Induction of C-Type Virus in Cell Lines Derived from Calf Form Bovine Lymphosarcoma

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Abstract For attempt to detect an etiological agent, cultures from bovine lymphosarcoma cases (adult form (ALS), calf form (CLS), and thymic form (TLS)) were maintained in vitro for over a 18 month period. In two cultures from ALS, bovine leukemia virus (BLV) antigen was constantly detected. On the other hand, BLV antigen remained negative in cultures from two CLS and one TLS cases up to 40 passages. The RNA dependent DNA polymerase activities in these cultures were also negative.

Treatment of a culture from CLS (3178) originated from liver tumor with 5'-ido-2'-deoxyuridine (IdU) and dexamethasone (DXM) resulted in production of an agent serologically and morphologically similar to BLV and in alteration of cell morphology. No virus was detected in culture from TLS after treatment with IdU and DXM.

The four forms of bovine lymphosarcoma; the adult (ALS), calf (CLS), thymic (TLS) and skin (SLS) forms, are clinically distinct from each other (3, 25). In a previous paper, we reported long-term monolayer cultures established from ALS, CLS and TLS (16). Bovine leukemia virus (BLV) antigen was detected in cells from most of the ALS cases, whereas no antigen was detected in cells from CLS and TLS up to 15 passages by serological methods (16). Serological and epidemiological studies suggest that CLS and TLS may not be etiologically related to ALS which has been associated with BLV. However, the possible association of a masked BLV genome in CLS and TLS which can not be detected by serological methods remains to be examined.

Long-term tissue culture cells from CLS and TLS cases appear to be non-virus producing. However, these tissue culture cells may contain proviral DNA of oncorna virus which is associated with CLS and TLS. In this paper, attempts were made to activate an etiological agent in tissue culture cells from CLS and TLS by treating cells with 5'-ido-2'-deoxyuridine (IdU) and the synthetic glucocorticoid dexamethasone (DXM), chemicals known as inducers of viruses in non-virus producing mouse cells (1, 10, 26). Three different methods, serological tests, electron microscopic observation and reverse transcriptase (RT) assay, were employed for the detection of etiological agent in the treated cells.
MATERIALS AND METHODS

1. Cell culture. Long-term monolayer cultures were established from 9 cases of bovine lymphosarcoma, including 6 cases of ALS, 2 cases of CLS and 1 case of TLS. The establishment and maintenance of these cultures were described previously (16, 18). Cells were grown in Eagle’s minimum essential medium containing 10% heat inactivated fetal calf serum (GIBCO).

In the present experiments, we mainly used the following cells; cultures from 3153 parotid lymph node and 3169 thymus for ALS cases, cultures from 3178 liver and 3182 thymus for CLS cases and culture from 3185 thymus for TLS case. The 3178 liver was infiltrated with many tumor cells. These cultures were maintained in vitro up to 35 to 50 passages. BLV positive A-77 thy+ cell line (20) and normal bovine fetal thymus cells were used as controls.

The culture fluid and/or cells of each culture were examined periodically for the presence of BLV antigen by serological methods. Also virus production was examined by electron microscopy (EM) and reverse transcriptase (RT) assay.

2. Serological tests. Two different serological tests including complement fixation (CF) (12, 19) and fluorescent antibody (FA) (15–17) were performed to detect BLV antigen. The standard anti-BLV serum (V34) (19) was used in all of the serological tests.

3. Induction and stimulation. Induction and stimulation experiments were performed as described by Wu et al (26). After subculture, the cells were treated with 40 μg of IdU per ml for 24 hr in the logarithmic phase (12 to 24 hr after subculture). Twenty-four hours later, the cells were washed with medium, and then the cells were treated with dexamethasone (DXM 10–6 M) for 48 hr. The culture fluid and/or cells were harvested at the third day after IdU treatment. The cultures were further maintained with growth media without these compounds.

4. Electron microscopy. The cells were fixed in 2.5% glutaraldehyde and 1% osmium tetroxide and embedded in Epon 812 as described by Okada et al (13). Ultrathin sections were stained with uranyl acetate and lead citrate and examined with HU-12A type electron microscope (Hitachi Co. Ltd). Virus particles were counted at the magnification of 20,000.

5. Reverse transcriptase (RT) assay. The RT assay was performed as described by Fujinaga and Green (8) with a slight modification. The synthetic template-primer poly (rA) Oligo (dT)12–18 was used.

Forty to 60 ml of culture fluid were centrifuged at 12,000 rpm for 10 min and the supernatant was further centrifuged at 66,000 × g for 90 min at 4 C. The resulting virus pellet was resuspended in 100 μl of Tris-HCL buffer, pH 7.4 containing 0.1 M NaCl and 0.001 M EDTA.

The virus suspension (5 μl) was added to a 20 μl reaction mixture to give the final concentration of 0.05 M Tris-HCl pH 7.8, 0.06 M KCl, 25 mM dithiothreitol, divalent cation, 0.02% NP 40, 0.5 A260 unit of poly rA-Oligo (dT)12–18 and 5 mM
Table 1. Detection of virus expression in cultures from bovine lymphosarcoma before (≤30 passages) and after (>30 passages) treatment with IdU and DXM

<table>
<thead>
<tr>
<th>Case</th>
<th>Tissue</th>
<th>Chemical treatment (IdU and DXM)</th>
<th>BLV antigen</th>
<th>Virus particle (total virus/counted cell)</th>
<th>Polymerase activity (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>no yes</td>
<td>≤30th&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;30th&lt;sup&gt;b&lt;/sup&gt;</td>
<td>≤30th&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>ALS 3153</td>
<td>lymph node</td>
<td>no yes</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>ALS 3169</td>
<td>thymus</td>
<td>no yes</td>
<td>+</td>
<td>+</td>
<td>168/49</td>
</tr>
<tr>
<td>CLS 3178</td>
<td>liver</td>
<td>no yes</td>
<td>–</td>
<td>–</td>
<td>0/108</td>
</tr>
<tr>
<td>CLS 3182</td>
<td>thymus</td>
<td>no yes</td>
<td>–</td>
<td>–</td>
<td>0/100</td>
</tr>
<tr>
<td>TLS 3185</td>
<td>thymus</td>
<td>no yes</td>
<td>–</td>
<td>–</td>
<td>4/101</td>
</tr>
<tr>
<td>A-77thy+</td>
<td></td>
<td>no yes</td>
<td>–</td>
<td>–</td>
<td>0/103</td>
</tr>
<tr>
<td>Normal bovine</td>
<td>thymus</td>
<td>no yes</td>
<td>–</td>
<td>–</td>
<td>285/54</td>
</tr>
<tr>
<td>fetus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>188/18</td>
</tr>
</tbody>
</table>

<sup>a</sup> Virus expression was tested in cultures at passage 20 to 30th, before or just after treatment with IdU and DXM.

<sup>b</sup> Virus expression was tested at passage 30 to 40th, then again 5 to 10 passages after treatment with IdU and DXM.

<sup>c</sup> Only mature BLV particles were counted. No immature or filamentous particles were included.

<sup>d</sup> The enzyme activity is expressed as cpm of <sup>3</sup>H-TTP incorporated into acid-insoluble fraction.

ND: not done.
\[ 3^H\text{-TTP}. \text{ The results are expressed as cpm of } 3^H\text{-TTP incorporated into acid-insoluble fraction during 30 min incubation at } 37\, \text{C.} \]

**RESULTS**

Long-term monolayer cultures established from ALS, CLS and TLS were maintained for more than 40 passages, over a 18 months. The proliferation of these cell lines became gradually slow with passage, especially after 25 passages. The morphology of these cell lines were epithelioid-like cells. As shown in Table 1, BLV antigen was constantly detected in chemically treated or non-treated cultures from ALS 3153 and 3169 by CF and FA tests, but the production of BLV antigen declined in chemically non-treated culture from ALS 3153. On the other hand, BLV antigen remained negative in non-treated cultures from CLS and TLS cases up to 40 passages.

In attempts to activate bovine C-type viruses, cultures from CLS and TLS were treated with IdU and DXM at the passage level of 25 to 30. Just after treatment with these compounds, no expression of C-type virus such as BLV antigen, RT activity or virus particle production was observed in any cultures. In culture from CLS 3178 which originated from lymphosarcoma cells and treated with IdU and DXM at 30th passage, morphological changes from epithelioid-like to fibroblast-like cells with focus formation were observed at 34th passage (Fig. 1 abc). After 35th passage, the percentage of FA positive cells reacted with anti-BLV serum (V34) gradually increased with passage (Fig. 2), and the proliferation of the cells became rapid. For elimination of the possibility of contamination during passage, the frozen culture from CLS 3178 (23rd passage level) was cultured again and treated by the same way. Similar antigen reacted with anti-BLV serum by FA test and morphological changes with focus formation was detected. However, neither morphological change nor antigen was found in the cultured cells which were not treated with IdU and DXM.

Culture from CLS 3182 was treated with these compounds at 28th passage. No virus expression was observed up to 34th passage. Although a weak and questionable antigen was detected by FA test slightly increased in chemically treated culture cells at 36th passage level (Table 1), the cell proliferation became very poor and further transfer of cells was unsuccessful at passage 37. Culture from TLS 3183 was also treated with these compounds at 26th passage. No virus expression was observed before and after treatment, and cells could not be transferred after 34th passage.

By EM, mature BLV particles could be easily detected in cells from BLV positive A-77thv + cell (20) (Figs. 4 and 6). Filamentous structures similar to these described previously (4, 7) were also found (Fig. 5). One hundred sixty eight mature BLV particles per 49 cells were detected in the culture from ALS 3169 (Table 1). In the cultured cells from CLS and TLS, more than one hundred cells were examined for virus particles by EM. A few virus-like particles similar to BLV (4 viruses per 102 cells) were detected in the culture from CLS 3182. No virus particles were detected in the cultured cells from CLS 3178 or TLS 3185. However, many C-type particles indistinguishable from BLV were evident in chemically treated culture
cells from CLS 3178 at 38th passage level (Fig. 3). All of the cells examined were negative for known virus particles from bovine origin.

To detect low virus production, RT assay was also carried out using concentrated culture fluids at different concentrations of Mg$^{++}$ and Mn$^{++}$. Mason-Pfizer monkey virus (MPMV) and endogenous rat leukemia virus (NRK-9) were used as controls. As shown in Table 2, the optimum concentration of divalent cation for the enzyme activity of BLV was 20 mM Mg$^{++}$. The optimum cation concentration for MPMV enzyme activity was 10 mM Mg$^{++}$. Mn$^{++}$, on the other hand, clearly stimulated NRK-9 enzyme activity. Therefore 20 mM Mg$^{++}$ was used at the divalent cation for BLV RT assay. As shown in Table 1, the polymerase activity of the concentrated culture fluid from BLV positive cell line (A-77thv+) was high, whereas the activity
Fig. 4. Cluster of extracellular BLV particles in ultrathin section of cell pellet prepared from the cell line A-77thy+. ×36,000

Fig. 5. Filamentous structures which have an outer envelope, inner layer and electron-dense materials. ×36,000

Fig. 6. Mature BLV particles and bud (arrow) forming from plasma membrane. ×117,500

Table 2. Comparison of cation concentration of reverse transcriptase from BLV, MPMV and NRK-9

<table>
<thead>
<tr>
<th>Viruses</th>
<th>Cation concentration (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mn&lt;sup&gt;++&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
</tr>
<tr>
<td>BLV</td>
<td>440&lt;sup&gt;a1&lt;/sup&gt;</td>
</tr>
<tr>
<td>MPMV</td>
<td>453</td>
</tr>
<tr>
<td>NRK-9</td>
<td>18250</td>
</tr>
</tbody>
</table>

<sup>a1</sup> The enzyme activity is expressed as cpm of <sup>3</sup>H-TTP incorporated into acid-insoluble fraction.
in the cultured cells from ALS 3153 was relatively low. The enzyme activity in cultures from CLS and TLS was less than one thousand cpm and considered to be negative. However, an enzyme activity was clearly detected in one of two cultured cells from CLS (3178). No enzyme activity was found in culture cells form TLS 3185 after chemical treatment.

DISCUSSION

Four types of bovine lymphosarcoma have been described on the basis of clinical and pathological features (3, 25). In an adult form (ALS), C-type BLV has been detected in cultured blood lymphocytes (11, 16, 17), whereas in the other forms including calf (CLS), thymic (TLS) and skin (SLS) forms, BLV has not been detected by the same way except one CLS case (14, 16, 17). In a previous paper, BLV antigen was detected in monolayer cultures from lymphoid tissues and mammary gland from 4 of 6 cases. However, in monolayer cultures from 2 CLS and 1 TLS cases BLV antigen remained negative up to 15th passage in vitro (16). In cultures from SLS established recently in Japan and from TLS obtained from Dr. C. Olson in USA, BLV antigen was also negative.

An epidemiological studies show that the ALS is enzootic, while CLS, TLS and SLS are sporadic (most common in single-incidence herds). Etiologies of CLS, TLS and SLS are still unknown. With regard to an etiological agent of CLS and its relation to BLV, the followings can be considered. First, the etiological agent of CLS may be different from ALS. Ressang (22) presented a hypothesis that CLS may be caused by an incomplete bovine sarcoma virus. Second, the etiology of CLS may be related to BLV from the evidences based on 1) In one CLS case 3178, antigen which reacted with anti-BLV serum was detected in peripheral blood lymphocytes (PBL) and tumor lymph node by short-term lymphocyte cultures (Dirscoll and Olson, unpublished data), 2) Calves, sheep and goats inoculated with materials from CLS occasionally produced antibody to BLV antigen (22), 3) In serum from one of twin calves with CLS, antibody to ether sensitive antigen of BLV was detected but no BLV antigens was found in cultured lymphocytes (5). However, the possibility that calves with CLS cases are super-infected with BLV, can not be ruled out.

Treatment of cultured cells from CLS 3178 with IdU and DXM resulted in production of virus particles which were indistinguishable from BLV and FA-positive cells reacted with anti-BLV serum after several passages. Rapid cell proliferation and alteration in morphology of cultured cells were also observed after treatment. On the other hand, cultured cells without treatment could not be transferred after 40th passage and these was no expression of C-type virus. The virus producing culture from CLS 3178 has been maintained for more than 60 passages, over 2 years period. Rapid cell proliferation and virus expression had been constantly observed.

In a murine system, IdU is known as an agent to induce the production of viruses in non-producing transformed or normal cells in vitro (1, 10), although no concrete evidence for transformation by IdU is known. Also DXM stimulated the synthesis
of oncorna viruses in vitro (2, 26). The mechanism by IdU induction of C-type virus remains to be clarified, although Teich et al (24) have shown that induction of virus by IdU appears to require the incorporation of IdU into DNA.

In murine and feline systems, the vertically transmitted virus is usually completely repressed but occasionally spontaneous virus release occurs. In such a case, activation of virus production was facilitated sometimes by treating cells with chemicals, such as IdU (6). BLV may be mainly horizontally transmitted from infected to non-infected animals (21) but also can be vertically transmitted from a positive dam to her offspring via the placenta and/or germinal cells (15, 23). Fetal infection has also been evidenced in CLS case (9). Since the dam of CLS 3178 had antibody to BLV, the possibility that BLV infection was acquired from the dam can not be ruled out. Thus, at the present time, we can not drive any firm conclusion for the etiology of CLS case.

Characterization of the induced virus particles and morphological changed culture from CLS 3178 should be performed. To be sure the etiology of CLS, similar experiments and molecular hybridization test will be required using additional CLS cases.

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REFERENCES

C-TYPE VIRUS FROM CALF LYMPHOSARCOMA 691


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