An Infectivity Assay for Bovine Leukemia Virus Using the Immunoperoxidase Technique

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ABSTRACT

This report describes the development of a 7-day infectivity assay for bovine leukemia virus (BLV) which is based on the induction of the major internal virion antigen (a protein with a molecular weight of 25,000) in susceptible indicator monolayer cell cultures. In this assay, the antigen is detected in the indicator cells by the immunoperoxidase antibody technique using a specific rabbit antiserum to a BLV protein with a molecular weight of 25,000. The immunoperoxidase infectivity assay is specific, quantitative, reproducible, and more sensitive than the previously described syncytia induction assay. The immunoperoxidase infectivity assay can be applied to the detection of BLV-infected cells and provides a reliable method for the direct diagnosis of BLV infection in cattle.

INTRODUCTION

It has been demonstrated that BLV3 as well as BLV-infected lymphocytes induce rapid syncytia formation in several cell cultures, particularly in early passages of BESP (3, 4). These observations led to the development of a sensitive, reproducible, and specific SIA for BLV and BLV-infected lymphocytes (4, 6).

The induction of viral antigens in susceptible indicator cells has provided the basis of several infectivity assays for leukemia viruses (2, 13-15). In this report, we describe an infectivity assay of this type for BLV in which the immunoperoxidase technique has been used to detect the major internal BLV antigen (p25) (11) in the indicator cells. The IPIA proved to have several advantages over the SIA.

MATERIALS AND METHODS

Cells and Virus. The source of BLV was a clone (clone 1) derived from a cell line, BLV-bat2, which produces large quantities of BLV particles and is free of other adventitious agents, including Mycoplasma (3, 9, 10). Supernatant fluids from clone 1 were collected on Day 4 or 7, filtered through a 0.45-µm Nalgene filter, and used as the BLV inoculum.

BLV-infected lymphocytes were obtained from Cow AJ-147 with persistent lymphocytosis in multiple-case Herd BF and frozen with 20% dimethyl sulfoxide in liquid nitrogen until used.

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: BLV, bovine leukemia virus; BESP, bovine embryonic spleen cells; CC81 cells were maintained in Roswell Park Memorial Institute tissue culture medium (RPMI 1640) supplemented with 15% heat-inactivated fetal calf serum, penicillin (100 µg/ml), and streptomycin (100 µg/ml). BESP were prepared as described previously (3). A feline kidney cell line designated CRFK (1) was supplied by Dr. R. Schultz (Cornell University, Ithaca, N.Y.). The human embryonic cell lines F2000 and F4000 were purchased from Flow Laboratories (Rockville, Md.). Whole human embryonic cells (FHu) were a gift from Dr. K. Irgens (Ecole Nationale Veterinaire, Alfort, France). TB1Lu bat lung cells and embryonic bovine tracheal cells (EBTr) were obtained from American Type Culture Collection (Rockville, Md.). The mouse sarcoma virus-infected feline cell line CC81 (8) was supplied by Dr. P. Fischinger (National Cancer Institute, Bethesda, Md.).

3 All monolayer cultures, with the exception of the CC81 cells, were grown in Eagle's minimum essential medium supplemented with 10% heat-inactivated (56° for 30 min) fetal calf serum, penicillin (100 µg/ml), and streptomycin (100 µg/ml). CC81 cells were maintained in Roswell Park Memorial Institute tissue culture medium supplemented with 15% heat-inactivated fetal calf serum, penicillin, and streptomycin.

4 SIA. The standard SIA was conducted as described in previous reports (3, 6) in 35-mm Falcon tissue culture dishes which were seeded with 1.5 x 10^3 indicator BESP per dish.

5 Immunoperoxidase Assay. Indicator cells were seeded, inoculated, and incubated as in the SIA. At the end of the incubation period, the indicator cells were washed in ice-cold 150 mm NaCl: 6.1 mm Na_2HPO_4: 7H_2O: 3.8 mm KH_2PO_4 (pH 7.2) and immediately fixed for 2 min in methanol. Subsequent steps of the immunoperoxidase staining procedure were conducted as described by Nexo (13), with slight modification. The plates were incubated for 20 min at room temperature with 1 ml of rabbit antiserum to BLV p25 diluted 1:100 in TEB buffer containing 10 mg of bovine serum albumin per ml (Fraction V; Sigma Chemical Company, St. Louis, Mo.). The preparation and characteristics of this antiserum have been described previously (11). Following incubation, the plates were washed twice with TEB buffer for 10 min and then incubated for 20 min at room temperature with 1 ml of peroxidase-coupled goat immunoglobulin against rabbit IgG (Miles Laboratories, Elkhart, Ind.) diluted 1:100 in TEB buffer containing 10 mg of bovine serum albumin per ml. The plates were then washed twice with TEB buffer and stained with 2 ml of freshly prepared staining solution for 10 to 15 min in the dark. The plates were washed in deionized water, dried, and examined under a light microscope.

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The staining solution was prepared by dissolving 20 mg of 3-amino-9-ethylcarbazol (Aldrich Chemical Co., Milwaukee, Wis.) in 5 ml N,N-dimethylformamide. Ninety-five ml of cold 50 mM sodium acetate buffer, pH 5.0, were added. The mixture was stirred at 4°C for 10 min in the dark and then filtered through a 0.20-μm Nalgene filter, and 0.05 ml of 30% hydrogen peroxide was added immediately before use.

The infectivity titer of the cell-free inocula was expressed in terms of AIU/ml. To minimize counting errors, AIU were determined from duplicate plates inoculated with a dilution of the virus preparation producing 100 to 300 stained cells per dish. The average number of stained cells in these plates was then multiplied by the reciprocal of the dilution.

RESULTS AND DISCUSSION

Fig. 1, A and B, shows a low magnification of 2 cultures of BESP, one of which was infected with BLV 6 days prior to the immunoperoxidase staining. The infected culture (Fig. 1A) shows evenly distributed dark spots, whereas the control uninfected culture (Fig. 1B) is uniformly and weakly stained. At a higher magnification (Fig. 1C), it can be seen that these dark spots consist of mononucleated or multinucleated (syncytial) cells with cytoplasmic staining. Nuclear staining was never observed. The nuclei in the positive cells are readily noticeable as white holes. As shown in Fig. 1D, no stained cells were seen in the uninfected control cultures. Stained cells were also not detected in infected cultures when the rabbit anti-p25 serum was replaced by either normal rabbit serum or a rabbit antiserum prepared against the protein (molecular weight, 30,000) of the Rauscher murine leukemia virus.

Table 1 shows the results of titration experiments on 2 different indicator cell cultures which were fixed and stained 7 days after inoculation. In each of these cultures, there was a direct and close correlation between the number of stained cells and the concentration of the BLV inoculum. In other titration experiments (not shown), it was found that virus preparations having titers of 5 × 10^4 AIU per ml or more produce autoinhibition at the lower dilution.

A series of titration experiments evaluated 7 days postinoculation showed that BESP and, particularly, CEHC are the most sensitive indicator cells. The sensitivity of the CRFK cells was moderate, whereas F2000, F4000, FHu, TbLu, EBrTr, and CC81 cells showed only a few stained cells.

The response of CEHC and BESP in the IPIA did not change significantly during the first 40 cell passages. In contrast, we found that the syncytia response of BESP to BLV decreases rapidly after the 10th passage, thus confirming the results of Diglio et al. (4). Furthermore, at the passage levels used in this study, CEHC respond poorly to the syncytia-inducing effect of BLV. Therefore, it seems clear that the ability of the indicator cells to form syncytia does not affect the sensitivity of the IPIA.

Table 2 summarizes the results of an experiment in which indicator CEHC, BESP, and CRFK cells were examined for the presence of the stained cells at various times after virus inoculation. The titers obtained at Days 5 and 7 postinfection were similar in both CEHC and BESP; these titers were approximately 10-fold higher than those obtained in the corresponding cells at Day 3 postinfection. Incubation for 10 days resulted in only a slight increase in titer. After longer incubation periods, the cell monolayers began to show signs of degeneration, thus precluding reliable evaluation. The titer in CRFK cells at Day 7 postinfection was approximately 100 times higher than at Day 3 postinfection.

The sensitivities of the IPIA and SIA were compared in titration experiments using 3 different cell-free BLV preparations. The results (Table 3) show that the titers in the IPIA were 2 to 5 times higher than those in the SIA.

Previous studies (6, 7) have shown that the detection of BLV-infected lymphocytes by the SIA in the peripheral blood provides a reliable method for the direct diagnosis of BLV infection in cattle. Titration experiments (Table 4) suggest that the IPIA is as sensitive as the SIA for the detection of BLV-infected peripheral blood lymphocytes.

In conclusion, the IPIA provides a new, sensitive, and reproducible method for the direct diagnosis of BLV infection in cattle. It is of particular value for the testing of peripheral blood lymphocytes.

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### Table 1

<table>
<thead>
<tr>
<th>Dilution of the BLV inoculum</th>
<th>Indicator cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CEHC</td>
</tr>
<tr>
<td>10^0</td>
<td>TNTC</td>
</tr>
<tr>
<td>10^-1</td>
<td>2231</td>
</tr>
<tr>
<td>10^-2</td>
<td>245</td>
</tr>
<tr>
<td>10^-3</td>
<td>19</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
</tr>
</tbody>
</table>

*a* Indicator monolayers were fixed and stained 7 days after virus inoculation. Results are the average number of stained cells in duplicate dishes. 

*b* TNTC, too numerous to count.

### Table 2

<table>
<thead>
<tr>
<th>Indicator cells</th>
<th>3 days</th>
<th>5 days</th>
<th>7 days</th>
<th>10 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEHC</td>
<td>3.0 × 10^3</td>
<td>3.6 × 10^3</td>
<td>4.4 × 10^3</td>
<td>7.9 × 10^3</td>
</tr>
<tr>
<td>BESP</td>
<td>2.5 × 10^3</td>
<td>2.7 × 10^3</td>
<td>2.9 × 10^3</td>
<td>4.6 × 10^3</td>
</tr>
<tr>
<td>CRFK</td>
<td>6.7 × 10^3</td>
<td>ND</td>
<td>5.6 × 10^3</td>
<td>ND</td>
</tr>
</tbody>
</table>

*a* AIU/ml were determined by titration of the virus inoculum in serial 10-fold dilutions. Each dilution was assayed in duplicate. 

*b* ND, not done.

### Table 3

<table>
<thead>
<tr>
<th>Experiment</th>
<th>IPIA a (AIU/ml)</th>
<th>SIA b (SFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>1.32 × 10^2</td>
<td>4.6 × 10^2</td>
</tr>
<tr>
<td>15</td>
<td>3.6 × 10^2</td>
<td>1.4 × 10^2</td>
</tr>
<tr>
<td>19</td>
<td>3.7 × 10^3</td>
<td>7.8 × 10^3</td>
</tr>
</tbody>
</table>

*a* IPIA was conducted on indicator CEHC. 

*b* SIA was conducted on indicator BESP cells.

### Table 4

<table>
<thead>
<tr>
<th>Lymphocytes inoculated</th>
<th>IPIA (stained cells/dish)</th>
<th>SIA (syncytia/dish)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLV-infected Cow AJ-147</td>
<td>2.5 × 10^4</td>
<td>TNTC</td>
</tr>
<tr>
<td></td>
<td>2.5 × 10^3</td>
<td>TNTC</td>
</tr>
<tr>
<td></td>
<td>2.5 × 10^2</td>
<td>604</td>
</tr>
<tr>
<td></td>
<td>2.5 × 10^1</td>
<td>578</td>
</tr>
<tr>
<td></td>
<td>2.5 × 10^0</td>
<td>85</td>
</tr>
<tr>
<td>BLV-free Cow BI-469</td>
<td>2.0 × 10^4</td>
<td>0</td>
</tr>
</tbody>
</table>

*a* TNTC, too numerous to count. Results are the average number of stained cells or syncytia in duplicate dishes.
ducible assay for the detection of infectious BLV or BLV-infected cells. The assay is quantitative, measures infectious virus, and is more sensitive than the SIA. Another advantage of the IPIA is that its sensitivity is not significantly affected by the passage level of the indicator cells. To ascertain the specificity of the results of the SIA, it is necessary to include inhibition controls with well-characterized sera in the assay. These controls are not necessary in the IPIA because the immunoperoxidase staining is conducted with a specific rabbit anti-BLV p25 serum. In this regard, it is important to note that BLV p25 is immunologically different from the antigens of other common bovine viruses and from the antigens of other RNA tumor viruses (5, 11, 12). Thus, the IPIA may be the method of choice for the detection of infectious BLV in materials that may be contaminated with adventitious agents.

ACKNOWLEDGMENTS

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REFERENCES


Fig. 1. A and C. a low (×4) and a high (×100) magnification of a BLV-infected BESP culture stained by the immunoperoxidase technique 7 days after BLV infection. B and D, the low and high magnifications, respectively, of a control uninfected culture stained by the immunoperoxidase technique.
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