Depression of B-Lymphocytes by Mastitis and Treatment with Levamisole

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ABSTRACT

Proportion of B-lymphocytes in milk and blood decreased simultaneously with development of mastitis induced with endotoxin and recovered with disappearance of clinical signs. However, the change in T-lymphocytes was slight. The reduced percentage of B-lymphocytes was caused by reduction in absolute number of such cells. When used orally, levamisole increased the proportion of B-lymphocytes of milk to the same as or more than that of similar cells in peripheral blood. This compound may enhance activity of the bovine mammary immune system and be of value for control of bovine mastitis.

INTRODUCTION

The total number of cells in milk is used as an indicator for mastitis. Considerable attention has been given to cell type although most emphasis is on the neutrophil.

The work of Smith and Schultz (24), Parmely and Beer (18), and Concha et al. (4) on bovine mammary secretions showed that lymphocytes in milk have an important role in immunoprotection of the mammary gland against microbial infection. In bovine mastitis, the pattern of T- and B-lymphocytes has not been evaluated sufficiently, and little is known about the relative importance and behavior of the two components of the immune system (28).

Levamisole has influenced host defense by modulating cell-mediated immune response (2, 5, 7, 11, 13, 20, 25, 26), humoral immunity response (13, 17, 21, 25), and enhancement of macrophage and polymorphonuclear cell function (2, 8, 12, 14, 16, 23, 27). We also found that the compound was of value in treatment of subclinical mastitis and observed a marked increase in immunoglobulin in normal quarter milk from treated cows (9).

It is, therefore, expected that the analysis of the distribution pattern of T- and B-lymphocytes is of use for understanding immune response to infectious agents and for controlling mastitis. We report decreased B-lymphocytes caused by mastitis and effect of treatment with levamisole.

MATERIALS AND METHODS

Animals, Treatments, and Sample Collection

Milk and peripheral blood B- and T-lymphocytes were determined for 16 Holstein cows, of which 8 were healthy and the rest were affected with mastitis accompanied by clinical signs. Milk samples were taken daily at the morning milking from individual quarters of four healthy cows in the following two separate experiments.

Mastitis was induced by intramammary infusion of the right front quarter with 10 ml sterile saline containing 10 µg endotoxin (lipopolysaccharide from Escherichia coli 055: B5, Sigma Chemical Co., St. Louis, MO) after the morning milking.

Levamisole hydrochloride (Lederle Japan, Tokyo, Japan) dissolved in water was administered orally at 7.5 mg/kg body weight. Equal volumes of milk from each quarter were pooled for subsequent analysis except for animals receiving intramammary infusion, for which we analyzed only the infused quarter.

Isolation of Milk Lymphocytes

About 1000 ml milk from each cow was collected and centrifuged at 400 x g for 20 min. The cell pellet was washed twice with phosphate buffered saline pH 7.2 (PBS), centrifuged at 400 x g for 10 min, and then suspended in 5 ml PBS. This was layered carefully onto a cushion of 3 ml Ficoll-Conray with solution density 1.077 g/cm³ in siliconized glass tube. The Ficoll-Conray solution was made by adding 24 parts of 9% Ficoll 400
(Pharmacia Fine Chemicals, Uppsala, Sweden) to 10 parts of 33.4% Conray 400 (Daichii Seiyaku Co., LTD., Tokyo, Japan). The tubes were centrifuged at 400 x g for 30 min, and cells in the interphase were collected, washed once with PBS, and resuspended to 1 x 10^6 cells/ml in Eagle minimum essential medium (Nissui Seiyaku Co., LTD., Tokyo, Japan) supplemented with 2 mM glutamine (MEM). This mononuclear cell suspension was enriched further for lymphocytes by depleting phagocytic cells by the iron powder method of Sanderson et al. (22, 28). Briefly, cell suspension was incubated with 4 mg carbonyl iron (Nakarai Chemicals LTD., Kyoto, Japan) per milliliter at 37°C for 60 min with intermittent mixing. Then a teflon-coated magnetic bar was put into this mixture and allowed to stand for 5 min at room temperature. Nonphagocytic cells unattached to the bar were decanted into conical tube, centrifuged, and resuspended in fresh MEM at a concentration of 1 x 10^6 cells/ml. The mononuclear cells so prepared consisted of 92 to 99% lymphocytes and less than 5% monocytes with virtually 100% viability by trypan blue exclusion test.

Isolation of Blood Lymphocytes

Heparinized blood (10 IU/ml) was diluted with PBS (1:1) and layered onto the Ficoll-Conray. Further procedure followed that described.

Erythrocyte-Antibody-Complement Rosette Assay

Proportion of B-lymphocytes in milk and blood was determined by erythrocyte-antibody-complement (EAC) rosetting technique of Aiuti et al. (1) with modification. Chicken erythrocytes (CRBC) were washed three times with gelatin veronal buffer pH 7.5 (GVB), and a 5% suspension of CRBC was sensitized at 37°C for 30 min with IgM fraction of rabbit anti-CRBC antiserum at a subagglutinating dose. Cells were washed twice and resuspended to 5% in GVB. An equal volume of horse serum diluted 1:20 with GVB was added as a source of complement. The cells were incubated at 37°C for 1 h, washed twice, and adjusted to .5% in MEM. The test procedure for EAC rosettes was equal volumes (.1 ml) of lymphocyte suspension and CRBC prepared as described were mixed, centrifuged at 200 x g for 5 min, and left at 37°C for 1 h. The cells were resuspended gently, and about 200 lymphocytes were scanned microscopically under a sealed cover slip. All of the lymphocytes binding more than three CRBC were considered positive.

Erythrocyte Rosette Assay

Percentage of T-lymphocytes in milk and blood was determined by erythrocyte (E)

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\begin{align*}
\text{EAC Rosetting Cells} & \\
\text{Normal Mastitis Milk} & \\
\text{Normal Mastitis Blood} & \\
\text{E Rosetting Cells} & \\
\text{Normal Mastitis Milk} & \\
\text{Normal Mastitis Blood} & \\
\end{align*}
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Figure 1. Percent erythrocyte-antibody-complement (EAC) (A) and erythrocyte (E) (B) rosetting lymphocytes of normal and mastitic cows. Bar represent the standard deviation of the mean.
Figure 2. Changes in the percentage of erythrocyte-antibody-complement (EAC) and erythrocyte (E) rosetting lymphocytes with the course of mastitis: o—o, milk EAC; •—•, blood EAC; □—□, milk E--; ■—■, blood E-rosetting cells; ................ , somatic cell count.
rosetting technique of Grewal and Babiuk (6) and Paul et al. (19). Sheep erythrocytes (SRBC) were washed three times with PBS and centrifuged at 400 × g for 10 min. One milliliter of washed cells was added to 4 ml of .1 M freshly prepared 2-aminoethylisothiouronium bromide hydrobromide (AET; Sigma Chemical Co., St. Louis, MO) solution (pH 9.0) and incubated at 37°C for 20 min. Cells were washed three times with cold saline and resuspended to 1% in MEM containing 6% dextran. For detection of E rosette-forming cells, equal volumes (.1 ml) of lymphocyte suspension and AET treated SRBC were mixed, incubated at 37°C for 10 min with intermittent mixing, and centrifuged at 200 × g for 5 min. After overnight incubation at 4°C, a drop of .5% trypan blue was added, and cells were resuspended gently. The cell analysis procedure followed that described for the EAC rosette assay.

Somatic Cell Count

Direct microscopic somatic cell count with Newman-Lampert stain was used to determine cell counts for milk samples.

Statistics

Statistical analysis of data was Student's t test.

RESULTS

As in Figure 1A, normal cows had 28.8 ± 4.8% EAC-rosetting lymphocytes in milk as compared with 18.5 ± 3.6% similar cells in mastitic quarters (P<.005) and 23.6 ± 4.8% in normal quarters (P<.05) of cows affected with mastitis. In peripheral blood, normal cows had 34.3 ± 2.9% EAC-rosetting cells against 20.6 ± 3.7% similar cells in mastitic cows (P<.005). Percentage T-lymphocytes determined by E-
rosetting technique is in Figure 1B. Normal cows had 43.5 ± 4.7% E-rosetting cells in milk in comparison with 40.6 ± 5.7% similar cells for mastitic quarters and 42.4 ± 3.7% for normal quarters of cows affected with mastitis. In peripheral blood, normal cows had 44.2 ± 6.1% E-rosetting cells against 45.5 ± 6.3% similar cells in mastitic cows. There were no noticeable differences in either total leukocyte or differential counts of peripheral blood between normal and affected cows.

Changes in proportion of lymphocyte subpopulations with the course of mastitis are in Figure 2. The marked decrease in proportion of EAC-rosetting cells in both milk and blood occurred simultaneously with development of mastitis although changes in E-rosetting cells were slight. Decreased EAC-rosetting cells recovered with disappearance of clinical signs of mastitis. These changes in EAC-rosetting cells were in contrast with those in number of milk cells. Furthermore, the response of healthy cows to levamisole treatment was evaluated (Figure 3). The compound had little influence upon proportion of blood EAC- and milk and blood E-rosetting cells, although it caused significant increases in milk EAC-rosetting cells (P<.001). These increases came to the same proportions as or more than those of similar cells in peripheral blood, and they remained until at least 63 days after treatment.

DISCUSSION

These studies showed there was a statistically significant (P<.005) reduction in percentage of B-lymphocytes in milk and blood of mastitic cows. The proportion of T-lymphocytes, however, changed least. These data were confirmed with mastitic cows with experimentally induced mastitis. Because there was no noticeable change in either number of leukocytes per cubic millimeter or differential counts of peripheral blood of mastitic cows as compared with normal cows, the reduced percentage of B-lymphocytes indicates a significant reduction in the absolute number of such cells. This may be explained partially as increases in lymphocytes of other types, such as null cells, which were newly formed and had not acquired their C3 receptors. The IgA system in the cow, especially in its mammary gland, is relatively poorly developed (10). The major immunoglobulins of all lacteal secretions are derived from circulation (15) whereas the local immune system of the udder is inactive (3, 15). We also observed lower percentage of B-lymphocytes in milk than that in blood, although the difference was not significant (P<.10).

In normal cows, levamisole induced the increase in proportion of milk B-lymphocytes. This is consistent with our observation that there was a marked increase in immunoglobulin in normal quarter milk after levamisole treatment (9). These findings suggest that the compound may enhance the activity of this local immune system and be of value in control of bovine mastitis.

REFERENCES