Integrated bovine leukosis proviral DNA in T helper and T cytotoxic/suppressor lymphocytes

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Bovine leukosis virus (BLV) is associated with the disease complex enzootic bovine leukemia. The infection may remain clinically silent in the form of an aleukaemic state or emerge as a persistent lymphocytosis and more rarely as lymphosarcoma. BLV has been considered classically to be a B lymphotropic virus, based upon the absolute increase in B lymphocytes in persistent lymphocytosis, the B lymphocyte phenotype of a majority of the cells making up lymphosarcomas and the identification of viral antigen expressed in B lymphocytes following in vitro culture of peripheral blood mononuclear leukocytes. This association of BLV with B lymphocytes is well established but the mechanism(s) of disease expression is not defined. To examine further the cellular tropism(s) of BLV, T lymphocyte subpopulations from 10 lymphocytotic cattle were established in vitro. Lymphocyte cultures were characterized by their subpopulation phenotype and DNA was extracted for identification of integrated provirus by Southern blot hybridization. Provirus was identified in T lymphocyte cultures derived from seven of 10 lymphocytotic cattle, with both T helper and T cytotoxic/suppressor subpopulations affected.

Introduction

Infection of cattle with bovine leukemia virus (BLV) is associated with persistent lymphocytosis (PL) or lymphosarcoma, both of which develop after extended latency periods of between 1 and 8 years (Onuma et al., 1979; Ghysdael et al., 1984; Burny et al., 1985). Animals develop a strong humoral response to BLV structural proteins at the time of infection that persists throughout the lifetime of the animal and appears to be ineffective in preventing disease progression. Between 30 and 70% of infected cattle develop PL that is characterized by an increase in circulating B lymphocytes; a much smaller number, 0.1 to 10%, eventually develop lymphoid tumours (Kumar et al., 1978; Onuma et al., 1979; Burny et al., 1985; Thurmond et al., 1985). The mechanism by which BLV remains latent and causes lymphocyte proliferation, and the parameters that define progression to the PL or tumour disease states, are as yet undefined.

A tropism of BLV for B lymphocytes has been indicated by a number of different experimental designs. In vitro, mitogen-induced expression of viral antigen from nylon wool-enriched B lymphocytes (Paul et al., 1977b) and syncytium formation in permissive cells upon cocultivation with enriched B lymphocytes (Paul et al., 1977a) have both indicated that viral replication occurs in this cell population. Integrated BLV provirus has been demonstrated in DNA obtained from BLV-induced lymphoid tumours (Kettmann et al., 1982, 1983; Onuma et al., 1982), as well as in peripheral blood mononuclear (PBM) leukocyte DNA from cattle with PL and lymphosarcoma (Kenyon & Piper, 1977; Kettmann et al., 1979, 1980a); all of these cell populations typically contain a high percentage of B lymphocytes.

The mechanism by which BLV causes B lymphocyte proliferation is unknown at present. The viral genome does not contain a transduced cellular oncogene and proviral integration into the host cell genome appears to be random; these facts argue against a viral effect upon host cell oncogenes that in turn could affect cellular proliferation (Kettmann et al., 1980a; Gelmann et al., 1984). The BLV genome contains sequences in the X region that encode proteins required for efficient transcription from the viral genome (Rosen et al., 1986; Katoh et al., 1987; Rice et al., 1987), as well as for regulation of virion protein production (Derse, 1988). It is possible that these gene products are involved in the regulation of lymphocyte proliferation, although there is little viral transcription in vivo (Kettmann et al., 1980b, 1982; Broeke et al., 1988).

Disease expression may also be influenced by proviral integration into additional PBM leukocyte subpopula-
tions. Utilizing monoclonal antibodies (MAbs) specific
for bovine leukocyte differentiation antigens and analy-
tical flow cytometry, a potential association of BLV with
T lymphocytes, based upon an increase in the absolute
number of such cells in both aleukaemic and lymphocy-
totic cattle, has been suggested (Williams et al., 1988a).
In studies of aleukaemic cattle, provirus has been
identified in PBM leukocyte populations that were
indirectly enriched for T lymphocytes by depletion of
surface immunoglobulin-bearing cells (Williams et al.,
1988b).

To examine further a possible BLV tropism for T
lymphocytes, as well as subsets within this cell popu-
lation, PBM cells from lymphocytotic cattle were expanded
in vitro using recombinant interleukin-2 (IL-2) and
mitogens. The cultures were periodically characterized
for cell phenotype using flow cytometry and MAbs
specific for bovine leukocyte differentiation antigens.
Restriction digests of DNA extracts, from cultures
determined to consist of T lymphocytes, were analysed
by Southern blot hybridization. The two major subpopu-
lations within bovine T lymphocytes, BoCD4 and
BoCD8, were found to possess integrated BLV proviral
DNA.

Methods

Experimental animals. This study included 13 Holstein cattle: 10 cows
seropositive for BLV, as determined by agar gel immunodiffusion using
gp51 as antigen (Thurmond et al., 1985), and two seronegative cows
and a bull as controls. Of the seropositive animals, five were
persistently lymphocytotic and the other five were lymphocytotic but
could not be defined as persistently so, because follow up times were
less than 3 months. A cow was determined to have lymphocytosis if
the number of lymphocytes per ml exceeded the upper 95% predicted value
for an animal of that age (Thurmond et al., 1990). None of the cattle
had antibodies to bovine immunodeficiency-like virus, as determined
by indirect immunofofluous antibody staining (Dr J. W. Black,
American Bio-Research, Milton, Tenn., U.S.A.). Three of the cattle,
373, 826 and 846, were positive for bovine respiratory syncytial virus as
determined by agar gel immunodiffusion (Dr M. J. Van Der Maaten,

Isolation of PBM cells. Blood was collected by jugular venipuncture
into heparinized vacutainer tubes (Becton Dickinson). Buffy coats
were diluted 1:2 in phosphate-buffered saline (PBS) pH 7.4, layered
over an equal volume of Histopaque-1077 (Sigma) and centrifuged for
30 min at 1000 g in a Beckman TJ-6 centrifuge. The mononuclear
leukocyte layer was washed in PBS, counted and a portion of the cells
were analysed by flow cytometry with the rest being placed in culture
for in vitro expansion.

Analytical flow cytometry. The relative percentages of lymphocyte
subpopulations, present in freshly isolated or in vitro cultures of PBM
lymphocytes, were determined by fluorescent antibody staining of bovine
lymphocyte cell surface antigens followed by analytical flow cytometry.
Labelling was conducted in microcentrifuge tubes with between 2 ×
10⁶ and 4 × 10⁶ cells/tube. All incubations were for 30 min on ice in a
volume of 100 μl, a PBS rinse was used between incubations and cells
were pelleted at 1300 g in a microfuge for 5 min. Cells expressing
surface immunoglobulin (B lymphocytes) were identified with a
polyclonal F(ab')₂ goat anti-bovine IgG (heavy and light chains
(H+L))-fluorescein isothiocyanate (FITC) conjugate (Pel-Freeze).
Lymphocyte subpopulations expressing major histocompatibility com-
plex (MHC) class II proteins (B lymphocytes), BoCD2 (pan T
lymphocyte marker) (Baldwin et al., 1988), BoCD4 (T helper
lymphocytes) (Baldwin et al., 1986) and BoCD8 (T cytotoxic/suppressor
lymphocytes) (Ellis et al., 1986) were identified by application of MAbs
(provided by the International Laboratory for Research on Animal
Disease, Nairobi, Kenya). Specific binding of MAbs was identified by
incubation with an F(ab')₂; polyclonal goat anti-mouse IgG (H+L)–
FITC conjugate (Tago). A MAb with an unrelated specificity was used as
a negative control. Following labelling, the cells were washed and
suspended in 500 μl PBS and analysed by flow cytometry. All labelled
cell suspensions were analysed on a FACStar Plus flow cytometry
(Becton Dickinson) using laser excitation of FITC at 488 nm. A
minimum of 10000 events was collected into list mode using a live gate.
The data collection gate was based upon forward and side-angle light
scatter; these parameters were used to gate-out erythrocytes, platelets,
granulocytes and cellular debris. Data were analysed using BD
FACStar Research software with the percentage of positive cells versus
negative cells being determined by single parameter (fluorescence)
histogram analysis.

In vitro culture. Freshly isolated PBM cells were diluted to 2 ×
10⁶/ml in Dulbecco’s MEM (Gibco) containing 10% foetal bovine
serum (Hyclone), 100 units/ml recombinant human IL-2 (Cetus) and
1 mg/ml of either leucosaheamagglutinin (PHA-L; Sigma), pokeweed
mitogen (PWM; Sigma) or concanavalin A (Con A; Sigma); cultures
were propagated in 25 cm² tissue culture flasks (Falcon) in a 5% CO₂,
37 °C incubator. After a minimum of 2 weeks in culture, the cell
phenotype was analysed as described above. Periodic phenotypic
analysis was continued until cultures were determined to consist solely
of T lymphocytes (within the limits of flow cytometry); these cultures
were then expanded for DNA extraction.

DNA extractions and restriction enzyme digests. Freshly isolated PBM
cells or cultured lymphocytes (5 × 10⁶ to 5 × 10⁷) were placed in 1 ml
TE buffer (10 mM-Tris-HCl pH 8.0, 1 mM-EDTA pH 8.0) containing
1% SDS and 500 μg proteinase K (Sigma) and incubated at 65 °C for
6 h. The cell lysates were extracted once with a 1:1 (v/v) mixture of
phenol and 100 mM-Tris–HCl pH 8.0 containing 0.1% β-hydroxyquinol-
one, once with a 1:1 (v/v) mixture containing phenol, chloroform
and TE buffer and once with a 24:1 mixture of chloroform and isooamyl
alcohol. DNA was ethanol-precipitated, resuspended in distilled water
(ΔH₂O) and the concentration determined by A₂₆₀/₂₈₀. DNA was
digested with HindIII or SacI (Boehringer Mannheim). A 10:1 ratio of
enzyme units to μg of DNA was used and digestions were carried out at
37 °C for 4 h followed by ethanol precipitation.

Gel electrophoresis and vacuum blotting. DNA digestes were electro-
phoresed in 0.8% agarose gels in TAE buffer. (0.04 M-Tris–acetate,
0.001 M-EDTA pH 8.3) at 45 V for 20 h at room temperature. DNA
(10 μg) in 30 μl ΔH₂O and 5 μl loading buffer (0.25% bromophenol
blue, 40% w/v sucrose in ΔH₂O) were loaded per well. DNA from foetal
lamb kidney (FLK) cells, persistently infected with BLV, was used as
a positive control; DNA extracts from freshly isolated PBM cells or
cultured lymphocytes from BLV seronegative cattle were used as
negative controls. DNA was vacuum-blotted using an LKB 2016
VacuGene (Pharmacia) onto Hybond-N nylon membranes (Amer-
sham) according to the manufacturer’s directions.

BLV cDNA probe. The probe was obtained from a BLV genomic
DNA clone (provided by Dr J. Casey, Cornell University, Ithaca, New
York, U.S.A.) representing a full-length viral genomic insert in a pUC8
vector. The plasmid was amplified in Escherichia coli strain JM109 and
purified by a modified miniscreen technique (Close & Rodriguez, 1982). The plasmid with the BLV insert was digested with Sacl which, according to published sequences (Sagata et al., 1985), cuts at five sites within the viral genome, twice within each long terminal repeat (LTR) and at a site 1-5 kb from the 3' end of the genome. This digestion yields viral fragments of approximately 1-2 and 6-8 kb when taking into account the loss of the distal ends of both LTRs due to the presence of Sacl sites in these regions. The 6-8 kb fragment represents the 5' LTR plus the gag, pol and env regions. These fragments were separated on a 1% agarose minigel at 100 V for 2 h. The 6-8 kb fragment was purified using the Schleicher & Schuell method for recovery of DNA by electrophoresis onto NA-45 DEAE membranes (Schleicher & Schuell Application Update no. 364). The 6-8 kb fragment (80 ng) was labelled with 50 μCi [32P]dCTP, specific activity 3000 Ci/mmol (Amersham), using a random primer oligonucleotide labelling kit (Amersham). The labelled probe was purified using Sephadex G-50 spun columns (Boehringer Mannheim).

Southern hybridization. Nylon membranes were prehybridized for 1 h in Rapid Hybridization Buffer (Amersham). Hybridizations were carried out at 65 °C in hybridization tubes supplied with the Robbins Scientific Hybridization Incubator (Robbins Scientific). The BLV probe, with 100 μl salmon sperm DNA (10 mg/ml), was added to 5 ml hybridization buffer, mixed and pipetted into a hybridization tube containing the membrane; hybridization was for between 18 and 24 h. All membrane rinses were at 65 °C and included two rinses in 2 × SSC (0-3 M-NaCl, 0-03 M-sodium citrate pH 7-0), 0-1% SDS for 15 min; one rinse in 1 × SSC, 0-5% SDS for 30 min; one rinse in 0-1 × SSC, 0-1% SDS for 60 min. Membranes were exposed at -70 °C to Kodak X-Omat AR film (Eastman Kodak) with intensifying screens for between 1 and 5 days and developed with a film processor.

Results

Phenotypic analysis of PBM leukocytes

Freshly isolated PBM leukocytes were analysed by analytical flow cytometry to determine relative T and B lymphocyte percentages. As illustrated in Table 1, lymphocytotic cattle had elevated numbers of B lymphocytes relative to T lymphocytes, with B lymphocyte percentages ranging from 54 to 90%. This is contrasted with values obtained from the three BLV-seronegative controls, in which the T lymphocytes predominated, and in which the percentage of B lymphocytes ranged from 27 to 34%. The analysis excluded cellular debris, platelets, erythrocytes and the majority of monocytes; cells not labelled with the T (BoCD2) or B (surface immunoglobulin and MHC class II protein) lymphocyte markers represented null cells and monocytes.

Phenotypic analysis of lymphocyte cultures

Lymphocytes grew rapidly in culture for a minimum of 1 month and a maximum of 6 months (average, 3 months), and cultures were generally split 1:10, between two and four times each week. The percentages of the lymphocyte subpopulations present in these cultures were determined with the same reagents as for the PBM leukocytes, with the exception of the anti-MHC class II reagent.

![Fig. 1. Single parameter (fluorescence) flow cytometry analysis of lymphocyte cultures established from animal 714 using IL-2 plus PWM (a) or PHA-L (b). Data are illustrated as two histogram overlays representing cell surface expression of surface immunoglobulin (1), BoCD2 (2), BoCD8 (3) and BoCD4 (4). Expression of T lymphocyte differentiation antigens was identified by indirect fluorescent (FITC) antibody staining and expression of surface Ig (B lymphocytes) was by direct fluorescent (FITC) antibody staining.](image)

Table 1. Phenotype of freshly isolated PBM leukocytes from BLV seropositive and seronegative cattle

<table>
<thead>
<tr>
<th>Animal</th>
<th>Clinical status*</th>
<th>B (%)†</th>
<th>T (%)‡</th>
<th>Leukocyte count§</th>
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<td>90</td>
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<td>14500</td>
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<tr>
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<td>PL</td>
<td>72</td>
<td>21</td>
<td>21700</td>
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<tr>
<td>555</td>
<td>L</td>
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<tr>
<td>564</td>
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<td>714</td>
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<td>73</td>
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<tr>
<td>826</td>
<td>PL</td>
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<td>23600</td>
</tr>
<tr>
<td>846</td>
<td>PL</td>
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<td>850</td>
<td>SN</td>
<td>27</td>
<td>50</td>
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</table>

* PL, Seropositive and persistently lymphocytotic; L, seropositive and lymphocytotic; SN, seronegative and not lymphocytotic.
† Percentage determined by labelling with a polyclonal goat anti-bovine IgG and an anti-bovine MHC class II MAb.
‡ Percentage determined by labelling with a MAb to the bovine pan T marker BoCD2.
§ Total leukocyte number/μl.
Table 2. Phenotypic and proviral status of PBM leukocyte cultures established from persistently lymphocytic cattle

<table>
<thead>
<tr>
<th>Animal and Days in</th>
<th>Mitogen*</th>
<th>BoCD2 (%)‡</th>
<th>BoCD4 (%)‡</th>
<th>BoCD8 (%)‡</th>
<th>Provirus§</th>
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<tbody>
<tr>
<td>373 Con A 28</td>
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<td>80</td>
<td>5</td>
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<tr>
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<td>79</td>
<td>18</td>
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<tr>
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<td>58</td>
<td>20</td>
<td>-</td>
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<tr>
<td>373 PWM 49</td>
<td></td>
<td>85</td>
<td>93</td>
<td>12</td>
<td>-</td>
</tr>
<tr>
<td>564 IL-2 50</td>
<td></td>
<td>99</td>
<td>0</td>
<td>98</td>
<td>+</td>
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<td>100</td>
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<td>+</td>
</tr>
<tr>
<td>826 Con A 16</td>
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<td>28</td>
<td>63</td>
<td>-</td>
</tr>
<tr>
<td>826 PHA-L 16</td>
<td></td>
<td>100</td>
<td>7</td>
<td>90</td>
<td>-</td>
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<tr>
<td>826 PWM 16</td>
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<td>98</td>
<td>72</td>
<td>90</td>
<td>-</td>
</tr>
<tr>
<td>846 Con A 16</td>
<td></td>
<td>96</td>
<td>87</td>
<td>12</td>
<td>+</td>
</tr>
<tr>
<td>846 PWM 19</td>
<td></td>
<td>95</td>
<td>93</td>
<td>8</td>
<td>+</td>
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<td>88</td>
<td>78</td>
<td>16</td>
<td>+</td>
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<td>940 PHA-L 16</td>
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<tr>
<td>940 PWM 16</td>
<td></td>
<td>97</td>
<td>85</td>
<td>12</td>
<td>+</td>
</tr>
<tr>
<td>940 PWM 49</td>
<td></td>
<td>99</td>
<td>99</td>
<td>2</td>
<td>+</td>
</tr>
</tbody>
</table>

* Mitogen refers to the type used to propagate each culture; all cultures received IL-2. Those cultures with IL-2 following the animal number received no mitogen.
† Refers to the day of culture on which the data presented were obtained.
‡ Percentages determined by labelling with MAbs to BoCD2, BoCD4 and BoCD8.
§ Presence or absence of integrated proviral DNA determined by Southern hybridization.

because these proteins were expressed on a variable percentage of activated T lymphocytes as well as B lymphocytes. Periodic analyses were begun after 2 weeks in culture and continued for the duration of each individual culture system. A rapid overgrowth of B lymphocytes by expanding T lymphocyte populations occurred; after 2 to 3 weeks in culture only weak staining of 0 to 4% of the cells by the anti-bovine immunoglobulin reagent was observed. With time, three general phenotypes emerged; the cultures became BoCD4-dominant (> 85% of the cells carried the marker), BoCD8-dominant or continued to consist of a mixture of BoCD4 and BoCD8 cells with neither cell type making up more than 85% of the culture (Table 2). Fig. 1 illustrates a typical histogram overlay identifying dominant lymphocyte subpopulation phenotypes, as determined by fluorescence analysis, in cultured cells established from animal 714. The phenotypic changes and growth characteristics reported for the lymphocyte cultures derived from lymphocytic cattle also were observed in cultures established from seronegative cattle.

**Southern hybridization analysis**

Only DNA extracts obtained from freshly isolated PBM cells and cultures determined to be greater than 95% T lymphocytes were analysed. The proviral status of six of the 10 lymphocytic cattle is shown in Table 2. At least one culture established from each of these six cattle was provirus-positive. The Con A culture from animal 7079 was also positive for provirus whereas cultures from animals 92, 161 and 555 were provirus-negative (data not shown).

The Fig. 2 autoradiograph is a Southern blot analysis of HindIII digests of PBM cell DNA samples derived from six BLV seropositive animals and a seronegative control (791). Lane 1, DNA extracts from FLK cells chronically infected with BLV; lane 2, 373 PBM DNA; lane 3, 564 PBM DNA; lane 4, 714 PBM DNA; lane 5, 826 PBM DNA; lane 6, 846 PBM DNA; lane 7, 940 PBM DNA; lane 8, 791 PBM DNA. Each lane contained approximately 10 μg DNA. Membranes were exposed to X-ray film for 1 to 3 days at -70 °C with an intensifying screen.

![Southern blot analysis](image-url)
Integrated BLV provirus in T lymphocytes

Fig. 3. Autoradiograph of a Southern blot analysis of HindIII digests of genomic DNA extracts from T lymphocyte cultures. Lane 1, DNA extracts from FLK cells chronically infected with BLV; lanes 2 to 10, 373 PHA-L, 564 PHA-L, 826 PHA-L, 714 Con A, 714 PWM, 846 Con A, 846 PWM, 940 PWM and 940 Con A DNAs, respectively. Animals 791 PWM (lane 11) and 850 PWM (lane 12) were seronegative controls. Mitogen refers to those used in each of the leukocyte cultures. Each lane contained approximately 10 μg DNA. Membranes were exposed to X-ray film for 1 to 3 days at -70 °C with an intensifying screen.

Random polyclonal proviral integration will result in a smear of hybridization because of the numerous bands in a wide spectrum of sizes generated by HindIII digestion. This is the case in Fig. 2, although in a few samples, notably animal 373, there appears to be a number of slightly more distinct bands.

DNA samples from T lymphocyte cultures were also digested with HindIII (Fig. 3). An entire range of clonality of proviral integration, from monoclonal to polyclonal, was demonstrated in these samples. The 714 Con A and 940 Con A samples contain smears of hybridization indicative of polyclonal integration, whereas integration in the 826 PHA-L culture appears to be predominantly monoclonal, with two distinct bands in the 7 to 9 kb range emerging from the polyclonal background. In other samples, for example 373 PHA-L and 564 PHA-L, the presence of a number of dominant bands in the hybridization background suggests that integration is partially limited to a few main sites.

In Fig. 4, DNA samples were digested with SacI. All samples appeared to contain the internal SacI site 7.2 kb downstream of the 5' end as well as the LTR sites, because the same proviral fragment of approximately 6-8 kb was detected in all provirus-positive T lymphocyte cultures and in the FLK control. Because the molecular probe utilized in this study represents only the 6-8 kb fragment that is 5’ to the internal SacI site, the 3’ 1-2 kb fragment also generated cannot be detected. Concentration of all proviral DNA into a single band by SacI digestion generated a strong hybridization signal in contrast to the spectrum of fragment sizes and, in many cases, a much weaker signal generated by HindIII digestion. This allowed a more definitive assessment of

Table 3. Summary of phenotypic and proviral data from PBM leukocyte cultures

<table>
<thead>
<tr>
<th>Phenotype*</th>
<th>Con A (8)t</th>
<th>PHA-L (5)</th>
<th>PWM (7)</th>
<th>IL-2 (3)</th>
<th>Provirus (+)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>6</td>
<td>0</td>
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<td>1</td>
<td>1</td>
<td>0</td>
<td>4</td>
</tr>
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</table>

* Final dominant phenotype of the lymphocytes at the end of the culture period
† Numbers in parentheses refer to the number of cultures established with each specific mitogen or IL-2 alone.
the proviral status of the T lymphocyte cultures; for example, culture 846 PWM, which gave a weak signal following HindIII digestion. No hybridization signal has been detected in DNA extracts from freshly isolated PBM leukocytes, or from lymphocytes expanded in vitro, that were derived from seronegative cattle expanded in vitro.

Discussion

Cattle with PL typically show a large increase in the absolute number of B lymphocytes, resulting in a highly inverted T:B lymphocyte ratio compared to aleukaemic or seronegative cattle (Kumar et al., 1978; Ghysdael et al., 1984; Burny et al., 1985). This dramatic B cell lymphocytosis in cattle with PL has directed the majority of BLV tropism studies to the B lymphocyte with little attention having been given to the T cell population. A potential association of BLV with T lymphocytes was first suggested indirectly by data describing an increase in the absolute number of T lymphocytes in cattle with PL (Williams et al., 1988a). Although the presence of provirus in T lymphocytes derived from BLV-infected aleukaemic cattle and enriched by immunoaffinity depletion of B lymphocytes has been suggested, the question of integrated versus extrachromosomal provirus has not been addressed (Williams et al., 1988b). The present study has extended these observations to BLV-infected lymphocytotic cattle and has identified integrated proviral DNA in both major subpopulations of T lymphocytes.

Because of the low percentage of T lymphocytes in the peripheral blood of lymphocytotic cattle (Table 1), the T lymphocyte population was selectively expanded in all cultures with concurrent loss of B lymphocytes. Dependent upon the culture, 0 to 4% of the cells stained weakly with the FITC-conjugated anti-bovine immunoglobulin. This small population of cells probably represents non-specific trapping of the conjugate by dead cells. The contention that B lymphocyte contamination was negligible is supported by our inability to expand or maintain B lymphocytes from the cattle with PL or normal cattle using culture conditions described in this report.

As summarized in Table 3, a correlation appeared to exist between the type of mitogen employed and the emergence of a particular T cell phenotype over time. Of those cultures in which a dominant phenotype was established, all PHA-P and IL-2 (no mitogen) cultures were predominantly BoCD8+; PWM cultures were BoCD4+ and Con A cultures were BoCD4+ (one culture) or BoCD8+ (four cultures). Those cultures in which a dominant phenotype had not been established at the time of analysis may well have acquired one if propagation had been continued. This contention is best illustrated by the four cultures (373 PWM, 564 Con A, 714 PWM and 940 PWM) in which cultures progressed from a mixed phenotype to a dominant phenotype (Table 2). In some cases, addition of the percentages of BoCD4 plus BoCD8 cells in a given culture did not add up to 100%. It is assumed that a population of double-negative cells, representing non-T or non-B lymphocytes, or possibly gamma/delta T cells, is responsible for this phenomenon. Two of these cultures with double-negative cells were cultured for additional time (373 PWM and 564 Con A) with subsequent loss of this null cell population. In the case of 826 PWM, the percentages of BoCD4 plus BoCD8 significantly exceeded 100%, suggesting the presence of double-positive T lymphocytes. The failure of all cells in some cultures to express BoCD2 is unexplained; however, since expression of this cell surface antigen is often modulated (Redelman, 1987), we speculate that some cells may down-regulate its expression after extended culture.

Provirus was detected in DNA extracts of all culture types, BoCD4-dominant, BoCD8-dominant and mixed phenotypes (Table 3). It is concluded that both T helper and T cytotoxic/suppressor cell populations are susceptible to BLV proviral integration because two provirus-positive cultures were 100% BoCD4 (714 PWM and 940 PWM) and two were 100% BoCD8 (564 IL-2 and 826 PHA-L). Not every culture from the same animal, regardless of T cell phenotype, was provirus-positive; it is therefore speculated that the proviral status of these cultures may be merely a matter of chance. T lymphocytes may have been infected in vivo and, since these cultures were initiated as bulk cultures, individual provirus-positive T lymphocytes may or may not have expanded in any particular culture system. Previous work has shown that BLV is expressed at high levels when PBM leukocytes are placed in short-term culture (Baliga & Ferrer, 1977; Driscoll & Olson, 1977; Wyatt et al., 1989); therefore, it is possible that the T lymphocytes in these cultures may have become infected in vitro due to the initial presence of BLV-infected B lymphocytes and, again, expansion of particular T lymphocytes would have been random. If such an in vitro infection of the T lymphocytes did occur, we might have expected a greater percentage of cultures to be provirus-positive; in addition, previous evidence suggesting the existence of provirus in T lymphocytes was obtained from T cells that had not been placed in culture. At this point in the present study the actual site of T lymphocyte infection cannot be proven, although the tropism of BLV for both BoCD4 and BoCD8 T lymphocyte subsets was established.

In all the provirus-positive DNA samples analysed after SacI digestion, the 6.8 kb fragment representing the
medial portion of the 5' LTR, the gag, pol and env regions of the genome have been present, indicating that truncation in these regions has not occurred. Since the 3' 1-2 kb region of the genome cannot be detected, the possibility of loss of these sequences during integration cannot be addressed; however, previous studies have found that the majority of deletions in integrated provirus are in the 5' end of the genome (Kettmann et al., 1982).

Smears of hybridization, indicating integration of a polyclonal nature, occurred in most of the PBM cell DNA samples digested with HindIII. Distinct bands in a few of the PBM cell samples, such as 373, may indicate the outgrowth of dominant cell clones containing only these specific integration sites, or the possibility exists that in some cases integration is of a more specific nature from the onset of infection. These same patterns of hybridization have been previously demonstrated in studies of PBM cell DNA of cattle with PL (Kettmann et al., 1980a); there is no evidence at this time that the occasional dominant cell clones emerging during PL are destined to become tumour clones (Kettmann et al., 1980b).

HindIII digestion of DNA from the lymphocyte cultures demonstrated a variance in the number of integration sites between the cultures. Smears of hybridization in the high M, region from 9 to 23 kb, as in the 940 Con A sample, indicated polyclonal integration, whereas monoclonal integration in the 826 PHA-L culture and oligoclonal integration in the 373 PHA-L and 564 PHA-L cultures was demonstrated. The majority of the distinct bands detected after HindIII digestion of DNA derived from the lymphocyte cultures were larger than 5 kb and would require the presence of viral-host fragments rather than viral fragments alone because digestion of the unintegrated viral genome yields fragments of smaller size. These data indicate that viral DNA is integrated rather than extrachromosomal in T lymphocytes. The partial to complete polyclonality of integration demonstrated in the T lymphocyte cultures suggests that proviral integration into the T cell genome is random and occurs at numerous sites. The predominantly monoclonal or oligoclonal integration found in many of the cultures is probably due to the preferential expansion of one or a few provirus-positive T lymphocytes rather than the preference of preferred integration sites in the T cell genome. Since the majority of the provirus-positive cultures display some degree of monoclonal or oligoclonal integration, the possibility exists that the presence of BLV provirus in select sites confers some type of selective growth advantage upon the cells in vitro.

The mechanism(s) by which BLV causes lymphocytosis and lymphosarcoma in cattle with an apparently low level of viral transcription remains unknown; BLV does not disrupt regulation of cellular proliferation by mechanisms common to other animal retroviruses. Proviral integration appears to be random, with most studies so far demonstrating polyclonal integration in PBM leukocytes from cattle with PL, whereas monoclonal or oligoclonal integration occurs within a single tumour with no common integration site found between different tumours (Onuma et al., 1982; Kettmann et al., 1980a, 1983; Gregoire et al., 1984). Chromosomal abnormalities have not been demonstrated in circulating lymphocytes from cattle with PL and, although changes in ploidy of tumour cells have been found, these do not follow any common theme (Hare et al., 1967). Many of the integrated proviral copies in tumours are truncated and the deletions appear to affect mainly the 5' half of the genome, which may be indicative of the importance of the 3' end of the genome in initiation or maintenance of the tumour state (Kettmann et al., 1980b, 1982).

BLV is closely related genetically and phenotypically to another tumorigenic retrovirus, human T cell leukaemia virus type I (HTLV-I) (Sagata et al., 1985). Like BLV, HTLV-I carries no cellular oncogenes, proviral integration is random and the 3' end of the genome contains X sequences which encode several proteins including tax, a trans-activation factor that is required for efficient transcription of the viral genome (Rosen et al., 1986; Rice et al., 1987). HTLV-I tax has the additional ability of indirectly inducing transcription of several cellular genes, including those encoding the IL-2 receptor α protein and IL-2. The tax-induced constitutive expression of the IL-2 receptor α gene results in an increase in T lymphocyte proliferation (Greene et al., 1986, 1989; Leung & Nabel, 1988). A similar phenomenon is perhaps associated with the lymphocytosis characteristic of BLV infections if the expression of genes encoding cytokines involved with regulation of B lymphocyte proliferation is affected. The ability of the BLV provirus to integrate into the genomes of both major subpopulations of T lymphocytes, as well as the increase in the absolute T cell number noted in animals with PL (Williams et al., 1988a; our unpublished data), would indicate that T lymphocytes are in fact affected during a BLV infection. A deregulation in the synthesis of specific T cell-derived cytokines during infection may well have an influence upon disease progression.

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