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Bactericidal Mechanisms of Human Breast Milk Leukocytes

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The functional capacity of human breast milk phagocytes was evaluated with both bactericidal and biochemical assays. Acridine orange was used as a vital stain for bacteria to directly visualize phagocytosis and killing. Bactericidal capabilities were further examined by colony count and chemiluminescent methods. Cytocentrifuged specimens stained for myeloperoxidase exhibited enzyme activity in breast milk leukocytes equal to that of peripheral neutrophils. A radioisotopic assay of hexose monophosphate shunt activity demonstrated metabolic activity in breast milk leukocytes greater than that in peripheral blood neutrophils. However, the chemiluminescent response of breast cells was negligible, apparently the result of quenching secondary to fat present in the milk; preincubation of human blood leukocytes with the fatty layer of breast milk produced similar inhibition in the chemiluminescence assay. By most parameters, breast milk phagocytes are at least equal to blood neutrophils.

Human milk contains antibodies, opsonins, and cellular components which can provide essential aspects of immunity for neonates, whose host defenses are deficient by many parameters (10, 15, 16, 18). A direct clinical correlate is the observation that infectious processes, particularly gastrointestinal disease, are less common in breast-fed infants (5, 7, 9, 13, 14). The present study was designed to define mechanisms by which breast milk cells phagocytize and kill bacteria and to better elucidate the methods by which breast milk might offer such protection.

We recently noted that leukocytes obtained from breast milk did not appear to generate a chemiluminescent response after initiation of phagocytosis of zymosan or a variety of bacteria. However, resting metabolic activity was actually greater than that of peripheral neutrophils, as measured by reduction of Nitro Blue Tetrazolium, and bactericidal capacity, as measured by colony count and acridine orange assays, was also excellent. These observations therefore suggested either that killing by breast milk cells is not accomplished by the usual mechanism of the generation of hydrogen peroxide and superoxide after oxidation of glucose via the hexose monophosphate (HMP) shunt or that these metabolic processes are in fact present in milk cells but that the measurement of a chemiluminescent response is being inhibited. Therefore, this study was designed to test whether HMP shunt activity is indeed present in breast milk cells and, if so, what factor(s) is responsible for the negligible chemiluminescence observed.

Our experimental data demonstrated the presence of HMP shunt activity in breast milk cells and suggested that the chemiluminescence normally present as a by-product of metabolic activity during phagocytosis and intracellular killing is being quenched by the large amount of ingested fat found in breast milk cells.

MATERIALS AND METHODS

Collection of colostrum and milk samples. Breast colostrum and milk were obtained from 60 lactating mothers by either manual or breast pump expression and placed in sterile plastic containers. These were transported at room temperature and were examined in the assays described below within 2 h.

Separation of peripheral blood leukocytes. Heparinized peripheral blood was obtained by venipuncture from normal volunteer donors. Leukocytes were isolated by 6% dextran sedimentation. Cells were washed twice in Gey balanced salt solution, quantitated in a hemacytometer, and suspended in Krebs-Ringer bicarbonate solution. A cell differential was accomplished with Wright stain of cytocentrifuged slides. The final cell concentration used was 5.0×10^6 polymorphonuclear leukocytes (PMNs) per ml. Assays with peripheral blood PMNs served as a basis of comparison for data collected from colostral and milk cells.

Quantitation of the cellular components in colostrum and breast milk. Sixty colostrum and milk samples were diluted 1:2 with Gey balanced salt solution and centrifuged at $500 \times g$ for 20 min at 12°C. During centrifugation, milk separates into three layers: a cell pellet, middle layer, and upper fat layer. The top two layers were aspirated, and the cell pellet was washed three times with Gey balanced salt solution and suspended in Krebs-Ringer bicarbonate solution. Cell differentials were determined with acridine orange stains of the suspensions. Cells were quantitated in a hemacytometer and diluted to a concentration of 5.0×10^6 PMNs/ml.

Assays of PMN function. Nitro Blue Tetrazolium dye reduction was performed by the semiquantitative slide method (8). Phagocytosis and killing of bacteria were first measured by the bactericidal method of Quie et al. (17). An assay employing acridine orange as a vital stain for bacteria was then used to directly visualize phagocytic and killing processes. Live bacteria stain green with acridine orange, and the color changes to bright red when the bacteria are killed by phagocytes. Details of this assay were previously reported (8a, 19). Briefly, monolayers were first prepared by placing 5 drops of colostrum, milk, or peripheral blood onto flat glass cover slips and allowing attachment of these cells during an incubation of 1 h at 37°C in 5% CO_2. The supernatant fluid was then removed by gentle washing with normal saline, and 2 drops of preopsonized bacterial suspension containing approximately 10^8 bacteria per ml in phenol-free Hanks balanced salt solution with 10% autologous human serum were placed on the cell monolayers. Cover slips were then incubated on a shaker at 37°C for 90 min. After incubation, monolayers were washed twice with normal saline and stained for 1 min with 0.14% acridine orange in Gey balanced salt solution. Cover slips were mounted face down and examined by fluorescent microscopy. Approximately 100 neutrophils were counted, and the total number of bacteria phagocytized and the percent killed (red) and percent alive (green) were recorded.

Measurement of HMP shunt activity. Experiments were carried out in Warburg flasks (20) at 37°C. In the main compartment of each flask, incubation mixtures were prepared in the following manner. To the blank, 5 ml of Krebs-Ringer bicarbonate solution and 1 μCi of [^{14}C]glucose at the C-1 or C-6 position (New England Nuclear Corp., Boston, Mass.) were added; to each resting flask, 4 ml of Krebs-Ringer bicarbonate solution, 1 ml of leukocyte suspension (5×10^6 PMNs/ml), and 1 μCi of [^{14}C]glucose were added; to the stimulated flask, 3 ml of Krebs-Ringer bicarbonate solution, 1 ml of leukocyte suspension (5×10^6 cells per ml), 1 μCi of [^{14}C]glucose, and 1 ml of zymosan or bacterial suspension were added. The blank, resting, and stimulated flasks were gassed for 30 s with 5% CO_2 and then stoppered with serum caps. Each of the serum caps was pierced with a 26-gauge needle for 30 s. The flasks were then placed in a 37°C incubator for 1 h and on a shaker (S/P Tektator V) for 55 min at 120 rpm. At the end of the incubation period, 0.3 ml of 2 N NaOH was added to the center well of each flask with a 2-in. (ca. 5.08-cm), 20-gauge needle. The reaction was terminated after 5 min with the addition of 0.2 ml of 6 N H_2SO_4. Liberated $^{14}CO_2$ was allowed to absorb into the NaOH for 60 min at 37°C on the shaker.

At the end of the incubation, 0.2 ml of NaOH was removed from the center well and added to 1.8 ml of distilled water. This solution was added to 8 ml of Bray scintillation fluid and counted on a Packard Tri-Carb scintillation counter. These experiments were repeated six times with similar results.

Preparation of bacteria. Stock cultures of bacteria stored at −70°C in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) with glycerol were thawed, isolated into 10 ml of Trypticase soy broth, and incubated for 18 h at 37°C. Cultures were centrifuged and washed twice in Gey balanced salt solution and suspended to 1 ml. The final concentration used was 0.5×10^9 to 1.0×10^9 bacteria per ml.

Measurement of chemiluminescent response. Chemiluminescent activity was measured on colostral and breast milk cells as described by Allen et al. (3) with zymosan, *Staphylococcus aureus*, and *Salmonella enteritidis* as phagocytized particles. Isolated peripheral PMNs were run simultaneously as a control. Peripheral PMNs incubated for 2 h with the fat layer of breast milk were also evaluated by chemiluminescence in an effort to ascertain whether the fat normally ingested by breast milk cells might be responsible for quenching normal responses.

After incubation with breast milk fat, peripheral cells were examined with Wright stain.

Myeloperoxidase assay. Myeloperoxidase was assayed with both breast milk cells and peripheral PMNs in a semiquantitative assay as described by Kaplow (11).

RESULTS

HMP shunt activity. Table 1 summarizes the HMP shunt activity for breast milk and peripheral PMNs stimulated by either zymosan or selected bacteria. In typical experiments, control cells (peripheral PMNs) generated CO_2 from

TABLE 1. *$^{14}CO_2$ production from [1-^{14}C]glucose and [6-^{14}C]glucose*

Glucose	cpm/10^6 cells per 10 min		% Change	C-1/C-6 ratio	
	Resting cells	Stimulated cells		Rest	Stimulated
[1-^{14}C]glucose					
Breast milk cells with zymosan	19,918	92,956	367		
Peripheral PMNs with zymosan	5,281	79,481	1,405		
Breast milk cells with *Salmonella*	58,080	152,781	163		
Breast milk cells with *S. aureus* 502A	19,651	48,936	149		
[6-^{14}C]glucose					
Breast milk cells with zymosan	1,722	4,864	182	11.6	19.1
Peripheral PMNs with zymosan	1,988	4,666	134	2.6	17.0
Breast milk cells with *Salmonella*	1,197	5,138	329	48.5	29.7
Breast milk cells with *S. aureus* 502A	1,533	2,206	43	12.8	22.2

glucose C-6 to a level 134% over the base line, whereas the conversion of glucose C-1 to CO_2 increased 1,405%; these results are consistent with previous studies (19). With breast milk cells, the conversion of glucose C-6 to CO_2 increased 182% as a result of phagocytosis, whereas the conversion of glucose C-1 to CO_2 increased 367% above the resting state. These data demonstrate that the resting values of HMP shunt activity in breast milk cells are significantly greater than those of peripheral blood leukocytes; this accounts for the smaller percent change from resting to stimulated cells and the higher C-1/C-6 ratio demonstrated for breast milk cells at rest.

Chemiluminescence. Figure 1 demonstrates only negligible chemiluminescence with zymosan for colostrum and breast milk phagocytes as compared with the control cells. Results were similar with *S. aureus* and *Salmonella*. This suggests a quenched chemiluminescent response by the control cells after being incubated for 2 h with the fatty layer of breast milk. Examination of Wright-stained smears revealed abundant ingested fat in these phagocytes.

Myeloperoxidase. Myeloperoxidase staining of cytocentrifuged prepared smears showed that the number of myeloperoxidase granules present in breast milk cells was comparable to that seen upon examination of smears of peripheral blood leukocytes.

Neutrophil function assays. Resting metabolic activity in colostral PMNs was greater than that for peripheral PMNs as measured by the Nitro Blue Tetrazolium assay. Averages of 24% (range, 11 to 34%) of colostral PMNs and 9% (range, 2 to 17%) of blood PMNs were Nitro Blue Tetrazolium positive.

Both bactericidal assays demonstrated vigorous phagocytic and killing capacity of colostral and milk cells against selected bacterial organisms (Table 2). The activity was comparable to that of peripheral PMNs with the colony count method, but the acridine orange assay, a bacterium-saturated system, revealed that under these experimental conditions breast cells phagocytize more bacteria than do peripheral PMNs.

DISCUSSION

Phagocytosis of peripheral PMNs is accompanied by an increase in glycolysis-lactate production, O_2 uptake, HMP shunt activity, and production of hydrogen peroxide, superoxide, singlet oxygen, and the hydroxyl radical. Results from past studies indicate that these changes are associated with normal intracellular bactericidal activity (1-4, 6). Other methods of killing involve glucose-6-phosphate dehydrogenase, myelo-

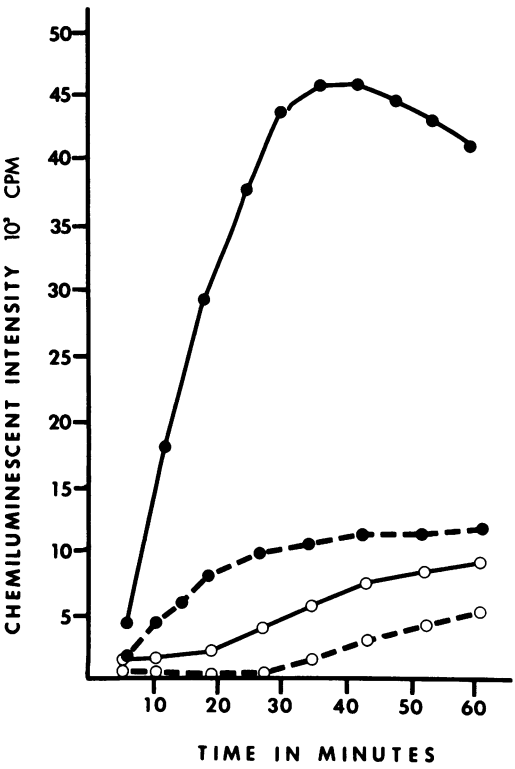

FIG. 1. *Temporal traces of chemiluminescence from 5 × 10^6 PMNs after the addition of opsonized zymosan. Zymosan added to buffer alone and then combined with the leukocytes yielded no chemiluminescent response at any time interval studied. Symbols: (●——●) peripheral PMNs; (○——○) peripheral PMNs preincubated with breast milk fat; (●- - -●) breast milk PMNs; (○- - -○) PMNs with inactivated serum (opsonin control). Each point is the mean of triplicate determinations from the same donor. Results were consistent for 12 similar experiments.*

TABLE 2. *Phagocytosis and killing of S. enteritidis by the colony count and acridine orange staining methods*

Cell source	% Killing by:			Phagocytic index[a] by acridine orange	
	Colony count	Acridine orange			
		Neutrophils	Macrophages	Neutrophils	Macrophages
Peripheral blood	95.7	87		1.9	
Colostrum	94.5	73	99	3.5	4.7
Milk	91.2	55	84	2.5	3.2

[a] Total number of intracellular bacteria divided by the number of cells counted. Results represent the mean values for nine normal donors, each done in triplicate.

peroxidase, and other enzymes in neutrophils. The relationship between HMP shunt activity, H_2O_2 formation, and intracellular killing of bacteria has been well defined (2, 4). It has been suggested that neutrophils are capable of converting reduced nicotinamide adenine dinucleotide phosphate in the presence of O_2 to H_2O_2 (2). The H_2O_2 combines with granule-bound myeloperoxidase and a halide to form a bactericidal complex. This myeloperoxidase-halide peroxide system has been demonstrated to produce chemiluminescence, the relaxation of electronically excited molecules resulting from the singlet oxygen-mediated oxidative destruction of the ingested organism (1). Chemiluminescence from phagocytizing PMNs has been shown to correlate well with glucose oxidation via the HMP shunt (4).

It was our purpose in the present study to examine the bactericidal mechanisms of human breast milk leukocytes by evaluating breast milk cells for the above changes in metabolic activity known to occur in peripheral blood leukocytes during phagocytosis. On the basis of a model system, the theoretical ratios of the $^{14}CO_2$ from [1-^{14}C]glucose and [6-^{14}C]glucose have been estimated as a function of the different pathways of metabolism of glucose. Through the increased HMP activity, phagocytosis induces a greater increase in CO_2 production from [1-^{14}C]glucose relative to [6-^{14}C]glucose (12, 20). For breast milk cells, the conversion of glucose C-6 to CO_2 increased 182% as a result of phagocytosis, whereas the conversion of glucose C-1 to CO_2 increased 367% from resting to stimulated cells. The resting values of HMP shunt activity in breast milk cells were greater than those of peripheral blood leukocytes. This accounts for the smaller percent change from resting to stimulated cells and also explains the larger C-1/C-6 ratio in resting breast milk cells. Our data also show elevated resting values for the Nitro Blue Tetrazolium dye reduction assay, indicating that phagocytes in colostrum and breast milk are more metabolically active than those in peripheral blood. This may be the result of the active phagocytosis of fat particles by breast milk cells. Also, essentially all colostrum and milk samples are colonized by bacteria, regardless of the care in collection, and such contamination may elevate phagocytic and resultant metabolic activity.

It is apparent that there is increased HMP shunt activity in breast milk phagocytes. Also, metabolic activity, as evidenced by the reduction of Nitro Blue Tetrazolium, and the killing of bacteria, as measured by a standard colony count assay and an acridine orange assay, were apparent. However, leukocytes obtained from breast milk failed to generate electronically excited molecules, as shown by a negligible chemiluminescent response after initiation of phagocytosis by zymosan or selected bacteria.

When peripheral PMNs were incubated with the fatty layer of breast milk and examined for chemiluminescent activity after phagocytosis, data clearly showed an inhibition of the normally vigorous response. This discrepancy may be attributed to the phenomenon known as quenching. This term is applied to any factor that reduces the light output or photon production in the system. Ingested fat in the PMN, as we have shown, quenches the reaction of chemiluminescence either by some chemical interaction of the compounds contained in the counting solution or more likely by the absorption by the sample itself of the light given off.

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