalin, and stained with Wright stain. The percentage of macrophages with phagocytized bacteria was calculated from observations of 100 cells, and the number of phagocytized bacteria per 100 cells was counted.

Statistical analysis. Experimental results were expressed as the mean ± standard deviation. The significance was evaluated by the Student *t* test.

RESULTS

Detection of FcR-bearing cells. The leukocytes from normal cavian milk were composed of approximately 30% polymorphonuclear lymphocytes (PMNs), 35% macrophages, and 35% lymphocytes (Fig. 1), whereas leukocytes from normal bovine milk were composed of 55% PMNS, 31% macrophages, and 14% lymphocytes. Approximately 50% of the leukocytes from normal cavian and bovine milk had FcRs on their surfaces. Of the cavian FcR-bearing leukocytes, 61 ± 1% were PMNs, 23 ± 11% were macrophages, and 15 ± 11% were lymphocytes. Bovine FcR-bearing leukocytes were composed of 30 ± 8% PMNs, 58 ± 9% macrophages, and 12 ± 4% lymphocytes. Approximately 50% of the bronchial leukocytes, 55% of the P-815 mastocytoma cells, 65% of the murine leukemia L1210 cells, and 0% of the human leukemia cells formed EA rosettes (indicator for FcR-bearing cells).

Blocking of FcRs by mastitic milk. Cavian skim milk and whey from normal milk (24 and 48 h preinjection) slightly inhibited EA rosette formation with bronchial leukocytes, P-815 cells, and leukocytes from normal milk (Table 1). The blocking of FcRs on milk leukocytes, bronchial leukocytes, and P-815 cells was significant ($P < 0.01$) with skim milk and whey from mastitic milk of eight guinea pigs collected at 6 to 23 h postinjection. Skim milk and whey from milk collected at 54 h postinjection possessed minimal blocking capacity. There was only a small difference in the blocking capacity between whey and skim milk (Table 1). Similar results were obtained with bovine skim milk and whey from normal (24 h preinjection) and mastitic (6 and 16 h postinjection) milk from 16 glands (Table 2). Cavian and bovine mastitic skim milk and whey from mastitic milk at a dilution to 1:2 significantly ($P < 0.05$) inhibited EA rosette formation with P-815 cells and bronchial leukocytes (Table 3).

TABLE 3. Blocking of FcRs on cells by various dilutions of skim milk and whey from bovine and cavian mastitic milk[a]

Milk (dilution)	% EA rosette formation by[b]:		
	Bovine		Cavian skim milk
	Skim milk	Whey	
Mastitic	25.3 ± 3.1	23.3 ± 4.6	20.0 ± 2.1
1:2	36.5 ± 1.3	33.3 ± 4.6	22.3 ± 4.3
1:4	38.3 ± 1.0	40.0 ± 1.8	41.2 ± 2.2
Normal	40.3 ± 3.0	39.0 ± 0.8	47.0 ± 3.5
Control (PBS)	45.0 ± 0.8	45.0 ± 0.8	53.3 ± 5.2

[a] Experiments with bovine skim milk and whey were performed with bronchial leukocytes, whereas experiments with cavian skim milk were performed with mastocytoma cells (P-815).
[b] Mean percentage ± standard deviation of seven samples.

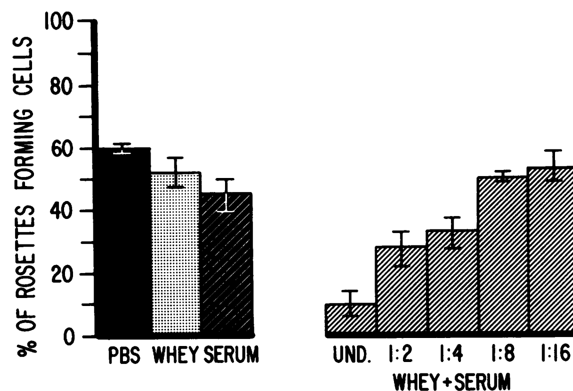

FIG. 2. Mean percent ± standard deviation of blocking of EA rosette formation with mastocytoma cells (P-815) by five preparations of Ic formed in vitro with cavian whey and cavian serum or its dilutions. Und, Undiluted.

TABLE 4. Binding of Ic from cavian mastitic whey to FcRs detected with FITC-labeled protein A or by inhibition of attachment of the aggregated IgG[a]

Incubation[b]	Binding of Ic or aggregated IgG detected with FITC-labeled[c]:	
	Protein A	Anti-IgG
Whey from mastitic milk with:		
Bronchial leukocytes	+	−
Mastocytoma cells	+	−
Murine leukemia cells	+	−
Human leukemia cells	−	−
Whey from normal milk with:		
Bronchial leukocytes	−	+
Mastocytoma cells	−	+
Murine leukemia cells	−	+
Human leukemia cells	−	−

[a] Some of the cells (after incubation with whey) were stained with FITC-labeled protein A, and the rest of the cells were subsequently incubated with the aggregated IgG and stained with FITC-labeled anti-IgG.

[b] Bronchial leukocytes, mastocytoma cells, and murine leukemia cells are FcR-bearing cells; human leukemia cells are FcR-negative cells.

[c] Positive (+) or negative (−) results from all seven tested samples.

Blocking of FcRs by in vitro-prepared Ic. The inactivated sera from females at dilutions of up to 1:32 (one serum at 1:8, three sera at 1:16, and one serum at 1:32) agglutinated the erythrocytes coated with whey from normal cavian milk. In contrast, only one of five sera from males agglutinated at a dilution of 1:2. The sera from females mixed with cavian whey from normal milk blocked FcRs on P-815 cells. The EA rosette formation was significantly ($P < 0.05$) inhibited by complexes formed with undiluted or diluted (1:4) serum and undiluted whey (Fig. 2). A 1:8 dilution of serum mixed with whey formed undetectable quantities of Ic by this assay. A mixture of whey from normal milk with sera from males did not inhibit EA rosette formation.

Detection of Ic or aggregated IgG by FITC-labeled protein A or anti-IgG. Ic were detected on bronchial leukocytes, P-815 cells, and L1210 cells but not on human leukemia cells after incubation with whey from cavian mastitic milk (Table 4). Incubation of these cells with whey from mastitic milk inhibited binding of aggregated IgG. In contrast, aggregated IgG readily bound to bronchial leukocytes, P-815 cells, and L1210 cells which had been incubated with whey from normal milk. The cells stained with FITC-labeled protein A were not observed after incubation with serum or medium.

Inhibition of phagocytosis by mastitic milk. Incubation of alveolar macrophages with whey from mastitic milk (6 and 16 h postinjection) significantly ($P < 0.01$) decreased the percentage of cells phagocytizing and the number of staphylococci ingested (Table 5). In contrast, there was no difference in the percentage of cells phagocytizing and the number of ingested staphylococci between cells incubated in medium and cells incubated in the whey from normal milk.

DISCUSSION

The blocking of FcRs for IgG by Ic on phagocytic (15, 17), lymphoid (18), and mastocytoma (19) cells is utilized in detecting soluble Ic in the body fluids. The Fc portion of IgG complexed with the corresponding antigen or heat-aggreated IgG selectively binds to heterologous or homologous FcR-bearing cells. Attachment of the uncomplexed, monomeric IgG molecules is limited (11). The binding of IgG to FcRs is detected by their blocking so indicator cells (IgG-sensitized erythrocytes) or labeled aggregated IgG cannot attach to FcRs. In the present study, mastitic skim milk or its whey significantly blocked FcRs on leukocytes, mastocytoma cells, and murine leukemia cells (Tables 1, 2, and 4), suggesting the presence of Ic. Binding of Ic from mastitic milk to FcRs was also detected with FITC-labeled protein A. In contrast, binding of Ic from mastitic milk to human leukemia cells (FcR negative) was not observed. This observation indicates the specificity of binding of Ic from mastitic milk to FcRs.

In vitro-prepared Ic were formed by mixing the homologous (containing antibody to milk components) serum with whey from normal milk. The concentration of serum antibody against whey components was relatively low, which was consistent with results from other studies (2, 3). These Ic blocked FcRs on leukocytes and P-815 cells in a manner similar to that of skim milk or whey from mastitic milk. However, the mixture of whey from normal milk with cavian serum from males (no antibody to milk components) did not block FcRs on leukocytes, P-815 cells, or L1210 cells, indicating the necessity of antibody for formation of a blocking factor. During inflammation of the mammary gland, a large quantity of serum components including antibodies against milk components penetrates into the secretion. Therefore, serum antibody in the mammary gland can react with mild components to form Ic. In normal milk, there is a very low concentration of serum components, and consequently, Ic are usually undetectable. In contrast, colostrum contains a large amount of serum components and, consequently, Ic (10). Although there was no direct evidence, these observations suggested that mastitic milk contained Ic composed of serum-IgG antibody and the corresponding antigen of lacteal origin.

Attachment of Ic to FcRs on leukocytes profoundly impairs their ability to phagocytize IgG-opsonized particles (6, 8, 14). Similar inhibition of the phagocytosis of opsonized staphylococci by macrophages pretreated with whey from mastitic milk was observed in this study. The percentage of alveolar macrophages with ingested staphylococci and the number of ingested bacteria was significantly reduced. The mechanism of the inhibition of phagocytosis of the IgG-opsonized particles by Ic is unknown. Michl et al. (14) suggested that the attachment of Ic to some FcRs inhibits the function of the remaining FcRs because of their relocation to the Ic-binding area of the plasma membrane.

On the surface of bovine and cavian macrophages, there are FcRs for the homologous IgG1 and IgG2 and FcRs for rabbit immunoglobulin (9, 12). The latter receptors were blocked by cavian and bovine mastitic milk and by in vitro-prepared Ic made of cavian whey (antigen) and serum (Tables 1 and 2; Fig. 2). Therefore, FcRs for rabbit IgG on bovine or cavian leukocytes could be one of the receptors for homologous immunoglobulin (IgG1 or IgG2) or could be located in close vicinity to both of them. Rabbit IgG ex-

TABLE 5. Inhibition of phagocytosis by cavian mastitic milk[a]

Sampling periods (h)	% of macrophages phagocytizing	No. of bacteria per 100 macrophages
−48	66.0 ± 15.7	202.5 ± 51.2
−24	66.3 ± 3.2	158.0 ± 51.7
+6	34.9 ± 16.1	70.1 ± 45.1
+16	25.0 ± 11.4	48.6 ± 25.0
+23	52.0 ± 11.3	192.0 ± 4.2
Control (PBS)	59.0 ± 16.8	202.0 ± 83.2

[a] Mean percentage ± standard deviation of seven milk samples in each period.

presses the widest spectrum of cross-reactivity with FcRs on heterologous leukocytes (11).

The results of this study contribute to a better understanding of the pathogenesis of inflammation in the mammary gland. A large amount of soluble blood components (including immunoglobulins) transudes to the mammary gland secretion during inflammation. Serum antibody against milk components (2, 3) and milk components may form Ic that apparently attach to FcR-bearing leukocytes in the milk. These leukocytes consequently have reduced capacity to phagocytize opsonized bacteria through FcRs. However, phagocytosis by use of nonspecific receptors should not be altered by Ic. In addition, the attachment and ingestion of Ic stimulates the release of proteolytic enzymes and metabolic products from phagocytes which subsequently may potentiate the severity of inflammation (21).

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