ABSTRACT

Blood leukocytes of cattle characterized in terms of bovine leukemia virus (BLV) infection and persistent lymphocytosis (PL) were examined for the presence of lymphocyte subpopulation markers and viral antigens. The percentages of cells with surface and intracytoplasmic immunoglobulin M (IgM) and erythrocyte-antibody-rosetting cells agreed closely in all infected cattle. This correlation and the results of double labeling experiments indicate that virtually all the surface IgM-positive B-lymphocytes in the blood of these animals carry Fc receptors. In PL cattle, the percentages of surface IgM-positive cells were more than twice those of normal cells and accounted for all the increase in peripheral blood lymphocytes. B-cells accounted for most of the increase in peripheral blood lymphocytes seen in cattle with PL. In contrast, most BLV-infected, nonlymphocytotic cattle had normal percentages of B-cells. Thus, the expansion of the B-cell population in blood, while being a conspicuous characteristic of PL, is not necessarily a consequence of BLV infection per se.

Comparisons of the percentages of IgM-positive and erythrocyte-antibody complement-rosetting cells, together with the results of double labeling experiments, indicate that about one-half of the B-cells in the blood of cattle with PL lacked C-3 receptors. The proportion of these cells (most likely immature B-lymphocytes) was smaller in the blood of BLV-infected nonlymphocytotic cattle.

Direct comparison showed that, in BLV-infected cattle with or without PL, and in BLV-free cattle, virtually all erythrocyte-rosetting blood cells had human agglutinin receptors. With only one exception, the numbers of erythrocyte-positive cells in the blood of BLV-infected cattle with or without PL were within normal values.

The "null" blood cell population, estimated as the difference between the IgM-positive and erythrocyte-positive populations, was essentially unaffected in BLV-infected cattle without PL, but it was absent in PL cattle.

The large majority of the B-lymphocytes present in the blood of cattle with PL were infected with BLV. The proportion of infected B-lymphocytes in the blood of BLV-positive nonlymphocytotic cattle was much lower. Even in cattle with low or moderate levels of BLV-infected blood lymphocytes, the percentages of these cells were remarkably constant during the 12-month period of the study. The data indicate that most of the BLV-infected B-lymphocytes of cattle with PL lack C-3 receptors.

INTRODUCTION

BLV infection in cattle is, as a rule, persistent even though virus-neutralizing antibodies are continuously present, usually in high titers, in all infected animals. Only a small fraction (probably less than 10%) of the BLV-infected cattle develop leukemia (lymphosarcoma). The rest of these animals either remain as asymptomatic carriers or develop a condition known as PL (for review, see Ref. 1). It has been assumed that PL represents a preleukemic stage. However, most cattle with PL are otherwise clinically normal and do not develop leukemia even when kept until advanced ages. Moreover, there is no evidence that these animals harbor neoplastic cells (2). Thus, PL in cattle should be regarded as an essentially benign response to BLV infection. The available evidence strongly suggests that the development of lymphosarcoma and PL in cattle depends to a large extent upon the host genetic constitution (3, 4).

Several workers (5-11) have found increased percentages of PBL bearing slg in cattle with PL, but they did not distinguish whether these cells had synthesized or absorbed the immunoglobulin. DeLima and Mitscherlich (12) showed that PL-positive cattle have elevated percentages of 1 PL bearing C-3 receptors, as determined by EAC rosetting. Kumar et al. (6) reported increases in the percentages of slg-positive cells and cells bearing C-3 receptors (as determined by binding of aggregates of human IgG) in the blood of 5 cows with PL. These 2 studies, however, do not clarify the relation of BLV infection to the observed increases in PBL bearing B-cell markers, because neither the control nor the PL-positive animals were examined for BLV.

Takashima et al. (11) showed that the concentration of slg-positive or EAC-positive PBL was higher in BLV-infected cattle with PL than in BLV-free cattle. Since BLV-infected cattle without PL were not included, this study also fails to clarify whether or not BLV infection per se was responsible for the changes in the concentration of PBL bearing B-cell markers. More conclusive information on this question was reported by Kenyon and Piper (5) who studied animals that had been examined for both BLV infection and PL. This study showed that, as compared with BLV-free cattle, about 65% of the BLV-infected cattle without PL had normal concentrations of slg-positive or EAC-positive PBL. In contrast, the percentages of these cells were greatly increased in all cattle with PL.

The available information on the changes in the concentration of T-cells in the blood of BLV-infected cattle is scant. In 3 PL cattle examined by Paul et al. (8), the percentages of PBL forming erythrocyte rosettes were about one-half of that of nonlympho-
cytotic cattle of unknown BLV status. On the other hand, Takashima et al. (11) found no significant differences between BLV-infected cattle with PL and noninfected cattle in regard to the concentration of erythrocyte-positive PBL.

From the studies of Kenyon and Piper (13) and Paul et al. (14), it appears that the BLV-infected PBL in cattle are predominantly, if not exclusively, B-cells. However, no information is available on the contribution of BLV-infected PBL to the PL status.

The purpose of the present study was to obtain more conclusive information on the changes in PBL populations associated with BLV infection and PL. With this purpose, the blood of a relatively large number of animals of the same breed, rigorously characterized in terms of BLV infection and PL and matched with regard to age, was examined for the presence of cells bearing IgM on the surface and cytoplasm; cells forming EA, EAC, or erythrocyte rosettes; and cells with receptors for PNA. Using a highly sensitive and specific assay developed recently (15), we have also compared the concentration of BLV-infected PBL in BLV-infected cattle with or without PL.

MATERIALS AND METHODS

Animals. Twenty-one adult cattle naturally infected with BLV, 10 of which had PL, and 5 BLV-free control animals of approximately the same ages were obtained from a high-incidence study herd (herd BF) maintained at the University of Pennsylvania. The characteristics of this herd have been described (3, 4). None of the experimental animals developed leukemia or any other clinically detectable abnormality during the observation period. The BLV-infected animals had antibodies against the virion glycoprotein antigen, as determined by radioimmunoassay (16), and were positive in an in vitro BLV infectivity assay (17). Animals were classified in regard to PL according to criteria previously established (4).

Preparation of Peripheral Blood Lymphocytes. The isolation, culture, and harvest of blood lymphocytes were done as described in the accompanying paper (15).

Immunofluorescence Assay for slg. PBL were washed 3 times and resuspended to a final concentration of $10^8$ viable cells/ml of PBS. One hundred $\mu$l of this suspension were mixed with 400 $\mu$l of normal goat serum to prevent nonspecific reactions. A monospecific rabbit antiserum against bovine IgM (Miles Laboratories, Inc.) was added to the mixture to a final concentration of 1:400. After incubation for 1 h at 4°C, the cells were washed 3 times and resuspended in 300 $\mu$l of PBS, pH 7.4. Fluoresceinated goat anti-rabbit IgG (heavy and light) (Miles Laboratories, Inc.) was then added to the cell suspension to a final concentration of 1:300. Following incubation for 45 min at 4°C, the cells were washed again 3 times with cold PBS and resuspended in a 25% (v/v) glycerol in 0.15 M NaCl. Each test included controls in which the rabbit antiserum was replaced with normal rabbit serum or PBS. At least 200 cells were counted using a Zeiss fluorescence microscope to determine the percentage of cells with slg.

Immunofluorescence Assay for Intracellular Immunoglobulin. The preparation of the cell smears and the performance of the tests were essentially the same as that of the AIP test described in the accompanying paper (15). The first incubation was done with monospecific rabbit antisera against bovine IgM, IgG1, IgG2, or IgA (Miles Laboratories, Inc.), which were previously diluted 1:300 in normal goat serum to prevent nonspecific reactions. For counterstaining, we used 0.02% Basic Blue 24 (Sigma Chemical Co.) for 10 min. In the control, the monospecific rabbit antisera were replaced with normal rabbit sera. As positive controls for IgG1 and IgG2, we used smears of cell suspensions prepared from the spleen of a BLV-free animal.

Erythrocyte, EA, and EAC Rosette Test Procedures. These were done as described in the accompanying paper (15).

Determination of PNA Receptors. The lymphocytes were isolated as described above and adjusted to $10^8$/ml. To 100 $\mu$l of cell suspension, fluorescein isothiocyanate-PNA (E.Y. Laboratories, Inc.) was added at the final concentration of 1:100, incubated at 4°C for 30 min, and washed 3 times with PBS (pH 7.4), and the cells were scored using a fluorescent microscope.

Simultaneous slg Assay and EA or EAC Rosetting. PBL were assayed for slg as described above, washed, and divided into 2 aliquots. One of these aliquots was used for EA rosetting, and the other was used for EAC rosetting following the procedures described earlier, except that the incubations were done at 4°C to prevent slg capping. Also, the ethidium bromide staining solution was not used. At least 200 cells were scored for slg and rosette formation using a fluorescent microscope. The numbers of cells forming EA or EAC rosettes were not influenced by preincubating them with the rabbit anti-IgM serum and corresponding conjugate or by incubating them at 4°C instead of 37°C.

AIP Assay. This test has been described in detail in the accompanying paper (15).

RESULTS

Table 1 shows the percentages of PBL carrying surface or intracytoplasmic IgM, as well as the percentages of PBL forming EA, EAC, and erythrocyte rosettes in 3 categories of cattle: BLV-free; BLV infected without PL; and BLV infected with PL. The numbers given in the table are the average of 2 (cells with slg) or 3 (cells forming EA, EAC, or erythrocyte rosettes) measurements performed at monthly intervals. These replicate measurements were remarkably constant for each animal. The results show that the percentages of PBL carrying slg were within normal values in 8 of 11 infected cattle without PL, and it was elevated significantly (3 or more SDs above the mean) in the remaining 3 animals. In contrast, in all cattle with PL, the blood concentration of these cells was markedly increased.

In the 2 groups of infected cattle, the percentages of PBL with intracellular IgM were in good agreement with those of slgM-bearing cells. Furthermore, less than 9% of the cells with intracellular immunoglobulin reacted with monospecific antisera against IgG1, IgG2, or IgA (results not shown). Thus, the large majority of the slg-bearing PBL were B-lymphocytes.

Table 1 also shows that, whereas all but one (BF-301) of the infected nonlymphocytotic cattle had normal levels of EA-rosetting PBL, the concentration of these cells was greatly increased in all cattle with PL. In nearly all infected cattle, the percentages of EA-rosetting and slg-positive PBL were in close agreement, thus suggesting that they represent the same B-cell population. This was confirmed in double labeling tests on 4 infected cattle, 2 of which had PL; 99% of the slg-bearing PBL of these animals formed EA rosettes. Thus, slg-positive EA-positive B-cells account for most of the increase in lymphocytes circulating in the blood of cattle with PL.

The percentages of PBL cells forming EAC rosettes were within normal values in infected cattle, even in those with PL (Table 1). It is clear, therefore, that a large proportion of the B-lymphocytes in the blood of cattle with PL were EAC negative. This was also shown directly in double labeling experiments in which about 50% of the slg-bearing PBL of the 2 BLV-infected cattle with PL examined formed EAC rosettes. In 2 BLV-infected nonlymphocytotic cattle, the proportion of EAC-rosetting slg-positive cells was about 80%.

In most of the cattle with PL and in one of the infected nonlymphocytotic cattle, the percentages of PBL forming eryth-
BLOOD POPULATION OF BLV-INFECTED CATTLE

Table 1
Percentages of PBL with various markers in BLV-free and BLV-infected cattle with and without PL

<table>
<thead>
<tr>
<th>Cattle</th>
<th>Total PBL/cu mm of blood</th>
<th>IgM</th>
<th></th>
<th>Rosettes</th>
<th></th>
<th></th>
<th>EAC</th>
<th>EAC</th>
<th>Erythrocyte</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>On the surface</td>
<td>Intracellular</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>EA</td>
<td>EAC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

BLV free

| BF-366 | 4,300 | 24 | ND<sup>a</sup> | 47 | 31 | 29 |
| BF-360 | 5,100 | 37 | ND | 38 | 32 | 36 |
| BF-359 | 4,000 | 32 | ND | 43 | 35 | 30 |
| BF-358 | 7,200 | 28 | ND | 46 | 41 | 26 |
| BF-352 | 5,200 | 23 | ND | 46 | 20 | 33 |

BLV infected, PL negative

| BF-362 | 3,600 | 39 | 51 | 45 | 43 | 26 |
| BF-349 | 5,000 | 42 | 49 | 44 | 31 | 29 |
| BF-338 | 3,500 | 44 | 61 | 51 | 43 | 24 |
| BF-335 | 3,700 | 21 | 27 | 26 | 15 | 37 |
| BF-330 | 4,200 | 35 | 40 | 39 | 29 | 30 |
| BF-309 | 4,600 | 54 | 47 | 45 | 35 | 34 |
| BF-304 | 4,200 | 55 | 52 | 48 | 37 | 37 |
| BF-301 | 5,500 | 73 | 85 | 68 | 44 | 16 |
| BF-284 | 1,700 | 31 | 40 | 35 | 29 | 34 |
| BF-281 | 2,700 | 38 | 49 | 44 | 32 | 26 |

BLV infected, PL positive

| BF-340 | 14,500 | 88 | 85 | 73 | 44 | 10 |
| BF-337 | 10,600 | 82 | 89 | 74 | 43 | 11 |
| BF-334 | 11,000 | 81 | 86 | 74 | 42 | 16 |
| BF-331 | 12,200 | 86 | 88 | 80 | 34 | 12 |
| BF-314 | 10,400 | 86 | 84 | 84 | 44 | 11 |
| BF-308 | 10,900 | 82 | 91 | 81 | 42 | 10 |
| BF-295 | 11,700 | 88 | 87 | 71 | 33 | 17 |
| BF-286 | 10,800 | 85 | 90 | 72 | 42 | 16 |
| BF-238 | 20,200 | 90 | ND | 77 | 29 | 43 |
| BF-177 | 11,400 | 63 | 82 | 83 | 45 | 9 |

|       | 85 ± 2.8 | 86.8 ± 2.7 | 76.9 ± 4.5 | 39.8 ± 5.3 | 13.5 ± 4.1 |

<sup>a</sup> ND, not done.
<sup>b</sup> Mean ± SE.

Erythrocyte rosettes were significantly lower than in the BLV-free controls. It is important to note, however, that the absolute number of erythrocyte-positive cells was within normal values in all but one of the PL animals. The exception was a cow (BF-238) whose PBL counts were by far the highest.

PBL samples of 3 cattle with PL, 3 BLV-infected cattle without PL, and 6 noninfected cows were examined in parallel in the erythrocyte rosette and PNA tests. The percentages of erythrocyte-positive and PNA-positive cells were virtually identical for all but one of the PL animals. The exception was a cow (BF-238) whose PBL counts were by far the highest.

Using the AIP test (15), we also examined the proportion of BLV-infected cells in PBL samples obtained at approximately monthly intervals during a 12-month period from all the infected animals. The results are summarized in Table 2. Cattle with PL consistently showed greater percentages of AIP-positive PBL than nonlymphocytic cattle. In both groups of animals, the percentages of infected PBL were remarkably constant during the entire observation period. Only 3 cattle (BF-362, BF-301, BF-304) showed deviations greater than the 95% confidence interval of the values obtained during the 12-month observation period. In 2 of them, the deviations were seen at parturition.

In both PL and non-PL cattle, the number of BLV-infected PBL correlated well with the number of slg-positive (Chart 1A) or EA-positive (Chart 1B) cells. The number of BLV-infected PBL also correlated well with the number of EAC-positive cells in non-PL cattle. However, in PL cattle, there was little or no correlation (Chart 1C). Thus, most of the BLV-infected B-lymphocytes in these cattle are EAC negative.

DISCUSSION

The presence of nonlabile, monomeric surface IgM is a characteristic shared by all the B-cells of the species examined. B-cells also have Fc and EAC receptors, but these are neither unique to B-cells nor are they present in all B-cells. There are also non-B-cells with Fc receptors that can bind serum immunoglobulin of all classes. Thus, to identify true B-cells, it is necessary to use antibody specific for subclass immunoglobulin or to show that the slg is not labile. There are also cells believed to be in the B-cell lineage that lack slgM but have intracellular IgM. Erythrocyte receptors appear to be a unique characteristic of T-cells, although it is not yet clear whether all T-cells of cattle have erythrocyte receptors (see Ref. 18).

Several investigators (5–11) have reported on the presence of slg on bovine PBL. However, they did not use antisera specific for IgM and made no attempt to demonstrate that the immunoglobulin was endogenous rather than cytophilic. In the present study, we have used class-specific antisera to show that the slg present on the PBL of BLV-infected cattle with or without PL, and in BLV-free cattle, is predominantly, if not exclusively, IgM.
The large majority of the slgM-positive PBL from infected cattle also had cytoplasmic IgM, thus confirming that they were B-lymphocytes. Consistent with this conclusion are also the results showing that most of these cells had Fc receptors, as determined by their ability to form EA rosettes. This latter observation is at variance with the results of Kumar et al. (6), who found that a significant proportion of the slg-bearing PBL of cattle with PL did not react with fluoresceinated heat-aggregated human immunoglobulin. It is likely that this discrepancy reflects a higher sensitivity of the EA-rosetting technique for the detection of bovine cells with Fc receptors.

Our results confirm the conclusion (5-12) that PL is associated with an increase in the percentage of circulating B-cells. Indeed, our data show that the expansion of the B-cell population accounts for virtually all of the increase in PBL associated with PL. A possible exception was Cow BF-238 which also showed a marked increase in the number of erythrocyte-positive PBL. BF-238 had, by far, the highest blood lymphocyte counts among the animals examined. Thus, the possibility should be considered that, in animals such as this, with exceptionally high numbers of PBL, the increase in lymphocyte subpopulations in blood is more generalized. It should be noted that the sum of slgM-positive and erythrocyte-positive PBL in BF-238 was 133%. Thus, it is also possible that some of the PBL of BF-238 had both slg and Fc receptors.

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erythrocyte receptors. Usinger and Splitter (19) have demonstrated a very small (less than 1%) subpopulation of cells bearing both slg (class unknown) and PNA receptors in undefined cattle. BF-238 was one of the cattle tested for both erythrocyte receptors and PNA receptors, and the percentages of cells with these markers were almost identical. Therefore, this cow could conceivably have had a substantial increase in the PNA-positive B-cell subpopulation described by Usinger and Splitter (19).

Other studies (5–11) have not shown as high percentages of slg-positive PBL in PL cattle as those found by us. A likely explanation is that anti-IgM antibody is more efficient in detecting slgM than the polyclonal antisera used in the other studies.

It is important to stress that most of the BLV-infected non-PL cattle examined by us, as well as those examined by Kenyon and Piper (5), had normal percentages of B-cells in blood. Thus, contrary to a commonly stated conclusion, BLV infection per se does not necessarily involve an expansion of the B-cell population in blood. A corollary of this finding is that the development of PL involves, in addition to BLV infection, other factors. There are data indicating that these factors are largely associated with the host’s genetic constitution (3, 4).

Approximately one-half of the circulating B-cells which we detected in cattle with PL lacked C-3 receptor, as shown by their inability to form EAC rosettes. Such cells, which most likely are relatively immature B-lymphocytes, were also present, but in a lower proportion, in the blood of BLV-infected nonlymphocytotic cattle. Kenyon and Piper (5) and Takashima et al. (11) did not find evidence for the presence of the slg-bearing lymphocytes lacking C-3 receptors in the blood of BLV-infected cattle. This may have been due to an overestimation of the percentage of EAC-positive cells in PL cattle. In the case of Kenyon and Piper (5), this overestimation was probably due to the use of trypan blue in counting rosettes. Since this dye, unlike the acridine-orange:ethidium bromide dye used by us, does not stain viable cells, the nonrosetting viable lymphocytes must be distinguished by size and morphology. In our experience, such identification is highly equivocal; it always leads to undercounting of nonrosetting lymphocytes, particularly in PL cattle, and hence to overestimation of the percentage of rosetting cells. The differences of our results with those of Takashima et al. (11) could be ascribed to the fact that we used a different complement in the EAC-rosetting technique.

The percentages of erythrocyte-rosetting cells detected by us in BLV-free and BLV-infected cattle are in good agreement with those reported by Takashima et al. (11), but they are considerably lower than those found by Paul et al. (8). It should be noted, however, that the non-PL cattle examined by Paul et al. (8) were only 1 to 2 years old, an age at which the PBL counts in cattle begin to gradually decrease. Also, these workers used trypan blue staining in counting rosettes and might have therefore overestimated the percentages of erythrocyte-rosetting cells because of the reasons mentioned earlier.

With the exception of Cow BF-238, the absolute numbers of erythrocyte-rosetting PBL were within normal values in BLV-infected cattle with or without PL. Thus, the erythrocyte-positive, presumably T-, cell population of cattle does not seem to be affected by either BLV infection per se or by the development of PL.

The sum of the percentages of IgM-bearing cells and erythrocyte-positive cells was about 100% (except in BF-238) in PL cattle, 70% in BLV-infected non-PL cattle, and 60% in BLV-free cattle. By this calculation, it would appear that PL, but not necessarily BLV infection per se, is associated with the loss of a “null” lymphocyte population.

The close correlation between the numbers of BLV-infected cells and the number of slg-positive or EA-positive cells in the blood of animals with or without PL implies that B-lymphocytes are the main, if not the only, target for BLV infection in cattle. From our results, it is clear that most of the PBL of cattle with PL were infected with BLV. The proportion of BLV-infected lymphocytes in the blood of nonlymphocytotic cattle was much lower and more variable. The data in Chart 1C indicate that the majority of the BLV-infected PBL in cattle with PL belong to the EAC-negative B-cell subpopulation. In contrast, all, or nearly all, BLV-infected PBL of non-PL cattle were EAC positive. This indicates that the development of PL is associated with the release into the blood stream of cells which do not usually circulate in significant numbers.

Previous studies (20) have shown that, as the consequence of a specific plasma-blocking protein, BLV particles are not synthesized or are seldom synthesized in vivo. If synthesized, BLV particles are most likely prevented from infecting other cells by the virus-neutralizing antibodies that are present, usually in high titers, in all BLV-infected cattle (10, 21). Thus, it is reasonable to assume that the BLV-infected PBL in cattle are derived from precursor B-lymphoid cells which are infected during the first phase of the infectious process. This assumption provides the most likely explanation for our observation that the concentration of BLV-infected PBL was remarkably constant for as many as 12 months, even in cattle with low or moderate levels of these cells. An alternative explanation is that new lymphocytes become infected periodically in vivo and are released into the blood stream at about the same rate at which previously infected cells are removed from circulation. Lymphocyte recirculation studies are needed to resolve this issue.

It is conceivable that the number of B-cell precursors that become infected with BLV and therefore the percentage of infected PBL depend to a large extent on the levels of the BLV plasma blocking factor that are present during the initial stages of infection. Thus, the animals that develop PL could be those that, following infection, synthesize the blocking factor less rapidly and efficiently.

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BLOOD POPULATION OF BLV-INFECTED CATTLE


Characterization of the Blood Lymphocyte Population in Cattle Infected with the Bovine Leukemia Virus

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