Depression of B-Lymphocyte Levels in the Peripheral Blood of Cows with Mastitis†

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The levels of B-lymphocytes in the peripheral blood of normal and mastitic cows were evaluated by fluorescent-antibody and erythrocyte-antibody–complement-rosetting techniques. Normal cows (N = 8) had 26.9 ± 4.4% surface membrane immunoglobulin-positive lymphocytes, whereas mastitic cows (N = 6) had only 16.3 ± 3.6% similar cells among lymphocyte preparations purified by the Ficoll-Paque density gradient separation and carbonyl iron phagocytosis methods. Studies on similar lymphocyte preparations by erythrocyte-antibody–complement-rosetting techniques showed that normal cows (N = 12) had 33.5 ± 7.1% erythrocyte-antibody–complement-rosetting lymphocytes, whereas mastitic cows (N = 6) had 20.5 ± 6.0% similar cells.

T- and B-lymphocytes in the peripheral blood of normal and pregnant individuals and patients with cancer and other diseases have been the subject of intensive quantitative studies in recent years (3, 8, 12, 13, 16, 17). In infectious diseases, however, the pattern of T- and B-lymphocytes has not been sufficiently evaluated, and little is known about the relative importance and behavior of the two compartments of immune responses in most of the common acute infections (1, 7, 14, 17).

In bovine mastitis, where acute local inflammation due to bacterial infection is the cardinal sign of the disease, the analysis of the distribution pattern of T- and B-lymphocytes will be of great use in understanding the immune response to infectious agents. In this communication we report that the percentage and the absolute number of peripheral blood B-lymphocytes are significantly lower in the cows with mastitis.

MATERIALS AND METHODS

Animals. Twelve normal and six mastitic cows from the University of Connecticut dairy herd were used. Diagnosis for mastitis was done both clinically and bacteriologically, and Streptococcus agalactiae was the etiological agent for the cases studied. All diseased cows had acute mastitis, and no treatment was instituted during the experimental period.

SRBC. Sheep erythrocytes (SRBC) were collected in equal volumes of Alsever solution and used within 2 weeks.

Tissue culture medium. RPMI 1640-HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) medium supplemented with 10% heat-inactivated fetal calf serum, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 2 mM glutamine was used for the preparation of lymphocyte suspensions.

For the rosette assay, the same medium supplemented with heat-inactivated fetal calf serum which had been preabsorbed with SRBC was used.

Peripheral blood lymphocyte suspension. Venous blood was collected in the morning into heparinized (preservative-free sodium heparin, Fellows Medical Manufacturing Co., Inc., Oak Park, Mich.) tubes. Portions (10 ml) of whole blood were diluted with 10 ml of Hanks buffered salt solution (HBSS) and layered over 10 ml of Ficoll-Paque (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.). This was centrifuged for 40 min at 400 x g, and the cells in the interface were collected, washed once with HBSS, and resuspended to 3 x 10⁶ cells per ml in RPMI 1640-HEPES medium. At this stage, the mononuclear cell preparations contained 45 to 87% lymphocytes with 12 to 32% monocytes as verified from Giemsa-stained cytocentrifuged cell smears. These mononuclear cell suspensions were further enriched for lymphocytes by depleting the phagocytic cells by the iron powder method of Sanderson et al. (10, 18). Briefly, carbonyl iron (40 mg, no. 44890, Tridom Chemical, Inc., Hauppauge, N.Y.) was suspended in distilled water, sonicated, washed, and resuspended in RPMI 1640-HEPES medium supplemented with 10% heat-inactivated fetal calf serum at a concentration of 20 mg/ml. After incubation at 37°C for at least 1 h, it was centrifuged immediately before use at 200 x g for 5 min, and the supernatant was decanted.

The mononuclear cell suspension (3 x 10⁶/ml) in medium was incubated with 4 mg of the treated iron powder per ml for 60 to 90 min at 37°C while mixing on a rocker platform (Belco Glass, Inc., Vineland, N.J.). The nonphagocytic cells were decanted with the help of a strong magnet into tubes which contained a Teflon-coated magnetic bar and were allowed to stand for 5 min at room temperature. They were then decanted into conical tubes, centrifuged, and resuspended in fresh medium at a concentration of 10⁷ cells per ml.

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The mononuclear cells so prepared consisted of 92 to 99% lymphocytes and less than 5% monocytes, with virtually 100% viability by the trypan blue exclusion test.

EAC rosette assay. For the erythrocyte-antibody-complement (EAC) rosette assay, the method of Kenyon and Piper (4) was employed. Briefly, 2 ml of subagglutinating dilutions of anti-SRBC hemolysin in HBSS was added to 0.01 ml of packed SRBC which had been prewashed with HBSS. After sensitization at room temperature for 15 min, 2 ml of 1:40 dilution of horse complement (nonlytic) was added, and the mixture was incubated for 1 h at 37°C. After centrifugation at 200 × g for 5 min, the EAC reagent was resuspended in 2 ml of tissue culture medium. For rosette assay, 0.25 ml of the EAC suspension was added to equal volumes of lymphocyte suspension (3 × 10^6/ml), centrifuged gently, incubated for 30 min at 37°C, and counted by the method of Sandilands et al. (2, 11).

Cell smears. Smears of cells and EAC rosettes were prepared by centrifugation of less than 0.5 ml of cell suspension onto glass slides for 10 min at 1,500 rpm in a Shandon cytocentrifuge (Shandon Southern Instruments, Inc., Sewickley, Pa.).

SmIg staining. The surface membrane immunoglobulin (SmIg)-containing cell population was determined by the membrane immunofluorescence method based on general procedures described by Winchester and Ross (15). Briefly, the lymphocyte suspensions depleted of monocytes with carbonyl iron phagocytosis were washed two times with HBSS and finally once in phosphate-buffered saline (PBS) containing 2% bovine serum albumin and 0.02% NaN₃ (PBS-BSA). Lymphocytes were then suspended in PBS-BSA to 2 × 10^7 to 2.5 × 10^7 cells per ml. Fluorescein isothiocyanate (FITC)-conjugated rabbit anti-bovine immunoglobulin G (IgG; G1,G2), IgA, and IgM antisera (IgG) were purchased from Miles Laboratories, Inc., Elk hart, Ind.

The FITC antisera were diluted in PBS-BSA with anti-IgG, 1:20, anti-IgA, 1:20, or anti-IgM, 1:20. Polyvalent antiserum was prepared by combining appropriate portions of anti-IgG, -IgA, and -IgM antisera with final dilutions consistent with the individual dilutions used. They were ultracentrifuged before use.

Equal volumes (0.05 ml) of diluted antisera and lymphocyte suspension were mixed quickly and incubated for 30 min at room temperature. (We did not detect differences between room temperature and 4°C incubation.) After the addition of 2 ml of cold PBS-BSA, they were centrifuged at 4°C for 5 min at 60 × g, and the supernatant was decanted. This was repeated twice, and then the cells were examined under a fluorescent microscope. For every sample, 200 to 500 cells were counted under the fluorescent microscope at 400×. As reported for human lymphocytes (5), the majority of SmIg-positive cells were of the IgM class, and the total population of B-lymphocytes was enumerated from the mixture of the FITC-conjugated anti-IgG, -IgA, and -IgM antisera.

Statistics. Statistical analyses of data were performed by the Student's t test.

RESULTS

EAC-rosetting cell populations. The percentage of lymphocytes with C3 receptors was determined by EAC-rosetting techniques on similarly purified lymphocyte preparations described above. As shown in Fig. 1A, normal cows (N = 12) had 33.5 ± 7.2% EAC-rosetting lymphocytes as compared with 20.5 ± 6.0% similar cells in the cows affected with mastitis (N = 6; P < 0.005); that is to say, there was a 13% reduction in EAC-rosetting cells among total lymphocytes or 38.8% among EAC-rosetting cells in mastitic cows.

SmIg-positive cell populations. The percentage of cells with SmIg among lymphocyte populations purified by the Ficoll-Paque separation and carbonyl iron phagocytosis techniques was determined by fluorescent-antibody staining of the viable lymphocytes with a mixture of FITC-conjugated rabbit anti-bovine-IgG, -IgM, and -IgA antisera. As shown in Fig. 1B, normal cows (N = 8) had 26.9 ± 4.4% of SmIg-positive lymphocytes, compared with 16.6 ± 3.6% similar cells in the cows affected with mastitis (N = 6; P < 0.005). There were no noticeable changes in either the total leukocyte or the differential counts of the peripheral blood of the affected cows, indicating that the 10.3% reduction in SmIg-positive cells among total lymphocytes or 38.3% reduction among SmIg-positive cells is significant.

DISCUSSION

The experiment showed that there was a statistically significant (P < 0.005) reduction in the percentage of B-lymphocytes as determined by SmIg and EAC-rosetting techniques among Ficoll-Paque-carbonyl iron phagocytosis-purified lymphocyte populations in mastitic cows. Since

![Fig. 1. Percent EAC-rosetting (A) and SmIg-positive (B) cells among peripheral blood lymphocytes of normal and mastitic cows. Bars represent the standard deviation of the mean.](http://iai.asm.org/Downloaded from http://iai.asm.org/ on November 6, 2017 by guest)
there were no noticeable changes in either the
total leukocyte or differential counts of the per-
ipheral blood of mastitic cows as compared with
normal cows, this reduction in the percentage of
B-lymphocytes indicates also a significant re-
duction in absolute number of such cells in the
peripheral blood.

The results with SmIg immunofluorescence
tests were comparable with values reported else-
where for normal cattle (6, 9) and were more
uniform than those with EAC-rosetting tech-
niques. The lymphocyte cell preparations, puri-
ified by Ficoll-Paque separation and carbonyl
iron treatment, contained less than 5% mono-
cytes, indicating that the 13% reduction of EAC-
rosetting cells among total lymphocytes, i.e., 20.5
± 6.0% versus 33.5 ± 7.1%, or 38.8% among EAC-
rosetting cells in mastitic cows, cannot be attrib-
uted solely to the contaminating monocytes (8).
They represent net B-cell and, possibly, also K-
cell depression. Furthermore, the EAC-rosetting
cells reported here represented those of the cows
at different stages of lactation. Our studies (un-
published data) indicate that the percentage of
EAC-rosetting lymphocytes in the peripheral
blood was higher in the normal cows during the
first 40 days postpartum (average of 37.3% with
a range of 28.3 to 43.3%) than that of the normal
cows at later stages of lactation (average of 28.4%
with a range of 19.5 to 33.4%). In mastitic cows,
the number of EAC-rosetting lymphocytes was
lower than that of normal cows throughout la-
tation (average of 20.5%, with a range of 13.1 to
29%).

In contrast to our findings, Niklasson
and Williams (7) reported that the percentage of
T-lymphocytes was depressed in most patients in
the early stages of both bacterial and viral or
mycoplasmal infections, whereas the B-lympho-
cyte proportion was usually elevated compared
with convalescence and controls. However, the
B-lymphocyte increase occurred earlier in a
small group of viral or mycoplasmal infections
than in bacterial infections. In parallel to our
observations, Wybran and Fudenberg (17) re-
ported decreased numbers of rosette-forming
lymphocytes in viral infections in contrast to
normal numbers of rosette-forming lymphocytes
in bacterial infection. Although certain viral dis-
ases are known to be associated with defects in
cellular immunity, which may reflect a decrease
in the number of T-lymphocytes, similar asso-
ciation between bacterial infections such as mas-
titis and a decrease in the number of B-lympho-
cytes attributable to defects in humoral immu-
nity seems not to be present in this study.

Since the number of lymphocytes per cubic
millimeter was not significantly different be-
tween the normal and mastitic cows tested, the
depression of B-lymphocytes that we observed
in mastitic cows is thus presumably due to a loss
of B-lymphocytes and simultaneous increase in
T- and/or other subpopulations of lymphocytes.
A unilateral change could have been reflected by
a change in the total lymphocyte count. How-
ever, the selective B-lymphocyte depression in
the peripheral blood cannot be construed as
evidence for decreased B-lymphocyte function,
and its significance needs to be elucidated. Al-
though depressed B-lymphocyte numbers may be
explained partially as increased T-lymphocy-
ectype or other types of lymphocytes, such as null
cells, which are newly formed and have not yet
acquired their SmIg or EAC receptors, there
seemed to be an additional B-lymphocyte de-
pression over and above the dilution effect, i.e.,
altered B-lymphocyte traffic patterns in the
presence of mastitis which result in reduced
numbers of the B-lymphocytes circulating
through the peripheral blood compartment sim-
lar to the pattern proposed by Wood and Neff
(16) for cancer patients. Whether a similar type
of circulation (sequestering) pattern occurs in
other types of localized lesions such as infections
remains to be investigated. Unlike the cancer
patient, the cows with mastitis were studied
previous to any treatment, indicating that the
low numbers of B-lymphocytes were not caused
by therapeutic procedures.

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BLOOD B-LYMPHOCYTE RESPONSE TO MASTITIS


