**B95a, a marmoset lymphoblastoid cell line, as a sensitive host for rinderpest virus**

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We reported earlier that B95a, an Epstein–Barr virus-transformed marmoset B lymphoblastoid cell line, is more susceptible to infection with measles virus than other cells. The cell line also was found to be susceptible to infection with the lapinized Nakamura III (L) strain of rinderpest virus and various strains derived from it. The B95a cell line was therefore the only host cell system available for the propagation and quantification of the L strain. In contrast to the adaptation of the L strain to Vero cells which results in a diminution of virulence in rabbits, the propagation of the virus in B95a cells preserved the virulence and some other properties in rabbits. Furthermore, when Vero cell-adapted variants of the L strain with diminished virulence were serially passaged in B95a cells, virulence in rabbits was gradually regained.

**Introduction**

Rinderpest virus (RPV), which causes an economically important cattle disease, belongs to the Morbillivirus genus together with measles, canine distemper and peste des petit ruminants viruses. The lapinized Nakamura III strain (L strain), an attenuated vaccine, was derived from a wild RPV strain through innumerable passages in rabbits (Nakamura & Miyamoto, 1953). The L strain, although avirulent in cattle, remains virulent in rabbits, causing extensive lymphoid necrosis and a severe, often fatal illness (Yamanouchi et al., 1974). No cell cultures were available for propagation and titration of the L strain. Adaptation of the L strain either first to chicken embryos and then to Vero cells (Shishido et al., 1976) or directly to Vero cells (Ishii et al., 1986) resulted in the ability of the virus to grow in Vero cells but also in a markedly diminished virulence in rabbits.

We have recently reported that the Epstein–Barr virus (EBV)-transformed marmoset B lymphoblastoid B95-8 and its derived B95a cell lines are uniquely susceptible to measles virus (Kobune et al., 1990). This communication will show that the B95a cell line is also susceptible to the L strain and other RPV strains derived from it and that in contrast to the passage in Vero cells, the passage of the L strain in B95a cells not only preserved its virulence in rabbits but restored virulence to variants rendered avirulent through Vero cell passage.

**Methods**

*Cells.* The B95a cell line was derived from B95-8, an EBV-transformed marmoset B lymphoblastoid cell line (Miller et al., 1972) and was adapted to growth by adherence to the vessel surface (Kobune et al., 1990). The cells were propagated in RPMI 1640 (Nissui Pharmaceutical) supplemented with 10% foetal bovine serum under a 5% CO₂ atmosphere. Vero cells were propagated in Eagle's MEM supplemented with 10% calf serum. The maintenance medium for B95a cells was RPMI 1640 supplemented with 5% foetal bovine serum and MEM supplemented with 2% calf serum.

*Virus strains.* The L strain of RPV, which had been passaged in rabbits, was cloned by three consecutive passages in B95a cells at limiting dilutions. The infected culture fluid from the third passage level was inoculated into rabbits. The 10⁻⁶ homogenate of mesenteric lymph nodes collected on day 4 was used as the stock virus for the L strain. The virus was adapted to Vero cells as follows. Peripheral blood mononuclear cells from a rabbit infected with the stock virus were stimulated with 5 μg/ml of concanavalin A for 48 h and about 5 × 10⁵ cells were mixed with 10⁶ Vero cells. The mixture was incubated at 37°C under a 5% CO₂ atmosphere. On day 4, when Vero cells exhibited c.p.e., the culture fluid was collected, diluted 1:100 and inoculated onto a fresh Vero cell culture for the second passage. Subsequent passages in Vero cells were done in a similar way for 32 passages. The virus from the 32nd Vero cell passage was designated LV32. LV32 was then passed serially back into B95a cells for 5, 10 and 15 passage levels and the virus strains obtained were designated LV32B5, LV32B10 and LV32B15, respectively. An additional series of passages of the stock virus were done in B95a cells until the 32nd passage level, when the virus strain was designated LB32. All passages were done at a virus dilution of 1/100 that of the preceding passage level.

*Infectivity titration.* Dilution endpoint assays were done in either B95a or Vero cell monolayers formed in 24-well cluster plates (well
diameter of 16 mm). A volume (0.1 ml) of 10-fold serially diluted sample was adsorbed to each of four wells for 30 min at 37 °C. Cells were overlaid with the maintenance medium and incubated under a 5% CO2 atmosphere at 37 °C for 7 days for B95a cells and 10 days for Vero cells under daily observation for the appearance of c.p.e. The infectivity was expressed as TCIDso. Plaque assays were performed in monolayer cultures of B95a cells formed in six-well cluster plates (Costar; well diameter of 35 mm). A volume (0.1 ml) of 10-fold serially diluted sample was adsorbed to each of two wells for 30 min at 37 °C. Cells were overlaid with 3 ml of RPMI 1640 containing 2% foetal bovine serum and 0.6% agarose and incubated under a 5% CO2 atmosphere at 37 °C. One ml of the secondary overlay containing 0.012% neutral red was added on day 3. Plaques were counted on day 5 or 6. Infectivity was expressed as p.f.u. Infectivity was also determined by the febrile response in rabbits as described below and expressed as ID50.

Monoclonal antibodies (MAbs). The following MAbs prepared with the Vero cell-adapted L strain were used. S-1 and 1d were directed to the HA protein; H21, O32, R23 and 2-1 were directed to the F protein; M22, K11, P31, G11, D12, B11, and Y1 were directed to the NP protein; and 2a and 7-1 were directed to the F protein (Sugiyama et al., 1989). MAbs A27, A42 and A184 directed to measles virus M protein were also employed (Sato et al., 1985).

Immunofluorescence. B95a cells were infected with various strains of RPV. When c.p.e. developed, cells were fixed with acetone and reacted with fourfold serially diluted ascitic fluids containing MAbs. Fluorescein-conjugated rabbit anti-mouse IgG (Cappel Laboratories) was used as the secondary antibody.

Animal experiments. Male Japanese White rabbits weighing 2.7 to 3.0 kg were used. For infectivity titration, a 1 ml volume of 10-fold serially diluted sample was intravenously inoculated into each of four rabbits. The rectal temperature was measured daily for 7 days. A fever exceeding 40 °C was regarded as a sign of infection. Virus growth in rabbits was studied after intravenous inoculation of 1000 TCID50 (determined in B95a cells) of the virus to be tested. Two to four rabbits were killed at various times after infection up to day 7 and the infectious virus content in mesenteric lymph nodes from individual rabbits was determined in B95a cells. Portions of lymphoid organs collected on day 4 were fixed in 10% formalin in phosphate-buffered saline and processed for histological examination. Sections were stained with haematoxylin and eosin.

Results

Growth characteristics of host cell-dependent variants in cell cultures

As the L strain of RPV does not grow well in the cell cultures so far tested, the propagation and quantification of the virus had to be done by infection of rabbits. We showed recently that the B95a cell line is more susceptible to measles virus than the Vero cells commonly used for its propagation (Kobune et al., 1990). We therefore tested the susceptibility of B95a cells to RPV. Fig. 1 shows the multicycle growth of the L strain in B95a and Vero cells. The virus grew very slowly and to a low titre in Vero cells without causing recognizable c.p.e. In contrast, it grew rapidly in B95a cells reaching a plateau by 72 h. C.p.e., characterized by syncytium formation, appeared 24 h after infection and the entire cell sheet came off within a week. Previous studies showed that the L strain is easily adapted to Vero cells by serial passage (Shishido et al., 1976; Ishii et al., 1986). The L strain was subjected to serial passage in Vero and B95a cells in parallel as described in Methods. After 32 passages in Vero cells, the virus (LV32) grew more efficiently in Vero cells than the original L strain and caused distinct c.p.e., whereas the virus passed in B95a cells (LB32) was as incapable of efficient growth in Vero cells as the original L strain (Table 1). When LV32 was passaged in B95a cells, the growth capability in Vero cells gradually diminished after 15 passages towards the level of the original L strain. The L strain and all strains derived from it grew equally well in B95a cells irrespective of whether they had been adapted to Vero cells.

Another feature that changed with passage in different host cells was the size of syncytia formed in B95a cells. The L and LB32 strains formed syncytia approximately 100 μm in diameter containing about 1000 nuclei by day 2, but those of LV32 were approximately 30 μm in diameter and contained about 100 nuclei (Fig. 2a).

Table 1. Infectivity titration of various strains of RPV in rabbits, Vero cells and B95a cells

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Rabbit*</th>
<th>Vero</th>
<th>B95a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ID50/ml</td>
<td>TCID50/ml</td>
<td>TCID50/ml</td>
</tr>
<tr>
<td>L</td>
<td>3.5</td>
<td>&lt;1.0</td>
<td>4.5</td>
</tr>
<tr>
<td>LV11</td>
<td>1.75</td>
<td>2.5</td>
<td>4.0</td>
</tr>
<tr>
<td>LV32</td>
<td>&lt;1.0</td>
<td>5.5</td>
<td>5.8</td>
</tr>
<tr>
<td>LB32</td>
<td>4.8</td>
<td>&lt;1.0</td>
<td>5.5</td>
</tr>
<tr>
<td>LV32B15</td>
<td>4.0</td>
<td>2.75</td>
<td>5.75</td>
</tr>
</tbody>
</table>

* A fever exceeding 40 °C was taken as the sign of infection.
† NT, Not tested.
Fig. 2. Syncytia formed by RPV-infected B95a cells. Cultures were fixed 48 h after infection with (a) LV32 and (b) LV32B15 and stained with haematoxylin and eosin. Bar markers represent 50 μm.
Passage of LV32 in B95a cells increased syncytium size and LV32B15 formed syncytia of the same size as the original L and LB32 strains (Fig. 2b). In spite of a marked variation in the size of syncytia, the size and morphology of plaques did not differ appreciably between viruses with different passage histories (data not shown), nor was there a noticeable difference between them in the electrophoretic pattern of five major virus structural proteins, i.e. the haemagglutinin protein, phosphoprotein, nucleoprotein and the fusion and matrix proteins, on an SDS–polyacrylamide gel (data not shown).

**Virulence in rabbits**

Febrile response is a prominent and reproducible feature in rabbits of infection with the L strain (Yamanouchi et al., 1974; Kurosawa et al., 1987). It develops 48 to 72 h after infection and lasts for 48 to 72 h (Fig. 3a). Due to the lack of suitable cell cultures permissive to the L strain, febrile response has been used as an indicator of infection in the quantification of the infectivity of the strain (Table 1). The virus adapted to Vero cells (LV32) no longer caused febrile response, and LB32 was similar to the progenitor L strain in this respect (Fig. 3).

Clinical manifestations and the outcome of infection are a reflection of virus growth in, and the resulting damage of lymphoid tissues, a major target of the RPV infection in rabbits (Yamanouchi et al., 1974). The virus content in the mesenteric lymph nodes from rabbits infected with the L strain is shown in Fig. 4. Virus growth reached its peak on day 4. The peak virus titre of LV32 was more than 1000-fold lower than those of the L and LB32 strains. Passage in B95a cells restored virulence to the attenuated LV32. Gradual acquisition of virulence during its readaptation is shown by a stepwise increment in virus contents in the mesenteric lymph nodes (Fig. 4). After 15 passages (LV32B15), the virulence had returned to the original level in lymphoid tissues as well as in febrile response (Fig. 3 and 4). Histological findings paralleled the clinical and virological features. Fig. 5 shows Peyer's patches from rabbits infected with the LV32 and LV32B15 strains. A mild to moderate depletion of lymphocytes from follicles (B cell area) is seen in the former but there are no necrotic lesions; extensive necrosis is evident in both T and B cell areas of the latter.

**Antigenic characterization**

Reactivity of the L, LV32, LB32 and LV32B20 strains with MAbs was examined by indirect immunofluorescence. B95a cells equally permissive to all RPV strains were used as host cells. The RPV MAbs employed were reactive with all of two, four and five antigenic sites defined on the HA, P and NP proteins, respectively (Sugiyama et al., 1989). The MAbs directed to the M protein of measles virus reacted with three out of five antigenic sites (Sato et al., 1985). Cells infected with the four RPV strains were stained with equal intensity by all the MAbs. No antigenic difference was noticed, therefore, among the four RPV strains. It was reported earlier that antigenic changes in the P, NP, F and M proteins.
occurred during adaptation of the L strain to Vero cells when examined with MAbs against measles virus (Ishii et al., 1987). Unfortunately, because of the unavailability of the same MAbs, it was not possible to confirm this finding.

Discussion

Thus far, the study of RPV has been hampered by the lack of suitable hosts. No susceptible cell cultures have been available for the progenitor L strain which is
virulent in rabbits, whereas cell culture-adapted variants of the L strain were incapable of efficient growth and were avirulent in rabbits, as shown before (Shishido et al., 1976; Ishii et al., 1986) and confirmed in this study. The use of B95a cells reported here is expected to facilitate the study of RPV, particularly pathogenetic and pathophysiological aspects of RPV infection.

The findings of this study can be explained by postulating the presence of two distinct subpopulations of RPV in the L strain. One is a subpopulation of the virus which grows well in B95a but very poorly in Vero cells, forms large syncytia in B95a cells and is highly virulent in rabbits. The second subpopulation of the virus grows in B95a and Vero cells equally well and forms small syncytia in B95a cells, grows less actively in rabbit lymphoid organs, and accordingly is less virulent. The former subpopulation becomes dominant over the second when the virus is propagated in rabbits and B95a cells, but the latter subpopulation outgrows the former during propagation in Vero cells. Neither of the two subpopulations is completely eliminated however, because passage in the opposite host gradually restored the dominance of the other subpopulation. Virus stocks of either subpopulation may contain a minority of virions of the other subpopulation. On the other hand, the fact that the virus of the second phenotype (Vero-adapted) emerged readily from a clonal stock of the L strain of the first phenotype suggests a ready interconversion between the two phenotypes, presumably as a result of a relatively high mutation rate. The two subpopulations of RPV are unlikely to be antigenically very different from each other.

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References


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