Studies on the Relationship between Infection with Bovine C-type Virus, Leukemia, and Persistent Lymphocytosis in Cattle

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SUMMARY

Leukemic cows and adult cattle in several herds under long-term surveillance were examined for the presence of antibodies to bovine C-type viruses (BLV) in order to investigate further the possible relationships between infection with this virus, bovine leukemia, and persistent lymphocytosis (PL). This latter condition is considered a preclinical stage of cattle leukemia by some investigators. Fluorescent antibodies reacting specifically with a virion antigen in the cytoplasm of BLV-infected cells were found in 90% of leukemic cows and in 80 to 100% of clinically normal cows with PL in multiple-case herds. The antibody titers of animals with PL were often lower than those of the leukemic cows. Clinically normal cattle with consistently normal lymphocyte counts in the multiple-case herds also showed fluorescent antibodies to BLV; however, in each of these herds the incidence (25 to 76%) and, in most cases, the titers of antibodies found in the nonlymphocytic animal groups were lower than those of the corresponding PL groups. The antibody was also present in one-third of the cattle in a single-case herd in which the PL rate was not significant. In contrast, antibody was found in only 4 out of 214 animals from leukemia-free herds. In two multiple-case herds in which pedigree data are available, it was observed that animals from high-risk families had fluorescent antibodies to BLV at a significantly higher frequency, and often at higher titers, than animals from minimal-risk families. These data are consistent with the hypothesis that BLV may be the etiological agent of bovine leukemia and perhaps of PL but that host and virus factors may play significant roles in the host's response to BLV infection.

Virus was detected electron microscopically in 19 out of 20 antibody-positive cattle and in none of 12 antibody-negative animals examined. This shows that the fluorescent antibody test is as sensitive as electron microscopy for the demonstration of current BLV infection.

The distribution of precipitating antibodies reacting in immunodiffusion tests with the intraspecies group-specific (gs-I) BLV antigen among our various cattle populations correlated closely with the distribution of the fluorescent antibodies. This and the observation that semipurified BLV gs-I antigen completely removes the specific fluorescent activity of our standard BLV reference serum indicates that the fluorescent and precipitating antibodies react with the same antigen. A comparison of the fluorescent and precipitating activities in the same bovine sera showed that the fluorescent antibody test is substantially more sensitive than the immunodiffusion test for the demonstration of BLV infection.

The significance of these data is discussed in terms of the value of the bovine system as a model for studies on the natural history of leukemia.

INTRODUCTION

Particles similar in morphology and size to the mature forms of the C-type leukemia viruses of other species were originally detected by Miller et al. (28) in short-term cultures of BC cells from leukemic cattle. Studies in our laboratory confirmed and extended this observation, demonstrating that the particles do in fact represent viruses (33). A C-type virus was also found in permanent cultures (NBC cell lines) established from peripheral lymphocytes of leukemic cows (10, 12, 13). We have shown that the viruses from the BC cultures and NBC cell lines share a virion antigen as demonstrated by the immunofluorescence technique in the cytoplasm of acetone-fixed cells as well as an antigen detected by gel diffusion experiments in ether-treated virus preparations or cell extracts of virus-infected cells (8, 9, 11). The presence of a precipitin antigen in the C-type virus isolated from the BC cultures was confirmed by Miller and Olson (29). Studies in our laboratory have also indicated that the precipitin antigen of the bovine virus most probably represents the intraspecies bovine gs-I antigenic determinant. While this antigen is ether resistant, soluble, and apparently located within the virion, its specificity differs from that of the murine and feline gs-I antigens (7, 8, 11).

The morphological and antigenic similarities of the viruses found in the short-term BC cultures and in the NBC cell lines indicate that these agents represent different...
isolates of the same bovine C-type virus, hereafter referred to as BLV.\(^3\)

Epidemiological and pedigree studies have shown that the adult form of bovine leukemia, the most frequent form of the disease, occurs in striking herd and familial aggregations (2, 6, 26). On the basis of the observation that many multiple-case herds contain some clinically normal animals with PL, Bendixen (4) proposed that PL is due to the same agent responsible for bovine leukemia and that it represents a subclinical form of the disease. On the other hand, long-term herd studies in the Eastern United States have shown that, although in many herds leukemia and PL aggregates in the same families, in other herds these aggregations do not coincide (1, 2, 26).

During the past 15 years we have intensively studied a large number of multiple-case, single-case, contact, and leukemia-free herds by means of periodic blood counts, pedigree analyses, clinical surveillance, and detailed post-mortem examination. These cattle populations have provided us with an extraordinary opportunity for large-scale seroepidemiological studies on the distribution of BLV infection and its possible relationship to bovine leukemia and PL. In a previous report we showed that fluorescent and precipitating antibodies to BLV occur in high incidence in 2 multiple-case herds, particularly in animals with leukemia and PL, whereas, in leukemia-free herds, these antibodies were either absent or found in a much lower percentage of animals (11). Subsequently, Olson et al. (30) reported similar results on the distribution of precipitating antibodies to BLV among various herds. Since then our seroepidemiological studies have been extended to include more animals from our various herd categories. Owing to extensive knowledge of the pedigrees and the hematological and clinical characteristics of these cattle populations, they are particularly well suited for studies on the possible relationship between BLV infection and PL.

**MATERIALS AND METHODS**

**Herd BB.** A purebred Guernsey herd of approximately 35 animals in which 4 cases of leukemia have been documented during a 14-year period. Approximately 33% of the cattle have PL.

**Herd BF.** A purebred Jersey herd of approximately 120 animals in which 47 cases of leukemia have been documented during a 15-year period. Approximately 40% of the cattle have PL.

**Herd BJ.** A purebred Jersey herd of approximately 225 animals in which 2 cases of leukemia have been documented during the past 3 years. This herd had been under our surveillance as a contact herd (2, 25) for 8 years prior to occurrence of the 1st leukemia case. Approximately 18% of the cattle have PL.

**Herd BG.** A purebred Holstein herd of approximately 220 animals that had been under our surveillance for 11 years as a leukemia-free control herd. Owing to the occurrence of 1 leukemia case in the summer of 1973, this herd is now classified as a single-case herd. Approximately 1.8% of the cattle have PL.

All leukemia cases in the above herds were adult female cattle.

**Herd BH.** A leukemia-free purebred Guernsey herd of approximately 35 animals that has been under our surveillance for 8 years. None of these cattle has ever had PL.

**Herd BI.** A leukemia-free purebred Guernsey herd of approximately 175 animals that has been under our surveillance for 8 years. None of these cattle has ever had PL.

**Herd GB.** A leukemia-free purebred Guernsey herd of approximately 145 animals that has been under our surveillance for 5 years. None of these cattle has ever had PL.

**Cell Cultures**

Cell line NBC-13 was established from peripheral lymphocytes of a cow with leukemia and is routinely maintained as a suspension culture in McCoy's 5A modified medium supplemented with 10 or 20% heat-inactivated fetal calf serum. Details on the derivation, maintenance, and characteristics of BLV production of this cell line have been reported earlier (13, 16).

The procedures for the short-term cultures of bovine BC cells have also been described in detail (33). PHA was used at the concentration of 0.01 or 0.02 ml/ml of medium. Virus and cells for antigen preparation or for electron microscopic examination were collected from these cultures 48 to 72 hr after they were set up.

**Preparation of Cells for Immunofluorescent Tests**

Target cells were obtained from NBC-13 cultures showing 15 to 20% of the cells with viral BLV antigen. The presence of this antigen was determined by immunofluorescent tests with our standard BLV reference serum obtained from a cow (27–125) in which leukemia had regressed spontaneously. The specificity and characteristics of this serum have been fully described in previous papers (7, 9). NBC-13 cells were washed twice in PBS at pH 7.2 by low-speed centrifugation (500 x g for 15 min), adjusted to a concentration of approximately 2 x 10^6 cells/ml of PBS.

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\(^3\) Final proof for the etiological role of BLV in cattle leukemia has yet to be obtained. The designation BLV is appropriate; however, on the basis of strong indirect evidence, i.e.: (a) morphologically, as in the case of the other known leukemia viruses, BLV is a C-type virus; (b) as shown in this paper and in previous studies (11, 28, 33), BLV infection and bovine leukemia are closely associated; and (c) transmission studies indicate that BLV is leukemogenic (31).
and added to special slides containing 8 wells and prepared as described by Hirshaut et al. (19). After the addition of the cells, the slides were air dried, fixed for 10 min in acetone at room temperature, and stored at −70° until used.

**Fluorescent Staining**

The indirect technique was used in this study. Approximately 0.05 ml of serum was placed in each well and incubated for 45 min at 37° in a humidified incubator. The slides were then washed 3 times in PBS (5-min intervals for each wash), dipped in distilled water, and air dried. Next, FITC-conjugated goat anti-bovine γS globulin diluted 1:10 with a solution of bovine serum albumin: rhodamine (1:30 in PBS), was added to each well, and the slides were incubated at 37° for 40 min. The procedures for the preparation and adsorption of the FITC-conjugate have been described previously (9). After incubation with the conjugate, the slides were again washed twice in PBS, dipped in distilled water, and air dried. Then, a small drop of glycerin, diluted 1:1 in PBS, was placed next to each well, and the slides were covered with a 24- x 50-mm coverslip.

As controls the cells were incubated with either a known positive serum (Reference Serum 27–125), a known negative serum (from cows in leukemia-free Herd BH), or PBS and then with the FITC-conjugate.

Slides were examined with a Zeiss fluorescent microscope with a dark-field condenser, FITC filter, barrier filters (65 and 50), and a quartz halogen bulb as the light source.

**Antigen for Immunodiffusion and for Immunofluorescence Absorption Tests**

Viral pellets were obtained from clarified (centrifugation at 2,000 × g for 15 min) culture fluids by centrifugation at 56,000 × g for 2 hr. The pellets were dispersed in a small volume of PBS and mixed with 5 ml of peroxide-free ether (anesthetic grade). The mixture was continuously agitated at room temperature until most of the ether evaporated. This procedure was repeated once more and then nitrogen gas was bubbled through the mixture until the ether completely evaporated. Ether-treated cell extracts were prepared as follows. Approximately 1 × 10⁷ cells were packed by centrifugation at 2,000 × g for 20 min, resuspended in 15 ml of distilled water, and then homogenized in a Polytron (Bronwill Scientific, Rochester, N. Y.) homogenizer in the cold at speed 30 for 5 min. This homogenate was then concentrated by lyophilization at about 3 ml and treated twice with 15 ml of ether as described for the viral pellets. The homogenate was then clarified by 2 cycles of centrifugation at 15,000 × g for 30 min and concentrated by lyophilization to about 1 ml.

Semipurified BLV γS antigen, kindly provided by our associate, Dr. Hugh McDonald, was prepared by placing an ether-treated and clarified BLV preparation on a G-100 Sephadex (bead form) (Pharmacia, Uppsala, Sweden) column (1.5 × 100 cm) equilibrated in 0.02 M Tris-HCl: 0.2 M NaCl: 0.02 M NaN₃, pH 7.8. The column flow rate was 6 to 8 ml/hr, and 1-ml fractions were collected; the void volume of the column was 53 ml. Antigen reactivity of the fractions was determined by immunodiffusion with Reference Serum 27–125. The antigen was eluted at approximately 71 ml. The final preparation used in the immunofluorescence absorption tests was a pool of the most active fractions.

**Immunofluorescence Absorption Tests.** Reference Serum 27–125 was diluted 1:64 (approximately two 2-fold dilutions below its end point) and mixed with an equal volume of the antigen preparation. After incubation at 37° for 1 hr with occasional agitation and remaining overnight in the cold, the mixture was centrifuged at 5000 × g for 15 min. The supernatant fluid was removed and a further 2-fold dilution was made in PBS. Samples of these undiluted and diluted supernatant fluids were tested by the indirect immunofluorescence technique on acetone-fixed NBC-13 cells.

**Immunodiffusion Ouchterlony Tests.** These were carried out in 2% Noble agar (Immunoplate, Pattern C, Hyland Laboratories, Los Angeles, Calif). The wells were filled twice with the reagents, and the plates were incubated at room temperature in a humidified chamber. The slides were examined at regular intervals with magnifying lenses under indirect illumination over a 4-day period. Optimal precipitation occurred by 18 or 24 hr and remained unchanged until at least the 4th day. Subsequently, the plates were immersed in sodium barbital buffer (pH 7.4) for 2 days and in distilled water for 1 day and then air dried at room temperature. The dried agar plates were stained for 10 min in 0.1% Amido black 10B (1.0 g Amido black 10B in a solution of 100 ml glacial acetic acid, 700 ml methanol, and 200 ml distilled water) and then destained in the same solution without Amido black 10B for 2 to 3 min. After this staining procedure, bands that were weak on the unstained plates usually became more noticeable.

For the seroepidemiological survey, the standard antigen preparation (ether-treated BLV obtained from cell line NBC-13) was placed in the center well and, as a positive control, Reference Serum 27–125 was added to alternate peripheral wells. All the positive sera found in this study formed precipitating lines showing reaction of complete identity with the line formed by Serum 27–125.

**Electron Microscopy.** Methods for the preparation of cell cultures for electron microscopy have been described elsewhere (9, 33). In order to ensure that each cell section viewed represented a different cell, only a single section on each block was examined.

**RESULTS**

**Fluorescent Antibodies.** All sera tested except the controls were coded in order to avoid bias in scoring the results. All positive sera gave a fluorescent reaction pattern similar to that observed with Reference Serum 27–125, i.e., bright staining which was either diffuse or granular and confined solely to the cytoplasm. Extensive studies have demonstrated that, in the NBC cell lines, the presence of the fluorescent antigen and the presence of the virus, as determined by electron microscopic examination, are closely correlated. Furthermore, in the present study, many of the sera that were found to react with BLV-producing NBC-13 cells were tested against cells from NBC-13 cultures maintained...
under conditions in which neither the virus nor its antigen are detected (9, 13). Negative results were obtained in all cases. Fluorescent cells were not observed in the control wells in which a known negative bovine serum and the conjugate or PBS and the conjugate were tested against BLV-producing NBC-13 cells.

As shown in Table 1, the majority (90%) of leukemic cattle referred to our veterinary hospital or occurring in our multiple-case study herds had fluorescent antibodies to BLV with titers that in most cases were relatively high. Clinically normal cattle with PL in the multiple-case herds had a similar incidence (80 to 100%) of antibody, but the titers were generally lower than in the leukemic group. Cattle with consistently normal lymphocyte counts in the multiple-case herds also had BLV antibody; however, in each of these herds the groups of nonlymphocytotic animals had lower incidences and usually lower titers than the corresponding groups with PL. In single-case Herd BG, which had a PL rate of only 1.8%, sera were collected for 3 years before the leukemia case occurred. Of the 54 Herd BG cattle tested, 18 (33%) had antibodies and most of these were in low titer. The reactors in Herd BG included the cow that subsequently developed leukemia (titer, 1:16) and 2 animals (titers, 1:4 and 1:8) which had lymphocyte counts above expectation but below the level required for classification as PL. The overall incidence of antibody in the animals from the multiple- and single-case herds studied was 115 out of 250 (46%). In contrast, antibodies were present in only 4 of the 214 (2%) animals tested in the 3 herds in which no leukemia or PL has ever been detected.

Correlation between Fluorescent Antibodies and Virus Infection. In order to determine whether or not the presence of fluorescent antibodies to BLV is associated with the actual presence of virus, a group of 32 cattle (25 from the multiple-case BF herd, 3 from the leukemia-free BI herd, and 4 leukemia referral cases) were examined for both BLV antibody and C-type virus particles in PHA-treated, short-term BC cultures. We have shown previously that the in vitro maintenance of these cells is essential for expression of BLV and that this is enhanced by the addition of PHA to the culture medium (33). Cells for electron microscopic examination were collected 48 to 72 hr after the cultures were initiated and in some cases at both intervals. A culture was not considered negative for virus particles until at least 300 cell sections, each representing a different cell, were examined. The results (Table 2) show that there is indeed a very close and direct correlation between the presence or absence of BLV antibody and the presence or absence of the virus; only 1 out of 20 antibody-positive animals tested was negative for C-type virus particles, and no virus particles were present in any of the cultures derived from antibody-negative animals.

Relationship between Leukemia Risk and BLV Infection. The availability of 2 multiple-case herds (BA and BF), in which previous studies demonstrated that the disease aggregates along family lines (2, 6), provided us with an opportunity further to examine the relationship between leukemia risk and virus infection. Table 3 shows that the incidence of fluorescent antibody (86%) among cattle in families with maximal leukemia risk is significantly higher than that (63%) found among animals from families with minimal risk ($\chi^2 = 8.513, d.f. = 1, p < 0.01$); moreover, the antibody titers of the animals with maximal risk were in most cases higher than the titers of the animals with minimal risk.

Precipitating Antibody. Many of the bovine sera that were

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**Table 1**

**Distribution of fluorescent antibodies to BLV among cattle**

<table>
<thead>
<tr>
<th>Cattle category</th>
<th>No. antibody positive/total no. tested</th>
<th>2-4</th>
<th>8-32</th>
<th>64-256</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukemic</td>
<td>62/69 (90)†</td>
<td>18 (29)‡</td>
<td>24 (39)‡</td>
<td>20 (32)‡</td>
</tr>
<tr>
<td>Multiple-case BA herd</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PL*</td>
<td>15/16 (94)</td>
<td>1 (7)</td>
<td>14 (93)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Non L</td>
<td>24/48 (50)</td>
<td>14 (58)</td>
<td>10 (42)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Multiple-case BB herd</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PL</td>
<td>8/10 (80)</td>
<td>6 (75)</td>
<td>2 (25)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Non L</td>
<td>2/8 (25)</td>
<td>2 (100)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Multiple-case BF herd</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PL</td>
<td>32/35 (91)</td>
<td>17 (53)</td>
<td>15 (47)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Non L</td>
<td>16/21 (76)</td>
<td>14 (88)</td>
<td>2 (12)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Multiple-case BJ herd</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PL</td>
<td>24/24 (100)</td>
<td>8 (33)</td>
<td>14 (59)</td>
<td>2 (8)</td>
</tr>
<tr>
<td>Non L</td>
<td>16/34 (47)</td>
<td>13 (81)</td>
<td>3 (19)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Single-case BG herd</td>
<td>18/54 (33)</td>
<td>12 (67)</td>
<td>6 (33)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Leukemia-free BH herd</td>
<td>0/32 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Leukemia-free BI herd</td>
<td>2/40 (5)</td>
<td>2 (100)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Leukemia-free GB herd</td>
<td>2/142 (1)</td>
<td>2 (100)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

* Histopathologically confirmed cases referred to our clinic or occurring in the multiple-case study herds.
† Reciprocal of the highest serum dilution showing fluorescent activity.
‡ Numbers in parentheses, % of total.
§ Numbers in parentheses, % of positive sera.

PL, clinically normal animals with PL; Non L, clinically normal, nonlymphocytotic animals.
tested for fluorescent antibodies to BLV were also tested for precipitating antibodies to the BLV gs-1 antigen. The association between the occurrence of the 2 types of antibodies among different groups of cattle is shown in Table 4. The incidence of precipitating antibodies was lower than that of fluorescent antibodies in most cases but followed the same trend, i.e., the incidence was high among the cattle with leukemia or PL, lower in the nonlymphocytotic animals from multiple-case herds, and rare in the animals from leukemia-free herds.

Relationship between Fluorescent and Precipitating Antibodies. Since the largest difference between the incidence of the 2 types of antibodies was found in the group in which the titers of fluorescent antibody were low (animals without PL) (Table 4), it was of interest to examine the relationship between the titers of fluorescent antibody and the precipitating activity of the same sera. The results (Table 5) clearly show that the higher the fluorescent antibody titer in a given serum the more likely it is to have precipitating activity. Thus, in considering the possibility that the 2 types of antibodies are directed against the same antigen, we tested the ability of various preparations containing the BLV-precipitating antigen to absorb the fluorescent antibodies from BLV Reference Serum 27-125. As shown in Table 6, the fluorescent activity of the reference serum for the viral antigen in acetone-fixed NBC-13 cells was totally removed after incubation with all of the preparations of BLV gs-1 antigen tested, including one that was partially purified by column chromatography.

Table 2

**Correlation between the presence of fluorescent antibodies to BLV and the presence of C-type virus particles in short-term BC cultures**

<table>
<thead>
<tr>
<th>Titer of fluorescent antibodies a</th>
<th>Presence of BLV in short-term BC cultures a</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;2</td>
<td>0/12</td>
</tr>
<tr>
<td>2-4</td>
<td>10/11</td>
</tr>
<tr>
<td>8-32</td>
<td>9/9</td>
</tr>
</tbody>
</table>

a Determined by the indirect immunofluorescence technique on acetone-fixed NBC-13 cells. Titers expressed as the reciprocal of the highest antiserum dilution showing distinct fluorescent activity.

b Determined by electron microscopic examination.

Table 3

**Correlation between leukemia risk and incidence and titers of fluorescent antibodies to BLV**

<table>
<thead>
<tr>
<th>Degree of leukemia risk a</th>
<th>No. antibody positive/total no. tested</th>
<th>Titer distribution of positive sera</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2-4</td>
<td>8-32</td>
</tr>
<tr>
<td>Maximal</td>
<td>59/69 (86)%</td>
<td>23 (39)%</td>
</tr>
<tr>
<td>Minimal</td>
<td>40/63 (63)%</td>
<td>26 (65)%</td>
</tr>
</tbody>
</table>

a Determined by pedigree evaluation of cattle from multiple-case herds BA and BF.

b Numbers in parentheses, % of total. Differ significantly ($\chi^2 = 8.513$, d.f. = 1, $p < 0.01$).

c Numbers in parentheses, % of positive sera.

**Table 4**

**Correlation between the incidence of fluorescent and precipitating antibodies to BLV**

<table>
<thead>
<tr>
<th>Cattle category</th>
<th>Fluorescent antibodies a (positive/total)</th>
<th>Precipitating antibodies b (positive/total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukemic c</td>
<td>55/60 (92)%</td>
<td>44/60 (73)%</td>
</tr>
<tr>
<td>Multiple and single-case herds</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PL</td>
<td>49/51 (96)%</td>
<td>38/51 (74)%</td>
</tr>
<tr>
<td>Non L</td>
<td>67/148 (45%)</td>
<td>23/148 (15%)</td>
</tr>
<tr>
<td>Leukemia-free herd</td>
<td>4/120 (3)</td>
<td>2/120 (2)</td>
</tr>
</tbody>
</table>

a Determined by the indirect immunofluorescence technique on acetone-fixed NBC-13 cells.

b Determined in Ouchterlony immunodiffusion tests with a standard reference BLV gs-1 antigen preparation.

c Leukemic, histopathologically confirmed cases referred to our clinic or occurring in 3 multiple-case study herds; PL, clinically normal animals with PL; Non L, clinically normal, nonlymphocytotic animals.

d Numbers in parentheses, % of total.

**Table 5**

**Correlation between the titer of fluorescent antibodies and precipitating activity of bovine sera**

<table>
<thead>
<tr>
<th>Fluorescent antibody titer a</th>
<th>Precipitating antibodies b (positive/total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;2</td>
<td>0/85 (0)%</td>
</tr>
<tr>
<td>2-4</td>
<td>15/65 (24)%</td>
</tr>
<tr>
<td>8-16</td>
<td>47/53 (89)%</td>
</tr>
<tr>
<td>&gt;32</td>
<td>37/37 (100)%</td>
</tr>
</tbody>
</table>

a Determined by the indirect immunofluorescence technique on acetone-fixed NBC-13 cells. Titers expressed as the reciprocal of the highest antiserum dilution showing distinct fluorescent activity.

b Determined in Ouchterlony immunodiffusion tests with a standard reference BLV gs-1 antigen preparation.

c Numbers in parentheses, % of total.

**Table 6**

**BLV gs-1 antigenic activity and immunofluorescence absorption activity of various antigen preparations**

<table>
<thead>
<tr>
<th>Antigen preparation</th>
<th>BLV gs-1 antigen activity a</th>
<th>Immunofluorescence absorption activity a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ether-treated BLV from cell line NBC-13</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ether-treated homogenate of BLV-infected NBC-13 cells</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ether-treated cell homogenate from a BLV-infected short-term BC culture a</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ether-treated cell homogenate from an uninfected short-term BC culture a</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

a Determined in Ouchterlony immunodiffusion tests with Reference Serum 27-125. All the precipitin lines of the positive antigen preparations showed reactions of identity with the line formed by a standard reference BLV gs-1 antigen preparation.

The immunofluorescence absorption tests were carried out with Reference Serum 27-125 which, after incubation with the antigen preparation, was tested for residual fluorescent activity against acetone-fixed NBC-13 cells. +, complete absorption.

b Derived from a cow with PL in a multiple-case herd.

c Derived from a normal cow in a leukemia-free herd.

BLV, Leukemia, and Lymphocytosis
DISCUSSION

Confirming previously reported results from this laboratory (9, 11), the present seroepidemiological survey shows that BLV infection, as evaluated by the presence of fluorescent antiviral antibodies, is highly prevalent in cattle with leukemia and in clinically normal animals from multiple-case and single-case herds, whereas it is infrequent or absent in leukemia-free herds. That the presence of fluorescent antibody to BLV does represent actual rather than past infection, at least in adult animals, is demonstrated by the fact that C-type virus particles were found in short-term BC cultures of 19 out of 20 animals examined. A possible exception to this relationship has emerged from unpublished studies on newborn calves in the multiple-case BF herd. These calves possess fluorescent antibodies to BLV which most likely have been acquired passively through colostrum.

The observation that all animals which yielded C-type virus-positive BC cultures had fluorescent antibodies indicates that, for the detection of BLV infection, the fluorescent antibody test is at least as sensitive as electron microscopic examination of PHA-treated BC cultures. In addition, the fluorescent antibody technique is simpler, faster, and less expensive than electron microscopic examination.

The pattern of distribution of BLV, as determined by the present seroepidemiological survey, among herds with different incidences of leukemia (Table 1) as well as within Herds BA and BF in which pedigree data demonstrate familial aggregations of the disease (Table 3), clearly establishes that there is a close association between leukemia or leukemia risk and BLV infection. This pattern and recently reported transmission studies suggesting that BLV is leukemogenic (31) strongly supports the view that BLV is the etiological agent of bovine leukemia. On the other hand, the incidence of leukemia in the multiple- and single-case herds studied (Ref. 2; “Materials and Methods”) is much lower than the incidence of BLV infection. In addition, in multiple-case Herds BA and BF we found that more than one-half of the animals with minimum risk of the disease are infected with the virus. These observations are consistent with the idea that, if BLV is in fact the causative agent of bovine leukemia, the development of the disease is strongly influenced by host genetic factors.

It is conceivable that, in general, the titer of fluorescent antibodies reflects the degree of BLV infection. If this is the case, the finding that the antibody titters of leukemic cattle are usually higher than those of clinically normal animals in multiple-case herds could be taken to indicate that the level of virus infection is also an important determinant in development of the disease. Also, in multiple-case Herds BA and BF, there seems to be a positive correlation between antibody titters and leukemia risk (Table 3).

It is also possible that the immune mechanism in some cattle may be impaired (leukemic infiltration, genetic factors, etc.) and that such animals would be incapable of producing antibody or would produce only low levels of antibody to BLV. This could explain the absence or low titters of antibody found in some of the leukemic and high-leukemia-risk animals in our study.

Our data showing that the incidence of fluorescent antibodies to BLV in clinically normal animals with PL in multiple-case herds is quite similar to that observed among leukemic cows are compatible with the view of Bendixen (4) that PL is due to the same agent that is responsible for leukemia. The fact that in most cases cattle with PL had lower titers of antibody than did leukemic animals may indicate that animals with PL are either genetically more resistant to BLV or have been exposed to a lower virus dose. The same reasoning could explain why cattle without PL in the multiple-case herds studied here have even a lower incidence as well as lower titers of BLV antibody. Thus, depending on host genetic susceptibility and/or virus dose, response to BLV infection could be the production of antibody alone, the production of antibody with PL, or the production of antibody with leukemia with or without PL. In any case, the fact that cattle without PL in multiple-case and single-case herds have antibodies to BLV clearly shows that PL cannot be used as a reliable indicator of BLV infection.

On the other hand, previous studies have shown that PL and leukemia may occur in different families within the same herd and, moreover, that 35% of leukemic cattle do not have histories of PL (2, 26). Thus, it is possible that bovine leukemia and PL may not have a common etiology. From a cow with lymphocytosis, Van Der Maaten et al. (34) have recently isolated a virus (R-29) that, upon injection into newborn calves, causes PL. Morphologically, R-29 is not a C-type virus and preliminary experiments in our laboratory show that it is antigenically different from BLV (D. M. Bhatt, C. Diglio, and J. F. Ferrer, unpublished experiments). Thus, it is possible that PL may be due to R-29 and that this virus may occur simultaneously with BLV in many herds as a result of a particularly favorable ecology.

The present data showing that precipitating antibodies to the BLV gs-l antigen are frequent in multiple- and single-case herds and very infrequent or absent in leukemia-free herds also confirm and extend results of a previous study (11). It should be clarified that in that communication we had reported a 17% incidence of precipitating antibody in a leukemia-free herd. This was Herd BG which, as mentioned in “Materials and Methods,” subsequently developed 1 case of leukemia and has been therefore reclassified as a single-case herd. The observation that the fluorescent activity of Reference Serum 27–125 on BLV-infected cells can be completely removed by incubation with semipurified and ether-treated BLV precipitin antigen indicates that the fluorescent antibodies found in this study are also reacting with BLV gs-l. It is important that the gs antigen of other C-type viruses can also induce both fluorescent and precipitating antibodies and that, of the known antigens of, or associated with, other leukemia viruses, only the gs antigen is either resistant and can be detected in the cytoplasm of acetone-fixed cells (5, 14, 15, 18, 20, 23).

Studies on the occurrence of fluorescent and precipitating antibodies in the same sera showed that, of the sera with
fluorescent antibody titers between 1:2 and 1:4, only 24% had precipitating activity (Table 5). Thus, it is clear that fluorescent antibodies are a much more sensitive marker for BLV infection than precipitating antibodies.

Our previous results (11) on the frequent occurrence of precipitating antibody to BLV in clinically normal animals from multiple-case herds has been recently confirmed by Olson et al. (30), using ether-extracted virus pellets from short-term BC cultures as antigen. These authors did not find any precipitating sera in a leukemia-free herd kept in isolation, an observation that is also in agreement with our results (Table 4) showing that the sera of only 2 out of 120 animals in leukemia-free herds had precipitating activity. On the other hand, Olson et al. found a significantly higher incidence (up to 16%) of precipitating antibody in nonisolated herds that have been free of the disease for at least 13 years. However, since 3 cases did occur in one of these herds prior to that time, it is perhaps not surprising that BLV infection, as indicated by the presence of antibodies, is still present in this herd. It is also possible that such herds, containing considerable numbers of infected animals, may develop the disease at some future time. This possibility is illustrated by our Herd BG in which no leukemia cases had been detected during 15 years of close observation. As discussed above, Herd BG, unlike our other leukemia-free herds, has a relatively high incidence of both fluorescent (33%) and precipitating (17%) antibodies and has recently developed a case of leukemia. This emphasizes the value of the BLV antibody test, particularly the detection of fluorescent antibodies, as an indicator of leukemia risk for a given herd.

In considering results on the distribution of antibodies to BLV in the cattle population, it is important to take into account that, as shown by Malmquist et al. (24), animals from multiple-case or leukemia-free herds frequently possess both fluorescent and precipitating antibodies against a ubiquitous BSV which can be isolated from BC cells. It is extremely unlikely that in our seroepidemiological survey antibodies to BSV could have been mistaken for BLV antibodies since the target cells for the immunofluorescence tests and the viral antigen preparations for the immunodiffusion experiments were obtained from cultures of cell line NBC-13 rather than from short-term bovine BC cultures. NBC-13 has been extensively examined by electron microscopy and by immunofluorescence, and in no instance have we observed particles resembling BSV or cells with nuclear fluorescent staining which predominate in cultures infected with this agent (7, 9, 10, 12, 13). By periodic testing we have also found that NBC-13 cells are free of other common indigenous bovine viruses, i.e., parainfluenza-3 (PI3), bovine viral diarrhea (BVD) and infectious bovine rhinotracheitis (IBR) viruses.

It has been well established that mice are immunologically tolerant to the gs antigen of their indigenous leukemia virus, most likely as a consequence of vertical transmission of the viral genome or of the whole virus early in intrauterine life (20, 21). The observation that most cattle with leukemia and those in multiple-case herds possess naturally occurring antibodies to the gs antigen of the putative etiological agent of the disease indicates that the virus-host, and perhaps the tumor-host, immunological interactions in bovine leukemia may be different from those of murine leukemia and that, unlike the murine leukemia virus, BLV is naturally transmitted to already immunologically competent hosts. Although bovine fetuses are capable of producing circulating antibodies (22, 32), we have failed to detect fluorescent antibody to BLV in most calves from multiple-case Herd BF before the ingestion of colostrum (unpublished observations). Thus, it appears likely that natural infection with BLV usually takes place either through the milk or by horizontal transmission later in life.

In view of these fundamental differences, it is necessary now to consider the possibility that the bovine system is a more relevant model than the murine system for studies on the natural history of human leukemia. At present, the bovine system represents the only available model of a C-type virus-associated leukemia occurring spontaneously and frequently in a large, relatively outbred population of genetically well-defined animals living in close contact with man and serving as a major source of human food. In this regard, 2 out of 6 chimpanzees fed since birth on milk from BF cows infected with BLV died with leukemia at 34 and 45 weeks of age, respectively (H. McClure, R. R. Marshak, and R. P. Custer, manuscript in preparation). Leukemia has never before been reported in this primate. If the suspicion that BLV is leukemogenic for chimpanzees is confirmed in future studies, it will then be important to consider seriously the possible public health significance of bovine leukemia.

The availability of multiple-case herds in which it is possible, on the basis of pedigree studies, to identify with a high degree of accuracy those cattle that are likely to develop leukemia and those that will not develop the disease, despite natural infection with BLV, offers a unique system for studies on the participation of immunological and genetic factors in the etiology and pathogenesis of a naturally occurring leukemia as well as an opportunity to develop immunoprophylactic approaches to the disease.

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900
CANCER RESEARCH VOL. 34

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