In vitro infection of cells of the monocytic/macrophage lineage with bovine leukaemia virus

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The oncogenic retrovirus bovine leukaemia virus (BLV) primarily infects B cells. Most infected animals remain asymptomatic for long periods of time before an increase in circulating B cells or localized tumours can be observed. This long clinical latency period may be explained by cells of the monocyte/macrophage lineage (M/M) becoming infected and acting as a reservoir for the virus, as shown for other retroviruses (human immunodeficiency virus-1, feline immunodeficiency virus). M/M cells in different stages of differentiation (HL-60, THP-1, U-937, J774, BGM, PM2, primary macrophages of sheep and cows) were cultured with BLV produced by permanently infected donor cells (FLKBLV and BLV-bat2). Donor cells were inhibited from multiplying by either irradiation or treatment with mitomycin C. In other experiments, supernatant from donor cells containing virus was used. In co-culture with the donor cells, the less differentiated monocytic cells showed severe cellular changes such as differentiation, vacuolization, cell lysis and membrane blebbing; apoptosis was a frequent phenomenon. Budding and extracellular viruses were also observed. The more differentiated macrophage cells, although they showed less signs of infection by microscopy, had a complete BLV protein profile, as seen by Western blotting; bands corresponding to p24CA (Gag) and its precursors were clearly seen. In addition, gp51SU was identified by syncytia formation assays. It is concluded that M/M cells may be infected by BLV, the consequences of the infection differing according to the type of cell.

Introduction

Enzootic bovine leukaemia is an infectious disease of cows produced by a lymphotropic retrovirus, bovine leukaemia virus (BLV) which, experimentally, may also infect other species (Burny et al., 1988). The encounter of an animal with BLV does not necessarily mean infection. If an animal is susceptible to BLV, a persistent infection occurs with the development of an immune response. Bovine leukaemia is characterized by a very long incubation period, ranging from 200 days to 7 years, during which animals are asymptomatic carriers. The mechanisms which mark the end of the asymptomatic stage are unclear. Some authors have suggested that it is not a real virus latency (Radke et al., 1992), as there is expression of the virus by some cells, as detected by in situ hybridization (Heeney et al., 1992; Powers & Radke, 1992), which stimulates antibody production.

Depending on the evolution of the disease, animals may be ascribed to one of three groups: (a) clinically healthy; (b) those with persistent lymphocytosis; or (c) those with lymphosarcoma or localized lymphoid tumours. The first group includes around 60% of infected animals (Kettmann et al., 1994), which are asymptomatic carriers of the virus, even though they may suffer a relative increase in B cells (Fossum et al., 1988). These animals are only identifiable by the presence of specific antibodies or by the more cumbersome molecular biology techniques. In the other two groups, there is a marked increase of the number of B lymphocytes, either in blood or localized in lymph nodes or in tissues (Burny et al., 1988).

The virus persists in mainly peripheral B cells (Paul et al., 1977), but results presented by several authors suggest that the virus may replicate in various sites, and possibly in some...
currrently unidentified tissues. T cells seem to decrease in number in animals with persistent lymphocytosis, which may be a result of BLV infection (Meiron et al., 1985; Fossum et al., 1988). Even though there have been reports indicating that there is no convincing evidence that T lymphocytes or monocytes are a major reservoir for the BLV provirus (Mirsy et al., 1996), BLV has been shown to be able to replicate in several areas associated with the presence of monocytes and macrophages (Reinacher et al., 1989; Radke, 1994). Lagarias & Radke (1989) detected viral mRNA in monocyte-like cells from experimentally infected asymptomatic sheep by in situ hybridization. Wyatt et al. (1989) observed BLV expression in peripheral blood multinucleated cells positive for non-specific esterase [i.e. cells of the monocyte/macrophage lineage (M/M)] obtained from infected rabbits. They concluded that circulating monocytes could be infected by the virus and act as a reservoir for BLV. Heeney et al. (1992) detected proviral DNA in circulating monocytes separated by FACS from infected cows and proposed that the infection of monocytes by BLV could explain the persistence of the virus in the animal, as well as the continuous antigenic stimulation. Schwartz et al. (1994) also detected viral RNA by PCR in monocytes and they suggested that BLV could have a larger in vivo tropism than expected initially. This also agrees with previous data reporting that human T-lymphotropic virus 1 (HTLV-1), which is very closely related to BLV, can infect M cells (see Hoffman et al., 1992; de Revel et al., 1993).

Some aspects of disease progression, such as altered immunological functions, could also be explained by infection of M/M cells. Schwartz et al. (1994) have suggested that the expression of some viral proteins could give rise to abnormal expression of some cellular genes, such as interleukins, thus altering normal cell interactions and leading to the expansion of B cells. This was further supported by findings by Pyeon et al. (1996) and Pyeon & Splitter (1998), mainly concerning IL-10 and IL-12, cytokines that are expressed in M/M cells. According to these authors, IL-10 mRNA expression is increased during the late stages of the disease (as compared with non-infected animals or in the early stages of disease) (Pyeon et al., 1996). In contrast, the levels of expression of IL-12 mRNA are lower than normal in the late stages of disease, even though in the early stages of infection they are higher than in non-infected animals (Pyeon & Splitter, 1998). Werling et al. (1995) determined that the production of IL-1 and TNF-α was increased in infected animals after stimulating monocytes in vitro with lipopolysaccharide. Recently, Werling et al. (1998) also reported alterations of surface antigen expression and a decreased phagocytic activity of M/M cells isolated from BLV-infected cows compared to those from healthy animals.

In this paper, we have studied the experimental transmission of BLV from cells persistently infected with BLV to several M/M cells (cell lines and primary cultures) by cell contact achieved by co-cultivation and by infection with filtered supernatants.

### Methods

#### Cells

Two cell lines persistently infected with BLV were used as donor cells: FLKBLV (Van der Maaten & Miller, 1976) and BLV-bat (Graves & Ferrer, 1976), kindly provided by A. Burny (University of Brussels, Belgium) and K. Radke (UC Davis, CA, USA), respectively. M/M cells used included the following cell lines (in order of increasing degrees of differentiation): human promyelocytic cell line HL-60 (ATCC CCL 240) (Collins et al., 1978); human monocyte cell line THP-1 (ATCC TIB 202) (Tsuciya et al., 1980); human monocyte-like cell line of histiocytic origin U-937 (ATCC CRL 1593) (Sandstrom & Nilsson, 1976); mouse monocyte-macrophage J774 A.1 (ATCC TIB 67) (Ralph & Nakoinz, 1975); African green monkey macrophage-like BGM (ATCC 03-240); and PM2, a porcine alveolar macrophage cell line obtained in the University of James Cook (Australia). HL-60, THP-1, U937 and J774 grew as a suspension of cells, while BGM and PM2 grew as an adherent monolayer.

Primary macrophages were isolated from 40–50 ml heparinized blood from sheep or cows. The white layer was obtained by centrifugation (3500 r.p.m. for 30 min at room temperature) and treated with sterile distilled water for 20 s to lyse remaining red blood cells. Cells were washed twice with Hanks’ balanced solution without Ca²⁺ or Mg²⁺ and separated through a Ficoll–Paque gradient (Pharmacia) at 2500 r.p.m. for 30 min at room temperature. White cells were washed twice in Hanks’ solution and resuspended in culture medium with 10% foetal calf serum (FCS). The medium, containing non-adherent cells, was removed after 2 h and periodically thereafter and replaced with fresh medium. Non-specific esterase was assayed initially and after 10 days of culture.

All cells in this study were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated FCS (Gibco BRL), penicillin (100 U/ml), streptomycin (100 mg/ml) and anti-PPLO agent (Gibco BRL) and incubated at 37 °C in a humidified 5% CO₂ atmosphere.

#### Infection of M/M cells

The mitotic activity of donor cells was irreversibly blocked either: (a) by incubation of the cells with 100 μg/ml mitomycin C for 30 min at 37 °C; or (b) by sublethal irradiation at 15,000 rads in a cobalt chamber. The cells were washed several times with medium and used for co-cultivation experiments. For co-cultivation, 10⁶ treated donor cells were mixed with 10⁶ recipient cells. During 3 weeks of co-cultivation, with three or four culture transfers, the mitomycin C-treated or irradiated donor cells were usually eliminated from the mixture (as judged by the control flasks with only treated donor cells) leaving the recipient cells only. In other experiments, the various M/M cells were infected with the filtered (0.45 μm) supernatant (containing BLV) from the same donor cells which had been used for the co-cultivation experiments.

Controls included: (a) M/M cells without the addition of treated donor cells (FLKBLV or BLV-bat) or supernatant containing BLV; (b) treated (irradiated or mitomycin C) donor cells; and (c) non-treated donor cells. Controls of M/M cells co-cultured with non-infected donor cells (FLK or bat cells) were not used as these cells were not available.

We named the co-cultures using three letter combinations. The first one represents the treatment of the donor cell (M. mitomycin C; R. irradiation; S. supernatant); the second, the recipient cell (J. J774; H. HL-60; T. THP-1; U. U-937; B. BGM; P. PM2; O. sheep macrophages; C. cow macrophages); and the third, the donor cell line (F. FLKBLV; B. BLV-bat). For purposes of brevity, ‘M/M + BLV’ is used to refer to any of these co-cultures.

Electron microscopy studies, syncytium induction assays (SIA) and Western blotting tests were performed with 3-week-old co-cultures.

#### Electron microscopy studies

Pellets were fixed for 1 h with 2%...
glutaraldehyde in 0.1 M sodium cacodylate at 4 °C and then with 1% OsO₄ in the same buffer for 45 min on crushed ice. The samples were dehydrated through an ethanol series and stained with 1% uranyl acetate in 70% ethanol. The samples were embedded in Epon–Araldite resin and polymerized for 48 h at 60 °C. The ultrathin sections were stained with uranyl acetate followed by lead citrate and were examined by electron microscope (JEOL 100B). The monolayer cells were fixed, dehydrated and stained directly in the bottle.

- Syncytium induction assay (SIA). We followed the protocol described previously (Domenech et al., 1997) using the line CC81 (Fischinger et al., 1974; a kind donation from A. Burny) as indicator cells. We used a ratio of 1:2 (M/M + BLV to CC81 cells). The mixture was incubated in 8-well Lab-Tek (Nunc) (3 x 10⁴ total cells/0.4 ml per well) or in 96-well plates (1 x 10⁴ total cells/0.2 ml per well) at 37 °C in CO₂. After 24 h, the supernatant was aspirated, the cells were air-dried and Giemsa-stained. Syncytia with more than five nuclei were scored in 30 fields at 200 x, and the number of syncytia per 100 cells was determined and compared to the positive control (FLKBLV + CC81). Syncytia inhibition was performed in a similar way, incubating M/M + BLV with BLV-positive cow serum (reference positive serum included in the AGID Test from Rhône Mérieux), which had been filtered (0.22 μm) and complement-inactivated, for 1 h at 37 °C before mixing them with CC81. The titre of the serum (1:44) was determined prior to the test.

- Western blot (WB) analysis. Cells were centrifuged and treated with lysis buffer (0.15 M NaCl, 0.05 M Tris–HCl pH 7.2, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF) (100 μl/1 x 10⁶ cells) for 1 h on ice (Domenech et al., 1997). After centrifuging at 35,000 r.p.m. at 4 °C for 1 h, samples were resolved on 12.5% polyacrylamide gels and 1% SDS in a Mini-Protean II system (Bio-Rad). Gels were sandwiched in between two nitrocellulose membranes (0.45 μm pore size) and proteins were passively transferred for 24–48 h at 4 °C. Membranes were blocked with 3% BSA in PBS for 1 h at 37 °C before being exposed consecutively to a 1:250 dilution of a pool of sera from naturally infected seropositive cows (Domenech et al., 1997), and to a 1:8,000 dilution of rabbit anti-bovine IgG labelled with peroxidase (Sigma). To precisely localize p24CA and gp51SU, MAbs against these proteins, kindly provided by D. Portetelle (Faculty of Agronomy, Gembloux, Belgium), were used instead of cow sera, continuing the reaction of rabbit anti-mouse peroxidase-labelled conjugate. Reactions were revealed by a substrate mixture consisting of H₂O₂ and diaminobenzidine.

Results

BLV-infected monocytes and macrophages, representing different stages of differentiation, were tested for signs of infection after 3 weeks of co-culture. Infection was followed by the study of several indicative parameters: morphological alterations (optical and microscope alterations) and production of viral proteins (Env-related proteins by SIA and Gag-related proteins by WB). At this time-point, the viability of treated donor cells (FLKBLV and BLV-bat₂) in co-cultures was almost negligible.

Morphological changes seen by electron microscope in some M/M + BLV included, among others, nuclear and nucleolar condensation, cytoplasmic vacuolization, swelling of the rough endoplasmic reticulum, mitochondrial degeneration and blebbing of the cytoplasmic membrane, suggesting the possibility of apoptosis.

Extracellular viral particles are more evident in adherent M/M cell lines

By electron microscopy, viral particles were difficult to find in M/M + BLV. They were dispersed in the extracellular spaces usually as single particles (Fig. 1a), in groups (Fig. 1b), or were in the phase of condensation on the cell membrane or budding on the cell surface (Fig. 1b). Further analysis using colloidal-gold-labelled anti-lg against BLV-specific MAbs confirmed that both extracellular and budding particles were unequivocally of BLV origin (L. Llames, J. Goyache, A. Doménech, A. Monaña, G. Suárez & E. Gómez-Lucía, unpublished results). Extracellular particles were seen most frequently in adherent M/M + BLV (BGM, PM2), followed by other cells in suspension (U-937, J774, THP-1). Viral particles were never seen within cytoplasmic vacuoles.

Monocytic cell lines are more susceptible to morphological alterations

As seen with an optical microscope, more morphological alterations were observed in suspended monocytes + BLV than in adherent macrophages + BLV (Table 1). Morphological changes in suspended cells included cellular differentiation to a macrophage-adherent stage (THP-1 and U-937), vacuolization (THP-1 and J774), development of giant cells four to eight times the normal size (THP-1, U-937 and J774) and cell lysis (HL-60). Thus, the cells seemed to respond differently to BLV infection, and some (THP-1 and U-937) remained mostly viable, while in others (HL-60), the infection tended to be lytic.

Ultrastructural alterations observed are related to apoptosis and are more prominent in less differentiated cells

In some co-cultures, chromatin condensed close to the nuclear envelope maintaining the integrity of the plasma membrane (Fig. 1c, d). These alterations were present in suspended monocytes + BLV, principally in U-937, and were associated with other changes such as vacuolization, surface blebbing and cellular death, which are linked with apoptosis (Table 2). The nuclear condensation usually occurred in dying cells, just before massive cellular death. Cell cultures which showed more nuclear changes were suspended monocytes (J774, HL-60, THP-1 and U-937) + BLV (Table 2). Apoptosis was seen most frequently in mitomycin C-treated co-cultures, followed by incubation with supernatant, and it was never seen in irradiated co-cultures. As it was also frequently seen in M/M cells infected with supernatant, proteins secreted by mitomycin C-treated cells might induce apoptosis were not suspected. No nuclear alterations were observed in
monolayer cells (BGM, PM2, sheep or cow macrophages). We did not observe apoptosis or any other alteration in control non-infected M/M cells or in controls of irradiated/mitomycin C-treated donor cells.

The cytoplasmic alteration most frequently seen was vacuolization, particularly in J774. Vacuoles, usually empty, were generally circular and quite big, but seldom enlarged the cell (Fig. 1d, e). In some cases, vacuoles concentrated in an area free of cellular organelles. In several of the samples of M/M cells co-cultivated with mitomycin C-treated cells or M/M cells incubated with filtered supernatant, we observed surface blebbing; the blebs could be full of vacuoles (Fig. 1f). In some M/M + BLV (J774, HL-60 and U-937), these alterations were associated with apoptosis. Apoptosis was confirmed by visualization of a characteristic ladder pattern of genomic DNA fragmentation on agarose gels 2–7 days post-infection (data not shown), following a standard protocol already described (Cascales et al., 1994). These bands, present in all co-cultures of suspended monocytic cells with donor cells, were not seen in any of the controls.

Other cytoplasmic alterations observed frequently by electron microscopy were syncytia, hypertrophy of the rough
Table 1. Morphological changes in M/M + BLV seen with optical microscope

All changes refer to modifications with respect to the uninfected cell lines (M/M). + +, Effect observed in > 75% of the experiments and/or > 75% of the M/M + BLV; +, effect observed in 25–75% of the experiments and/or 25–75% of the M/M + BLV; ±, effect observed in < 25% of the experiments and/or < 25% of the M/M + BLV; −, effect was never observed.

<table>
<thead>
<tr>
<th>M/M + BLV</th>
<th>Cell differentiation</th>
<th>Vacuolization</th>
<th>Giant cells</th>
<th>Cell lysis</th>
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<td>±</td>
</tr>
<tr>
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<tr>
<td>M/cow§</td>
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<td>±</td>
<td>±</td>
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</table>

* Only observed in co-cultures with FLKBLV.
|| Only observed when incubated with supernatant.
‡ M/sh: sheep primary macrophages.
§ M/cow: cow primary macrophages.
NA, Not applicable.

endoplasmic reticulum with cisternal swelling, degeneration of mitochondria, tubulo-reticular structures (associated with virus replication) and areas of condensed proteic matter. Further analysis using BLV-specific MAbs and colloidal-gold-labelled anti-Ig detected BLV proteins in mitochondria and areas of condensed proteinaceous matter, which confirmed our previous association of these alterations with an active virus infection (L. Llames, J. Goyache, A. Doménech, A. Montañá, G. Suárez & E. Gómez-Lucía, unpublished results). Mitosis was almost non-existent.

These observations suggested that less differentiated monocytes underwent severe morphological changes as a consequence of infection; these changes were not so evident in more differentiated cells. Even though the presence of budding and extracellular virus indicated virus production, the synthesis of viral proteins was studied.

Only differentiated cow and sheep primary macrophages sustain a clear synthesis of BLV envelope and Gag proteins

SIA was used to determine the presence of the envelope glycoprotein gp51SU in the plasma membrane. Positive cases were confirmed by specific inhibition with a BLV-positive serum, which had been titred and used at a 1:44 dilution. Cow or sheep primary macrophages + BLV were positive in most experiments (Table 3). Syncytia seen with these co-cultures were similar to those observed in FLKBLV controls, i.e. syncytia with abundant cytoplasm and many nuclei, sometimes arranged in parallel lines. With the other M/M + BLV, the results were weak (BGM and PM2), unclear (J774, THP-1 and U-937) or clearly negative, and syncytia did not include as many nuclei and were more difficult to identify. A very low number of syncytia (background) was seen in sheep and cow M/M cells used as controls, whereas they could not be seen in the other M/M cell controls.

Table 2. Morphological changes in M/M + BLV seen with electron microscope

All changes refer to modifications with respect to the uninfected cell lines (M/M). + +, Morphological changes observed in > 75% of the experiments and/or > 75% of the M/M + BLV; +, morphological changes observed in 25–75% of the experiments and/or 25–75% of the M/M + BLV; ±, morphological changes observed in < 25% of the experiments and/or < 25% of the M/M + BLV; −, morphological changes were never observed.

<table>
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<tr>
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* Syncytia seen with optical microscope.
‡ M/sh: sheep primary macrophages.
§ M/cow: cow primary macrophages.
Table 3. Syncytia induction assay in M/M + BLV

Values correspond to the number of syncytia in 30 fields at 200 ×. Control columns, M/M infected with FLKBLV or BLV-bat₂ (M/M + BLV) or FLKBLV without adding CC81; + CC81 columns, (M/M + BLV) or FLKBLV incubated with CC81; + serum columns, (M/M + BLV) or FLKBLV incubated with bovine serum positive to BLV (1:44) for 1 h before mixing them with CC81. —, No syncytia observed.

<table>
<thead>
<tr>
<th></th>
<th>Infected with FLKBLV</th>
<th>Infected with BLV-bat₂</th>
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<td>24</td>
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</table>

* M/sh: sheep primary macrophages.
† M/cow: cow primary macrophages.
‡ FLKBLV: permanently infected cell line used as positive control.

Cell lysates from M/M + BLV were analysed by WB using a pool of cow sera positive to BLV. Reference specific bands, identified in lysates from FLKBLV and BLV-bat₂, had apparent molecular masses of 25, 32, 40, 50 and 62 kDa. These same bands were recognized by the MAb against p24CA and correspond to p24CA and its precursors (A. Burny, personal...
communication). Four additional bands of 28, 41, 76 and 89 kDa (Doménech et al., 1997) were also seen. None of these nine bands were seen in lysates of non-BLV-infected M/M cells.

A complete profile of proteins was only seen in cow and sheep primary macrophages (Fig. 2). p24CA was the protein produced in largest amounts, as judged from the intensity of the band, followed by the protein giving a band of 32 kDa. Some of these M/M + BLV expressed only Gag precursors but no p24CA (identified with the MAb against p24CA). Regarding suspended monocytes, the results were variable. In U-937, p24 was observed in all cases when FLKBLV were used as donor cells (MUF, SUF and RUF) and in co-cultures with irradiated BLV-bat2 (RUB); nevertheless, Gag precursors were not detected (Fig. 2c). The level of protein expression was lower than in primary macrophages. In the other suspended M/M + BLV, p24CA was sporadically detected. BGM and PM2 rendered very few specific bands, possibly because of the low protein concentration due to the limited amount of cells that could be used for the lysate (data not shown).

Discussion

The recipient M/M cells used represent cells of varying degrees of differentiation, from the least differentiated HL-60 (promyelocytic) to primary macrophages, passing through THP-1, U-937 and J774 (suspended cells), and BGM and PM2 (macrophage-like) in ascending order of differentiation, as described in the Methods. We wanted to determine whether, as stated for other retroviruses, the level of cell differentiation affected susceptibility to infection. To determine the degree of infection, we analysed several indicative parameters: (a) extracellular viral particles, which would mean that the cell was sustaining synthesis of structural proteins and others necessary for replication; (b) morphological alterations seen in cells; and (c) expression of viral proteins which would also indicate efficient integration and an active replication cycle.

Extracellular BLV particles

The number of viral particles and budding activity were low. It has been proposed for other retroviruses that the degree of cell infection does not always coincide with a larger extracellular presence, which may also be true for BLV. Moreover, the number of budding particles has been calculated as 2–9 per cell (Calafat & Ressang, 1977), which is lower than in other retroviruses (Mussgay & Kaaden, 1978; Dekegel, 1987). Thus, the budding activity of M/M + BLV could be considered to be normal. Budding also depends on the cell type; very few viral particles have been seen budding from lymphocytes and granulocytes in peripheral blood (Calafat & Ressang, 1977). We also observed that budding takes place mostly in localized areas of the membrane, where particles may be shed very close to each other. Budding should be considered as an indication of active infection of M/M cells, the cell being able to sustain all stages of virus replication.

The absence of extracellular viral particles in sheep and cow macrophages + BLV may indicate an apparent restriction of virus morphogenesis and release under in vitro conditions, described in other cell types of bovine origin, through a mechanism which remains to be elucidated. This restriction may be overcome in other species, as these same primary macrophages + BLV induced a high humoral response when inoculated in rabbits (Doménech et al., 1997).

Expression of BLV proteins by M/M + BLV

The protein profile seen by WB was dependent on the degree of differentiation of the recipient M/M cells. Suspended monocytes (HL-60, THP-1, U-937 and J774) had very incomplete profiles, whereas the more differentiated primary macrophages had most of the standard bands. In sheep and cow macrophages + BLV, the amount of viral core protein (Gag) expressed was high; the intensity of the bands was comparable to those observed in the persistently infected cell line FLKBLV (Fig. 2), even when the M/M + BLV lysates contained two-thirds of the total amount of cells that FLKBLV had. In addition, the expression of membrane gp51SU was high, judging from the SIA values (Table 3). These results indicate that primary macrophages + BLV were able to produce a complete BLV protein pattern, comparable to that observed in permanently infected cells. On the contrary, suspended cells + BLV expressed few BLV proteins. In some co-cultures, p24 was seen in the absence of precursors, whereas in others, precursors were present but no p24 was observed. The fact that Gag precursors were seen in the absence of p24CA may indicate that either the protease, or the proteolytic route is lacking and that precursors are inefficiently cleaved to mature proteins.

Morphological alterations in infected cells

Ultrastructural changes in some M/M + BLV resembled those described in other retrovirus-infected cells, and are typical of programmed cell death or apoptosis, which is distinct from the usual necrosis of cytotoxic virus-infected cells.

In general, cells undergoing apoptosis displayed profound structural changes, including blebbing of the plasma membrane. This surface blebbing has also been described in infections of human immunodeficiency virus (HIV) (Gougeon & Montagnier, 1993) and feline leukaemia virus (FeLV) (Rojko et al., 1992). In HIV, it has been suggested that surface blebbing may play a role in the direct cell-to-cell spread of the virus (Karimi et al., 1989).

The presence of apoptosis seems to be linked to virus infection as non-BLV-infected controls of the M/M cells never
presented apoptotic bodies. Neither controls of irradiated or mitomycin C-treated donor cells presented chromatin condensation in their nuclei. Moreover, apoptosis figures were also seen in M/M cells incubated with filtered supernatant of FLKBLV and BLV-bat; thus, apoptosis does not seem to be due to the blocking treatment of the donor cell lines (which might promote secretion of certain apoptosis-inducing proteins) but a consequence of virus infection.

Apoptosis has been related to the pathogenesis of several retroviruses, such as HIV (Gougeon & Montagnier, 1993; Dittmer et al., 1996), simian immunodeficiency virus (Dittmer et al., 1996), felineline immunodeficiency virus (FIV) (Momoi et al., 1996) and FeLV (Rojko et al., 1992). It has been observed in in vitro cultures of U-937 cells infected with HIV (Malorni et al., 1993) and T cells from cats infected with FIV (Momoi et al., 1996) and with FeLV (Rojko et al., 1992). Candidate HIV proteins which may modulate apoptosis include the surface glycoprotein, gp120SU (Gougeon & Montagnier, 1993), and the regulatory protein Tat (Li et al., 1995). HTLV-I Tax, which is very closely related to BLV Tax, has been reported to have different effects on the regulation of apoptosis. Some authors have reported that it may promote apoptosis in Rat-1 cells (Yamada et al., 1994), possibly through the activation of c-myc and c-fos (Fujita & Shiku, 1995), whereas others (Tsukahara et al., 1999) have suggested that HTLV-I Tax may prevent apoptosis.

Regarding BLV, early reports describe the presence of fragmented nuclei in infected bovine cells associated with virus activity in these cultures (Van der Maaten et al., 1974; Mussgay & Kaaden, 1978), which could be what is termed apoptosis today. Later, Pyone et al. (1996) determined that, in BLV infection, there is an increase in secretion of IL-10 mRNA by M/M cells, which could lead to apoptosis.

Susceptibility to apoptosis associated with BLV infection seems to vary from one species to another. B cells from lymphocytotic sheep appear to be protected from ex vivo spontaneous programmed cell death (Dequiedt et al., 1997; Schwartz-Cornil et al., 1997), while PBMCs from persistently lymphocytotic BLV-infected cows are more susceptible to ex vivo apoptosis than PBMCs from uninfected or aleukaemic animals (Dequiedt et al., 1999). According to our results, primary macrophages from uninfected sheep and cows, infected in vitro with BLV, were similarly resistant to apoptosis.

The mechanism by which BLV interferes with apoptosis is unknown. It may be either a direct consequence of BLV gene expression, or the result of viral or cellular factors secreted in the medium by BLV-infected cells. Several authors have presented data that could argue for a possible role of virus gene expression in protection against programmed cell death (Schwartz-Cornil et al., 1997). Favouring the second hypothesis, Dequiedt et al. (1997) described that even strongly attenuated BLVs protected PBMCs from apoptosis, and suggested that a viral or cellular factor might be secreted by the infected cells which protects uninfected cells. Their findings indicated that the decreased susceptibility to cell death in infected sheep PBMCs does not depend on the total amount of viral proteins expressed in short-term cultures. Our results suggest that in cultures in which the expression of BLV proteins was more complete were protected from apoptosis, which agrees with data presented by Schwartz-Cornil et al. (1997), even though the secretion of an apoptosis-protecting factor should not be disregarded. On the contrary, cells where apoptosis was seen did not express a complete BLV protein pattern.

Conclusions

In view of our results, we may conclude that M/M cells may be infected by BLV, as they are able to express viral proteins. However, the degree of infection seems to depend on the M/M cell type. Thus, in the primary sheep and cow macrophages, the expression level is high as inferred from the induction of syncytia (gp51SU) and WB (Gag proteins). On the other hand, in suspended M/M cells, which are less differentiated, the expression level is lower. If functional protein expression results are compared to morphological results, it appears that in the group of cells (suspended and macrophage-like) where more morphological alterations were seen, the infection did not culminate in a typical protein profile. Thus, cells may fall into one of two categories: ‘restrictive’ or ‘permissive’ (Narayan & Zink, 1988). Restrictive infection would be characterized by the absence of gp51SU, and the presence of morphological changes related to apoptosis. On the other hand, permissive infection characteristics could include high expression of P24CA and other Gag proteins, as well as membrane-associated gp51SU, few extracellular particles and a lack of apoptosis-related morphological alterations of the infected cells. According to our results, cells belonging to the first category (restrictive) would correspond to the less differentiated monocytes or suspended cells, and those in the second category (permissive) would be adherent primary macrophages. This is a surprising conclusion as, unlike lentiviruses, oncoviruses seem to replicate better in less differentiated cells. However, the presence of regulatory genes in BLV and HTLV may relate these two oncoviruses to lentiviruses.
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References


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