Cellular distribution of bovine leukemia virus proteins gp51SU, $Pr72^{env}$, and $Pr66^{gag\text{-}pro}$ in persistently infected cells

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Abstract

Monoclonal antibodies (mAbs) against bovine leukemia virus (BLV) mature proteins and precursors were used to map the localization of these proteins in persistently infected non-lymphocytic cell lines using immunofluorescence assay (IFA) and immuno-electron microscopy. IFA staining was observed in the basolateral surface of live FLK-BLV cells. When using a mAb against $Pr66^{gag\text{-}pro}$, mottled pinpoint fluorescence was seen in the cell surface of polarized cells, but no reaction was observed in cells undergoing mitosis. However, a mAb against $Pr72^{env}$ stained only mitotic cells and cellular fragments. Additionally, in these dividing cells, this envelope (Env) precursor polyprotein was not evenly distributed but concentrated predominantly in only one daughter cell. To the best of our knowledge, this observation has not been reported previously, either for BLV or for other retroviruses. The results of immunogold electron microscopy confirmed the specificity of the mAbs in the intracellular level. In infected cells, $Pr72^{env}$ and gp51SU were seen in proximity at the plasma membrane in incipient budding sites. Additionally, the mAb against $Pr72^{env}$ also reacted with Env precursor polyproteins in the mitochondria of BLV-bat_2 ultrathin sections. These mAbs may be used as a tool for mapping virus excretion sites in the cell surface of naturally or in vitro infected cells in the different stages of the cell cycle.

Keywords: BLV; Morphogenesis; Immunofluorescence; Immuno-electron microscopy

Introduction

The causative agent of Enzootic Bovine Leuko- sis (EBL) is the Bovine Leukemia Virus (BLV), a lymphotropic retrovirus. It is a chronic disease, characterized by a long latency period which may be followed by the development of tumors. BLV infection is persistent for life. The predominant target of BLV are B cells (Boonstra et al., 1987), but other cellular types, such as T cells or even macrophages, might be infected (Schwartz et al., 1994; Domenech et al., 1997, 1998, 2000). BLV-infected cells usually do not produce viral particles in vivo (Kettmann et al., 1994), and viremia has not been convincingly demonstrated in BLV infected cattle. However, some virogenesis may happen as antibody titers are persistent in infected animals (Heeney et al., 1992), and diagnosis is usually based on their detection.

BLV is a spherical virion which is 50 – 180 nm in diameter. The centrally located icosahedral or round dense core is enclosed by a double membranous envelope (Calafat and Ressang, 1977; Burny et al., 1980) with projections approximately 114 A0in length (Weiland and Ueberschär, 1976; Calafat and Ressang, 1977; Burny et al., 1980). The diameter of the core ranges between 40 and 90 nm, possibly in relation to the maturation stage of the particle. BLV particles are very fragile and are frequently somewhat deformed when pre- pared using high speed centrifugation (Burny et al., 1980). The virion has a buoyant density of 1.15–1.17 g/ml (Burny et al., 1980).

By conventional electron microscopy, BLV has been detected budding from the cell membrane, alone or in groups or packets of virions, with cell debris and in the vacuoles of infected cells (Calafat and Ressang,1977; Burny et al., 1980; Chung et al., 1984; Dekegel, 1987; Roussev et al., 1993; Domenech et al., 2000). When cultivated, BLV proteins and particles can be detected 3 – 6 h after the initiation of cellular cultures but are most abundant after 24 – 48 h (Kettmann et al., 1994). More recently we have shown that BLV protein excretion occurs in cycles with peaks at ca. 8 and 16 using various BLV

specific mAbs in persistently infected cell line FLK-BLV (Llames et al., 2001).

The replication cycle of BLV is assumed to be similar to other type C retroviruses (Coffin, 1992), including the decapsidation, dsDNA synthesis, and integration in the host genome. The viral precursors for the capsid proteins (Gag), or for the capsid plus enzymatic proteins (Gag-Pro-Pol) assemble at the cellular membrane with the pre- cursors for the envelope proteins (Env) and the viral RNA. This process leads to the budding of the virion from the cell and is followed by the cleavage of the Gag and Gag-Pro-Pol precursors to separate the various domains from one another. Cleavage is accompanied by the condensation of the core, along with other less obvious changes (Coffin, 1992).

The use of colloidal gold as an electron dense marker for immuno-cyto-chemical labeling was developed by Tokuyasu in 1973 (Boonstra et al., 1987). Immunolabeling of biological material for the examination at the ultrastructural level re- quires the maintenance of full antigenicity and the preservation of ultrastructural detail (Boonstra et al., 1987). The main advantage of this method over others is that it enables post-sectioning labeling, and thus, all cytoplasmic and cell surface-located antigens are, in principle, fully accessible to the antibodies and gold labels (Boonstra et al., 1987).

A new panel of BLV-specific mAbs has been produced successfully from various crude BLV antigen preparations (Llames et al., 2000) and includes anti-BLV precursor proteins (Pr72env and Pr66gag-pro).

The aim of the present work was to study cell surface distribution and morphogenesis of BLV in live cells and using a panel of specific mAbs against different BLV proteins (Llames et al., 2000) using immunofluorescence and immunogold electron microscopy labeling techniques.

Methods

Cell lines

Fetal lamb kidney cells (FLK-BLV) and bat lung fibroblasts (BLV-bat$_2$) permanently infected with BLV, were a kind donation from Dr Burny (Gembloux, Belgium) and Dr Radke (Davis, CA, USA). Fetal bovine lung (FBL), kindly provided by Dr Levy (Alfort, France) was used as negative control. All cells were grown in RPMI-1640 (Gibco) containing 10% inactivated fetal calf serum (Difco) (FCS), and maintained with 5% CO_2 at 37°C.

Antibodies used for the immunological techniques

A panel of 59 monoclonal antibodies (mAbs) was produced following standard procedures (Llames et al., 2000). Antigens used for hyperim- munizing Balb/c mice were obtained from infected FLK-BLV or BLV-bat$_2$ cells, including lysates, supernatant, formalin-fixed or whole cells. Screen- ing was done using both indirect ELISA and dot-ELISA with five different antigen prepara- tions. The specific viral proteins with which they reacted were determined by Western Blot (Llames et al., 2000). The mAbs chosen for this study against both mature proteins and precursors are listed in Table 1, along with their most important characteristics.

As negative controls we employed NP3 myeloma supernatant, PBS, and a mAb specific for staphylococcal enterotoxin B (Goyache et al., 1992).

Indirect immunofluorescence assay (IFA)using live FLK-BLV cultured cells

Methanol washed and autoclaved sterile cover- slips (10 mm in diameter) were placed in each well of a 24-well plate (Costar). FLK-BLV cells (5×10^4) were plated in each well and incubated overnight in complete media. Grow-To-Die culture supernatants (Llames et al., 2000), produced by growing the cloned hybridomas for 1 – 2 weeks until the cells died (GTD) (500 µl), and FITC-labeled rabbit *anti*-mouse immunoglobulins (Sigma) (1:200) were added to the wells. Thereafter, the cells were fixed with cold methanol and rinsed three times with PBS then with deionized distilled water. The specimens were then mounted into glass slides. The cells surface basolateral side (attached to the cover slips) were observed using an epifluorescence microscope.

Immunogold electron microscopy

Free viral particles from the BLV-bat$_2$ cell culture supernatant were prepared by passing 5 ml of the supernatant into 0.45 μm (Gelmann Science) to eliminate remaining cell debris and minimizing FCS. The filtrate was then centrifuged at 14 000 × *g* for 10 min. Ten microliters of the lower fraction of the eppendorf tube containing the virus was adsorbed in Formvar^R-Carbon- coated nickel grids for 5 min at room temperature. The grids were incubated sequentially with
10 μl of the mAbs (Table 1) for 45 min. The specimens were washed three times with PBS- 0.05% Tween 20 (PBS-T) then reacted with colloidal gold labeled *anti*-mouse immunoglobulins (*anti*-mouse IgM conjugated with 5 nm gold particles, and *anti*-mouse IgG with 15 nm gold particles, Biocell) diluted at 1:100 in PBS-1% bovine serum albumin (BSA) and left to react for an hour. The samples were again washed three times

with PBS-T then negatively stained with 1% Uranyl acetate (Spi-Chem) for 30 s. After rinsing three times with deionized distilled water, the specimens were blot-dried in filter paper and observed using a Jeol model 100B transmission electron microscope.

Intracellular staining of BLV-bat$_2$ cells using immunogold-labeled mAbs

BLV-bat$_2$ cells were grown in 25 cm^2 culture flasks (Costar) with complete media. After 3 days of culture cells were fixed for 2 h with 2.5% paraformaldehyde, and 0.5% glutaraldehyde in
0.1 M phosphate buffer. After, they were dehydrated with increasing concentrations of ethanol and embedded in LR White resins. The sections were obtained in a LKB ultratome III, mounted in nickel grids and labeled with colloidal gold using mAbs against BLV, as described above for supernatants.

Results

Immunofluorescence assay (IFA)

Several mAbs reacted positively against viral surface proteins in the basolateral side of live FLK-BLV cells. When using mAb SCF.I.11 (specific against BLV Pr66$^{gag\text{-}pro}$) the cell surface of resting non-dividing cells was stained with a mottled pinpoint fluorescence (Fig. 1a, rc). Another mAb, PVF.II.25 (specific for Pr72^{env}), showed a different characteristic as compared to SCF.I.11. This mAb intensely stained the cells during division, most likely in the late anaphase and early telophase stages (Fig. 1b and c). The basolateral cell surface of the two forming daughter cells (d_1, d_2) were stained un- equally (Fig. 1b and c). PVF.II.25 only stained cells that were in mitosis, dying cells (dc) (Fig. 1d and e), fragmented cells (cf) and cellular debris (Fig. 1d and e). Cellular fragments still contained high levels of viral proteins, indicated by the intense staining. The monoclonal antibody PVF.II.25 did not stain normal resting cells.

Electron microscopy

Initial results obtained without colloidal-gold labeling included the observation of a grayish magma, organized in circular areas in the cyto- plasm accompanied by the presence of intracytoplasmatic annulate lamellae which could be a possible site for protein synthesis (Fig. 2a and b). The relationship between these alterations and BLV infection was determined with the use of gold labeling.

In this study, both single and double immunogold labeling were performed. Two gold particle sizes were used for labeling *anti*-mouse immunoglobulins (*anti*-IgM conjugated with 5 nm gold particles, and *anti*-IgG with 15 nm gold particles). As most of the mAbs were produced using FLK-BLV for hyperimmunizing Balb/c mice, BLV-bat$_2$ was used to study the presence of BLV in the supernatant and in cells, to eliminate the possibility of non-specific binding due to cellular reactions. No gold particles were found in any of the negative controls confirming the specificity of this technique (Fig. 4a–c).

Immunogold-labeling of free viral particles

Gold-labeled mAbs reacted to BLV particles harvested from BLV-bat$_2$. The size of the particles was

measured to be between 100 and 150 nm, as seen in transmission electron microscopy (TEM). Intact free viruses were labeled by gold particles when using mAbs against gp51SU (CEF.6, CEF.7, and SCF.I.54) (Fig. 3a, b). Monoclonal antibodies against $Pr72^{env}$ (SCF.II.30, SCF.II.40 and PVF.II.25) rarely recognized intact free particles, but instead reacted with fragmented and partially disrupted BLV particles. The mAb SCF.I.11 (specific against BLV $Pr66^{gag\text{-}pro}$) was also observed, though seldom, attached to morphologically deformed viral particles (data not shown). It is unlikely that the virus particles observed in Fig. 3 were artifacts (i.e. cellular membrane fragments and vesicles), since the virus preparation described here (see Section 2) had been previously centrifuged to eliminate cells and cell debris and was further filtered (0.45 μm).

Immunogold-labeling of intracellular BLV proteins in the BLV-bat$_2$ cell line

When using PVF.II.25 (anti-$Pr72^{env}$), groups of 2 – 7 gold particles were seen in the cytoplasm of infected cells (Fig. 4d– f), coinciding with the areas of grayish magma observed previously with electron microscopy (Fig. 2a and b). Similar results were obtained with another mAb directed against $Pr72^{env}$ (SCF.II.30), but fewer gold particles were observed (data not shown). *Anti*-$Pr72^{env}$ mAb (PVF.II.25) were also seen reacting with the mitochondria crests (Fig. 4g). This reaction was not observed in negative controls, either with non-infected cells, or with the negative antibodies as mentioned in Section 2 (Fig. 4a–c).

Anti-gp51SU mAbs (CEF.7 and SCF.I.54) scarcely reacted inside the infected cells. If present, gp51SU was lined-up around the interior of the cellular membrane. With double labeling, clusters of PVF.II.25 (*anti*-$Pr72^{env}$ mAb) and CEF.7 or SCF.I.54 (*anti*-gp51SU mAbs) were observed grouped together within the cell mem- brane while in the process of budding (Fig. 4d and e).

Monoclonal antibodies against BLV Gag-Pro precursors also reacted with proteins in the cyto- plasm of infected cells. *Anti*-$Pr66^{pro}$ mAb (SCF.I.11) was detected scattered in the cyto- plasm in undefined groups or patches (Fig. 4f). This coincided with previous electron microscopy observations where cytoplasmic magma was presumed to be a possible site for the accumulation of viral protein (Fig. 2a).

Discussion

Immunofluorescence assays (IFA) have been used by various authors to determine the tropism of BLV (Yoshikawa, 1991; Rovnak et al., 1993). Using a newly generated panel of BLV specific mAbs (Llames et al., 2000), we have detected the distribution of BLV proteins in the plasma mem- brane surface of live FLK-BLV cells. As seen in Fig. 3a, the monoclonal antibodies used reacted specifically with the virus; it may be assumed that in the cells they recognized only viral components, and not cellular proteins. This is further confi- rmed by the observations shown in Fig. 4d and e, in which the mAbs bound to a structure on the cellular membrane resembling a budding particle. In addition, to minimize the cellular reactivity, two different cell lines were used for mAb production (FLK-BLV) and for virogenesis (BLV-bat$_2$).

Env and Gag-Pro precursors were mapped in localized areas on the basolateral side of the cell (see Section 2 regarding the orientation of the cells). This seems to agree with data from human retroviruses (Lodge et al., 1997a,b; Descham- beault et al., 1999) regarding the polarization targeting of retroviral Env proteins in the basolat- eral membrane surface of the progeny cell which could play a role in transmission, pathogenesis (Lodge et al., 1997b) and escape recognition by the host immune response.

In general, mAbs against BLV used in combi- nation with IFA have rendered similar observations to findings in the present work. Using mAbs against gp51SU, fluorescence has been seen both in the cytoplasm (Tanaka et al., 1988), and in the electron microscopy (Fig. 2a and b). Similar re- sults were obtained with another mAb directed against $Pr72^{env}$ (SCF.II.30), but fewer gold particles were observed (data not shown). *Anti*-$Pr72^{env}$ mAb (PVF.II.25) were also seen reacting with the mitochondria crests (Fig. 4g). This reaction was not observed in negative controls, either with non-infected cells, or with the negative antibodies as mentioned in Section 2 (Fig. 4a–c).

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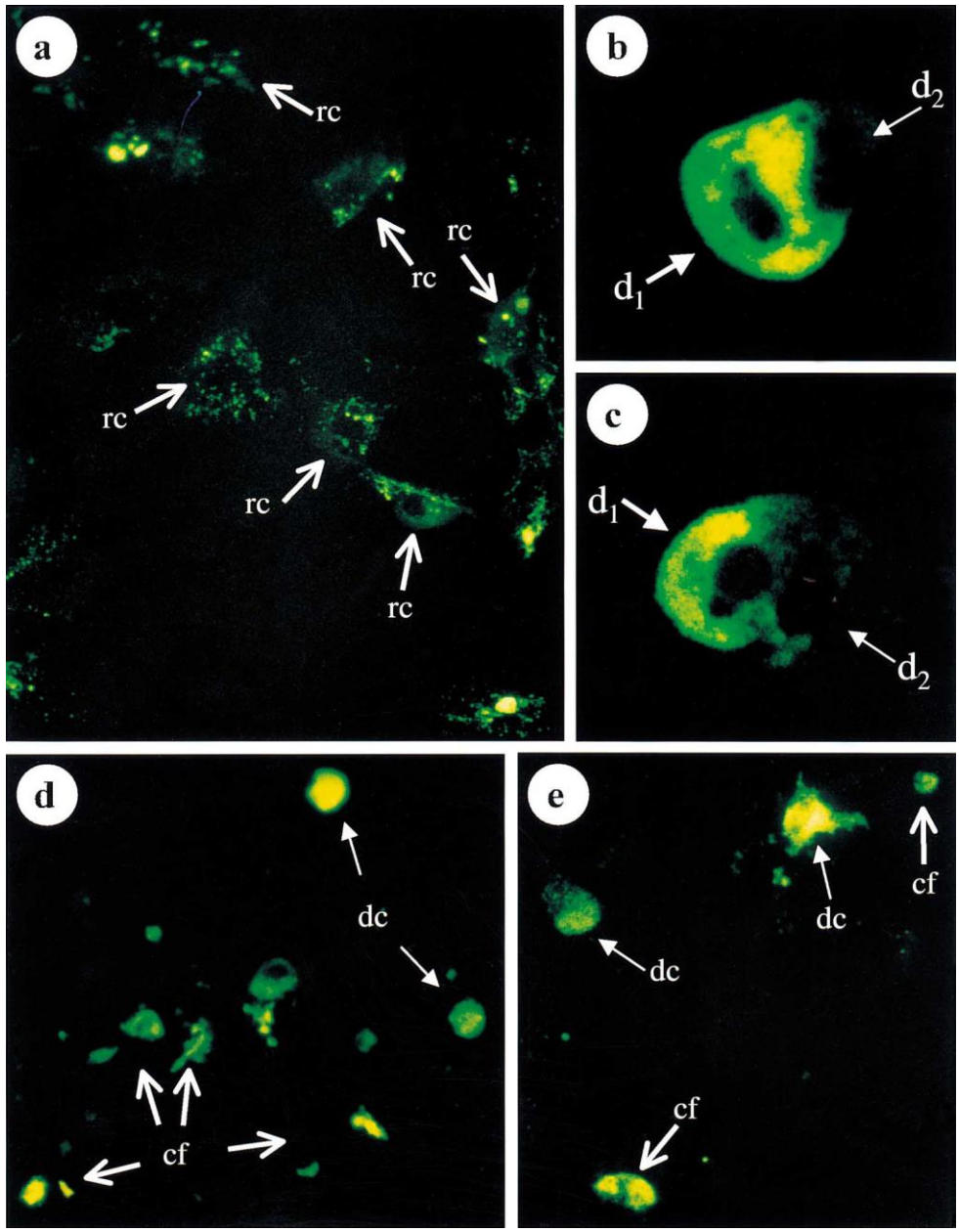

Fig. 1. Indirect immunofluorescence assay (IFA) of the surface basolateral side of live FLK-BLV cells incubated with 'grow to die' supernatant from antibody producing myelomas (GTD) and FITC-labeled rabbit *anti*-mouse immunoglobulins. Monoclonal antibodies used were: (a) SCF.I.11 (*anti*-$Pr66^{gag\text{-}pro}$), and (b, c, d, and e) PVF.II.25 (*anti*-$Pr72^{env}$). rc, resting cells (with mottled fluorescence); d_1 and d_2, daughter cells (showing the unequal staining); dc, dying cells; cf, cellular fragments.

was lined up around the interior of the cellular membrane. With double labeling, clusters of PVF.II.25 (*anti*-$Pr72^{env}$ mAb) and CEF.7 or SCF.I.54 (*anti*-gp51SU mAbs) were observed grouped together within the cell mem- brane while in the process of budding (Fig. 4d and e).

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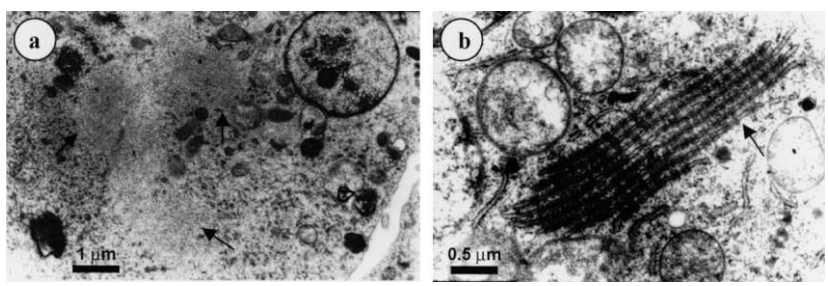

Fig. 2. Electron microscope images of cytoplasmic features of BLV-infected BLV-bat_2 cells: (a) greyish magma, identified by immunogold electron microscopy (Fig. 4f) as related to BLV protein synthesis (×10,000); and (b) intracytoplasmic annulate lamellae (×20,000).

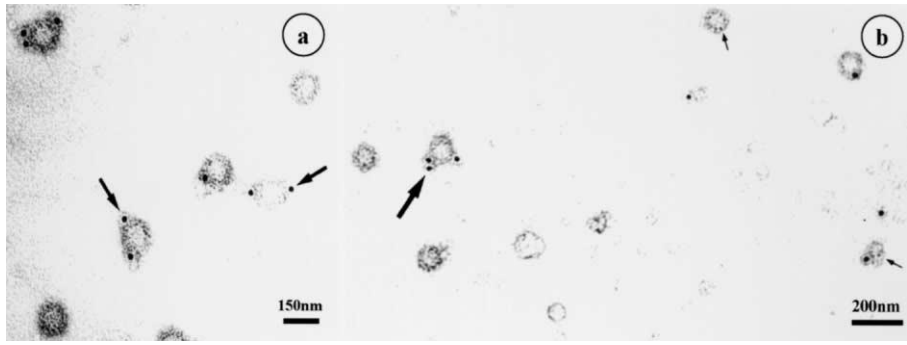

Fig. 3. Immunogold electron microscopy of free viral particles in the supernatant from BLV-bat$_2$ cultures using: (a) CEF.7 (*anti*-gp51SU), labeled with 5 nm gold particles (×47,000); and (b) SCF.I.54 (*anti*-gp51SU, labeled with 15 nm gold particles, bold arrow) and PVF.II.25 (*anti*-Pr72^{env}, labeled with 5 nm gold particles, thin arrows) (×55,000).

cell membrane (Rovnak et al., 1991), and with mAbs against the Gag protein p24CA, fluorescence was reported in the cytoplasm of BLV-infected cells (Rovnak et al., 1991).

Results of the present study also agree with previous observations (Stock and Ferrer, 1972; Calafat and Ressang, 1977; Chung et al., 1984; Dekegel, 1987) that cell fragments and debris contain abundant BLV proteins, since the greatest fluorescence when using mAb PVF.II.25, corresponding to Pr72^{env}, was seen in degenerated and fragmented cells.

Interestingly, results of this study showed that in dividing FLK-BLV cells Env proteins (or at least, derived from the Pr72^{env}) were not equally dis- tributed between the plasma membrane of the two daughter cells in the anaphase and telophase stages. Labeling of Gag-Pro proteins in dividing cells was not observed in this study. Our observation that antibodies detected Env protein in the basolateral surface of mitotic cells that segregate predominantly in only one daughter cell is truly interesting and could be further complemented with data from synchronizing cells.

As regards electron microscopy, the im- munogold labeled monoclonal antibodies (Llames et al., 2000) reacted both with free viral particles and with BLV-bat$_2$ cells. The mAbs reacting with free viral BLV particles included not only mAbs against gp51SU, but also those against the precursors of Env and Gag-Pro. The mAbs SCF.II.30 and SCF.II.40 were considered to be mAbs against Pr72^{env} since when using WB, they reacted with both gp51SU and gp30TM (Llames et al., 2000). Concerning the mAb against Gag-Pro precursors used, SCF.I.11, it was observed by WB that it recognized both the mature p24CA and its precursors (Llames et al., 2000). By immunoelectron microscopy, *anti*-Gag-Pro mAbs were ob- served attached to disrupted viral particles, in which it could be recognizing either exposed p24CA in particles which had already suffered maturation, or the precursor in immature parti- cles. This assumption is based on the fact that even purified virus may contain non-cleaved Gag-Pro precursors, aside from the expected structural proteins (Walker et al., 1987; Heeney et al., 1988; Radke et al., 1990; Domenech et al., 1998). However, utilizing different staining techniques, Pr66$^{gag\text{-}pro}$ had previously been found only in infected cells (Mamoun et al., 1983, 1984;Yoshinaka et al., 1986).

Type C-like particles assemble directly at the cell membrane with no visible cytoplasmic inter- mediate. After budding there may be an immature form with a large, open spherical core, which matures into a centrally located condensed form, with surface projections (Coffin, 1992). However, some authors observed the condensation of electron-dense material within the cytoplasm, resembling virus particles in the first stage of budding (Stock and Ferrer, 1972; Calafat and Ressang, 1977; proposed two paths for the morphogenesis of BLV. In the first path, the assembly and processing of the viral proteins take place in the budding site, releasing an immature particle, although the electron-dense nucleoid may be seen prior to budding in some cases. In the second path, the budding takes place in cytoplasmic vesicles and particles would be released in the cytoplasm, being shed from the cell by unknown mechanisms. One possible explanation is that the virus particles are freed from the cell as a consequence of cell degeneration, which

could explain why the viral particles outside the leukocytes are frequently seen together with cell debris, and why cellular debris was seen so in- tensely stained by immunofluorescence in our experiments. Nevertheless, these observations have not been confirmed by other authors, and during the current study BLV was not observed in vesicles.

Immunogold labeling studies reported here showed that BLV polyproteins, in particular $Pr72^{env}$ and $Pr66^{gag\text{-}pro}$, were located intracellularly in the cell cytoplasm in BLV-bat_2. Stanislawski (1983) also observed the precursor protein 76 ($Pr76^{gag}$) of avian leukosarcoma virus on ribosomes located initially near the nucleus, and later in the periphery of the cell, using the immunoperoxidase technique. However, Dekegel (1987) was able to detect BLV p24CA or gp51SU using specific mAbs only scattered irregularly in the proximity of the cell membrane but not intracellular. Even though the mAbs used had low affinity and the antigens were relatively inaccessible, he favored the idea that the integration of the viral proteins and their processing take place at the very site of virion synthesis, the budding site.

Results presented in this paper show viral protein presence in areas of grayish magma and annulate lamellae in the cytoplasm, an observation previously reviewed by Stock and Ferrer (1972). The intracytoplasmatic annulate lamellae are structures seen in certain cell types during viral infections, associated with the nuclear envelope and with the rough endoplasmic reticulum, but the function is not clear (Doane and Ander- son, 1987). These alterations could be related to a higher protein synthesis, probably a consequence of viral replication. During this study the presence of BLV Env proteins (identified by mAb PVF.II.25, *anti*-$Pr72^{env}$) in the mitochondria of BLV-bat_2 was observed, a fact previously described by Manolescu et al. (1977) for BLV, and by Kara et al. (1977) for Rous sarcoma virus and Rauscher murine leukemia virus. Ciminale et al. (1999) also found HTLV-1 proteins in mitochondria, specifically $p13^{II}$, a protein codified in the x-II ORF of the viral genome,and suggest it might be related to apoptosis. Our observation about the presence of Env precursors in mitochondria suggests that this organelle may play an essential role in the protein folding and assembly process of BLV. However, this assumption has to be further elucidated in detail.

Immunofluorescence assay (IFA) and Immunogold-labeling (single or double) of viral proteins using specific mAbs proved to be useful tools in studying BLV-infected cells. PVF.II.25 (*anti*-$Pr72^{env}$) mAb, among others, seems to be a useful BLV-specific surface and intracellular marker as shown in our preliminary studies which could be utilized further in substantial investigations and future in depth studies on the kinetics of virus entry, intracellular trafficking, protein folding and virus excretion of BLV-infected cells.

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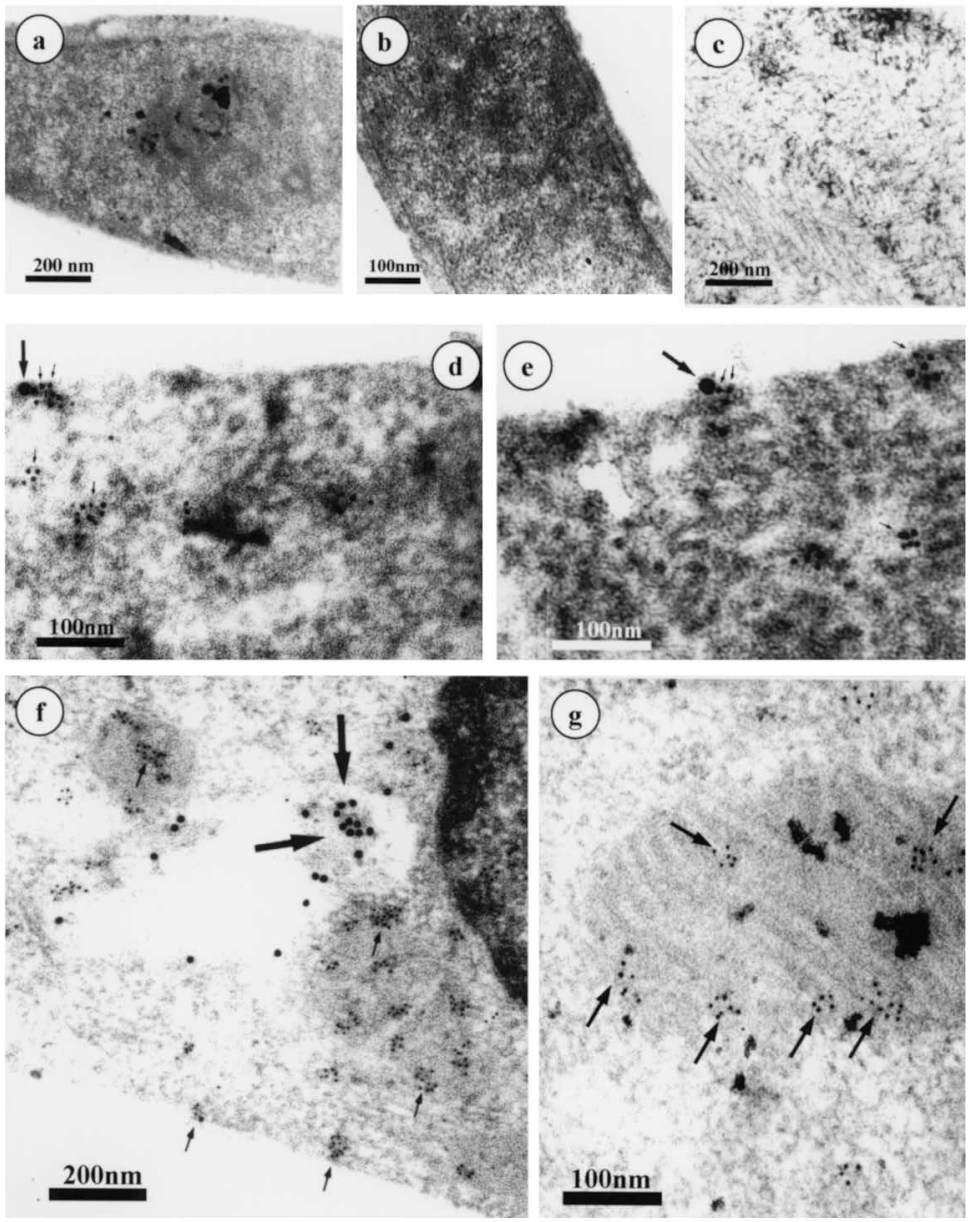

Fig. 4.

Fig. 4. Immunogold electron microscopy of BLV-bat$_2$ cell line persistently infected by BLV using different monoclonal antibodies (mAbs) to recognize the viral proteins. PVF.II.25 (*anti*-Pr72^{env}, mAb of the IgM class, labeled with 5 nm gold particles, thin arrows) was used in d, e, and f. A second mAb was used in each of these three images, but all of these were of the IgG class, and labeled with 15 nm gold particles (bold arrows): CEF.7 (*anti*-gp51SU, d), SCF.II.54 (*anti*-gp51SU, e), and SCF.I.11 (*anti*-Pr66$^{gag\text{-}pro}$, f). Incipient budding may be observed in d and e, while viral proteins can be seen dispersed in clusters or groups in the cytoplasm in f. Letter g corresponds to reaction of mitochondrial crests with mAb PVF.II.25. Letters a, b, and c correspond to negative controls using PBS, undiluted supernatant from myeloma cells allowed to grow for 1 – 2 weeks without medium replacement (GTD), and a mAb against *Staphylococcus* enterotoxin type B, respectively. (The magnifications of the pictures are: (a) 60,000; (b) 50,000; (c) 60,000; (d) 150,000; (e) 120,000; (f) 85,000; and (g) 180,000.

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