An Amplified Immunoperoxidase Assay to Detect Bovine Leukemia Virus Expression: Development and Comparison with Other Assays

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ABSTRACT

An amplified immunoperoxidase (AIP) assay using an avidin:biotin complex was developed to detect bovine leukemia virus (BLV) antigen expression in lymphocytes which had been cultured 24 h and fixed with acetone. Nonspecific reactions were eliminated by absorbing the test serum with calf embryonic heart cells and by diluting the absorbed serum with 100% horse or cow serum. DNA synthesis inhibition did not decrease the number of AIP-positive cells, and there were no apparent preferential losses of major lymphocyte subpopulations during culture. Both viable and nonviable BLV-expressing cells were detected. Thus, the number of AIP-positive cells seems to be a good estimate of the minimum number of infected lymphocytes present in the uncultured blood cells. In direct comparisons, twice as many BLV-expressing cells were detected with the AIP assay as with an indirect immunofluorescence test. The AIP assay is as sensitive as the syncytia infectivity assay and only slightly less sensitive than an immunoperoxidase infectivity assay for detecting BLV-infected lymphocytes in the blood of infected cattle that were in early stages of infection and/or had low titers of antiviral antibodies.

The AIP assay is the most sensitive, rapid, and reproducible procedure available for the identification of individual cells infected with BLV. This assay may be of great value in studies on the biology of BLV infection.

INTRODUCTION

BLV, a horizontally transmitted retrovirus, is regarded as the causative agent of enzootic bovine leukemia (lymphosarcoma), the most common cancer of cattle (for review, see Ref. 1).

Lymphocytes are the only cells known to become infected with BLV in vivo. In vitro cultivation of the infected lymphocytes is required for the detection of BLV particles (2), BLV antigens (3, 4), and BLV RNA (4, 5). This is attributable to the fact that, in vivo, the expression of the BLV genome is blocked at the transcriptional level by a protein which is present in the plasma, in the uncultured blood cells.

Thus, serological tests are commonly used for the diagnosis of BLV infection in cattle. BLV-infected cattle can also be identified directly by demonstrating infected cells in blood. Since the BLV genome is usually repressed in vivo, all direct methods for the identification of infected cells require short-term in vitro culture of the blood leukocytes. The best standardized among these methods is the SIA, which is based on the ability of the BLV-infected lymphocytes to undergo cell fusion with certain indicator cells (8), and the IPIA, which is based on the induction of BLV p25 antigen in highly susceptible monolayer cell cultures (9). BLV antigens have been demonstrated in the cytoplasm of infected, short-term cultured lymphocytes by several procedures, including the CRIA (7, 10), and the indirect IF test on acetone-fixed cells (3). Aside from electron microscopy, the IF test is the only available procedure that allows the identification of individual BLV-infected lymphocytes. Experience in other systems has shown that, as compared with IF tests, amplified immunocassays performed under stringent conditions are, as a rule, more sensitive and can be scored more consistently because of the better contrast between positive and negative reactions.

We report here the development of a specific, sensitive, and rapid amplified immunoperoxidase assay for the identification and quantitation of BLV antigen-expressing cells in 24-h cultures of blood lymphocytes. The number of AIP-positive cells was unaffected by a DNA synthesis inhibitor or by virus-neutralizing antibody, and there were no significant selective losses of major lymphocyte subpopulations during the 24-h culture. Both viable and nonviable BLV-expressing cells were detected in the AIP assay. Thus, this assay allows a close estimate of the percentage of BLV-infected blood lymphocytes in vivo. Direct comparisons of the AIP assay were made with other procedures to detect BLV-infected cells in blood.

MATERIALS AND METHODS

Animals. All cattle originated in 2 high-incidence leukemia herds (11) and were 1 to 2 years old. All had anti-BLV gp antibodies, as determined by radioimmunoassay (7).

Blood Lymphocyte Isolation. Ten ml of heparinized blood (Panheparin, 10 U.S.P. units/ml; Abbott Laboratories) were placed in a 15-ml polystyrene conical tube and centrifuged at 1000 x g for 20 min at 4°C. The Buffy coat was collected, diluted with 7 ml of MEM (suspension), and layered on an isosmophic:Lypholyte-M gradient (Gallard-Schlessinger Chemical Manufacturing Corp.) previously prepared in a 15-ml conical tube using a mixture of 2 ml of isosmoph and 2 ml of Lympholyte-M. After centrifugation at 1000 x g for 20 min at 20°C, the interface was
aspirated with a Pasteur pipet, placed in a round-bottomed tube, diluted in MEM, and centrifuged at 600 x g for 10 min at 20°C. Erythrocytes were eliminated from the cell pellet by hypotonic shock as described (2). The lymphocytes were suspended in MEM, and their viability was determined by staining with an acridine-orange:ethidium bromide solution (12).

**Lymphocyte Culture.** The culture medium used was MEM (supersision) supplemented with 20% heat-inactivated (56°C, 30 min) fetal bovine serum and penicillin G-streptomycin sulfate (100 units/0.1 mg/ml). PHA (Wellcome Reagents, Ltd.; Lot K1340) was added to the medium at a final concentration of 1:50. The cell suspension was adjusted to 3 x 10^5/ml and cultured in 50-ml Erlenmeyer glass or in 30-ml Corning tissue culture flasks. The incubation was done at 37°C in a humidified incubator with 5% CO₂ during 16 to 24 h.

After 16- to 24-h incubation, the lymphocytes were washed 3 times with PBS, pH 7.4, by centrifugation (600 x g for 10 min at 4°C), and their viability was determined as described above.

**Smears Preparation.** Lymphocyte suspensions were adjusted to 4 to 5 x 10^6/ml, and 1 ml of the suspension was added to the 2 central holes of a 1024 cytology bucket assembly (Coulter Electronics, Inc.). After centrifugation at 300 x g for 5 min, the supernatants were aspirated through the side holes. The smears were fixed while damp in acetone for 10 min, air dried, and, if not used immediately, stored at -20°C.

**Reference Sera.** The preparation and characteristics of the monospecific rabbit antiserum against BLV p25 antigen have been described (10). A control serum was obtained from a healthy young adult rabbit.

**AIP, IPRA, and SIA Assays.** For the AIP assay, the smears were washed first in PBS and then in H₂O, dried, and placed in a humidified chamber. Eighty μl of the monospecific rabbit anti-BLV p25 serum or normal rabbit serum, diluted 1:200 in 100% normal horse or bovine serum, were placed in one well. After incubation for 2 h at 37°C in a humidified incubator, the slides were washed for 15 min in PBS and then in H₂O, air dried, and placed in a humidified chamber again. Eighty μl of biotinylated goat anti-rabbit antibody (Vector Laboratories, Inc., Burlingame, CA), diluted 1:300 in PBS containing 10% BSA, were added to each well. After incubation for 1 h at 37°C, the slides were washed 3 times with PBS and once with H₂O and air dried. Each well was then incubated for 1 h at 37°C with 80 μl of the avidin:biotin complex reagent (Vector Laboratories), which was prepared 15 min earlier by mixing Reagents A and B in PBS plus 10% BSA at a 1:200 dilution. After incubation, the slides were washed 3 times with PBS and then submerged in a solution of prewarmed (37°C) 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Co.) (1 mg/ml), PBS (pH 7.4), and 0.67 μl of 30% H₂O₂ per ml. They were maintained in the dark in this solution for 10 min at 37°C and were subsequently washed 3 times with H₂O, counterstained in 0.01% aqueous methyl green for 5 min, washed 3 times with H₂O, air dried, and mounted with Preservavide.

The results were scored using a Leitz microscope (×400). At least 200 lymphocytes were counted in each well.

The IPRA (9) and SIA (8) tests were performed in 35- to 10-mm tissue culture dishes with 2-mm grids using the procedures detailed previously. Each dish was inoculated with 2 x 10⁶ viable lymphocytes.

**Indirect IF Test.** The procedure described for the AIP assay up to and including the first incubation, wash, and drying was used for preparing the smears. The slides were then placed in a humidified chamber, and both wells were incubated with fluorescein isothiocyanate:goat anti-rabbit IgG (Miles-Yeda, Ltd.) and diluted 1:200 in PBS plus 0.2% BSA:rhodamine for 30 min at 37°C. After washing them 3 times with PBS and once with H₂O, the slides were air dried and mounted in 50% glycerine in PBS, pH 7.4. Scoring was done immediately using a Zeiss microscope with epi-fluorescence excitation and a HBO 50-watt mercury lamp. At least 200 lymphocytes were counted in each slide.

**Mitomycin C Treatment of the Lymphocytes.** The lymphocytes were isolated as described before and suspended in MEM supplemented with 20% heat-inactivated fetal bovine serum. The cell concentration was adjusted to 2 x 10⁶/ml and mitomycin C (Sigma Chemical Co.) was added to a concentration of 50 μg/ml. After incubation for 30 min at 37°C, the cells were washed 3 times in MEM and cultured with or without PHA as described above. [³H]Thymidine incorporation was determined in the cultures as reported by Thorn et al. (13).

**Erythrocyte, EA, and EAC Rosette Test Procedures.** For erythrocyte rosettes, 2-aminoethylisothiouronium bromide hydrobromide (Sigma Chemical Co.)-treated sheep erythrocytes were used as described before (14).

For EA rosettes, the procedure was similar to that described for EAC rosettes (14), except that a subagglutinating concentration of the IgG of rabbit anti-sheep erythrocytes (Cappel Laboratories) was used for coating sheep erythrocytes, and complement was not added. Less than 5% of the lymphocytes formed rosettes if the RBC were not coated with the IgG.

For EAC rosettes, sheep erythrocytes were sensitized with a subagglutinating concentration of rabbit anti-sheep erythrocytes IgM (Grand Island Biological Co.). Horse serum at 1:40 dilution was used as the complement source. Details of the EAC rosette procedure have been reported (15).

Erythrocyte, EA, and EAC rosettes were counted using a fluorescence microscope after staining the lymphocytes with a solution of acridine-orange:ethidium bromide.

**RESULTS**

**Development of the AIP Assay.** We achieved consistent and specific detection of BLV p25 antigen-expressing cells following the procedure described in "Materials and Methods."

The most crucial step for the specificity of the AIP assay was the use of an adequately prepared monospecific rabbit anti-p25 serum. Assays in which anti-BLV p25 or control rabbit sera were diluted in PBS or in PBS with 3% or 10% BSA gave strong nonspecific staining. Repeated absorptions with calf liver powder (acetone dried) and/or with normal lymphocytes did not reduce significantly the nonspecific reactions. Complete removal of these reactions was achieved, however, by absorbing the primary sera with calf embryo heart cells (9) (20 x 10⁶ cells/ml of serum for 24 h at 4°C), followed by dilution of the absorbed serum with 100% normal horse or bovine serum. Both steps were necessary. Lower dilution of the absorbed rabbit sera in horse or bovine serum still gave nonspecific staining. Fig. 1A shows an example of the cytoplasmic staining obtained on cultures of lymphocytes from a BLV-infected cow using rabbit anti-p25 serum absorbed with calf embryo heart cells and diluted in 100% bovine serum. Most positive cells show an intense brown staining uniformly distributed throughout the whole cytoplasm; in other cells, the staining was restricted to some sectors of the cytoplasm and/or appeared as aggregates of granules. As shown in Fig. 1B, after absorption and dilution in 100% bovine (or horse) serum, the rabbit anti-p25 serum did not react with cultured lymphocytes from BLV-free cattle. Likewise, no staining reactions were seen in cultured BLV-infected lymphocytes tested with negative control rabbit serum absorbed with calf embryo heart cells and diluted in 100% bovine serum.

We also found that the staining reactions in the AIP assay were considerably less intense if the lymphocytes were not treated by hypotonic shock before culture. The reasons for this are unknown. Preparation of the cell smears by cytocentrifugation gave the best results. Fixing the smears while they were still damp also resulted in a greater intensity of the specific staining reactions.

In a series of experiments with blood lymphocytes from more than 20 cows, the cell viability of the cultures after 16 to 24 h of
incubation under the conditions described in "Materials and Methods" ranged between 52 and 66%. Total cell recovery (viable plus nonviable) after 16 to 24 h of culture was about 100%. Cultivation for 48 h or longer resulted in much lower cell viability without significant increase in the number of AIP-positive cells.

The addition of PHA to short-term cultures of blood lymphocytes from BLV-infected cattle usually results in an increase of cells producing BLV particles, as determined by electron microscopy (2), and of cells reacting in the immunofluorescence test for BLV p25 (3). However, this enhancing effect is somewhat inconsistent (16). Using the AIP assay, we have further examined the effect of PHA in short-term lymphocyte cultures from BLV-infected cattle. In the absence of PHA, the percentage of BLV antigen-expressing cells ranged from 10 to 50%. Parallel cultures with PHA always had more, often up to twice as many, antigen-expressing cells. Based on these results, PHA was used routinely in the AIP assay.

There were no significant differences in the viability and total number of cells recovered from cultures with or without PHA.

The results in Table 1 show that mitomycin C treatment of the lymphocytes did not impair their ability to express BLV p25 following short-term cultivation. No [3H]thymidine incorporation was observed in the mitomycin C-treated cultures. Thus, BLV antigen expression in the lymphocyte cultures does not seem to depend on DNA synthesis.

In another experiment, lymphocytes from a BLV-infected cow were grown for 24 h in 100% serum from a BLV-infected cow, and each mixture was tested in the AIP, SIA, and IPIA assays.

<table>
<thead>
<tr>
<th>Donor cattle</th>
<th>AIP</th>
<th>SIA</th>
<th>IPIA</th>
</tr>
</thead>
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<tr>
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<td>68</td>
<td>39</td>
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<td>BF338</td>
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<td>33</td>
<td>11</td>
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<td>BF281</td>
<td>30</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>BF253</td>
<td>19</td>
<td>9</td>
<td></td>
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</tbody>
</table>

The AIP, IPIA, and the SIA assays were also compared using uninfected lymphocytes as controls. The AIP assay was also compared with the SIA and IPIA.

<table>
<thead>
<tr>
<th>Donor cattle</th>
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<th>SIA</th>
<th>IPIA</th>
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<tr>
<td>BF295</td>
<td>7%</td>
<td>2,900</td>
<td>25,000</td>
</tr>
<tr>
<td>BF334</td>
<td>81%</td>
<td>2,300</td>
<td>16,400</td>
</tr>
<tr>
<td>BF281</td>
<td>40%</td>
<td>30,200</td>
<td>14,250</td>
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</table>

Table 2 shows that the AIP assay consistently detected more BLV p25-expressing cells than the indirect IF test.

The AIP assay was also compared with the SIA and IPIA. Uncultured lymphocytes from various BLV-infected cattle were mixed in varying proportions with lymphocytes from a BLV-free cow, and each mixture was tested in the AIP, SIA, and IPIA assays. The results (Table 4) show that the lowest numbers of BLV-infected lymphocytes detected by the AIP and SIA assays were the same. However, both procedures consistently detected less BLV-infected lymphocytes than the IPIA assay.

The AIP, IPIA, and the SIA assays were also compared using blood lymphocytes of 14 naturally infected cattle with relatively high titers of BLV neutralizing antibodies (17), in 100% serum from a BLV-free cow, or in MEM (suspension) supplemented with 20% fetal bovine serum. Viability, cell recovery, and number of AIP-positive cells in the 3 cultures were very similar.
low titers (20 to 40% precipitation of labeled antigen at a 1:50 dilution) in the radioimmunoassay for BLV gp. Six of these cattle were 1 to 2 years of age, and they had been in contact with infected animals for only a few weeks. They were, therefore, in early stages of infection. As shown in Table 5, all 14 cattle were positive in the SIA; only one, GT2245, was negative in the AIP assay. However, 2 x 10^6 lymphocytes from this animal produced only 3 infected positive indicator cells in the SIA. GT2245 was also negative in the SIA.

The excellent reproducibility of the AIP assay was confirmed in studies in which the peripheral blood lymphocytes of BLV-infected cattle were tested at regular intervals during a 12-month period (19).

**DISCUSSION**

This paper describes a new, specific, and reproducible AIP assay for the individual detection of cells expressing BLV antigen. The consistency and specificity of the assay were critically dependent on careful lymphocyte isolation and cell smears preparation. The most crucial requirement, however, was the blocking of nonspecific reactions by absorbing the primary antiserum and diluting it in 100% horse or cow serum. Fixation of the smears while damp was also essential.

The sensitivity of the AIP assay is dependent on the conditions used to induce BLV p25 expression. PHA is known to enhance the induction of BLV expression in blood lymphocyte cultures, but some results have suggested that the mitogen may be occasionally ineffective or detrimental (16). We found, however, that doses of PHA supraoptimal for mitogenicity consistently improved the sensitivity of the AIP assay in the 8 cattle tested, 2 of which had very low numbers of BLV-infected lymphocytes in blood.

The enhancing effect of PHA on BLV p25 expression does not seem to be a function of its mitogenic activity. The optimal mitogenic dose for the PHA lot used was 1:2000, whereas its optimal dose for induction of BLV p25 was 1:50. At the latter dose, there was no PHA-dependent thymidine incorporation before 36 h of culture.6

Several lines of evidence indicate that the number of AIP-positive cells in the 16- to 24-h cultures provides a good estimate of the minimum number of BLV-infected lymphocytes in the blood before culture. (a) The numbers of AIP-positive cells in cultures with or without mitomycin C were similar, thus showing that BLV antigens expressing lymphocytes do not proliferate in the 24-h culture or that proliferation is not necessary for expression. (b) As shown by the results in Table 2, none of the major blood lymphocyte subpopulations of infected cattle is preferentially expanded or reduced after culture for 24 h. (c) The total number of lymphocytes recovered after 24 h of culture is close to 100%, and the AIP assay detects BLV-infected cells regardless of whether or not they are viable. (d) Virus neutralizing antibodies did not reduce the number of AIP-positive cells in lymphocyte cultures, and Cy values for BLV proviral DNA from cultured and uncultured lymphocytes were found to be the same (4). Therefore, there is little or no spread of BLV during the 24 h of culture.

The AIP assay gave very consistent results over a 4-month period at least. Initial results show, however, that in some cows, the number of AIP-positive cells changes immediately before parturition. The reasons for this variation are unknown.

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E. N. Esteban, R. M. Thorn, and J. F. Ferrer, unpublished observations.

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### Table 5

<table>
<thead>
<tr>
<th>Donor cattle</th>
<th>Age (yr)</th>
<th>IPIA</th>
<th>SIA</th>
<th>AIP (%)</th>
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<tr>
<td>GT118</td>
<td>4</td>
<td>21,800^5</td>
<td>ND^2</td>
<td>59</td>
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<tr>
<td>GT153</td>
<td>3</td>
<td>10,400</td>
<td>ND</td>
<td>54</td>
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<td>GT122</td>
<td>9</td>
<td>18,180</td>
<td>1,400</td>
<td>51</td>
</tr>
<tr>
<td>GT230</td>
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<td>1,380</td>
<td>50</td>
</tr>
<tr>
<td>GT234</td>
<td>2</td>
<td>8,040</td>
<td>1,584</td>
<td>47</td>
</tr>
<tr>
<td>GT4</td>
<td>12</td>
<td>3,900</td>
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<td>192</td>
<td>3</td>
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<td>19</td>
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<td>3,240</td>
<td>502</td>
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<td>1</td>
<td>2,500</td>
<td>310</td>
<td>7</td>
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<td>GT244</td>
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<td>4</td>
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<td>GT245</td>
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<td>3</td>
<td>0</td>
<td>0</td>
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</table>

^5 Cattle with low anti-BLV gp antibody titers in the radioimmunoassay (20 to 40% precipitation of radiolabeled antigen at a 1:50 dilution).

^6 Number of BLV p25-positive indicator cells per 35- x 10-mm dish inoculated with 2 x 10^6 viable lymphocytes.

^2 ND, not done.

^3 Number of syncytia inhibited specifically by reference BLV serum per 35- x 10-mm dish inoculated with 2 x 10^6 viable cells.

It is possible to detect confidently as few as 0.5% BLVexpressing cells by the AIP assay. At that level, the assay is as sensitive as the SIA and, by inference, the CRIA (7, 10). The AIP assay is more rapid than either test.

As determined by dose-response experiments with serially diluted BLV-infected lymphocytes, the AIP assay was less sensitive than the SIA. However, the sensitivity of both assays for determining whether or not infected cells were present in the blood of cattle did not differ significantly.

The results in Table 5 also show that 33 to 120 BLV p25-positive lymphocytes in the AIP assay are required to induce one infectious focus in the SIA assay. This suggests that some of the BLV p25-positive lymphocytes do not release BLV that is infectious for the indicator cells used in the SIA. According to the above calculations, the AIP assay would potentially be more efficient than the SIA for the detection of BLV-infected lymphocytes in blood. The fact that the results in Table 4 show the opposite is most likely because of the differences in the number of lymphocytes assayed in the SIA (2 x 10^6) and in the AIP assay (500 or less).

The AIP assay is also more rapid than the SIA. However, the most important advantage of the AIP assay over the other procedures is that it allows a direct and accurate identification of individual cells expressing BLV. This feature makes the AIP assay of particular value for studies on the biology of BLV infection. The only other available procedure to identify individual BLV antigen-expressing cells is the indirect IF test. However, as shown by the present results, this test is much less sensitive than the AIP assay. This is probably due to the avidin:biotin amplification complex. Indeed, in parallel comparisons, we found that positive lymphocytes were fewer and less intensely stained than the AIP assay. This also contributes to the greater sensitivity of the AIP assay. In the accompanying paper, we have made use of the AIP assay for studying variations of the BLV-infected lymphocyte population in cattle with or without persistent lymphocytosis.
ACKNOWLEDGMENTS

We thank Betty Thompson for secretarial help.

REFERENCES


Fig. 1. A, lymphocytes cultured for 24 h and tested in the AIP assay using monospecific rabbit anti-p25 serum absorbed with calf embryo heart cells and diluted in 100% bovine serum; B, same cells as in A but using control rabbit serum absorbed and diluted in 100% bovine serum.

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