Effect of mammary secretions on functions of polymorphonuclear leukocytes in pigs

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Objective—To determine the effects of porcine mammary secretions on polymorphonuclear (PMN) leukocyte function and to relate concentrations of estradiol-17β and cortisol in mammary secretions to PMN cell function.

Sample Population—Mammary secretions from 10 healthy sows and blood PMN leukocytes from 27 healthy sows.

Procedure—Mammary secretions were collected within 24 hours after parturition (colostrum) and 12 to 13 days later (milk). Chemoattractant properties were assessed by use of a cell migration assay. Phagocytic capacity of PMN cells in colostrum and milk was assessed by recording chemiluminescence following phagocytosis of Escherichia coli or zymosan. Estradiol-17β and cortisol concentrations were determined by use of radioimmunoassays.

Results—Chemoattractant properties of colostrum and milk were significantly greater than that of zymosan-activated serum. However, chemoattractant properties did not differ significantly between the 2 types of secretions. The capacity of PMN cells in colostrum to phagocytose either zymosan or E. coli was less, compared with cells in milk, and the ability of cells in either type of mammary secretion to phagocytose E. coli was greater than the ability to phagocytose zymosan. Concentrations of estradiol-17β and cortisol were greater in colostrum, compared with milk. No clear relation was evident between PMN cell activity and hormone concentrations in mammary secretions.

Conclusions and Clinical Relevance—Although chemoattractant properties of colostrum and milk did not differ, the phagocytic capacity of PMN cells in colostrum was significantly less than that of cells in milk. This may predispose sows to coliform mastitis during the early postpartum period. (Am J Vet Res 2001;62:1250–1254)

Polymorphonuclear (PMN) leukocytes are of vital importance in the defense against bacterial infections. This is illustrated by the fact that PMN leukocyte dysfunction or lack of PMN leukocytes can lead to recurrent and life-threatening infections.1-4 In healthy animals, mature PMN cells comprise circulating and marginating pools from which cells are recruited to fight a local infection. Within minutes, PMN cells attach to the endothelium, migrate through the tissues toward a chemoattractant gradient to the site of infection, and finally phagocytose and destroy the microbe.3

Leukocyte numbers and function and, consequently, host defense mechanisms against infection with microorganisms can vary according to nutritional state, genetic factors, and hormonal concentrations.5-8 Certain immune functions are regulated by steroid hormones, which in turn are known to vary in concentration according to reproductive status.9-11 Reproductive status may thus affect host immune functions, which can lead to an increase or decrease in resistance to infections. Indeed, in pigs, coliform mastitis develops exclusively at parturition.

Results of previous studies in pigs indicate that blood leukocyte count and leukocyte immune functions change considerably around parturition. For instance, there is evidence that monocyte and lymphocyte function is impaired at parturition.12 Functions and numbers of leukocytes in mammary secretions also change over time.13-14 However, changes affecting cells in mammary secretions do not always parallel those affecting cells in blood.10,11 This emphasizes the need to assess local defense mechanisms in the mammary gland when studying the pathogenesis of coliform mastitis in sows.

The reasons for differences in functional capacity of PMN leukocytes in mammary secretions and blood are not fully understood. Mammary secretions may have a direct effect on PMN cells, or cells may be affected by factors in mammary gland tissue. Alternatively, PMN cell function may be altered as a result of changes within the cell caused by the migration of the cell from the blood stream to the lumen of the mammary gland. In cows, casein and fat in mammary secretions are reported to impair PMN leukocyte function.14-15 Likewise, blocking cell-surface Fc receptors and diapedesis16 can reduce activity of bovine PMN cells. However, to our knowledge, there are no reports on the direct effect of mammary secretions on PMN function in sows.

Against this background, we compared the effect of mammary secretions on 2 essential PMN cell functions, namely migration across a chemoattractant gradient and phagocytosis. Samples of mammary secretions from healthy sows were collected at parturition (a time when sows often develop coliform mastitis) and 2 weeks after parturition (a time when sows rarely develop coliform mastitis). In an attempt to relate PMN cell activity to steroid hormone concentrations, concentrations of estradiol-17β and cortisol were also determined in mammary secretions.

Materials and Methods

Animals—Ten lactating Swedish Yorkshire sows were used in this study. Sows were housed in individual pens and fed 3 times a day. At parturition, sows were fed 2.8 to 3.2 kg of crushed pellets/d (energy content, 12.4 MJ/kg). Two weeks after...
parturition, feed intake was increased so that each sow received 3.0 kg of crushed pellets plus an additional 0.4 kg/piglet. Sows farrowed naturally, and no sow developed clinical signs of disease at either sampling time. There were no bacteriologic indications of infection in any sample of mammary secretions.

Experimental design and sample collection—The experimental design was approved by the Ethical Committee for Animal Experiments, Uppsala, Sweden. Mammary secretions were collected from each sow within 24 hours after parturition (colostrum) and 12 to 13 days later (milk). The samples were collected as described by Magnusson et al. In brief, piglets were separated from sows 20 minutes before sampling, and each sow’s teats were carefully washed and disinfected with ethanol. Sows were given 25 U of oxytocin by IM injection, and mammary secretions were collected by hand. Samples collected from 1 to 3 teats were pooled and centrifuged at room temperature (approx 21 °C) at 715 g for 10 minutes. The fat layer and supernatant were collected and homogenized to yield cell-free (whole) secretion samples that were stored at -70 °C until assayed. For hormone analyses, both whole and fat-depleted secretion samples were used. Samples collected at both time points (ie, colostrum and milk) from any 1 sow were analyzed in the same assay.

Isolation of PMN leukocytes—Blood PMN leukocytes were obtained from 27 healthy adult nonpregnant nonlactating female pigs not used elsewhere in this study. Blood samples were collected by jugular venipuncture into tubes containing heparin and diluted with an equal volume of Krebs Ringer buffer (KRB). Diluted blood was layered onto 2.5 ml of a low-viscosity sodium diatrizoate solution1 and centrifuged at 375 g for 30 minutes. The supernatant with its layer of mononuclear cells and platelets was discarded together with the upper 1.5 ml of the solution. The remaining 1 ml of the solution contained PMN leukocytes and erythrocytes and was collected and washed twice in KRB and pelleted at 260 X g for 10 minutes. Erythrocytes were lysed with distilled water, and remaining PMN cells were washed in PBS solution and pelleted at 260 X g for 10 minutes; this lysis procedure was repeated once. The final centrifugation resulted in a pellet of PMN cells that was resuspended in KRB. For cell migration and phagocytic assays, PMN cells were diluted in KRB to a concentration of 5 X 106 cells/ml. From a pilot experiment (n = 8), this procedure gave all cells were first applied) was focused initially, and thereafter, the most advanced layer in which at least 2 PMN cells could be observed was focused. This difference between these 2 layers constituted the migrated distance.

Each sample (ie, mammary secretions and positive and negative controls) was set in a triplicate of wells, each wells filter counted at 3 sites, and a mean value was calculated from the triplicates. Values were obtained with PMN cells from 2 indicator animals, and for statistical analysis, the mean calculated from values for both pigs was regarded as the chemotaxatric property of the mammary secretion sample or the positive control. Migration toward the negative control was regarded as the random migration.

Phagocytosis assay—The effect of mammary secretions on phagocytosis was assessed by recording chemiluminescence of PMN cells following phagocytosis of opsonized zymosan or Escherichia coli in cell-free colostrum and milk, as described for fat-depleted mammary secretions by Magnusson et al with some modifications described here.

All samples were assayed in triplicate, and each assay included a positive control (KRB). A mixture of 20 µl of pooled saline serum and 30 µl of zymosan or E coli test wells in darkness at 38 °C for 15 minutes. Sixty microliters of each mammary secretion sample or KRB, 40 µl of luminol, and 40 µl of a suspension of PMN cells were then added to each well, and chemiluminescence, measured as light emission (relative light units [RLU]), was recorded at 45-second intervals for 80 cycles. The effect of mammary secretions on spontaneous chemiluminescence of PMN cells was determined by replacing zymosan or E coli with 30 µl of KRB.

Phagocytic capacity of PMN cells in each sample was measured as the mean area under the chemiluminescence versus time curve for triplicate wells. All mammary secretion samples were analyzed 3 times, using PMN cells from 3 indicator sows. A final mean chemiluminescence value was calculated from mean values determined for each set of cells. To investigate a possible difference in quench effect between colostrum and milk, the light absorption of mammary secretion samples was monitored at the wavelength at which luminol emits light (ie, 411 nm); significant (P < 0.05) differences were not found between types of mammary secretions.

Determination of estradiol-17ß and cortisol concentrations—Estradiol-17ß and cortisol concentrations in whole and fat-depleted mammary secretions were determined by use of solid-phase radioimmunoassays1 validated for use with fat-depleted mammary secretions. For estradiol-17ß, the intra-assay coefficient of variation, calculated from the precision profiles of 13 assays, was < 11.8% for estradiol-17ß concentrations between 144 and 1,430 pmol/L. The corresponding interassay coefficients of variation for 2 quality control samples were 21.4% (137 ± 32 pmol/L) and 16.1% (1,494 ± 244 pmol/L). To increase sensitivity when analyzing fat-depleted mammary secretions, the lowest standard was diluted 1:2. The minimum detectable concentration of estradiol-17ß was 22 pmol/L, defined as the mean of values obtained in 12 assays plus 3 SD for a standard containing no estradiol-17ß. The intra-assay coefficient of variation for the cortisol assay, calculated from the precision profiles of 18 assays, was < 12.1% for cortisol concentrations between 40 and 538 nmol/L. The corresponding interassay coefficients of variation for 3 quality control samples were 8.8% (40 ± 5.9 nmol/L), 5.4% (86 ± 8.5 nmol/L), and 5.9% (338 ± 32 nmol/L). The minimum detectable concentration of cortisol was 10 nmol/L, defined as the mean of the values obtained in 11 assays plus 3 SD for a standard containing no cortisol.

In the present study both whole and fat-depleted secretion samples were analyzed. Cell-depleted mammary secretions that had been stored at -70 °C were thawed, warmed to 37 °C, and homogenized, and the whole samples were then analyzed directly. Fat-depleted samples were analyzed after centrifugation at room temperature and removal of the fat layer.
Results for serial dilutions of whole secretion samples with high estradiol-17β concentrations were parallel to the standard curve provided with the assay. As a biological validation, results of analysis of whole and fat-depleted secretions from the same sampling time were compared. Concentrations of estradiol-17β in fat-depleted secretion samples were approximately 60% of the concentration in whole secretion samples. Hence, only estradiol-17β data from analysis of whole secretion samples were reported. Validation of the cortisol assay for use with whole secretion samples indicated that the measured values were low and did not allow for serial diluting. Consequently, only cortisol data from analysis of fat-depleted secretions were reported.

Statistical analyses—Normality of distribution of data was assessed, using the univariate procedure. Estradiol-17β concentrations required logarithmic transformation before analysis. Paired Student t-tests were used to compare data between groups (ie, type of secretion sample [colostrum, milk]). Hormone concentrations in mammary secretions were reported as mean ± SEM. Differences were considered significant at P < 0.05.

Results

Chemoattractant properties of mammary secretions—The front of cells that migrated toward the positive control (ie, zymosan-activated serum) was dense, whereas the front of cells that migrated toward either colostrum or milk was less dense, and in filters from the negative-control wells (ie, KRB), only a few scattered cells were apparent in the front. The chemoattractant properties of colostrum and milk were significantly (P = 0.0024 and P < 0.001, respectively) greater, compared with zymosan-activated serum, but no significant difference in chemoattractant properties was found between the 2 types of mammary secretions (Fig 1). Random migration, that is, migration toward KRB, was significantly (P < 0.001) less, compared with migration toward either type of mammary secretion or zymosan-activated serum.

Effect of mammary secretions on phagocytic capacity of PMN leukocytes—The capacity of PMN leukocytes in colostrum to phagocytose zymosan or E coli was significantly (P = 0.0014 and P < 0.001, respectively) less, compared with that of cells in milk (Fig 2). Moreover, phagocytic capacity (ie, chemiluminescence) of PMN cells in mammary secretions was approximately 10-fold less, compared with cells in KRB. The capacity of PMN cells in either type of mammary secretion to phagocytose E coli was significantly (P < 0.001) greater than the capacity to phagocytose zymosan. However, the opposite was true for cells in KRB. Spontaneous chemiluminescence of PMN leukocytes (ie, without target particles) was significantly less in colostrum, compared with milk and KRB.

Concentration of estradiol-17β and cortisol in mammary secretions—Concentration of estradiol-17β in whole cell-depleted samples of colostrum (885 ± 337 pmol/L) was significantly (P < 0.001) greater than in milk (< 22 pmol/L). Cortisol concentration in fat-depleted samples of colostrum (31 ± 7 nmol/L) was also significantly greater than in milk (15 ± 3 nmol/L).

Discussion

In the present study, we found that the capacity of PMN leukocytes to phagocytose E coli was less when cells were exposed to colostrum rather than milk, whereas no difference was found in chemoattractant properties of the 2 types of mammary secretions. In addition, concentrations of estradiol-17β and cortisol were greater in colostrum, compared with milk, suggesting these 2 hormones are not the major regulators of PMN cell chemotaxis.

The results from the cell migration assay revealed that the 2 types of mammary secretions had greater chemoattractant properties for PMN cells, compared with zymosan-activated serum. In addition, cells that migrated toward mammary secretions formed a dense front in the same manner as those that migrated toward activated serum. This is in contrast to the loose front of few migrating cells that formed toward KRB. The difference that we observed in distribution of cells migrating toward a chemotactic substance, compared with cells migrating randomly, is consistent with that reported for human PMN cells. These results imply...
that mammary secretions from healthy sows are chemotactic for PMN leukocytes. Thus, recruitment of PMN cells from the blood to the mammary gland lumen can occur even in the absence of chemotactic signals induced by microorganisms. However, we found no difference in chemotactic properties of colostrum and milk, giving no explanation as to why there are more PMN cells in colostrum than milk.\(^{32,33}\)

Chemiluminescence of PMN cells in colostrum following phagocytosis of either \(E\) \(coli\) and zymosan was less, compared with cells in milk, which may indicate that overall bacterial resistance of colostrum is also less. However, phagocytic capacity of PMN cells isolated from mammary secretions of sows around parturition has been reported not to vary.\(^{20,21}\) Several factors may have contributed to the difference in results between our study, which examined the in vitro effects of mammary secretions on PMN cells isolated from blood, and studies that examined the function of PMN cells isolated directly from mammary secretions. Diapedesis of PMN cells as they migrate from the bloodstream to the mammary lumen has been shown to reduce both phagocytic ability and oxidative burst activity.\(^{19}\) Phagocytosis of fat and casein in mammary secretions by PMN cells that have reached the mammary gland has a similar effect on phagocytic ability and oxidative burst activity.\(^{22,23}\) Also, PMN cells derived from mammary secretions have, as a population, been exposed to mammary secretions for a variable but longer period, compared with PMN cells isolated from blood and exposed in vitro to mammary secretions for a fixed period of time.

The capacity of PMN cells in KRB to phagocytose \(E\) \(coli\) was less than the capacity to phagocytose zymosan, whereas the opposite was true for cells in either type of mammary secretion. This suggests that mammary secretions are better adapted to support phagocytosis of prevalent microorganisms, compared with nonpathogenic substances. However, in a previous study\(^{34}\) that compared the effect of blood and mammary secretions on phagocytic capacity of PMN cells, no difference was found in the capacity of PMN cells to phagocytose either target particle.

Physiologic concentrations of steroid hormones may affect various PMN functions in vitro as well as in vivo. In rats, an increased plasma concentration of cortisol is associated with decreased diapedesis of PMN cells,\(^{25}\) and physiologic concentrations of estradiol-17\(\beta\) can reduce chemotaxis of human PMN cells in vitro.\(^{35}\) Phagocytic function may also be affected. Phagocytic function of blood leukocytes is increased in pigs treated with estradiol-17\(\beta,\)\(^{36}\) whereas in vitro exposure of porcine PMN cells to prepartum concentrations of estradiol-17\(\beta\) (3,000 pmol/L) can suppress the same function.\(^{37}\) Phagocytic capacity of bovine PMN cells can be enhanced by exposure to physiologic concentrations of corticosteroids, whereas treatment of cows with corticosteroids can result in decreased bacterial ingestion by both blood and milk PMN cells.\(^{38}\) In the present study, we found significantly higher concentrations of estradiol-17\(\beta\) and cortisol in colostrum, compared with milk, which is in agreement with earlier observations.\(^{39}\) The lack of difference in chemotactic properties between colostrum and milk suggest that estradiol-17\(\beta\) and cortisol in mammary secretions do not play a major role in regulation of chemotaxis of PMN cells to the mammary gland. However, we found a low phagocytic capacity concomitant with high hormone concentrations. No clear relation was evident between estradiol-17\(\beta\) and cortisol concentrations in mammary secretions and PMN leukocyte activity.

We hypothesized that coliform mastitis may develop in sows if the number of functional PMN cells is inadequate in the lactating mammary gland. However, results from this in vitro study are ambiguous. Data from the cell migration assay do not support the hypothesis that the increased susceptibility of sows to coliform mastitis at parturition is attributable to an impaired recruitment of PMN cells to the mammary lumen. On the other hand, the phagocytic capacity of PMN cells in colostrum was significantly less than in milk, which may predispose sows to coliform mastitis during the early postpartum period.

References

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