Peripheral B Lymphocyte Percentage as an Indicator of Subclinical Progression of Bovine Leukemia Virus Infection

HARRIS A. LEWIN, MING-CHE WU, TIMOTHY J. NOLAN, and JULIE A. STEWART
Laboratory of Immunogenetics
Department of Animal Sciences
University of Illinois
Urbana 61801

ABSTRACT
The relationship between percentage of B cells in peripheral blood and subclinical bovine leukemia virus infection was examined in a herd of 240 Holstein-Friesian cows. Absolute leucocyte count and absolute lymphocyte count were significantly positively correlated with B cell percentage in cows that were seropositive to bovine leukemia virus envelope glycoprotein, but these parameters were not correlated in seronegative cows. The B cell percentage was not affected by age. Cows that had persistent lymphocytosis and hematologically normal seropositive cows had greater mean B cell percentages (78 and 45%, respectively) than did seronegative herdmates (37%). To evaluate B cell percentage as a means of detecting subclinical progression of bovine leukemia virus infection, an index was developed based upon the distribution of B cell percentages in seronegative cows. When this index was compared with a standard hematological key (the European Community’s Leukosis Key), which is based on absolute lymphocyte count and age, 29% of seropositive, hematologically normal cows had B cell percentages two standard deviations above the mean of their seronegative herdmates. The B cell percentage was thus shown to be more effective than absolute lymphocyte count for detecting subclinical progression of bovine leukemia virus infection in individual cows.

INTRODUCTION
Bovine leukemia virus (BLV) is recognized as the etiological agent of the adult or enzootic type of bovine lymphosarcoma [EBL, (3)]. Molecular cloning and sequencing have demonstrated that BLV belongs to a group of retrovirinae that includes human T cell leukemia virus (HTLV) types I and II (19). The major biological difference between BLV and HTLV-I and HTLV-II is that the primary target-cell of BLV infection is the B lymphocyte, whereas the HTLV infect primarily T cells.

Hematological keys based on absolute lymphocyte count provided the initial means of identifying cattle at risk for developing EBL (3, 15). Using these keys, approximately half of the cattle that developed tumors passed through a phase of subclinical BLV infection termed persistent lymphocytosis [PL (6)]. Persistent lymphocytosis is characterized by a polyclonal expansion of surface membrane immunoglobulin M (slgM)-bearing (9, 11, 16) BLV-infected B cells (5). In contrast, the tumor phase of BLV infection is characterized by a clonal expansion of immature, predominantly slg-negative B cells (18), which contain BLV provirus (10). Despite the clear association between B cells and PL, the precise quantitative relationship between the antibody response to BLV envelope glycoproteins and B cell percentage has not yet been established. Furthermore, very little is known about the cellular events that occur between the time of BLV infection and the development of PL.

The objectives of this study were 1) to elucidate the relationship between the percentage of B cells and absolute lymphocyte count in cows that were seronegative and seropositive.
seropositive to BLV; and 2) to evaluate B cell percentage as a marker of subclinical progression of BLV infection in individual animals.

**MATERIALS AND METHODS**

**Animals**

Subclinical parameters of BLV infection were monitored under natural conditions of exposure in a herd of 240 Holstein-Friesian cattle (234 cows and 6 pregnant heifers) that ranged from 1 to 10 yr of age. Animals were bled within a 1-mo period for 2 consecutive yr. All assays were performed on the same day that blood was collected. The herd is maintained at research facilities operated by the Department of Animal Sciences, University of Illinois.

**Detection of Antibodies to Bovine Leukemia Virus**

Serum samples were tested in duplicate each year for precipitating antibodies to commercially prepared BLV glycoprotein(s) (Pitman-Moore, Atlanta, GA) using the agar gel immunodiffusion (AGID) test, performed according to the manufacturer's instructions. This antigen preparation contains the Mr 51,000 BLV envelope glycoprotein (BLV-gp51) and a small amount of the Mr 24,000 viral core protein. A cow was classified as seronegative to BLV-gp51 only if AGID test results were negative in both years. This serological test does not distinguish between animals that are merely exposed to the virus and those that are in advanced stages of BLV infection (8).

**Leukocyte Counts and Differentials**

Blood samples were collected in vacutainer tubes containing acid-citrate-dextrose solution (Becton Dickinson, Rutherford, NJ). Buffy coat leukocytes were purified by centrifugation of whole blood for 10 min at 600 × g and transfer of the buffy coat to 17 × 100-mm polystyrene tubes (VWR, San Francisco, CA) containing 5.0 ml of red blood cell lysing solution (.155 M ammonium chloride solution supplemented with .1 mM EDTA, pH 7.4). Following centrifugation for 5 min at 500 × g, cells were resuspended in 1.0 ml of lysing solution, transferred to 1.3-ml plastic tubes (Robbins Scientific, Mountain View, CA), and centrifuged for 1 min at 1000 × g. Cells were washed twice with 1.0 ml of 90% Eagle's Minimum Essential Medium for suspension culture (S-MEM, pH 7.4; Sigma, St. Louis, MO) and 10% citrate solution (.13 M). Lymphocyte percentages ranged from 70% to 90% in these preparations as assessed by Wright-Giemsa staining.

A fluorescein isothiocyanate (FITC)-conjugated F(ab')2 fragment of rabbit anti-bovine IgG (heavy and light chains specific; Cappel, Malvern, PA) was used to directly label slg-bearing cells. For each sample, 2 × 10^6 cells were incubated with 50 μl of a 1:16 dilution of this reagent in .15 M phosphate-buffered saline (PBS) for 30 min at 4°C. Cells plus an FITC-conjugated F(ab')2 fragment of rabbit antimouse Ig (IgM + IgG + IgA; Cappel, Malvern, PA) were included as controls. Cell suspensions were washed 3x in cold S-MEM and fixed with 2% formalin in .9% NaCl for 3 min, washed 2x, and resuspended in PBS. The bovine T cell lymphoblastoid cell lines EBL1 and EBL2 (4, 18) were negative for slg and less than 10% of granulocytes purified from red blood cell fractions stained positive with either reagent under identical assay conditions. As additional controls, lymphocytes from randomly selected individuals were tested against the antibovine IgG reagent that was first absorbed with EBL1 and the percentage of positive cells did not

Journal of Dairy Science Vol. 71, No. 9, 1988
differ significantly from results obtained with the unabsorbed reagent or by preincubation of lymphocytes for 1 h at 37°C.

**Flow Cytometry**

A laser-based flow cytometer (EPICS C, Coulter Electronics, Hialeah, FL) equipped with a 2-W argon laser tuned to 488 nm was used to enumerate the percentage of slg+ cells. Laser power was 400 mW and high voltage on the photomultiplier tube (PMT) detecting green fluorescence was set at 1700 V. Green fluorescence was measured on a logarithmic scale. Gain on the forward angle light scatter detector was set at 20 and high voltage of the PMT detecting 90° light scatter was set at 650 V with a gain setting of 2. The percentage of B cells was calculated as the percentage of slg+ cells in the bit-mapped lymphocyte population (>95% lymphocytes), which was gated on forward angle and 90° light scatter properties (4, 13). The histogram marker was set such that 1% or less were positive in control samples, and a total of 10,000 cells in the gated region were counted and analyzed for FITC emission.

**Statistical Analysis**

Values for animal age, absolute leukocyte count, absolute lymphocyte count, and B cell percentage at the time of the second bleeding were used for the analyses. The 1st-yr blood samples were used only for a serological test to BLV-gp51 and for determination of hematological status based on the EC Leukosis Key. The relationship of B cell percentage to absolute leukocyte count and absolute lymphocyte count in peripheral blood of seropositive cows (Figure 1). In seronegative cows, there was no correlation between the percentage of B cells and these hematological parameters.

### TABLE 1. Hematological parameters in cows seronegative and seropositive to BLV-gp51.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Seronegativea (n = 67)</th>
<th>Seropositive (n = 173)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>X</td>
<td>SE</td>
</tr>
<tr>
<td>Leukocytes/μl</td>
<td>7716</td>
<td>501</td>
</tr>
<tr>
<td>Lymphocytes/μl</td>
<td>4002</td>
<td>463</td>
</tr>
<tr>
<td>% slg+ lymphocytes (B cells)</td>
<td>37</td>
<td>2</td>
</tr>
<tr>
<td>Age, yr</td>
<td>3.0</td>
<td>.2</td>
</tr>
</tbody>
</table>

aAll means in column are different from corresponding means of seropositive cows (P<.005 for each comparison).

### RESULTS

**Hematological Parameters in Seropositive and Seronegative Cows**

There were no animals that were seropositive to BLV-gp51 on the first test date and negative on the second; 29 animals that were seronegative the 1st yr seroconverted and were considered as seropositive to BLV-gp51 for statistical analyses. Absolute leukocyte count, absolute lymphocyte count, percentage of B cells, and age were all significantly greater in seropositive cows (Table 1). Regression analyses demonstrated linear positive correlations between the percentage of B cells and absolute leukocyte and lymphocyte counts in peripheral blood of seropositive cows (Figure 1). In seronegative cows, there was no correlation between the percentage of B cells and these hematological parameters.

**B Cell Percentage as a Function of Bovine Leukemia Infection and Age**

Age did not have an effect on B cell percentage in seronegative cows (Table 2). In contrast, absolute lymphocyte counts decreased significantly with age in seronegative animals, consistent with previously published results (15). Among seropositive cows, there was an upward trend in the percentage of B cells with
Figure 1. The relationship of B cell percentage to absolute leukocyte count (top) and absolute lymphocyte count (bottom) in seropositive (○) and seronegative (△) cows. Absolute leukocyte and lymphocyte counts are on a logarithmic scale. For all regression models, B cell percentage was the dependent variable (y). Panel A: in seropositive cows \( y = 0.0025x + 28.0, \ r = 0.67, \ P < 0.0001; \) in seronegative cows \( y = -0.0011x + 45.0, \ r = -0.24, \ P > 0.05. \) Panel B: in seropositive cows \( y = 0.003x + 34.9, \ r = 0.72, \ P < 0.0001; \) in seronegative cows \( y = -0.003x + 47.5, \ r = -0.39, \ P > 0.05. \) Slopes were different \( (P < 0.05) \) between seropositive and seronegative cows for each comparison.
TABLE 2. Effect of age on B cell percentage in cows seronegative and seropositive to BLV-gp51.

| Age (yr) | Seronegative | | | Seropositive | | |
|----------|--------------|-----------------|-----------------|-----------------|-----------------|
|          | n | Lymphocytes/μl | % B cells | n | Lymphocytes/μl | % B cells |
| 1 to 2   | 5 | 5070 ± 12 | 512* | 1 | 5338 ± 14 | 4384c,d |
| 2 to 3   | 41 | 4180 ± 33 | 335**a | 36 | 2 ± 4a | 53 | 5649 ± 60b,c,d |
| 3 to 4   | 9 | 3444 ± 57 | 312a | 41 | 5a | 56 | 6163 ± 58b,c,d |
| 4 to 5   | 7 | 3262 ± 35 | 355 | 40 | 6a | 27 | 7937 ± 84c |
| >5       | 5 | 3512 ± 42 | 397 | 36 | 5510 ± 73d |

* Means in row within measurements with different superscripts differ (P<.05).
** Means in column with different superscripts differ (P<.05).
1 Mean is different from all other means in column (P<.05 for each comparison).
2 Mean is different from means of 3 to 4 and 4 to 5 yr age groups (P<.05 for each comparison).

Age. Seropositive cows in the 2 to 3, 3 to 4, and 4 to 5 yr age groups had significantly greater absolute lymphocyte counts and percentage of B cells compared with seronegative cows. Above 5 yr of age, lymphocyte count and B cell percentage did not differ significantly between seronegative and seropositive cows, although both parameters tended to be greater in seropositives.

B Cell Percentages in Cows Classified According to the EC Leukosis Key

Each cow was classified according to its hematological status based on the EC Leukosis Key, and means of age, absolute lymphocyte count, and B cell percentage were compared (Table 3). Seroconversion and progression of BLV infection to PL were clearly age depen-

TABLE 3. The B cell percentages, lymphocyte counts, and ages of cows classified according to the European Community's (EC) Leukosis Key.

<table>
<thead>
<tr>
<th>AGID reaction (EC key)</th>
<th>Hematology</th>
<th>n</th>
<th>Age</th>
<th>Lymphocytes/μl</th>
<th>% B cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seronegative Normal</td>
<td>67</td>
<td>3.0</td>
<td>.2a</td>
<td>4002 ± 269b</td>
<td>37</td>
</tr>
<tr>
<td>Seropositive Normal</td>
<td>125</td>
<td>3.8</td>
<td>.1</td>
<td>4144 ± 197</td>
<td>45</td>
</tr>
<tr>
<td>Seropositive Dubious</td>
<td>17</td>
<td>3.6</td>
<td>.3</td>
<td>8241 ± 534</td>
<td>69</td>
</tr>
<tr>
<td>Seropositive Lymphocytotic</td>
<td>15</td>
<td>4.4</td>
<td>.4</td>
<td>10,277 ± 568</td>
<td>69</td>
</tr>
<tr>
<td>Seropositive PL 1</td>
<td>16</td>
<td>5.0</td>
<td>.3d</td>
<td>15,645 ± 550</td>
<td>78</td>
</tr>
</tbody>
</table>

* Mean is different from all other means in column (P<.05 for each comparison) except for the mean of seropositive dubious cows (P>.05).
1 All means in column are different (P<.05 for each comparison) except for the means of seronegative and seropositive hematologically normal cows (P>.05).
2 All means in column are different (P<.05 for each comparison) except for the means of seropositive dubious and seropositive cows with lymphocytosis (P>.05).
3 Mean is different from all other means in column (P<.05 for each comparison) except for mean of seropositive lymphocytotic cows (P>.05).
4 Persistent lymphocytosis.
dent. All seronegative cows had lymphocyte counts in the normal range as defined by the EC Leukosis Key. However, seropositive cows that were hematologically normal had a significantly greater mean percentage of B cells as compared with seronegatives (45 and 37%). Thus, in this group of seropositive cows, B cell expansion was detected without a concomitant increase in total lymphocyte count. Seropositive cows that fell into the dubious, lymphocytotic, and PL categories also had elevated percentages of B cells in peripheral blood (69, 69, and 78%).

The European Community's Leukosis Key Compared with an Index Based on B Cell Percentage

To evaluate B cell percentage as a means of detecting subclinical progression of bovine leukemia virus infection, an index was developed based upon the distribution of B cell percentages in seronegative cows (Table 4). Out of 125 seropositive cows with normal lymphocyte counts, 10 and 18% had B cell percentages that were 2 and 3 SD above the mean percentage of B cells in seronegative cows, whereas all cows with PL had B cell percentages at least 3 SD above this mean. Most cows in the dubious and lymphocytotic categories also had B cell percentages greater than 3 SD above the mean B cell percentage of seronegative herd-mates. These results demonstrate that at the time of the second blood sampling, B cell percentage resolved the subclinical status of individual animals that were classified in indeterminate categories of the EC Leukosis Key.

The B cell percentage and absolute lymphocyte count were negatively correlated in seropositive cows that had 16 to 22% B cells, but these results should be regarded with caution because of the small sample size (Table 4). Increases in absolute lymphocyte count in these cows thus appeared to be due to increases in non-B cells. There were significant positive correlations between B cell percentage and absolute lymphocyte count in seropositive cows that had 23 to 52%, 53 to 59%, and ≥60% B cells. The index appears to be conservative in that there are likely to be cows with advancing subclinical BLV infection in the 23 to 52% group (as would be expected because the tolerance limit in this B cell range is 95%). For example, when cows with less than 44% B cells were considered (±1 SD), the correlation between B cell percentage and absolute lymphocyte count was not significant (data not shown).

**DISCUSSION**

Around 20 yr ago, the relationship between absolute leukocyte count, percentage of leukocyte subtypes, and total lymphocyte count

---

**TABLE 4. Comparison of the European Community's (EC) Leukosis Key to an index based on percentage of B cells in peripheral blood.**

<table>
<thead>
<tr>
<th>AGID</th>
<th>Hematology (EC key)</th>
<th>16 to 22%</th>
<th>23 to 52%</th>
<th>53 to 59%</th>
<th>≥60%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seronegative</td>
<td>Normal</td>
<td>3</td>
<td>64</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Seropositive</td>
<td>Normal</td>
<td>4</td>
<td>85</td>
<td>13</td>
<td>23</td>
</tr>
<tr>
<td>Seropositive</td>
<td>Dubious</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>14</td>
</tr>
<tr>
<td>Seropositive</td>
<td>Lymphocytotic</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>12</td>
</tr>
<tr>
<td>Seropositive</td>
<td>PL²</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
<td>r³</td>
<td>-95*</td>
<td>.38*</td>
<td>.25†</td>
<td>.59*</td>
<td></td>
</tr>
</tbody>
</table>

1Index is based on general mean and SD in seronegative cows in the herd: 16 to 22% is 2 to 3 SD below mean; 23 to 52% is ±2 SD from mean; 53 to 59% is 2 to 3 SD above mean; ≥60% represents >3 SD above mean. One SD = 7.2%. Ranges in index were rounded to nearest percent. Numbers in table are numbers of animals in each category.

²Persistent lymphocytosis.

Correlation between B cell percentage and absolute lymphocyte count in seropositive cows only (*P<.05, †P<.06).
was described (1). These studies led to the establishment of “leukosis keys” for cattle based on absolute lymphocyte count and animal age (1, 15). In these studies, cattle were not grouped according to serological status, because a standardized serological test for BLV infection was not available. Comparison of our data with absolute leukocyte and lymphocyte counts reported by Mammerickx et al. (15) for Holstein cattle revealed that their values fall between means we report here for individuals seropositive and seronegative to BLV-gp51. These data strongly suggest that normal values for absolute lymphocyte count established in the EC Leukosis Key would have been significantly lower had a serological test for BLV infection been available and the serological status for BLV infection in individual animals been taken into account. We also consider it important that B cell percentages were not significantly different between age-grouped seronegative cows (Table 2), although the number of seronegative animals available in most age groups was limited. These results contrast with the well-documented decline in absolute lymphocyte counts that occurs with age (15) that has made the use of the established hematological keys somewhat cumbersome.

The significance of the PL phase of BLV infection has been a matter of debate (6). The major question is whether PL is a benign or preleukemic stage of BLV infection. The facts concerning PL are that 1) only around 30% of all infected cattle develop PL, 2) not all cows that develop PL develop tumors (10 to 50%), and 3) not all cows that develop tumors pass through PL. [10 to 50% (6)]. We agree that PL is a subclinical stage of BLV-infection that identifies a BLV-infected population at greater risk for tumorigenesis than seropositive cattle without PL. Based on our results, we propose that the percentage of B cells, compared with absolute lymphocyte count, is a more sensitive measure of early subclinical progression of BLV infection, because only the relevant lymphocyte population is considered. Furthermore, we propose that animals with increased percentages of B cells constitute the entire population at risk for tumor development. In two recent tumor cases that we studied, neither had PL but both had significantly elevated percentages of B cells in peripheral blood (92 and 84%, respectively, unpublished data).

Although the correlation between B cell percentage and total lymphocyte count was marked when all seropositive cows were examined (r = .72; Figure 1), the degree of correlation between these two traits was different in animals grouped according to the EC Leukosis Key and the B cell index (Table 4). These data have important implications concerning the possible use of an index based on B cell percentage for evaluating subclinical progression of BLV infection. 1) As subclinical BLV infection progresses, B cell percentage apparently becomes more highly correlated with absolute lymphocyte count. The B cell percentage was significantly correlated with absolute lymphocyte count in the group with >60% B cells (r = .59). 2) It is highly unlikely that an increase in B cells above 60% can be due to a cause other than BLV infection. The only factor reported to increase the percentage of B cells in bovine peripheral blood other than BLV is infection with bovine viral diarrhea virus [BVD, (2)]. However, in BVD-infected animals, absolute counts of T and B cells were also depressed. 3) The B cell percentages between 53 and 59% are clearly associated with BLV infection, as no seronegative animals had B cell percentages in this range (Table 4).

It is our ultimate aim to recognize better the different subclinical stages of BLV infection so that more informative studies on the genetics of resistance and susceptibility to EBL can be performed. Indeed, our recent work in this and other herds has shown that one part of genetic control of susceptibility to BLV infection is the BLV-infected host’s ability to limit the polyclonal expansion of B cells in peripheral blood (12, 14). Using a combination of the AGID test, determination of the total lymphocyte count and assay of B cell percentage, results presented herein demonstrate that we can now unambiguously distinguish BLV-exposed cattle that do not have detectable B cell proliferation from BLV-infected cattle with early and advanced subclinical infection. This is shown in Table 4; 29% (29/135) of seropositive cows with normal absolute lymphocyte counts had increased percentages of B cells. Furthermore, our study suggests that if, on a given day, a cow is seropositive and also has an
increased percentage of B cells, it is irrelevant whether or not lymphocytosis is persistent. This point is also illustrated in Table 4; 81% (26/32) of cows classified as dubious or lymphocytotic had greater than 60% B cells, as did cows with PL, although the 1 yr between differentials probably exaggerated this effect. Had the differentials been performed closer in time to the second bleeding (a 3-mo minimum is recommended) more of these animals likely would have been classified as PL.

Using our methods, the mean (37%) and range (19 to 52%) of B cell percentages for seronegative cattle were somewhat higher than normal values reported by others (7, 17, 21). Different values for B cell percentages in our study could be due to the relative sensitivity of flow cytometry compared with microscopic evaluation and other methods of assay of bovine B cells. Because B cell percentage appears to be a more sensitive assay of subclinical progression of BLV infection, it is important that methodologies and reagents for determining B cell percentages be standardized. Hence, the B cell index presented here is meant to provide a general guideline until more data are available from other laboratories and for different breeds of cattle.

ACKNOWLEDGMENTS

The authors thank G. McCoy and R. Milsap for their assistance in organizing bleeding of animals. The helpful comments of D. Bernoco, K. W. Kelley, and L. B. Schook are also appreciated.

REFERENCES


