Adaptation of the Lapinized Rinderpest Virus to in vitro Growth and Attenuation of Its Virulence in Rabbits

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SUMMARY

A lapinized rinderpest virus, the L strain, which is virulent in rabbits and had been grown only in rabbits, was adapted to grow in Vero cells by the fusion of Vero cells with virus-infected rabbit spleen cells in the presence of polyethylene glycol, and subsequently passaged in Vero cells by co-culture technique. After several passages, free virus was produced at high titre. The Vero cell-adapted virus acquired the ability to infect several cell lines which were non-permissive to the unadapted virus. Analysis of virus proteins by immunofluorescence using monoclonal antibodies revealed that marked changes occurred in F, P, NP and M proteins by passage in Vero cells. In parallel to the adaptation to cell culture in vitro, the virulence of the virus measured in terms of clinical signs and histological lesions in the lymphoid tissues decreased in its severity whereas its immunosuppressive capacity was maintained unaltered. Thus, rinderpest virus with different degrees of virulence is now available for study in vitro.

INTRODUCTION

Rinderpest virus, which is classified in the genus Morbillivirus of the family Paramyxoviridae, causes acute infection in cattle with high morbidity and mortality. The lapinized Nakamura III strain (L strain) (Nakamura & Miyamoto, 1953) was shown to have high virulence in rabbits causing severe clinical signs, lymphoid necrosis, immunosuppression and autoimmunity (Fukuda & Yamanouchi, 1976; Fukusho & Nakamura, 1940; Kobune et al., 1976; Yamanouchi et al., 1974a, b). On the other hand, the LA strain which had been attenuated by passage of the L strain in chicken embryos and further adapted to grow in a Vero cell culture (Shishido et al., 1976) does not show virulence in rabbits. These two strains, one virulent and one avirulent, provide useful models for the investigation of the virological properties of rinderpest virus in vivo and in vitro. However, comparative studies of the L and LA strains have been limited to a system in vivo, because of the lack of a suitable cell culture system for the L strain.

In the present study, the in vitro Vero cell system was developed for growth of the L strain and the virulence of the L strain was shown to be decreased by passage in vitro.

METHODS

Virus. Details of the preparation of stock virus have been described previously (Yamanouchi et al., 1974a). Briefly, a stock of the L strain of rinderpest virus was prepared as a 10% homogenate of the mesenteric lymph nodes of the virus-infected rabbits and had a titre of 10³-⁴ ID₅₀/ml when titrated in rabbits. The LA strain was grown in a Vero cell culture and its stock had a titre of 10⁶-⁷ TCID₅₀/ml.

Cell culture. HEL and KB cell lines were maintained in DM-160 culture medium (Kyokuto Pharmaceutical Co. Ltd., Tokyo, Japan) supplemented with 5% foetal calf serum (FCS) and kanamycin. Vero, CV-1, RK13, SIRC and BHK21 cells were cultured in Eagle’s MEM containing 10% calf serum and tryptose phosphate broth (TPB).

Rabbits. Male New Zealand White rabbits with an average body weight of 2.5 kg were purchased from a commercial breeder.
Infectivity titration. Vero, CV-1, RK13, SIRC, BHK21, HEL and KB cells were cultured in 24-well plastic dishes (Falcon 4047). One-tenth ml of serially 10-fold diluted virus was inoculated into each well. After virus adsorption at 37 °C for 1 h in a CO₂ incubator, 1 ml 0.9% agarose medium was added and virus-induced plaques were microscopically counted 7 days after inoculation.

Recovery of virus in cell cultures from L strain-infected rabbits. One ml of the L strain virus stock was intravenously inoculated into rabbits. Five days later, the spleen was removed, minced in serum-free Eagle’s MEM to prepare a single-cell suspension and used for virus recovery by the following methods. (i) Virus adsorption method: a splenic lymphocyte suspension (1 × 10⁸ cells/0.5 ml) was disrupted by 2 cycles of sonication for 5 s (Micro ultrasonic cell disruptor, Kontes, K-881440), freeze–thawed once at −80 °C and centrifuged at 3000 r.p.m. for 10 min. The supernatant was inoculated onto a monolayer of Vero cells (1 × 10⁶ cells). After adsorption at 37 °C for 1 h, the medium was changed and the cell culture was maintained in a CO₂ incubator. (ii) Co-culture method: splenic lymphocytes (1 × 10⁶ cells) and Vero cells (5 × 10⁶ cells) were mixed and cultured in a 250 ml culture glass bottle as above. (iii) Cell fusion method: a mixture of splenic lymphocytes (1 × 10⁶ cells) and Vero cells (5 × 10⁶ cells) was prepared in Eagle’s MEM and centrifuged in a 50 ml tube (Falcon 2070) at low speed. The supernatant was removed and 1 ml 42.5% (w/v) polyethylene glycol 1500 (PEG; Wako Pure Chemical Co., Osaka, Japan) in serum-free Eagle’s MEM was added slowly. The cell suspension was gently mixed for 1 min, and centrifuged at 2000 r.p.m. for 1 min. The supernatant was removed and 10 ml serum-free Eagle’s MEM was added slowly over a period of 5 min. The cells were centrifuged and resuspended in 10 ml Eagle’s MEM containing 10% FCS and 10% TPB. The cell suspension was placed in a 250 ml culture glass bottle and incubated at 37 °C.

Immunofluorescence (IF) test. Production of virus protein in Vero cells and spleen cells of rabbits infected with the L strain of rinderpest virus was examined by indirect IF methods as described elsewhere (Sakaguchi et al., 1984). Monoclonal antibodies against measles virus proteins, i.e. three clones of H, two clones of NP, one clone of F and two clones of M, which were shown to cross-react with structural proteins of rinderpest virus, were supplied by Dr K. Takeda, Department of Neurological Virus Disease, Institute for Virus Research, Kyoto University, and used as the first serum. Anti-mouse IgG goat serum conjugated with fluorescein isothiocyanate was used as the second serum.

Virus inoculation in rabbits. Rabbits were inoculated intravenously with 1 ml of the virus, and observed for clinical signs such as diarrhoea and fever.

Histological examination. Various lymphoid tissues were obtained 5 days after inoculation, fixed in 10% formalin, dehydrated, embedded in paraffin and thinly sectioned. The sections were stained by haematoxylin and eosin.

Estimation of immune capacity of rabbits. The immune capacity of virus-infected rabbits was examined by testing the blastogenic response of peripheral blood lymphocytes (2 × 10⁶/ml) in the presence of 1 μg/ml phytohaemagglutinin (PHA-P; Difco). Incorporation of [³H]thymidine was measured using a Beckman liquid scintillation counter. Details of the procedure have been described previously (Taniguchi et al., 1982).

RESULTS

Adaptation of the L strain to Vero cells

Infection of Vero cells with the spleen cells of L strain rinderpest virus-infected rabbits was attempted by the three different approaches described in Methods. The cell fusion method resulted in the most efficient recovery of virus, approximately 100 infectious centres being obtained on Vero cells. Small numbers of infectious centres were obtained by the co-culture technique; infection by free virus was unsuccessful. Five to 7 days after cell fusion, when a monolayer of cells was formed, the culture was passed by treatment with 0.05% trypsin containing 0.02% EDTA. After passage, the culture showed marked c.p.e. on the next day. Since free virus production was not observed, subsequent passages of the virus were carried out every 3 days by co-cultivation with normal Vero cells.

Free virus was not produced until the third passage. At the fourth passage, free virus was found at a titre of 10⁻⁵ TCID₅₀/ml and its titre exponentially increased in subsequent passages. At the 11th passage, virus yield reached a maximum titre of 10²⁻° TCID₅₀/ml.

In the early passages in Vero cells, the virus induced small and strand-form c.p.e. After a few more passages, fusion-type c.p.e. became predominant and tended to cover wide areas of the cell monolayer.

In vitro growth characteristics of the Vero cell-adapted L strain

The growth characteristics of the L strain at the 11th passage in Vero cells were compared with those of the LA strain. The growth curve of the Vero cell-adapted L strain was similar to
Adaptation of the lapinized rinderpest virus

Fig. 1. Immunofluorescent analysis by P-1 antibody in Vero cells infected with L strain virus at various in vitro passage levels. (a) 3rd passage. No specific fluorescence in giant-cell type c.p.e. (b) 5th passage. Specific fluorescence is seen in a localized area. (c) 11th passage. Specific fluorescence is widely spread.

Table 1. Production of virus proteins by infection with unadapted L strain, Vero cell-adapted L strain and LA strain

<table>
<thead>
<tr>
<th>Cells infected</th>
<th>Virus strain</th>
<th>In vitro passage level of virus</th>
<th>Reaction with monoclonal antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit spleen</td>
<td>Unadapted L strain</td>
<td>0</td>
<td>H-1 H-2 H-3 P-1 NP-1 NP-2 F-1 F-2 M-1 M-2</td>
</tr>
<tr>
<td>Vero</td>
<td>Vero cell-adapted L strain</td>
<td>1</td>
<td>- - - + + + + - -</td>
</tr>
<tr>
<td>Vero</td>
<td>Vero cell-adapted L strain</td>
<td>3</td>
<td>- - - + + + + - -</td>
</tr>
<tr>
<td>Vero</td>
<td>Vero cell-adapted L strain</td>
<td>5</td>
<td>- - - + + + + - -</td>
</tr>
<tr>
<td>Vero</td>
<td>Vero cell-adapted L strain</td>
<td>7</td>
<td>- - - + + + + - -</td>
</tr>
<tr>
<td>Vero</td>
<td>LA strain</td>
<td>9</td>
<td>- - - + + + + - +</td>
</tr>
<tr>
<td>Vero</td>
<td>LA strain</td>
<td>11</td>
<td>- - - + + + + - +</td>
</tr>
<tr>
<td>Vero</td>
<td>LA strain</td>
<td>30</td>
<td>- - - + + + + - +</td>
</tr>
</tbody>
</table>

that of the LA strain; in both virus infections, the maximum virus yield with an infectivity titre of $10^{5.5}$ TCID$_{50}$/ml was observed 72 h post inoculation and c.p.e. appeared at 56 h spreading over nearly 100% of the cell monolayer by 120 h.

The unadapted and Vero cell-adapted L strains and the LA strain were compared for their capacity to grow in various cell lines including HEL, KB, Vero, CV-1, RK13, SIRC and BHK21 cells. The unadapted L strain failed to grow in any of the cells, whereas the Vero cell-adapted L strain had acquired the capacity to grow in several cell types by the 11th passage, the maximum titres being $10^{4.5}$ TCID$_{50}$ in BHK21 cells, $10^{2.7}$ TCID$_{50}$ in CV-1 cells and $10^{1.7}$ TCID$_{50}$ in RK13 cells. The LA strain was able to grow in all the cells tested except KB cells.

Production of virus proteins in Vero cells infected with the L strain at different passage levels in vitro and with the LA strain was examined by IF using monoclonal antibodies. As summarized in Table 1, virus proteins reacting with P-1, NP-1 and NP-2 antibodies were demonstrated in the spleen cells of rabbits infected with the unadapted L strain. Virus proteins reacting with P-1 and NP-2 antibodies disappeared by the 1st and 3rd passages in Vero cells, while virus proteins reacting with F-1 and F-2 antibodies appeared. After further passages, virus protein reacting with M-2 antibody appeared in addition to the reappearance of virus proteins reacting with P-1 and NP-2 antibodies (Fig. 1). The LA strain reacted differently with H-1, NP-2 and M-2 antibodies compared with the highly adapted L strain. Thus, changes were evident in expression of antigenic epitopes in F, P, NP and M proteins by adaptation of the L strain to Vero cells.
Fig. 2. Lymphoid necrosis in mesenteric lymph node caused by L strain virus at various passage levels in Vero cells. (a) Unadapted virus; (b) 3rd passage; (c) 11th passage; (d) 30th passage.

Table 2. *Virulence of the unadapted and Vero cell-adapted L strains and of the LA strain in rabbits*

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Clinical signs</th>
<th>Blasting response of lymphocytes to PHA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diarrhoea</td>
<td>Fever</td>
</tr>
<tr>
<td>Unadapted L strain</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Vero cell-adapted L-strain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3rd passage</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>11th passage</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>30th passage</td>
<td>-</td>
<td>±</td>
</tr>
<tr>
<td>LA strain</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Uninfected control</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* C.p.m. in culture with PHA/c.p.m. in culture without PHA.

*Virulence of the Vero cell-adapted L strain in rabbits*

Rabbits were inoculated intravenously with the unadapted L strain, the Vero cell-adapted virus at the 3rd, 11th and 30th passages, and the LA strain. The virulence of these viruses was examined in terms of clinical signs, histological lesions and degree of immunosuppression. The results are summarized in Table 2. The L strain at the 3rd passage showed the same degree of virulence as the unadapted virus, causing diarrhoea, fever, severe histological lesions of lymphoid necrosis and marked immunosuppression. In contrast, the LA strain showed attenuated characteristics except for mild immunosuppression. At increased passage levels, the virulence of the L strain tended to decrease. Diarrhoea was not found at the 11th passage. Lymphoid necrosis, which is the typical lesion induced by the unadapted L strain, decreased in its severity with increasing passage, and became undetectable at the 30th passage (Fig. 2). The immunosuppressive capacity of the L strain was not altered by the 30th passage level.
DISCUSSION

The L strain of rinderpest virus, which is virulent in rabbits and has been grown only in rabbits, was successfully adapted to grow in Vero cell cultures by fusing spleen cells of virus-infected rabbits with normal Vero cells. Although the mechanisms for infection of Vero cells by fusion with virus-infected cells have not been examined in this study, it is plausible that cell fusion bypassed the adsorption and penetration steps, resulting in direct introduction of the virus genome into a cytoplasmic microenvironment which is apparently permissive for growth of the L strain. The similar phenomenon of virus infection of non-permissive cells being facilitated by PEG is well known for retroviruses (Rohde et al., 1978).

It is worth mentioning that the virus was cell-associated at early passages in Vero cells. A similar situation is seen in measles and subacute sclerosing panencephalitis viruses (Fraser & Martin, 1978; Katz, 1977), both of which are morbilliviruses like rinderpest virus. The L and LA strains have served as good models for the comparative study of viral pathogenesis. The former causes acute infection of rabbits with histological lesions. Marked suppression of immune capacity as well as development of autoantibodies are also induced. The LA strain is avirulent and does not show such pathogenic effects as the L strain except for mild immunosuppression (Fukuda & Yamanouchi, 1981). Therefore, it was of interest to see whether the L strain adapted to cell culture still maintained the original virulence. The Vero cell-adapted L strain at early passages maintained virulence in rabbits to the same degree as that of the unadapted virus. At increasing passage levels, the virus gradually lost virulence and finally became as avirulent as the LA strain except for the conservation of immunosuppressive capacity, even up to the 30th passage. Thus, attenuation seemed to occur in parallel with the adaptation of the virus to Vero cells.

In order to obtain an understanding of the attenuation and adaptation processes during passages in Vero cells, virus proteins were analysed by immunofluorescence using monoclonal antibodies. After a single passage in Vero cells, marked changes occurred in P, NP and F proteins. By the 5th passage in Vero cells, further changes were observed in P, NP and M proteins. At this passage level, production of free virus was noted. Involvement of core proteins in the adaptation process has not been reported before. Whether the changes in epitopes of core proteins and production of free virus are correlated will be the subject of future studies. The proteolytic cleavage of F protein by host enzyme has been shown to determine growth capacity of paramyxoviruses (Homma & Ohuchi, 1973; Scheid & Choppin, 1974; Nagai et al., 1970; Nagai & Klenk, 1977). In this respect, the appearance of epitopes of F protein in the Vero cell-adapted L strain might be related to successful growth in vitro. The acquisition of a capacity to grow in several cell lines in addition to Vero cells suggests that functional or structural changes in envelope proteins also occurred during adaptation to Vero cells.

Infection of rabbits with the L strain has been used as a model for the study of viral pathogenesis, including such phenomena as host defence mechanisms, virus-induced immunosuppression and virus-induced autoimmunity (Yamanouchi et al., 1974a, b; Fukuda & Yamanouchi, 1976). Viruses that can grow in the same in vitro system and show different degrees of virulence in rabbits have been made available by the present study. These viruses will be useful tools for the molecular analysis of virus virulence.

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REFERENCES


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