SHORT COMMUNICATIONS

Cytokine Activity in Bovine Mammary Gland Secretions during the Periparturient Period

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ABSTRACT

The presence of cytokine activity in periparturient bovine mammary secretions was evaluated. Mammary secretions were modified for use in biological assays for interleukin-2 (IL-2) like and antiviral activity. The level of IL-2 like activity in mammary gland secretions was lower during the last week of gestation when compared to levels detected approximately two weeks prepartum. Antiviral titers gradually increased as parturition approached. Results from Western blots indicated that the antiviral activity observed in prepartum secretions may be due to tumor necrosis factor (TNF). Interferons (IFN) were not detected in the colostrum samples.

RÉSUMÉ

Le but de la présente expérience était de mesurer la présence d'une activité cytokinique à partir des sécrétions mammaires chez les vaches en période péripartum. L'activité antivirale ainsi que les niveaux des substances ayant une activité similaire à l'interleukine-2 (IL-2) ont été mesurés par essais biologiques. Le niveau d'activité IL-2 des sécrétions mammaires était supérieur deux semaines avant la parturition comparativement à la dernière semaine de gestation tandis que les titres antiviraux ont augmenté graduellement jusqu'à la parturition. L'activité antivirale observée en période prépartum peut être associée à la présence du facteur tumoral de nécrose (tumour necrosis factor). À partir des échantillons de colostrum, on n'a pu détecter la présence d'interféron. (*Traduit par Dr Pascal Dubreuil*)

The bovine mammary gland has a natural ability to prevent bacterial invasion, but various physiological events can inhibit this capability. Dairy cattle are highly susceptible to new intramammary infections following the cessation of lactation and during the periparturient period (1). Incidence of clinical mastitis is highest during early lactation and often results from new intramammary infections acquired during the nonlactating period (1). Increased susceptibility during these times is most likely due to a combination of increased exposure of teat ends to mastitis-causing pathogens and diminished host defense mechanisms during functional transitions of the mammary gland (2). Enhancing an otherwise diminished mammary gland defense system may provide an effective barrier against new intramammary infections during periods of increased susceptibility to disease.

Cytokines are naturally-produced proteins that play a pivotal role in immune regulation (3). Very little is known about the function of cytokines in the bovine mammary gland and their potential importance in preventing mastitis. Delineating normal levels of cytokines in mammary gland secretions will provide crucial information as to how these substances may be manipulated to overcome compromised mammary gland immunity.

Seven pregnant dairy cows from the University of Saskatchewan Research Herd were used. All cows were in their third to fourth lactation and had no history of mastitis in the previous lactation. Quarter mammary gland secretion samples (n = 28) were collected at approximately 14 and 7 days prepartum and at parturition. Standard microbiological procedures were used to determine infection status of each quarter throughout the experimental period (4).

Mammary gland secretions were centrifuged for 30 min at 3,000 *g* to remove fat and cellular debris. Samples were delipidized further with an extraction mixture containing four parts diethyl ether and one part butanol. Briefly, 4 mL of mammary gland secretion were added to polypropylene tubes containing 10 mL of the extraction mixture. Tubes were rotated for 1.5 h at room temperature followed by centrifugation for 20 min (250 *g*). The delipidized portion was removed from the semi-solid lipid layer with a Pasteur pipette. Lower molecular weight proteins (<60,000 Da) were separated from higher molecular weight proteins by gel filtration through columns filled with Biogel P60 (Bio-Rad Laboratories, Mississauga, Ontario) and subsequently concentrated by lyophilization. The effect of the extraction procedure on cytokine activity was investigated in a preliminary study. Cocktail mixtures of ultrahigh temperature pasteurized milk and known concentrations of recombinant cytokines were delipidized as described above. Results from biological assays indicated that cytokine activity was maintained through the delipidization procedure.

The interleukin-2 (IL-2) activity in mammary gland secretions was

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TABLE I. Cytokine activity in bovine mammary secretions during the periparturient period

Biological assay[1]		Sample period[2] C-14	C-7	C-0
Interleukin-2	X	6.53[a]	1.95[b]	3.79[ab]
	SEM	1.66	0.87	1.27
Antiviral	X	1474.4[a]	1368.8[a]	1933.8[a]
	SEM	275.0	210.0	214.0

[a,b]Means with different superscripts differ ($p \leq 0.05$)
[1]Data in the IL-2 assay are expressed as a stimulation index and data in the antiviral assay are expressed as units of activity per mg of mammary secretion protein. Values are means ± standard error of the mean (SEM)
[2]Days preceding calving (C)

measured in a proliferation assay using[^{3}H]-methyl thymidine (^{3}HTdr, Amersham, Oakville, Ontario) incorporation by an IL-2 dependent bovine T-cell line. Briefly, lymphoblastoid cells (1×10^5) were added to each well of 96-well microtiter plates in a 100 μL volume of RPMI-1640 (Gibco, Grand Island, New York) containing 5% fetal bovine serum (FBS) and 50 mH HEPES (Sigma Chemical Company, St. Louis, Missouri). Delipidized samples of mammary secretion (100 μL) were added to triplicate wells and incubated at 37°C in a 5% CO_2 atmosphere for 72 h. Cultures were labelled with 1 μCi/well of ^{3}HTdr for the last 16 h of culture. The amount of radioactivity incorporated by cells was quantitated by liquid scintillation counting and expressed as mean counts per minute (CPM) of triplicate cultures. The level of IL-2 activity in mammary gland secretions was expressed as a stimulation index (SI) calculated from the following equation:

$$SI = \frac{\text{CPM in the presence of mammary secretions}}{\text{CPM in the absence of mammary secretions}}$$

Antiviral activity, indicative of either tumor necrosis factor (TNF) or interferon (IFN) function, was detected and titrated in a virus inhibition assay employing Georgia bovine kidney (GBK) cells and vesicular stomatitis virus (VSV). Briefly, GBK cells were grown to confluence in 96-well flat bottomed microtiter plates and treated with 100 μL aliquots of the delipidized mammary secretion samples. After overnight incubation at 37°C, the culture medium was removed and 100 μL of fresh culture media containing 100 plaque forming units (PFU) of VSV were added to each well. The virus inoculum was removed after 2 h and the wells were overlaid with 200 μL of methyl cellulose in modified Eagle's medium (Gibco) with 10% FBS. Culture plates were incubated further for 24 h and stained with crystal violet. The antiviral titer in mammary secretion samples was estimated by extrapolation from a standard PFU curve generated with known concentrations of recombinant bovine (rBo) IFN-gamma (CIBA-GEIGY Canada Limited, Mississauga, Ontario).

Proteins in mammary gland secretions and standards of recombinant cytokines were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% acrylamide gel run under reducing conditions using the methods described by Redmond (5). Replica gels were transferred electrophoretically to Immobilon membranes (Millipore, Mississauga, Ontario) and probed with rabbit antisera raised against TNF, IFN-alpha and IFN-gamma. Antibody binding was detected by using a horseradish peroxidase labelled secondary antibody and 4-chloronaphthol (5).

Data were analyzed by least squares analysis of variance using general linear model procedure. Statistical significance was determined by Duncan's Multiple Range Test.

Bacteriological evaluation of foremilk samples revealed that all quarters were free of intramammary infection during the entire sampling period. The toxic side-effects of whole mammary gland secretions on responder cells in the biological assays were removed through the delipidization and gel-filtration procedures.

Results of the biological assays on the processed samples are summarized in Table I. Stimulation indices calculated from the proliferation assay indicated that the highest levels of IL-2 activity were in mammary gland secretions obtained at approximately 14 days prior to expected parturition. Levels of IL-2 activity were considerably lower ($p \leq 0.05$) in colostrum samples collected during the last week of gestation and at parturition.

Data from the antiviral assay are expressed as units of activity per mg of mammary gland secretion. The highest antiviral titers were detected in colostrum samples obtained at parturition. Levels of antiviral activity were lower in mammary secretion samples collected at 14 and 7 days prior to expected parturition. Since antiviral activity is indicative of either TNF or IFN function, mammary gland secretion samples were subjected to gel electrophoresis (Fig. 1) and probed with antisera raised against TNF and IFN. Results from Western blot analysis of milk secretion indicate that the antiviral activity detected in prepartum secretions may be TNF (Fig. 2). Antisera against TNF reacted with a protein of apparent molecular mass of 25,000, which may represent either the fully glycosylated form of the cytokine (unglycosylated molecular mass is 17,000) (6) or the precursor form of TNF of apparent molecular mass 26,000 (7). Faint reactivity was also observed with a smaller protein with an apparent molecular mass slightly lower than the recombinant TNF standard. Antibodies against either bovine recombinant IFN-gamma or alpha showed no reactivity following electrophoretic transfer onto Immobilon membranes.

Attempts to confirm the source of antiviral activity in colostrum samples using a TNF specific biological assay were unsuccessful. Functional TNF assays using WEHI cells (8) could not be modified for use in determining antiviral activity due to TNF in mammary gland secretions. Mammary gland secretions were toxic to the WEHI cell line.

Humoral and cellular defence mechanisms in the bovine mammary gland vary markedly throughout the lactation cycle. Previous studies have

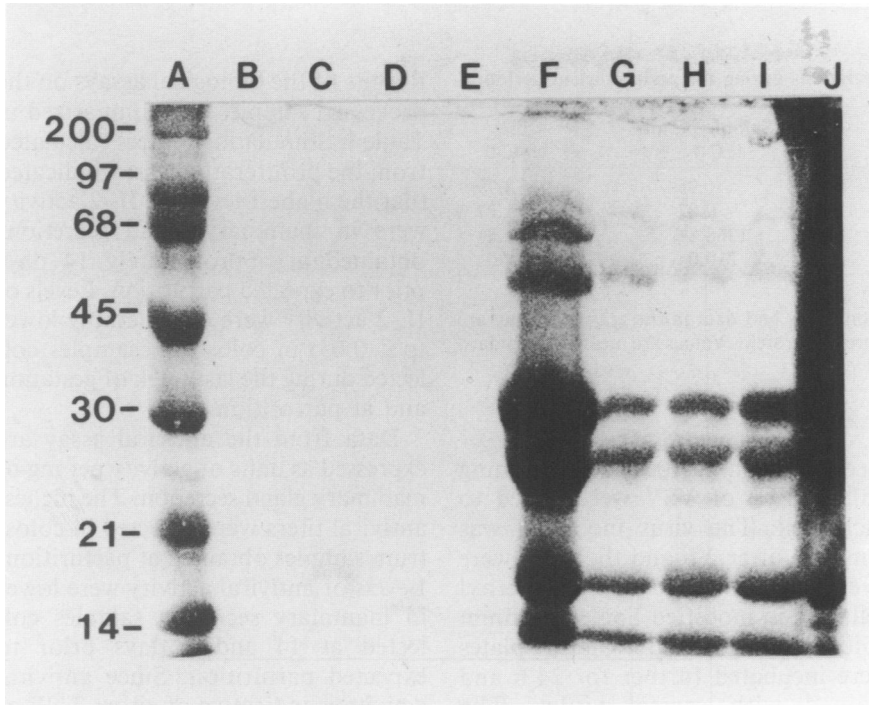

Fig. 1. Analysis of mammary secretion proteins. A 10% polyacrylamide gel was stained for proteins with Coomassie Blue. Lane A contained high molecular weight protein standards; lanes B-E contained recombinant cytokine standards; lanes F-I contained processed mammary gland secretion samples; and lane J contained whole mammary gland secretions. The high lipid and protein content of the whole sample interfered with the separation of the proteins by SDS-PAGE and this made further analysis difficult. However, the processed samples resolved well and although cytokines were undetectable by conventional protein staining techniques, the gels were suitable for further analysis using Western blotting techniques.

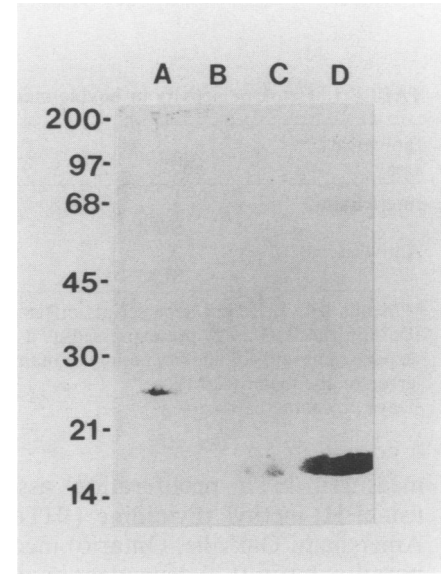

Fig. 2. Western blot probed with rabbit antisera to TNF and visualized with a horseradish-peroxidase secondary antibody-NBT system. Lane A contained a sample with antiviral activity; lane B contained a sample without antiviral activity; lane C contained rBoIFN-gamma; and lane D contained rBoTNF alpha. The presence of black bands indicated a positive reaction. The results showed immunoreactivity with the rBoTNF standard and a 25 K protein found in the mammary gland secretion with antiviral activity. A replica blot when probed with antibodies directed to IFN-gamma indicated immunoreactivity with only the rBoIFN-gamma standard.

shown that proliferative responses and functional activity of mammary gland leukocyte populations are diminished during lactogenesis (2,9,10). This reduced activity has been suggested to play a role in the development of new intramammary infections (2). Deficiencies in local immune responses during the periparturient period may at least partially be explained by variations in endogenous cytokine levels present in mammary gland secretions.

Ruminant mammary gland tissue is infiltrated heavily with lymphoid cells, particularly during the periparturient period (11). Studies involving monoclonal antibodies revealed that the majority of lymphocytes in both tissues and secretion were T-cells (12). However, the contribution of lymphocytes to mammary immune function and the potential of enhancing their function to increase resistance to mastitis is speculative at this time. Cytokine production is a major function of T-cells and there is evidence to suggest that mammary gland lymphocytes are as functionally capable of producing these immunological mediators as blood lymphocytes (13).

Interleukin-2 is one of the many T-cell-derived cytokines of major importance in the regulation of a variety of immune responses. There is evidence to suggest that altered IL-2 production contributes greatly to diminished immune capability which can lead to the development of both viral and bacterial diseases (14). Consequently, we were interested in examining the levels of IL-2 activity in bovine colostrum samples when diminished immune cell functions have been reported (9,10). Colostrum samples obtained during the last week of gestation had considerably lower levels of IL-2 activity when compared with samples obtained at approximately 14 days prior to parturition. Decreased levels of this important immunoregulatory cytokine correlated with diminished immune cell function and increased susceptibility to mastitis reported previously (2,9,10).

Interferon-gamma is another T-cell derived cytokine often produced in response to antigen or mitogen stimulation. This cytokine is a potent immunomodulatory factor to many aspects of the immune system (3). The pronounced influences of IFN-gamma on phagocytic cell populations suggests possible clinical application in the prevention of bacterial infections, such as mastitis. Results from previous studies have shown that human milk lymphocytes obtained during the first week of lactation were capable of producing IFN following mitogen stimulation (13). However, we were unable to detect the presence of IFN in bovine colostrum samples. Although speculative at this point, low levels of endogenous IFN may be a contributing factor to the diminished antibacterial capacity of mammary gland neutrophil and macrophage populations during the periparturient period.

Macrophages are the cellular source for TNF. Apart from its antiviral effects, TNF has been implicated in tumor cell cytotoxicity, B-cell maturation, induction of natural killer cells, maturation of thymocytes, activation of neutrophils, and in the orchestration of the inflammatory response (3). Macrophage numbers in bovine mammary tissues gradually increase from early involution to the last week of gestation where they are at their

highest concentrations (11). Since TNF is produced by stimulated macrophages, it follows that bovine colostrum samples also have relatively high levels of this cytokine. The biological significance of elevated TNF levels during periods of diminished immune capability is subject to conjecture. However, in a recent study, chronic administration of recombinant bovine TNF to cattle resulted in neutropenia, inhibition of *in vitro* migration, diminished production of reactive oxygen species, and absence of inflammatory reactions by neutrophil populations (15). Perhaps the high physiological levels of TNF in colostrum samples contributes to impaired function characteristic of mammary neutrophil populations during the onset of lactation.

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