

# Consequences of Interference of Milk with Chemoattractants for Enzyme-Linked Immunosorbent Assay Quantifications<sup>∇</sup>

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**Concentrations of the chemoattractants CXCL1, CXCL2, CXCL3, CXCL8, and C5a in milk were reduced by the preparation of milk whey by high-speed centrifugation or with rennet. About half of the chemoattractants (35 to 65%) were associated with the casein micelle sediment, except when whey was prepared by acidification. Consequently, quantification of chemoattractants should be carried out preferentially with skimmed milk samples or, whenever whey is needed, with acidic whey samples. The interference of milk or milk whey with the enzyme-linked immunosorbent assays (ELISAs) used to quantify the chemoattractants was moderate, as long as tetramethylbenzidine (TMB), not ABTS [2,2'-azino-bis-(3-ethylbenzthiazoline-sulfonate)], was used as the substrate of peroxidase. These considerations will help to assess more precisely a component of the immune response of the mammary gland to infection.**

Mastitis, an inflammation of the mammary gland provoked mainly by bacterial infections, is one of the most prevalent and costly diseases for the dairy industry (22). Experimentally induced mastitis has been used widely to explore the inflammatory and immune response of the mammary gland, with a view to gaining a better understanding of this pathology and finally improving its control. The mammary gland lends itself to kinetic studies, owing to the ease of collecting sizable milk samples, allowing investigators to analyze cellular and humoral mediators of immunity. Many investigations have taken advantage of this asset, by using either living bacteria or bacterial products to induce mastitis and to monitor the time course of the inflammatory and immune responses (4, 8, 20, 21, 23, 24). In these studies, variations of concentrations of several immune mediators were measured by enzyme-linked immunosorbent assays (ELISAs). The interference of milk with ELISAs designed to measure chemokines and C5a in milk has been reported (13, 18, 19). The reason for this quenching phenomenon has not been reported.

Milk is a complex and heterogeneous medium, comprising fat globules, casein micelles, and cells in suspension in the fluid phase. It is often necessary to process milk before freezing and storage to separate the cells and fat from the skimmed milk. Although devoid of cells and fat, skim milk is likely to exert a matrix effect, owing to its high protein content. The caseins are major components of milk and are secreted as large colloidal aggregates termed micelles. In milk, caseins are at equilibrium between the bulk micellar and the small diffusible fractions, the extent of dissociation depending on both pH and temperature (10). Caseins have been reported to bind to proteins, such as plasminogen or aflatoxin (6, 27). Indeed, it has been shown that caseins are endowed with chaperone-like activity (7, 28). Like many other chaperones, caseins do not contain cysteine but have a lot of proline residues, they are amphipathic with an

N-terminal hydrophilic polar domain and a C-terminal hydrophobic domain, they have a flexible tertiary structure, and they can form oligomers in solution (7, 26, 28). The flexibility of the structure and the presence of hydrophobic stretches would favor association with other proteins. Another feature of caseins is that their N-terminal hydrophilic polar domain is negatively charged. The polar domain of  $\alpha$ -casein and  $\beta$ -casein contain anionic clusters comprising phosphoserine residues, and the polar domain of  $\kappa$ -casein, although devoid of phosphoserine clusters, is also strongly anionic (26). The phosphate groups of the phosphoserine residues are the main binding sites of cations, which are exchangeable between the bound and diffusible fractions (10). These properties may have a bearing with chemoattractants for neutrophils in milk, because these small proteins are cationic molecules.

Chemokines which target neutrophils belong to the subgroup characterized by the presence of the Glu-Leu-Arg (ELR) motif preceding the first two cysteines which are separated by one amino acid, the ELR+CXC chemokines (1). Of note, ELR+CXC chemokines are cationic molecules, with several basic amino acids (arginine and lysine) at their C-terminal end. Another chemoattractant for neutrophils, the complement fragment C5a, has a high density of charges at the N-terminal end, with a cluster of cationic lysine residues (11). These chemoattractants have been given attention in the setting of mastitis, because neutrophils constitute a major defense of the mammary gland against infections, and the promptness of neutrophil recruitment is a major determinant of the outcome of infection (9, 17). Several studies have shown that interleukin-8 (IL-8) (CXCL8), the prototypic ELR+CXC chemokine, and C5a are not detected in normal bovine milk but are secreted in sizable concentrations in mastitic milk (2). Other ELR+CXC chemokines, such as CXCL1, CXCL2, and CXCL3, are secreted in milk during mastitis (16). Recently, it has been shown that bovine milk contains a high concentration of CXCL3 (about 200 ng/ml), which is secreted constitutively by mammary epithelial cells, and that this chemokine is responsible for the chemotactic activity of normal milk to neutrophils (18).

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Several procedures are commonly used to process milk samples before storage and analysis, but the effect of these procedures on the results of ELISA measurements has not been assessed. Several modes of preparation of mammary secretion samples are used, such as simple centrifugation to sediment cells and remove fat globules to prepare skim milk and such as ultracentrifugation or destabilization of casein micelles by lowering the pH below 4.6 to prepare milk whey (4, 15, 20, 21). Whey can also be obtained by using rennet, an extract of calf abomasum containing chymosin, a protease-cleaving  $\kappa$ -casein, which provokes casein micelle destabilization and precipitation (5). If certain milk components interact with chemoattractants, then the question of the interference of these components with not only their quantification but also their chemotactic activity arises.

The studies reported here were devised to address the question of a possible interference of milk with the determination of concentrations of chemoattractants for neutrophils in milk.

#### MATERIALS AND METHODS

**Reagents.** Chymosin (rennet) was from Cooper (Melun, France). The recombinant bovine chemokines CXCL1/GRO $\alpha$ , CXCL2/GRO $\beta$ , CXCL3/GRO $\gamma$ , and CXCL8/IL-8 were prepared in *Escherichia coli* using the vector pGEX-2T (GE Healthcare) and purified as previously described (18). The chemoattractant complement fragment C5a-desArg was purified from bovine serum as described previously (14).

**Preparation of skim milk and milk wheys.** Eight healthy Holstein cows of the institutional dairy herd at the institutional facility (PFIE, INRA, Nouzilly, France) were used for these studies. Milk samples were obtained from one uninfected, low-cell-count (12,000- to 25,000-cell/ml range) quarter per cow. The milk was immediately skimmed by centrifugation at  $1,500 \times g$  for 30 min at 4°C. Skim milk was harvested between the supernatant (fat layer) and the pellet (debris and cells). Milk whey was prepared by using four different techniques. Ultracentrifugation whey was prepared by ultracentrifugation at  $100,000 \times g$  for 30 min at 4°C in an Optima TLX ultracentrifuge and associated TLA 100.4 rotor (Beckman Coulter Inc., Fullerton, CA). Centrifugation whey was prepared by centrifugation at  $18,000 \times g$  for 90 min at 4°C. Acidic whey was obtained by milk acidification to pH 4.3 with 6.0 N HCl, centrifugation at  $2,500 \times g$  for 15 min at 4°C, and neutralization to pH 7.0 with 6.0 N NaOH. Rennet whey was prepared by incubating chymosin with skimmed milk (0.3  $\mu$ g active chymosin/ml milk) at 37°C for 3 h before centrifugation at  $2,500 \times g$  for 15 min. Skim milk and whey samples were stored in portions at -18°C.

**ELISAs for quantification of bovine chemoattractants.** The effect of milk and whey on the sandwich ELISAs used to measure concentrations of neutrophil chemoattractants was investigated by comparing the standard curves obtained with antigens diluted in phosphate-buffered saline (pH 7.0) supplemented with 0.5% (wt/vol) fish gelatin (PBS-G), milk, or whey. Sandwich ELISAs for the ELR+CXC chemokines CXCL1, CXCL2, CXCL3, and CXCL8 and for the complement chemoattractant C5a were described elsewhere (18, 19). Series of 2-fold dilutions of recombinant (chemokine) or purified (C5a) antigens from 10 ng/ml to 39 pg/ml were prepared in PBS-G, 25% skimmed milk, or 25% acidic whey in PBS-G and distributed in the same ELISA plates to reduce factors of variation. The chromogenic reagent tetramethylbenzidine (TMB) was used to reveal the binding of the peroxidase-conjugated second antibody (Ab), except in some experiments in which 2,2'-azino-bis-(3-ethylbenzothiazoline-sulfonate) (ABTS) was used.

**Statistical analysis.** The effect of milk treatments on chemokine concentrations was tested with the nonparametric test of Friedman, which was followed by the Bonferroni multiple-comparisons test (12, 25). *P* values of less than 0.05 were considered to be significant.

#### RESULTS

**Effect of techniques to prepare milk wheys on chemokine concentrations.** Pilot experiments showed that concentrations of the two constitutive milk chemokines, CXCL1 and CXCL3, were not affected by the centrifugation of milk to sediment

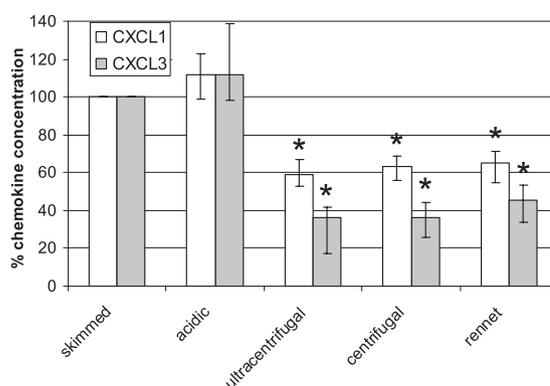


FIG. 1. Percentage of the constitutive milk chemokine CXCL1 and CXCL3 concentrations (100%, concentrations in skim milk) as a function of milk treatment. Milk wheys were prepared by lowering the pH to 4.3 and bringing it back to 7.0 (acidic), by ultracentrifugation ( $100,000 \times g$  for 30 min), by centrifugation ( $18,000 \times g$  for 90 min), or with rennet. Chemokine concentrations were measured by ELISAs. Results are medians from 8 milk samples (bars are for the upper and lower deciles). Asterisks indicate statistical significance ( $P < 0.05$ ) of difference from concentrations in skim milk (Friedman test followed by multiple comparisons).

cells and to float cream. Quarter milk samples from eight cows were tested for CXCL1 and CXCL3 contents before or after centrifugation at  $1,500 \times g$  for 30 min at 4°C (whole milk versus skim milk tested in the same ELISA plates), and ELISA results indicated that CXCL1 and CXCL3 concentrations were systematically higher in skim milk than in whole milk, by about 10%. This indicated that these chemokines did not tend to associate with milk fat. Consequently, skim milk was used as the reference to assess the effects of the currently used methods of milk whey preparation on the concentrations of chemoattractants.

The effect of the mode of preparation of milk samples on the apparent concentration of chemokines was investigated by measuring concentrations recovered by ELISAs in samples treated to obtain skim milk or to obtain whey by ultracentrifugation, high-speed centrifugation, or the addition of rennet (for acidic whey). Concentrations of the two constitutive ELR+CXC milk chemokines CXCL1 and CXCL3 were substantially and significantly ( $P < 0.05$ ) reduced by centrifugation, ultracentrifugation, or rennet treatment (Fig. 1). These three treatments had about the same effect on chemokine concentrations. In contrast, whey obtained by acidic treatment of milk contained slightly higher concentrations of the two chemokines (Fig. 1). These results indicated that CXCL1 and CXCL3 are partly associated with the casein milk fraction and suggested that this association was broken by acidification of milk to pH 4.3. The rennet preparation could also have a direct effect on the chemokines or on the capture Ab used in the ELISAs. To check this possibility, CXCL1, CXCL3, and CXCL8 diluted in RPMI to concentrations of 50 ng/ml were treated with rennet under the conditions used for the preparation of whey (concentration, time, and temperature). Then, the concentrations of the chemokines were determined by ELISAs. The experiment was repeated three times. Mean concentrations of CXCL1, CXCL3, and CXCL8 were 45.4 ng/ml, 52.6 ng/ml, and 46.5 ng/ml, respectively, after rennet treat-

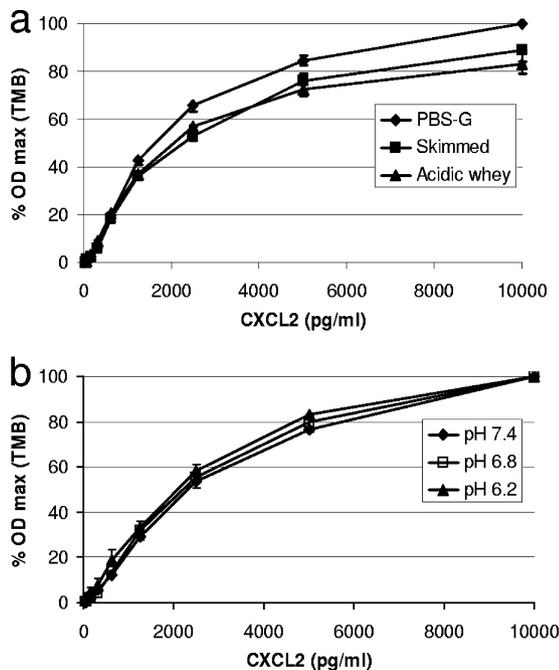


FIG. 2. Effect of skim milk or whey prepared by acidification on CXCL2 ELISA results. (a) Various concentrations of CXCL2 were prepared in either PBS-G, skim milk, or acidic whey, as they were for establishing reference curves. The ELISAs were performed as described in Materials and Methods. (b) The same concentrations of CXCL2 were prepared in parallel in PBS-G at pH 6.2, 6.8, and 7.4, and these samples were used in the ELISA to determine the effect of sample pH on the results. The chromogenic substrate was TMB. The three conditions were tested in parallel in the same ELISA plate to reduce factors of variation. Results are from 4 experiments made in duplicate and are expressed as median values. Variation bars indicate the first and third quartiles.

ment. It was considered that these chemokines and the capture antibodies were not susceptible to rennet.

The ability of the casein milk sediment to trap chemokines was checked by adding CXCL8, which is not present in normal milk, to skim milk before the removal of casein. Skim milk was spiked with 10 ng/ml recombinant bovine CXCL8, a concentration which can be reached during clinical mastitis, and incubated for 2 h at 37°C before either centrifugation at low speed (as for skimming) or preparation of centrifugation whey, acidic whey, and rennet whey. Then, an ELISA was carried out to measure the concentrations of CXCL8 in spiked whey or milk. The recovery of CXCL8 added to skim milk was 91% after centrifugation at  $1,000 \times g$  for 30 min at 20°C, 75% after acidic treatment, 44% after rennet treatment, and 41% after ultracentrifugation (median values,  $n = 3$ ). These results are in keeping with the trapping of more than half of CXCL8 in the casein milk fraction. Purified C5a (10 ng/ml) was added to skim milk ( $n = 3$ ) and incubated for 2 h at 37°C, and part of the skim milk was centrifuged ( $18,000 \times g$  for 90 min at 4°C). The mean C5a concentration in centrifuged milk was 45% of the concentration found in skim milk.

**Effect of skim milk and whey on measurements of chemoattractant concentrations by ELISAs.** Since chemokines appeared to interact with the casein milk fraction, it could be hypothesized that skim milk would interfere with determina-

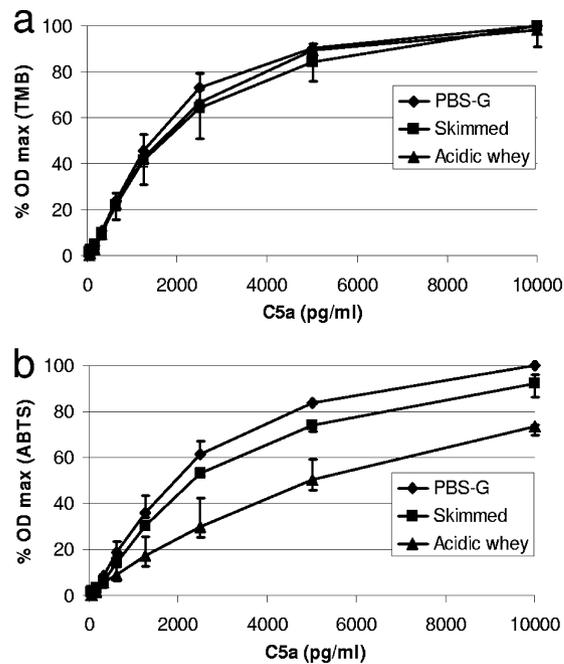


FIG. 3. Effect of skim milk or whey prepared by acidification on C5a ELISA results. (a) Various concentrations of C5a were prepared in either PBS-G, skim milk, or acidic whey, as they were for establishing reference curves. The ELISAs were performed as described in Materials and Methods. (b) Conditions were the same as those described above for panel a, except that ABTS was used as the chromogenic substrate instead of TMB.

tions of chemokine concentrations by the ELISA, whereas whey would not. To test this possibility, standard curves of the ELISA were performed by diluting the recombinant or purified chemoattractants with either skim milk or acidic whey. Comparison was made between dilutions of standard proteins in PBS-G and in skim milk or acidic whey half diluted in PBS-G. There was some reduction of the ELISA signals for CXCL2 with skim milk and acidic whey compared to PBS-G (Fig. 2a). This would result in some underestimation of milk concentrations, in case the standard curve were established by diluting the reference protein in PBS-G (Fig. 2a). The possibility that sample acidity modified the interaction of CXCL2 with the capture antibody was tested by comparing the standard curves established by diluting recombinant CXCL2 in PBS-G buffered at three pH levels: 7.4 as possibly in mastitic milk, 6.8 as in normal milk, and 6.2 to assess the effect of a slightly acidic pH possibly resulting from an incomplete neutralization of acidic whey. Results showed that within this pH range there was no change in ELISA optical density (OD) values (Fig. 2b), indicating that the lessened ELISA values obtained in whey or skim milk were not likely to result from sample pH.

Unexpectedly, the C5a ELISA was not affected by milk or acidic whey (Fig. 3a). This contradicted the previous observation that milk reduced the C5a ELISA signal (19). Since the only change in the protocol was the use of TMB instead of ABTS as the peroxidase substrate, the effect of milk and acidic whey was tested by using the ABTS substrate in the ELISA protocol. This resulted in a reduced signal (Fig. 3b), suggesting

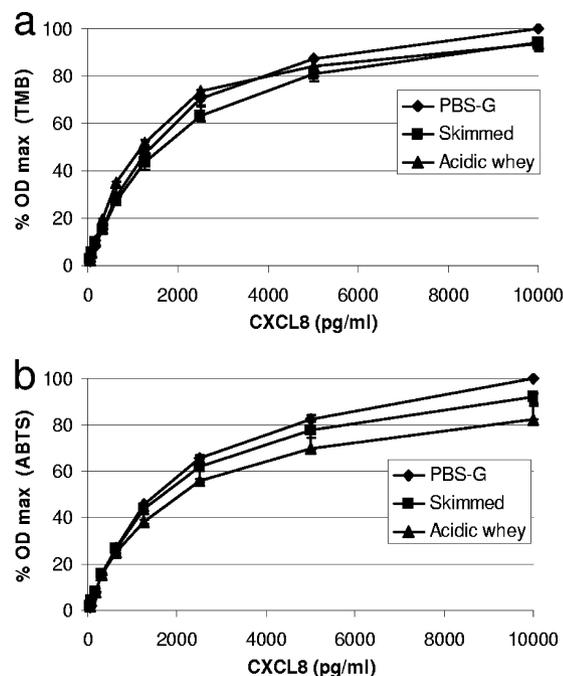


FIG. 4. Effect of skim milk or whey prepared by acidification on CXCL8 ELISA results. (a) Various concentrations of CXCL8 were prepared in either PBS-G, skim milk, or acidic whey, as they were for establishing reference curves. The ELISAs were performed as described in Materials and Methods. (b) Conditions were the same as those described above for panel a, except that ABTS was used as the chromogenic substrate instead of TMB.

that milk and whey did not impair Ab binding but that some milk components adsorbed to the wells and interfered with the peroxidase substrate (ABTS). Whether the same observation applies to the CXCL8 ELISA was investigated using the same approach as for C5a. Skim milk and acidic whey did not perturb the CXCL8 ELISA (Fig. 4a). Again with ABTS, there was a slight reduction in OD values with skim milk and acidic whey samples, although less pronounced than with the C5a ELISA (Fig. 4b). This experiment was not carried out with the constitutive bovine milk chemokines CXCL1 and CXCL3 because of their constitutive high concentrations in undiluted milk and wheys (18).

## DISCUSSION

From a practical standpoint, the main conclusion of this study is that the treatment of milk samples before storage and analysis has a marked effect on the measured concentrations of the chemoattractant C5a and ELR+CXC chemokines. This may result from the association of more than 50% of chemoattractant molecules with the casein sediment removed from the samples after centrifugation (Fig. 1). Nevertheless, lowering the pH to 4.3 to destabilize casein micelles prevented the loss of chemoattractants from the whey fraction. This may be because the low pH, by altering the charges of the chemoattractants or the trapping component of the solid sediment, freed the chemoattractants in the fluid phase. These results indicate that acidic treatment can be used if whey rather than milk is required, as far as the chemoattractant content of the samples

is concerned. Notwithstanding, this acidic treatment may alter the activity of other molecules. As a general rule, using skim milk for storage and subsequent analysis appeared to be the commendable procedure. Yet, a problem is likely to arise when dealing with mastitic milk, because overt inflammation is often accompanied by the clotting of casein. Also, after one cycle of freeze-thawing, the destabilized casein micelles in mastitis milk samples tend to aggregate and clog up pipette tips, which requires centrifugation to sediment the curdled casein. When this happens, a sizable proportion of chemokines can be trapped in the pellet, and the concentrations assessed from the supernatant are likely to be underestimated. This could be particularly worrying with CXCL3, which is constitutively secreted in milk at a high concentration (about 200 ng/ml), because the loss of CXCL3 from the supernatant by trapping in the casein pellet could completely offset an increase due to the inflammatory response (P. Rainard, F. B. Gilbert, A. Fromageau, and P. Cunha, unpublished observation). This also happens with CXCL8, but, as this chemokine is not found in normal milk, the fraction of CXCL8 remaining in whey is detected as an increase from the baseline, and the underestimation goes unnoticed.

Preparation of samples with rennin (5), by centrifugation (3, 20, 23), or by ultracentrifugation (21) were and are still of current use, which is likely to have resulted in the underestimation of chemoattractant concentrations in normal or mastitic milk. On the other hand, on the basis of the slight reduction in ELISA signals, it can be said that interference by milk, or the milk “matrix effect,” in the capture ELISAs is not a major concern. Consequently, skim milk is appropriate for quantifying chemoattractants in milk.

The use of ABTS as the peroxidase-conjugated second antibody is not commendable when milk or milk whey samples are concerned. The observation that ABTS is a much better substrate of lactoperoxidase than is TMB may have a bearing on the higher background and lower sensitivity of some ELISAs, but there is no obvious explanation for the decreased OD signal observed in this study (Fig. 3b and 4b). Although not tested, the use of reconstituted dry milk to block unoccupied sites of ELISA plates may not be the best choice because of the possible interaction with chemoattractants in the samples and because of the presence of CXCL3, the constitutive chemokine of bovine milk (18).

The most simple explanation for the reduction of chemoattractant concentrations in ultracentrifugation whey compared to skimmed milk is that part of the chemoattractants are bound to casein micelles. That concentrations of chemoattractants were not reduced in acidic whey would be a result of the perturbation of the casein micelles at acidic pH. The release of chemoattractants by acidification of milk would suggest that electrostatic forces are involved in the association of chemoattractants with casein micelles. Caseins expose clusters of phosphoserine residues, which are negatively charged. The phosphate groups of the phosphoserine residues constitute the main binding sites of cations in the casein micelles (10). It is plausible that the chemoattractants tested in this study, which expose patches of charged amino acids, could interact with casein micelles. For example, the N-terminal sequence of bovine C5a (Met-Leu-Lys-Lys-Lys-Ile-Glu-Glu-Ala-Ala-Lys-) presents both positively (lysine residues) and negatively (glutamic acid residues) charged patches (11), making possible

electrostatic interactions. This possibility needs to be demonstrated, and the effect of an association of chemokines with caseins on chemotactic activity would be worth investigating. Whatever it may be, it is worth noting that the binding of chemoattractants to casein micelles, if it occurs, did not preclude the binding to ELISA capture antibody or the binding of the detection antibody to the captured chemoattractant. The fortunate consequence is that milk does not preclude the quantification of chemoattractants, provided a few precautions are taken.

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