Alterations in Humoral Immune Response to Bovine Leukaemia Virus Antigens in Cattle with Lymphoma

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SUMMARY

Sera collected from cattle with enzootic bovine lymphoma (EBL) were compared to sera from clinically normal bovine leukaemia virus (BLV)-infected cattle for immunoglobulin concentration and for antibodies detecting BLV proteins tested using three different viral isolates. Monoclonal antibodies to bovine immunoglobulin isotypes were used in Western blot analysis to identify isotype reactivity to specific viral antigens. IgG titres to BLV were determined by ELISA. Serum immunoglobulin (G1, G2 and M) concentrations were assessed by radial immunodiffusion. Although EBL was associated with reduced total immunoglobulin production, sera from cattle with EBL had significantly (P<0.001) higher specific IgG titres and produced antibodies against a greater and more varied number of BLV proteins than did sera from clinically normal BLV-infected cattle. Variations were consistent within groups of cattle and did not depend on the viral isolate used.

INTRODUCTION

Lymphomas in adult dairy cattle are associated with bovine leukaemia virus (BLV) infection of B lymphocytes (Paul et al., 1977a, b; Kettman et al., 1980a; Esteban et al., 1985). Infection is lifelong and acquired by horizontal transmission by many natural or iatrogenic vectors (Larson et al., 1970; Miller & Van der Maaten, 1979; Onuma et al., 1980; Roberts et al., 1982; Romero et al., 1983; Buxton et al., 1985). BLV is a chronically transforming virus causing tumours in probably less than 5% of infected cows (Burny et al., 1980).

Infection with BLV is persistent with transcriptional blocking of viral expression, apparently due to a blocking factor in the plasma but not the serum of infected cattle (Gupta & Ferrer, 1982). The pathogenesis of infection is not well understood. Transmission from infected cattle may occur with as few as 2.5 × 10^3 lymphocytes or 5 × 10^-4 ml of blood transferred by a number of possible routes to uninfected recipients (Miller & Van der Maaten, 1979; Roberts et al., 1986). Cells which express BLV antigens have not been identified in vivo; however, peripheral blood lymphocytes from an infected donor which possess surface immunoglobulin will express BLV antigens in vitro if cultured in the absence of plasma proteins and in the presence of the mitogen phytohaemagglutinin (Miller et al., 1969; Esteban et al., 1985).

The mechanism of transformation by chronic transforming retroviruses such as BLV and human T cell leukaemia virus (HTLV) types I and II is not as yet understood. Expression of certain viral genes is believed to be necessary for transformation by these viruses; however, neither viral RNA nor viral proteins have been detected in cases of enzootic bovine lymphoma (EBL) (Kettmann et al., 1980b, 1982). Southern blot analysis of infected, non-transformed lymphocytes has demonstrated a large number of provirus integration sites per cell (Kettmann et al., 1980a). The tumours are clonal and transformed cells contain relatively fewer (one to four)

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integrated provirus copies which may be complete or less frequently occur with deletions in the 5' end of the proviral sequence (Kettmann et al., 1981).

The humoral immune response is strong (Mussgay & Kaaden, 1978; Miller & Van der Maaten, 1979; Burny et al., 1980; Ferrer, 1980) with high blood levels of cytotoxic antibodies to cells expressing BLV proteins (Portetelle et al., 1978). Rising antibody titres appear to be stimulated by virus persistence and replication. An increasing number of infected lymphocytes can eventually be detected by Southern blotting at 6 months to 1 year post-infection (Kettmann et al., 1980a). However, neither the spectrum of target cells nor the immunopathogenesis to the various chronic disease states are yet understood. Infection with BLV appears to be immunosuppressive despite the obvious absence of debilitating disease (Meiron et al., 1985). Cattle with lymphoma have decreased serum concentrations of IgM but not IgG or IgA (Trainin et al., 1968, 1976; Jacobs et al., 1980). Abnormalities in the ratio of peripheral B cells to T cells in infected cattle and a large increase in benign peripheral B cells in cases of persistent lymphocytosis have also been reported (Esteban et al., 1985; Meiron et al., 1985).

A strong association between the presence of antibodies to the major gag protein p24 of BLV and the presence of malignant lymphoma in cows with anti-envelope gp51 antibodies has been determined in a large sero-epidemiological study (Thurmond et al., 1985). This finding conflicts with reports that infected cattle possess antibodies to most BLV proteins (Wuu et al., 1977; Mussgay & Kaaden, 1978; Deshayes et al., 1980; Ferrer, 1980). It is not known whether there are alterations in expression of BLV genes in infected or transformed cells or if cattle with lymphoma have a response different to that of clinically normal infected cattle.

We have compared the humoral responses to various specific BLV proteins of clinically normal BLV-infected cattle and tumour-bearing cattle in order to identify differences in BLV proteins expressed and identified by antibodies of cows with BLV-associated lymphoma. We also identified the isotypes produced to various BLV proteins and measured the serum Ig isotype concentrations in each case.

METHODS

Animals and serum samples. Cattle with lymphoma were acquired through the Ontario Veterinary Teaching Hospital, University of Guelph. Clinical and histopathological examination confirmed lymphoma without other disease problems. Blood samples were collected immediately prior to euthanasia and complete necropsy. Sixteen cases of enzootic lymphoma were confirmed serologically by the standard agar gel immunodiffusion (AGID) test for BLV (Van der Maaten & Miller, 1979). Sixteen cases of sporadic bovine lymphoma (SBL) were confirmed seronegative for BLV by AGID tests. An additional 16 negative and 16 positive serum samples from clinically normal cattle were randomly selected from samples submitted to Veterinary Laboratory Services (VLS), Ontario Ministry of Agriculture and Food for routine BLV testing.

Virus isolates. Peripheral blood lymphocytes were collected from one clinically normal chronically infected cow and one case of BLV-positive lymphoma. To establish BLV-producing cell lines, infected lymphocytes from these two cases were co-cultivated with uninfected monolayer foetal lamb kidney (FLK) cells. The third viral isolate was derived from a previously established laboratory strain of BLV-infected ELK cells (Van der Maaten & Miller, 1975). After multiple passages, cells were cloned by serial dilution. The BLV isolates from each case were purified as previously described (Bex et al., 1979) and compared by Western blot analysis.

Western blot analysis. BLV proteins were separated by 10% SDS-PAGE (Laemmli, 1970) and transferred to nitrocellulose with a Trans-Blot apparatus (Bio-Rad) at 100 V for 2.5 h (Towbin et al., 1979). Nitrocellulose paper was blocked with 3% gelatin in 10 mM-Tris-HCl pH 7.4, 150 mM-NaCl (TBS) for 1 h at room temperature. The wash solution consisted of 0.02% Tween 20 in TBS. From each case, serial 10-fold dilutions of sera in 1% gelatin-TBS were applied to strips overnight at 4 °C. After rigorous washing, a 1/500 dilution of affinity-purified, goat anti-mouse IgG conjugated to peroxidase and reacted with the substrate solution described above.

Assays for bovine immunoglobulin isotypes. Monoclonal antibodies to bovine IgG1, IgG2, IgM, IgA and light chain (Nielsen et al., 1986) were used in Western blot analysis as follows. Each monoclonal antibody, diluted 1/500 to 1/2000 in 1% gelatin in TBS, was incubated for 1 h at 37 °C. After washing, strips were incubated for 1 h at 37 °C in 1/500 dilutions of affinity-purified goat anti-mouse IgG conjugated to peroxidase and reacted with the substrate solution described above.
**Determination of antibody titre to BLV.** Microtitre plates were coated with BLV using an established HTLV-I ELISA protocol (Saxinger & Gallo, 1983). Briefly, pelleted virus at 10 mg/ml in 3 M-KCl was disrupted with 10% Triton X-100 in H2O (final concentration approx. 7.6 mg/ml of virus). Optimal dilutions (greater than 1/1000) for coating microtitre plates were determined from positive and negative reference sera (VLS). BLV-coated plates (Probind, Becton-Dickinson) were prepared using approximately 5 µg/ml of disrupted virus in 50 mM-NaHCO3 (pH 9.6), 0.1 ml per well and incubated overnight at 4 °C. The above protocol for indirect ELISA was followed, using appropriate standards for each plate, with affinity-purified, alkaline phosphatase-conjugated rabbit antibody IgG at a 1/1000 dilution as the secondary antiserum.

**Estimation of total serum immunoglobulins.** Polyclonal rabbit antisera specific to bovine IgG1, IgG2 and IgM were used to estimate serum Ig levels by the overnight Fahey technique using standardized radial immunodiffusion kits (Veterinary Medical Research Diagnostics, Pullman, Wash., U.S.A.). Data were analysed by one-way analysis of variance with Duncan's post-test.

**RESULTS**

**Western blot analysis of virus isolates**

Since viruses with different pathological potential are occasionally identified in the retrovirus family, three different BLV isolates were examined for possible variations in proteins expressed and antigens recognized by autologous and allogeneic sera. Each serum showed the same Western blot profile regardless of the BLV isolate selected (Fig. 1). However, different sera had different profiles on Western blot analysis and in general seropositive cattle with lymphoma reacted to more BLV antigens than infected healthy cattle (Fig. 1). Variation in the intensity of gp51 staining was considerable and not related to the serum dilution used in the Western blot analysis. All positive cattle tested had antibodies to the major BLV antigens p24 gag and gp51 env. Sera from seronegative cattle failed to react with any proteins on nitrocellulose. Cattle with

![Western blot analysis of BLV proteins](image-url)

**Fig. 1.** Western blot analysis of BLV proteins derived from infected FLK cells separated in 10% (w/v) SDS–polyacrylamide gels under reducing conditions and blotted on to nitrocellulose. Strips were incubated with serum from BLV lymphomas (L), clinically normal BLV-infected cattle (I) or normal uninfected cattle (N). Affinity-purified peroxidase-conjugated rabbit anti-bovine IgG (1/500 dilution) was used.
EBL had antibodies to two low Mr proteins with approximate Mr of 12K and 15K; 12 of 16 cases reacted visibly to these proteins at dilutions of \( \geq 1/1000 \). Only three of 16 normal BLV-infected cattle had titres to these proteins of 1/10 or greater. A protein of approximately 15K was identified by sera of six EBL cases at 1/10 dilutions or greater. Antibody reacting with the 24K protein was identified in all BLV-positive cases, although some reacted only weakly. In many cases, reactivity with 45K and 70K proteins could also be detected.

All seropositive cattle in this study had antibodies to a diffusely banding protein ranging from 51K to 60K; tumour-bearing cattle reacted most strongly even at high dilutions (\( > 10000 \)). All cases with lymphoma reacted to another broadly banding protein ranging from 30K to 35K but with variable intensity. Fewer than half of normal infected cattle had detectable antibodies to this antigen which was usually only weakly detectable even at relatively low serum dilutions. A protein of 38K was detected by the majority of sera from cattle with BLV lymphoma and rarely identified using sera from clinically normal BLV-infected cattle at various titres.

**Antibody isotypes identifying BLV proteins**

All cattle tested had IgG responses to BLV antigens. Western blot profiles of antibody isotypes were similar when determined by polyclonal sera or with monoclonal antibodies to bovine light chain except that the latter had weaker staining and less apparent reactivity to the 51K protein (Fig. 2). Eleven of 16 cases of EBL reacted with isotypes other than IgG1 to the 24K, 51K or 35K antigens and occasionally the 15K antigen. Five EBL sera contained IgG2 antibodies to the 35K antigen; five had IgA responses to the 24K, 51K and other antigens. Three cases had IgG1, IgG2 and IgA antibodies, two with IgG1 and IgG2. In normal infected cattle, identification of BLV antigens with isotypes other than IgG1 or IgM was rare; three showed IgA anti-24K activity.

**ELISA titres and serum immunoglobulin levels**

Cases of EBL had significantly higher (\( P < 0.001 \)) IgG titres to total BLV antigens than BLV-infected cattle as determined by analysis of variance (Fig. 3). There was no consistent
relationship between cases with the highest titres and the number of BLV antigens identified or the number of types of isotypes which reacted with BLV antigens.

Serum IgM levels were lowest in BLV-positive cattle with lymphoma (Fig. 3). Normal BLV-infected cattle also had low IgM levels but not significantly lower than normal cattle. IgG2 levels were not altered in either group, however IgG1 levels were reduced in cases of EBL but not in normal BLV-infected cattle or cases of SBL. There were no consistent relationships between total serum Ig levels and the spectrum of BLV antigens identified by various isotypes. However, the EBL group with highest BLV titres had the lowest IgM levels.

**DISCUSSION**

The antigens identified in this study correspond well with the previously described $gag$ and $env$ gene products of BLV (Ghysdael et al., 1978; Mamoun et al., 1983). The expression of the $gag$ gene from 5′ to 3′ results in four major non-glycosylated proteins p24, p14, p12 and p10 (Burny et al., 1985). All the $gag$ gene products were resolved by Western blot analysis in this study. High titres to p10 and p12 were identified in the majority of cases of EBL but were found only occasionally in clinically normal BLV-infected cases. A 15K protein identified by some EBL sera corresponds to a similar $M_r$ protein reported as p14 by Mamoun et al. (1983) and p15 by Bex et al. (1979). Antigens with $M_r$ of 45K and 70K were also recognized. Since the crude BLV preparation used for Western blot analysis was derived from a chronically infected BLV–FLK cell line, it is possible that the 45K and 70K proteins frequently identified in this study represent the intermediate precursor proteins Pr45 and Pr70 produced and processed by these cells.
Two glycosylated gp51 and gp30 proteins are encoded by the BLV env gene. They are the major envelope protein and transmembrane protein respectively and have migration patterns similar to those in this study with the exception of gp30 which migrated broadly to 35K in our gels. Both of these proteins are derived from glycosylated 70K and 72K precursor(s), gPr70/72 (Ghysdael et al., 1978; Mamoun et al., 1983). Proteins of approximately 70K were occasionally identified by antibodies of both EBL and normal BLV-infected cases. However, it is not clear whether they are against Pr70 or gPr70/72. All cases contained antibodies to gp51.

Recently the Px (also called lor or tat) gene has been identified and partially characterized for BLV (Sagata et al., 1985); the products of this gene mediate trans-acting transcriptional activation (Felber et al., 1985). The products (p38, p17 and p14) are found most frequently in the nucleus of an infected cell where they have a short half-life (Sagata et al., 1985). In many EBL cases (Fig. 1) we identified IgG antibodies to a 38K protein which could be the BLV p38 tat.

Further studies will be required to confirm this finding and to determine whether non-structural proteins and other intermediate precursor BLV proteins are released into the FLK culture supernatant as are mature intact virus particles.

In general, sera from cattle with BLV-associated lymphoma clearly recognized most gag and env gene products. EBL cases had higher titres than normal infected cattle. This finding may be related to the chronic state of infection or alternatively might reflect occasional expression of provirus.

Isotype-specific responses to certain BLV antigens were detected by Western blot analysis. Infected cattle without tumours often possessed IgG1 and IgM antibodies to the major virus antigens p24 and gp51. IgM responses to BLV were more frequently detected in normal infected cattle but rarely in the cases of EBL we examined. IgG2 and IgA responses were detected to some antigens in several cases of lymphoma but were detected infrequently in healthy BLV-infected animals.

Total serum immunoglobulin concentrations of the IgM and IgG1 types were decreased in cases of EBL. Low IgM levels were also detected in many healthy BLV-infected cases. Previous reports have suggested that BLV-infected cattle are immunosuppressed with specific depression of IgM-producing cells (Meiron et al., 1985). Low IgM levels may indicate an altered or depleted subpopulation of B cells or an impairment in B and T cell interactions resulting in reduced IgM production. However, immunosuppressive disease caused by BLV is not clinically recognized; it can be subtle and susceptibility to disease may be limited to specific indolent diseases (e.g. subclinical mastitis). It is clear that the humoral immune system continues to respond to persistent BLV expression and that BLV expression is highly repressed but not totally blocked (Burny et al., 1985).

Our studies with three different viral isolates confirm the spectrum of viral antigens recognized (Burny et al., 1985). However, Bruck et al. (1984) demonstrated variability in detection of antigenic epitopes of gp51. In our study, antibodies to gp51 from different cases were variable in their ability to identify this antigen by Western blotting. Restriction endonuclease mapping of BLV (Kettmann et al., 1981) has demonstrated variant viruses and the possibility of antigenic drift as in other retrovirus diseases of domestic animals (Narayan et al., 1978). It is possible that variations in the BLV genome could occur and influence the course of the disease but might not be reflected antigenically. Evidence for alteration of the BLV genome has been demonstrated in some cases of EBL in which deletions of portions of the 5' region of the integrated provirus have been found (Kettmann et al., 1978). This may explain why the gag gene products p12 and p15 were not detected by some sera from cases of lymphoma in cattle in this study.

We have demonstrated that cattle with EBL have a strong but variable humoral response to BLV antigens with a pleomorphism of isotype reactivity. Further studies will be required to demonstrate that BLV lymphomas express BLV antigens and that deletions in the provirus are reflected as an absence of specific immune response to these proteins. Further confirmation is also necessary to determine whether cattle with BLV-associated lymphoma identify non-structural BLV-encoded proteins such as those associated with trans-activation.
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REFERENCES


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