STUDIES ON CELL LINES DERIVED FROM CALF, THYMIC AND SKIN FORMS OF BOVINE LYMPHOSARCOMA

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Résumé

ÉTUDE DE LIGNÉES CELLULAIRES DÉRIVÉES DE DIFFÉRENTES FORMES DE LYMPHOSARCOME BOVIN : LYMPHOSARCOME DU VEAU, DU THYMUS ET DE LA PEAU. — L'étiologie de la leucose bovine sporadique (SBL) est inconnue. On a réalisé des cultures en couche monocellulaire à partir de cas de leucose du veau (CLS), et de formes thymiques (TLS) et cutanées (SLS), et on a essayé de détecter un agent étiologique par des tests sérologiques, l'observation en microscopie électronique et des tests de transcriptase inverse.
L'antigène du virus de la leucose bovine (BLV) et l'activité de la transcriptase inverse sont restés négatifs dans les cultures provenant de SBL. Le traitement d'une culture de CLS 3178 par la 5'-iodo-2'-deoxyuridine et la dexamethasone a provoqué une altération de la morphologie cellulaire et la production de BLV, dont l'origine pourrait être la mère infectée du cas CLS 3178. La formation de foyers dans des cultures en couche monocellulaire et la formation de colonies dans les cultures sur agar mou ont été observés avec cette lignée cellulaire traitée. Les cellules fibroblastiques de poumon foetal humain cocultivées avec les cellules de SBL provoquaient une prolifération rapide des cellules avec formation accrue de foyers.

Introduction.

The four forms of bovine lymphosarcoma ; the adult (ALS), calf (CLS), thymic (TLS) and skin (SLS) forms, are clinically distinct from each other. In a previous paper, BLV antigen was detected in long-term monolayer culture cells from most of the ALS cases, whereas no BLV antigen was detected in cells from CLS and TLS by serological methods (Onuma and Olson, 1977). Furthermore, no virus was detected in these cultured cells up to 40 passages (Onuma *et al.*, 1978). However, treatment of a culture from CLS 3178 which originated from a liver tumor with 5'-iodo-2'-deoxyuridine (IdU) and dexamethasone (DXM) resulted in production of an agent and in an alteration of cell morphology (Onuma *et al.*, 1978). Long-term tissue cultures established from 4 CLS, 2 TLS and 2 SLS cases were used in this experiment. Three different methods, serological tests, electron microscopic observation and reverse transcriptase (RT) assay were employed for the detection of etiological

agent. This paper will describe the character of the induced virus and morphological alteration of cell culture from CLS 3178, and discuss the possible involvement of a bovine sarcoma virus infection in cultures from SBL.

Materials and Methods.

1. *Cell cultures.*

Long-term monolayer cultures were established from 4 cases of CLS, one case of TLS and 2 cases of SLS. Cultures from 3178, 3182 and 3185 were previously described (Onuma *et al.*, 1978 ; Onuma and Olson, 1977). The CLS 3178 liver was infiltrated with many tumor cells. The dam of this case had antibody to BLV. Two cases (128 and 129) of CLS and two cases (114 and 126) of SLS were provided by Dr. M. Sonoda, College of Dairy Agriculture, Ebetsu, Japan and Dr. S. Ichijo, Obihiro University, Agriculture and Veterinary Medicine, Obihiro, Japan, respectively. BLV positive A-77thv+ cell line (Onuma *et al.*, 1976) 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.

Human fetal lung fibroblast (HFL) cells were obtained from Dr. K. Yamamoto, Department of Virology, Cancer Institute, Hokkaido University School of Medicine, Sapporo, Japan.

2. *Serological tests.*

Two different serological tests including complement fixation (CF) (Miller and Van Der Maaten, 1974) and fluorescent antibody (FA) (Onuma *et al.*, 1977) were performed to detect BLV antigen. The standard anti-BLV serum (V34) (Onuma *et al.*, 1977) was used in all of the serological tests.

3. *Induction and stimulation.*

Induction and stimulation experiments were performed as described previously (Onuma *et al.*, 1978 ; Wu *et al.*, 1974). Cultures were treated with IdU (40 μg/ml) for 24 hours and then treated with DXM (10^{-6}M) for 48 hours. The culture fluid and/or cells were harvested at the third day after IdU treatment. The cultures were furtheromaintained with growth media without these compounds.

4. *Reverse transcriptase (RT) assay.*

The RT assay was performed as described previously (Onuma *et al.*, 1978). The exogenous template-primer poly (rA) oligo $(dT)_{12\text{-}18}$ and magnesium acetate (20 mM/reaction mixture) were used. The results are expressed as cpm of ^{3}H-TTP incorporated into acid-insoluble fraction during 30 minutes incubation at 37 °C.

Table 1. — Detection of virus expression in cultures from sporadic leukosis.

Case	Lymphocyte counts (cells/mm^3)	Age (month)	BLV		Virus Particles (total virus/counted cells)	Polymerase activity (cpm)
			Antigen	Antibody		
CLS 3178	15,600	4	— (a)	—	0/108	620
CLS 3182	13,100	2	—	—	4/101	730
CLS 128	23,814	5	—	—	ND	686
CLS 129	16,450	5	—	—	ND	726
TLS 3185	9,776	12	—	—	0/103	380
SLS 114	3,748	32	—	—	0/100	480
SLS 127	2,430	78	—	—	ND	683
A-77thv+			+		285/54	8,415
Normal bovine thymus			—		0/100	450

(a) BLV was detected in short-term lymphocyte culture, but not in long-term monolayer culture.

Table 2. — Detection of virus expression in cultures from sporadic leukosis after treatment with IdU and DXM.

Case	BLV antigen	Virus Particles (total virus/ counted cells)	Polymerase activity (cpm)
CLS 3178	+	118/103	2,586
CLS 3182	±	4/103	1,207
TLS 3185	—	0/103	360
SLS 114	—	ND	315
A-77thv+	+	ND	18,818
Bovine fetal thymus	—	ND	219

Table 3. — Comparison of population density and doubling time.

Case	In vitro passage No.	Percentage of BLV positive cells	Population density (per 75 cm²)	Population doublig time (hr)
CLS 3178	97	10	6×10^7	~23
TLS 3203 + BLV (a)	50	0.2	4.5×10^7	~23
CLS 3182	30	—	3×10^6	~48
SLS 114	20	—	3×10^6	~48
A-77thv+	40	15	3.5×10^6	~48
Bovine fetal thymus	20	—	5×10^6	~36

(a) BLV super-infected culture.

Table 4. — Comparison of focus number in cultures from sporadic leukosis and in cultures cocultivated with HFL.

Cultures	Number of foci (a)	Cocultivated cultures (b)	Number of foci (a)
CLS 128	5	HFL-CLS 128	70
CLS 128 + BLV (c)	12	HFL-CLS 128 + BLV	161
SLS 114	13	HFL-SLS 114	162
SLS 114 + BLV (c)	17	HFL-SLS 114 + BLV	231

(a) Mean number of foci per quarter of 75 cm² plastic flask.
(b) Cultures from sporadic leukosis were cocultivated with HFL cells.
(c) Cell culture was infected with cell-free BLV.

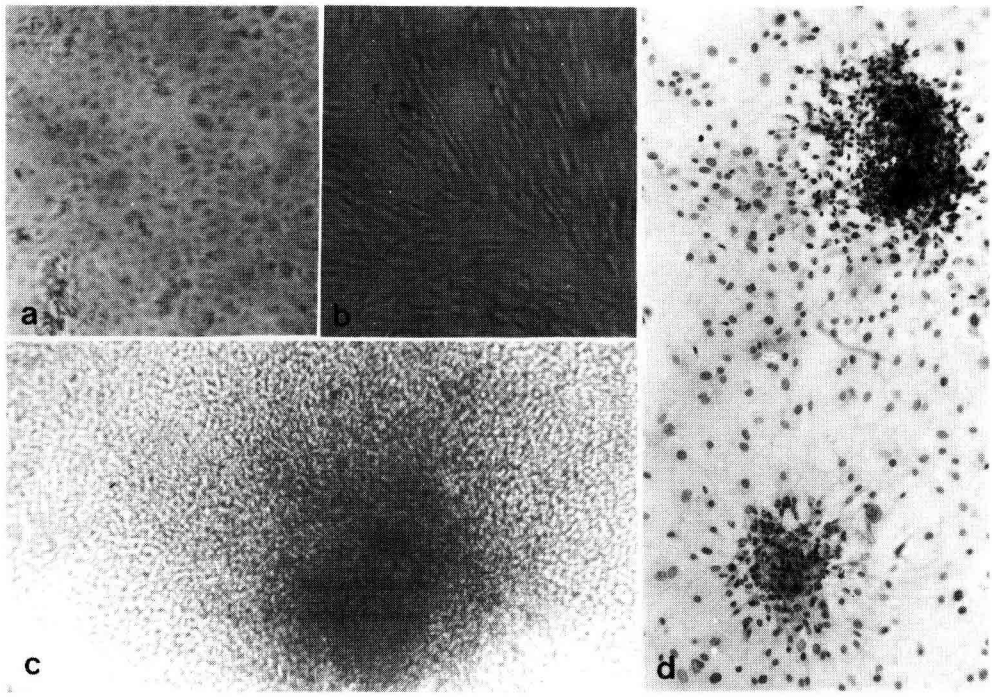

Fig. 1. — Monolayer culture from CLS 3178.
a) At 23rd passage level showing an area of epithelioid cells before treatment with IdU and DXM. Cell proliferation was very poor at this passage level.
b) At 35th passage level showing an area of spindle cells after treatment with IdU and DXM.
c) At 40 th assage level showing an area of focus formation. The cell proliferation was very rapid.
d) Focus formation observed in monolayer culture of CLS 3178. Cells were stained with Giemsa.

5. *Soft agar culture.*

Soft agar medium consisted of Eagle's medium with nonessential amino acids and supplemented with 10 % Bacto tryptose phosphate broth, 15 % fetal calf serum, 100 μg penicillin/ml, 100 μg dihydrostreptomycin/ml, 2.5 μg fungazone/ml plus 0.35 % Bacto Agar (Difco). A soft agar medium containing appropriate amount of cells was seeded in petri plates (4 cm in diameter). After solidification the plates were incubated in a CO_2 incubator at 37 °C.

6. *Description of focus assay.*

Trypsinized cells were seeded in 75 cm^2 Falcon culture flask at a cell concentration of 1 — 5 × 10^6 cells per flask which were sufficient in number to form a confluent sheet after 3 to 5 days. Upon confluency, the visible foci (Fig. 1c) were counted.

Results.

Long term monolayer cultures established from CLS 3178, CLS 3182 and TLS 3185 were maintained for more than 40 passages, over an 18 month period. Cell culture from SLS 114 was maintained for more than 20 passages, whereas cell cultures from CLS 128, CLS 129 and SLS 127 were maintained for less than 10 passages. After 20 passages, the proliferation of these cultures became gradually more slow with each passage.

As shown in Table 1 no BLV antigen and virus particles were detected in these cultures except for a few virus-like particles similar to BLV (4 virions per 101 cells) detected in the cell culture from CLS 3182. However, mature BLV particles could be easily detected in cells from the BLV positive A-77thv+ cell line. RT assay was also carried out to detect low virus production. The polymerase activity of the

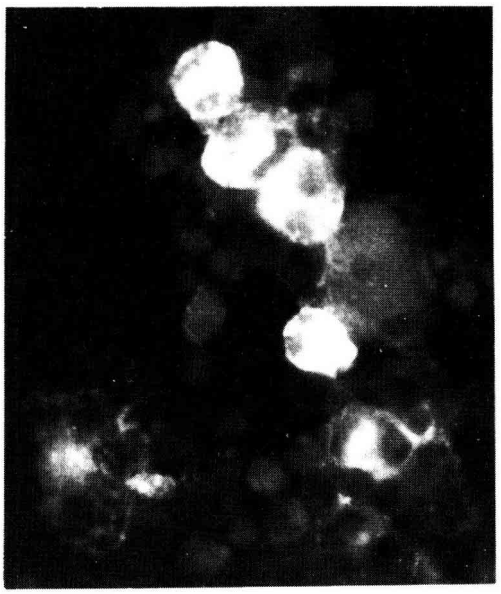

Fig. 2. — Acetone fixed cells from CLS 3178 at passage 38 showing cytoplasmic fluorescence when reacted with anti-BLV serum. (× 1800).

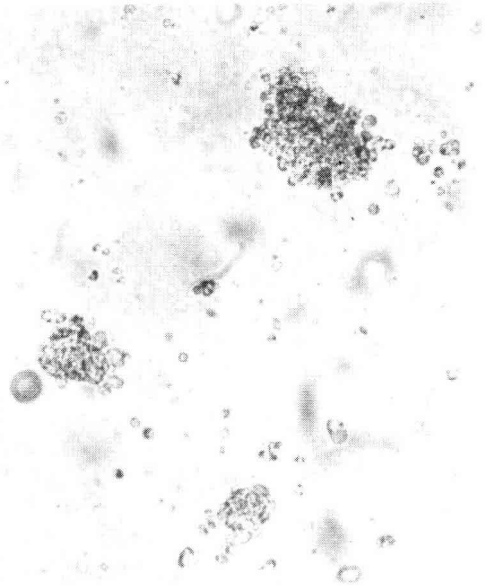

Fig. 3. — Colony formation observed in soft agar culture of CLS 3178.

concentrated culture fluid from BLV positive cell line (A-77thv+) was high, whereas the enzyme activity in cultures from CLS, TLS and SLS was less than one thousand cpm and was considered to be negative.

In attempts to activate bovine C-type viruses, cultures from 3178. 3182, 3185 and 114 were treated with IdU and DXM at the passage level of 20 to 30 (Table 2). No expression of C-type virus such as BLV antigen, RT activity or virus particle production was observed in cultures from 3185 and 114 after treatment with these compounds. However, in the culture from CLS 3178, morphological changes from epithelioid to fibroblast like cells with focus formation were observed after this treatment (Fig. 1). The percentage of FA positive cells which reacted with anti-BLV serum gradually increased with each passage (Fig. 2). Both ether-treated and non-treated antigen derived from this culture fluid showed lines of identity to p24 and glycoprotein antigens of BLV. Although a weak and questionable BLV antigen, detected by FA test, slightly increased in the chemically treated culture from 3182, the cell proliferation became very poor and further transfer of cells was unsuccessful at passage 37.

Rapid cell proliferation and virus expression have been constantly observed in culture cells from CLS 3178 for more than 100 passages, over a 2 year period. The population density was increased and the population doubling time was reduced in this culture as shown in Table 3. In soft agar culture, recognizable cell colonies were readily obtained in around 2 weeks when 10^4 to 10^6 viable cells were seeded per plate (Fig. 3). The successful establishment of the clone was achived by picking a single colony. A TLS 3203 culture which was provided by Drs. I. Takashima and C. Olson in Wisconsin, was negative for BLV antigen during 10 passages. After super-infection with cell free BLV material, rapid cell proliferation with focus formation and colony formation in soft agar were observed in the TLS 3203 culture (Table 3).

Because Ressang (1976) presented a hypothesis that CLS may be caused by an incomplete bovine sarcoma virus, HFL were cocultivated with cultures from sporadic leukosis. Either BLV superinfected or non-infected cultures from CLS 128, and SLS 114 were cocultivated with HFL cells. Focus formation in cocultivated cultures with HFL was increased to more than 10 times compared to non-

Table 5. — Characterization of HFL cells infected with culture fluids from SLS 114.

	BLV antigen	Polymerase activity (cpm)	Population density (per 75 cm²)	Population doubling time (hr)	No. of foci (b)
SLS 114	—	480	3.0×10^6	~48	13
HFL	—	310	4.0×10^6	~36	0
H F L (SLS 114) (a)	—	1,270	1.6×10^7	~28	155

(a) HFL cells infected with concentrated cell-free culture fluids from SLS 114.
(b) Mean number of foci per quarter of 75 cm² plastic flask.

cocultivated cultures, especially in HFL cells cocultivated with BLV-infected CLS or SLS cultures (Table 4). In one HFL cells infected with concentrated culture fluids from SLS 114 culture, rapid cell proliferation with increased focus formation was observed as shown in Table 5. No BLV antigen was detected in this infected culture, however, increased polymerase activity was observed.

Discussion.

Bovine leukoses are lymphoproliferative diseases and are classified into two types ; the enzootic and sporadic types. Enzootic bovine leukosis is a contagious disease (Bendixen, 1965 ; Theilen *et al.,* 1964) induced by bovine leukosis virus (BLV) (Miller *et al.,* 1969). Three main clinical types of sporadic bovine lymphosarcoma are known ; the calf form (CLS), thymic form (TLS) and skin form (SLS). No virus was detected in either short-term or long-term cultures of lymphoid cells (Onuma and Olson, 1977). Antibodies to BLV antigens were not detected in cattle with these diseases (Onuma and Olson, 1977). If the sporadic cases happen to be in a herd with enzootic cases, it is confusing to distinguish a BLV from an etiological agent of sporadic cases, because BLV is mainly horizontally transmitted from an infected to a non-infected animal (Piper *et al.,* 1975) but also can be vertically transmitted from a positive dam to her offspring via the placenta and/or germinal cells (Onuma *et al.,* 1977).

In the present study, no polymerase activity was detected in cultures from CLS, TLS and SLS without chemical treatment. However, treatment of culture cells from CLS 3178 with IdU and DXM resulted in production of BLV. Since the dam of CLS 3178 had antibody to BLV, this induced BLV may have been transmitted from the dam to CLS 3178 and repressed in the cells. Focus formation in monolayer culture and colony formation in soft agar culture were observed in this treated culture. When the frozen culture from CLS 3178 (23rd passage level) was cultured and treated by the same way, BLV production and morphological changes with focus formation were also observed. This result shows that the possibility of the contamination during passages could be ruled out.

Although no concrete evidence for transformation by IdU is known, IdU is known as an agent to induce the production of viruses in non-producing transformed or normal mouse cells *in vitro* (Aaronson *et al.,* 1971 ; Lowy *et al.,* 1971). DXM stimulated the synthesis ofoncorna viruses *in vitro* (Ahmed *et al.,* 1977 ; Wu *et al.,* 1974). The mechanism by IdU induction of C-type virus remains to be clarified, although Teich *et al.* (1973) have shown that induction of virus by IdU appears to require the incorporation of IdU into DNA.

BLV super-infected TLS 3203 culture showed an increased rate of growth and an alteration of cell morphology, as compared to the uninfected culture. An increased focus formation was also observed in HFL cells cocultivated with CLS or SLS cultures, especially when cocultivated with BLV-infected cultures of CLS or SLS. Furthermore, sera from CLS cases reacted with the culture fluid from BLV-infected cultures of CLS but not with BLV-free cultures from CLS in the preliminary experiments (unpublished results). These observations suggest that the cultures from sporadic leukosis contain an agent which is defective and has a transforming ability. This agent may be a defective bovine sarcoma virus and these observations may help to further substantiate the Ressang's hypothesis (1976).

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Summary

The etiology of sporadic bovine leukosis (SBL) is not known. Long-term monolayer cultures were made from calf (CLS), thymic (TLS) and skin (SLS) forms, and serological tests, electron microscopic observations and reverse transcriptase assays were employed for the detection of an etiological agent.

Bovine leukosis virus (BLV) antigen and reverse transcriptase activities remained negative in cultures from SBL cases. Treatment of a culture from CLS 3178 with 5'-iodo-2'-deoxyuridine and dexamethasone resulted in production of BLV which may have been acquired from the BLV-infected dam of CLS 3178, and in an alteration of cell morphology. Focus formation in monolayer cultures and colony formation in soft agar cultures were observed in this treated cell line. Human fetal lung fibroblast cells cocultivated with the cultures from SBL resulted in rapid proliferation of cells with an increased focus formation.

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