Characteristics of the Major Internal Protein and RNA-Dependent DNA Polymerase of Bovine Leukaemia Virus

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SUMMARY

A virus designated bovine leukaemia virus (BLV), associated with leukaemia in cattle and previously demonstrated to induce the disease in sheep, was purified from chronically infected sheep cell cultures. Electrophoretic analysis showed a major protein of mol. wt. about 24,000 (p24) which reacted in gel diffusion and complement-fixation tests with sera from naturally infected cattle, experimentally infected sheep, and guinea pigs immunized with p24. BLV p24 has an isoelectric point of 8.6. Interspecies antigenic reactivities characteristic of mammalian Type C virus p30s were not detected in disrupted BLV or on p24. Sheep and guinea pig antisera to BLV, reactive with p24, also did not precipitate several Type C virus p30s in radioimmunoassays. BLV is also distinguished from Type C viruses and resembles mouse mammary tumour virus and Mason–Pfizer virus in having an RNA-dependent DNA polymerase which is preferentially active in the presence of Mg²⁺ when synthetic templates are used. Along with previously published morphological data, the above indicates that BLV is not a Type C virus as classically defined. Four hundred and forty one human sera from cancer patients and matched controls were non-reactive with disrupted BLV, BLV infected cells, and BLV p24 in complement-fixation tests.

INTRODUCTION

Several laboratories have described ‘Type C’ viruses associated with bovine lymphosarcoma which are generally induced from peripheral blood leukocytes stimulated by mitogens (Miller et al. 1969; Dutta et al. 1970; Kawakami et al. 1970; Stock & Ferrer, 1972). A key problem in characterization of these viruses has been the lack of permanent cultures which continue to release adequate numbers of virus particles. Recently, Van der Maaten, Miller & Boothe (1974) have established a long-term culture by co-cultivation of virus-producing bovine cells with foetal lamb cells. The virus produced by this culture is immunologically identical to previously described bovine ‘Type C’ viruses based on precipitation and complement-fixation tests (Miller & Olson, 1972; Miller & Van der Maaten, 1974), and readily distinguished from bovine syncytial virus and a bovine Visna-like virus (Van der Maaten et al. 1974; Miller & Van der Maaten, 1974). The bovine virus
has not shown interspecies reactions shared by all other mammalian Type C viruses (Ferrer, 1972) and is morphologically somewhat different from typical Type C viruses (Calafat, Hageman & Ressang, 1974; Van der Maaten et al. 1974). Because this agent is not a typical Type C virus but does apparently induce leukaemia upon inoculation into sheep (Olsen et al. 1972) and is associated with bovine leukaemia, it is now commonly referred to as bovine leukaemia virus (BLV).

Using the virus present in the culture system developed by Van der Maaten et al. (1974), we have identified the protein which is reactive with reference bovine and sheep antisera and used this protein to prepare antibody in guinea pigs. The various antisera were tested for cross-reactivity with Type C viral proteins by several immunological procedures including radioimmunoassay.

The RNA-dependent DNA polymerase of BLV was also characterized as to cation preference in the presence of a synthetic template. Our results are consistent with the conclusion that BLV should not be considered a Type C virus, although it possesses pathogenic potential similar to members of this virus group.

**METHODS**

**Virus.** BLV was produced in foetal lamb spleen cultures as previously described (Van der Maaten et al. 1974). These cultures and purified BLV were non-reactive in tests for bovine syncytial virus and a Visna-like virus of bovine origin. Cultures were constantly monitored for BLV production by the complement-fixation (CF) test using sheep antiserum to BLV whose reactivity has been characterized previously (Miller & Van der Maaten, 1974).

**Virus purification.** The procedures of Olpin, Oroszlan & Gilden (1974) were followed for a pool of 12.5 l harvests of fluid from the BLV producing culture. Fluids were clarified by filtration through a 1.2 μm pore size membrane filter (Millipore Corp.) and virus concentrated by isopycnic banding in tris buffered (0.01 M, pH 7.4) sucrose gradients (20 to 50 %). The CF-32 continuous flow rotor was used in a L-350 ultracentrifuge (Spinco), operated at 102000 g at a flow rate of 4 l/h. Fractions (25 ml) were collected and tested for reactivity in the CF test using sheep antiserum to BLV. Fractions with CF activity were pooled (4 fractions around the peak at about 1.16 g/ml) and pelleted in a fixed angle rotor in the L-350 ultracentrifuge at 100000 g for 1 h. The pellet was resuspended in 0.01 M-tris-acetate buffer, pH 7.8, containing 0.1 M-NaCl to a final volume of 3 ml. Thus a concentration factor of slightly over 16000 was effected compared to the original fluid volume.

**Isoelectric focusing.** Virus pellets were prepared for isoelectric focusing according to procedure B as described in detail previously (Oroszlan et al. 1974). In brief, the resuspended virus which contained 20 mg protein was mixed with 100 mg sodium dodecyl sulphate (SDS) and 2-mercaptoethanol (0.01 M final concentration) for 1 h at room temperature. The solution was made 6 M in recrystallized urea and incubated at room temperature for 30 min. SDS and viral RNA were removed on a Dowex AG-1X2 anion exchange column (Bio-Rad Labs, Richmond, California). Isoelectric focusing was made in 2 % ampholine with pH ranges of 3 to 10 initially and 7 to 10 after initial results were available. Focusing was made for 48 h using 600 V at 5 °C. The pH gradient was stabilized by a 0 to 40 % sucrose gradient. Fractions (~2 ml) collected from the bottom of the column were monitored for pH, extinction determined at 280 nm using a Uvicord II ultraviolet analyzer, and tested for CF reactivity using sheep antiserum to BLV.

**SDS-polyacrylamide gel electrophoresis.** The procedure of Weber & Osborn (1969) was used as described in the legend to Fig. 1. Approx. 50 μg total protein (Lowry et al. 1951)
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of disrupted virus (prepared as described above) was applied to gels in 10 µl volumes. 10 µl of fractions from the isoelectric focusing separations were also analyzed. For evaluations of serological activity 1 mm gel thickness fractions were prepared by a mechanical slicer and eluted in buffer.

Immunodiffusion. Double immunodiffusion was done in plates that contained 0.8 % agarose, pH 7.4, ionic strength 0.15. In preparing plates, 8 g of agarose, 9.3 g of tris 2-amino-2-(hydroxymethyl)-1,3-propanediol, 74 ml of 1 N-HCl, and 7.0 g of NaCl were made up to 11 with distilled water (Crowle, 1961). Merthiolate (1:10000) was added as a preservative. Plates were kept at room temperature, observed for 72 h, and photographed when optimum precipitation lines developed.

RNA-dependent DNA polymerase. Concentrated supernatant fluids and purified virus were assayed for RNA-dependent DNA polymerase activity using the synthetic template-primer poly rA:dT₁₂₋₁₈ (Collaborative Research, Boston, Mass.). Conditions of the assay are described in Table 1.

Radioimmunoprecipitation (RIP). The assay used was similar to that previously reported Charman et al. 1974b) with several modifications: Labelled antigen was diluted in 0.01 M-tris, pH 7.2, containing 2 % BSA rather than 1 % normal rabbit serum. Test sera were diluted in tris buffer containing 10 % normal rabbit serum. Assays were incubated 3 h at 37 °C and then overnight at 4 °C. An equal vol. of 78 % saturated ammonium sulphate was then added with rapid vigorous mixing and tubes further incubated 5 h at 4 °C. Precipitates were then separated by centrifuging at 6000 rev/min for 45 min in a Beckman J-21 centrifuge using JA-14 fixed angle rotors equipped with plastic inserts (Beckman). Supernatant fluids were decanted to waste, tubes inverted, drained on absorbent paper for 2 to 4 h and counted in a Nuclear Chicago Model 1185 autogamma counter.

RESULTS

Electron microscopic examination of infected cells or viral pellets by thin section techniques showed particles with a morphology as previously described (e.g. Fig. 6 in Van der Maaten et al. 1974). The viral pellets used for protein purification contained 5 x 10¹¹ particles/ml based on evaluation of negative stained preparations using polystyrene latex spheres of known concentration as control (Olpin et al. 1974). This preparation also contained obvious cell debris so that some portion of the total protein was probably non-viral. Further purification of the intact virus was not made based on the aim of this study, namely to identify and characterize the protein reactive with bovine and sheep antiserum.

Isoelectric focusing of BLV protein

The initial purification procedure used was isoelectric focusing after first disrupting the virus with SDS and 2-mercaptoethanol and removal of SDS and nucleic acids on Dowex AG-1X2 columns. Separations were first made on a pH gradient of 3 to 10. In this separation the major extinction peak (280 nm) occurred at pH 8-6 which coincided with CF activity using sheep anti-BLV. Fractions showing peak activity were pooled and re-electrofocused on a pH 7 to 10 gradient. Again, serological activity coincided with a major extinction peak with an isoelectric point of 8-6 (Fig. 1). Approx. 1 mg of total protein was recovered which represents 5 % of the total protein of the crude viral pellet. When applied to 7.5 % polyacrylamide gels a single band with an estimated mol. wt. of 24000 relative to standards was found.

In Fig. 2 (inset) the electrophoretic pattern of the crude viral pellet is shown relative to
Fig. 1. Isoelectric focusing of partially purified BLV protein. Fractions from a pH 3 to 10 gradient reactive in the CF test with sheep anti-BLV were pooled and focused on a pH 7 to 10 gradient. $E_{280}$ nm was continuously monitored by a Uvicord II ultraviolet analyzer at double sensitivity. 2 ml fractions were collected and pH determined every 4th fraction and serological activity with sheep anti-BLV every 2nd fraction. Peak extinction and serological activity were found at pH 8.6.

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three mol. wt. standards. Multiple protein bands were resolved with an obvious major peak corresponding to a mol. wt. of 24,000. To confirm that p24 was the protein showing serological reactivity (as deduced from the isoelectric focusing results), acrylamide gels of disrupted BLV were divided into 1 mm sections, eluted, and tested for serological activity with reference sheep anti-BLV. As shown in Fig. 2, CF activity co-electrophoresed with p24. No CF activity was recovered at any other position in the gel.

### Lack of Type C virus interspecies determinants in BLV

Purified p24 from the isoelectric focusing experiment was used to immunize a group of three guinea pigs. Four inoculations were given at weekly intervals, the first two with Freund's complete adjuvant subcutaneously and in the footpad, the final two without adjuvant intraperitoneally. A total of about 0.2 mg was given to each animal. The responses of the individual animals were qualitatively similar and results with the best responses will be described here.

Gel diffusion analyses with disrupted BLV and a variety of Type C viruses and their respective antisera are shown in Fig. 3. The guinea pig anti-p24 reacted only with BLV and not with mammalian or avian Type C viruses. Disrupted BLV did not show reactions with sera specific for Type C virus internal proteins (designated here as p30; August et al. 1974), nor did this virus react with a potent interspecies p30 antiserum prepared by successive immunization with different p30s (R. V. Gilden, unpublished data). This procedure selects for enhanced synthesis of antibodies to shared determinants on the respective p30s as for other protein antigens (Gilden, 1963). As shown in Fig. 3 this antiserum reacts strongly with mouse and rat Type C viruses whose proteins were not in the immunization series. In further confirmation of the assertion that p24 was the major protein reactive with sheep and cow
Fig. 2. Mol. wt. of BLV internal antigen. The inset shows SDS-polyacrylamide gel patterns (7.5 %, 10 cm; Weber & Osborn, 1969) of disrupted bovine virus and a mixture of aldolase (mol. wt. 40 000), carbonic anhydrase (mol. wt. 29 000), and myoglobin (mol. wt. 17 000). The gel buffer was 0.1 M-phosphate buffer, pH 7.2, containing 0.2 % SDS. Electrophoresis was for 4 h 20 min at 10 mA and staining was made with Coomassie brilliant blue. The position of the tracking dye is indicated by thin needles (migration from left to right). There is a heavy staining band in BLV with a mol. wt. of approx. 24 000 based on relative mobility of standards. When parallel gels were eluted CF activity co-migrated with this band. The figure shows a similar experiment with gels run for 4 h. The position of the standards is indicated (○) as is the main band in BLV (●). Parallel gels were divided with a mechanical gel slicer and each fraction eluted in 0.1 ml of 0.01 M-tris buffer, pH 7.4, containing 0.15 M-NaCl and 0.02 % sodium azide. CF tests were made with 4 units of sheep antiserum to the bovine virus. Six positive fractions (●—●) were found which superimposed on the stained band. A mol. wt. of 24 000 was calculated based on absolute mobility versus log₁₀ (mol. wt. of standards).

anti-BLV, an identity reaction is seen between the sheep reference serum and the guinea pig anti-BLV p24 when diffused against disrupted BLV. Data with cow antiserum are not shown but gave equivalent results to sheep serum in gel diffusion (Fig. 7 of Van der Maaten et al. 1974).

In complement fixation tests the guinea pig antiserum proved specific for BLV-infected cells and did not react with Type C virus preparations (purified virus or infected cells) or uninfected hamster, mouse, cat, rat, human, bovine, or sheep cells. The latter tests utilized 20 % (v/v) cell suspensions. Our experience with guinea pig antiserum to purified viral components has been that such sera are remarkably free of ‘non-specific’ reactions in complement fixation tests with a wide variety of uninfected cell cultures from different species and thus are most useful in antigen-induction assays. Other animal sera, including rabbit, goat, and sheep, do, with varying frequency, contain natural antibody reactive with tissue culture cells and thus without absorption may give spurious results. Thus, assays of human cell cultures for BLV related antigens using guinea pig anti-BLV p24 have been clearly negative while the reference sheep anti-BLV has given sporadic positive results. This is relevant to...
Fig. 3. For legend see opposite page.
Table 1. Lack of antibody to Type C virus p30 in BLV immune sera

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Test - [3H] p30</th>
<th>GaLV</th>
<th>MuLV</th>
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<tbody>
<tr>
<td>Anti-RD 114* p30</td>
<td>350000†</td>
<td>12 800</td>
<td>N.T.;</td>
</tr>
<tr>
<td>Anti-GaLV p30</td>
<td>400</td>
<td>25 000</td>
<td>N.T.</td>
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<tr>
<td>Anti-MuLV p30</td>
<td>1 600</td>
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<tr>
<td>Anti-BLV p24</td>
<td>&lt; 10§</td>
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<td>Sheep anti-BLV</td>
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* The first 4 sera were prepared in guinea pigs, all having homologous CF titres of 1:80 to 160 vs. 4 units of homologous antigen. GaLV, Gibbon ape Type C virus; MuLV, murine leukaemia virus.
† Reciprocal of highest dilution giving 10 % binding of about 1 ng of respective p30, about 10000 ct/min input in each assay.
§ N.T. = Not tested.
|| Eight individual sheep antisera with CF and precipitating antibody to BLV.

studies which are now in progress aimed at detecting relationships among viruses containing non-Type C reverse transcriptase now in progress.

While the gel diffusion results of Fig. 3 clearly indicate the absence of determinants related to Type C virus p30s on BLV p24, a more sensitive procedure for detection of distant relationship is provided by the radioimmunoassay. Attempts to detect related determinants in a p30 interspecies inhibition assay (Charman, Kim & Gilden, 1974a) were clearly negative as were assays for antibody to p30 in both guinea pig and sheep antiserum (Table 1). The antibody assay is based on the separation of free from antibody-bound antigen by ammonium sulphate at 39 to 42 % saturation which is optimal for the various p30s (Charman et al., 1974b). This test is especially valuable for detection of low avidity antibodies as may be expected in cross-reacting antisera (Minden, Antony & Farr, 1969). Thus, by reasonably rigorous procedures, and in support of previous conclusions (Ferrer, 1972), BLV and BLV p24 has not revealed antigenic determinants cross-reactive with mammalian Type C virus p30s.

**BLV RNA-dependent DNA polymerase**

Another characteristic of mammalian Type C viruses is the presence of an RNA-dependent DNA polymerase (RDP) whose activity in the presence of synthetic templates is maximal with manganese as the divalent cation. This property contrasts with the mouse mammary tumour virus and Mason-Pfizer virus whose RDP is maximally active with magnesium (Scolnick et al., 1970; Howk et al., 1973). The latter two viruses are considered members of the retra-virus family (Parks et al., 1973) which like BLV do not share antigenic
determinants with mammalian Type C viruses. For the present purposes we consider polymerization of TTP using poly rA:dT₁₂₋₁₈ as template-primer to indicate RDP activity. This procedure is most useful for detection of virus replication by analysis of cell-free supernatant fluids (Kelloff, Hatanaka & Gilden, 1972), but by itself does not constitute definitive evidence for reverse transcriptase activity. Rigorous evidence for this activity would require demonstration of endogenous synthesis of a DNA transcript of the viral genome. For the present purposes the following constitutes the presumptive evidence for reverse transcriptase in BLV: (1) presence of TTP polymerizing activity in purified virions and tissue culture fluids using poly rA:dT₁₂₋₁₈ as an indicator of cellular DNA polymerase activity; (2) lack of activity using poly dA:dT₁₂ as an indicator of viral DNA polymerase activity; (3) lack of activity in uninfected culture fluids from the sheep cells used to propagate BLV; and (4) co-purification of enzyme activity with purified virus and a requirement for disruption of the virus by NP₄₀ to demonstrate activity. We note that such criteria have been accepted as evidence of a reverse transcriptase in other viruses, e.g. a hamster 'foamy' virus (Hruska & Takemoto, 1975) in the absence of hybridization data.

The divalent cation preference of the BLV polymerase was determined in multiple analyses of culture fluids and purified virus. This included virus produced and purified at the USDA laboratory (Van der Maaten et al. 1974) and at Flow Laboratories as described herein. A representative result is shown in Table 2 which includes RD 114 (a Type C virus) and the Mason-Pfizer virus for comparison. The BLV enzyme was clearly preferentially active in the presence of Mg⁺⁺ and under the conditions shown no activity was found with Mn⁺⁺.

Lack of antibody to BLV p₂₄ in human sera

Four hundred human sera consisting of cancer patient and matched controls (Gilden et al. 1970) stored in samples at -70 °C were tested for complement fixing activity utilizing purified disrupted BLV, infected tissue cultures and purified p₂₄. Antigens were used at 4 to 8 units based on titres with reference sheep antisera and guinea pig anti-BLV p₂₄. No positive reactions were obtained. A breakdown of the cancer patient categories represented in the serological survey made in one laboratory (Flow) was as follows: adenocarcinoma, 93; squamous cell carcinoma, 51; undifferentiated carcinoma, 9; malignant melanoma, 11; small cell carcinoma, 7; Hodgkins disease, 14; lymphosarcoma, 3; and sarcomas, 15. Human sera from 41 patients with various types of leukaemia were also tested versus BLV.
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at the USDA laboratory with negative results. These results agree with similar findings of Olson (1974) regarding lack of antibody activity to this virus in human sera.

Based on presence of CF and precipitating antibody in naturally infected cattle and experimentally infected sheep, these results imply that infection of humans with the bovine virus is not a common event, if it occurs at all.

DISCUSSION

BLV has previously been referred to as a Type C virus, although obvious differences in morphological properties have been noted (Van der Maaten et al. 1974; Calafat et al. 1974). A major mammalian Type C viral internal protein, designated p30, is a product of homologous genes in the various viruses as inferred from immunological cross-reactivity and directly shown by amino acid sequence homology (Oroszlan et al. 1973, 1975). Disrupted BLV did not show immunological cross-reactivity with a potent interspecies p30 serum nor did the obvious candidate homologue BLV p24 induce cross-reactive antibody in guinea pigs. Distant homology is not ruled out by these procedures and final evidence of relationship will await detailed primary structure comparisons; however, in terms of classification criteria the lack of cross-reactivity is a strong indicator of separation of BLV from Type C viruses.

Another property common to mammalian Type C viruses is the preferential activity of the virion RDP in the presence of Mn\(^{++}\) when synthetic templates are used. This contrasts with the RDP of the mouse mammary tumour virus and Mason–Pfizer virus which are preferentially active with Mg\(^{++}\) as the divalent cation. The BLV enzyme is similar to that of the latter two viruses in Mg\(^{++}\) preference which while an additional differentiating factor from mammalian Type C viruses is not necessarily an indicator of genetic relationship with other viruses whose enzymes prefer Mg\(^{++}\). At present there is no evidence indicating such a relationship and decisive studies remain to be reported. Thus, the current evidence suggests that BLV should be considered a retra-virus (Parks et al. 1973) without a definitive relationship to other members of the group.

The bovine virus does appear to induce leukaemia in inoculated sheep (Olson et al. 1972), thus while clearly not a Type C virus based on several criteria, it nevertheless appears to be an important virus for further study, especially with regard to leukaemia in cattle. The lack of antibody to BLV in human sera is taken by us to indicate that transmission to man must be a rare event, if it occurs at all. Further studies on this point will be made by more sensitive immunological tests and molecular hybridization when appropriate probes are available.

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