Characteristics of Parker's rat coronavirus (PRC) replicated in L-2 cells

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Summary. Parker's rat coronavirus (PRC) is a naturally-occurring viral infection of the laboratory rat. On the first passage, ATCC strain 8190 of PRC replicated in L-2 cells. Using the tenth passage of PRC in L-2 cells, the characteristics of the virus were compared with previous studies of sialodacryoadenitis virus (SDAV) replicated in L-2 cells. Based on light and immunofluorescence microscopic examination of control and inoculated cell cultures, PRC-associated CPE was frequently confined primarily to individual cells, and there were relatively few syncytial giant cells. Maximum titers were recovered at 36 h post inoculation (pi). Infectious virus was demonstrated at pH values ranging from 6.0 to 9.0 and a pH of 7.5 was determined to produce the highest titers of PRC. The optimum temperature for viral replication was 33 °C. Up to 15 passages of PRC in L-929 cells failed to produce detectable virus. However, after adaptation in L-2 cells (20th passage), PRC replicated to high titers in L-929 cells. Previously, in vitro studies of rat coronaviruses have been hampered by the lack of an identified continuous cell line to replicate these viruses in the laboratory. L-2 cells represent a readily-available continuous cell line that can support the replication of relatively high titers of PRC.

Introduction

Parker's rat coronavirus (PRC) and sialodacryoadenitis virus (SDAV) are two naturally-occurring coronaviruses in the laboratory rat that have been identified and partially characterized [1, 9]. In the current literature they are associated with different patterns of disease. PRC is considered to produce a disease primarily of the respiratory tract [2, 9], while SDAV infections are associated with lesions of the salivary and lacrimal glands [7], and respiratory tract [13]. However, the viruses are closely related antigenically [1]. Thus conventional serological techniques do not serve to differentiate between the different rat coronaviral strains. Serological surveys indicate that the incidence of colonies positive for rat coronaviruses in a conventional setting may be over 50% [3,

8]. In the past, the characterization of rat coronaviruses has been hampered by the difficulties in replicating virus other than in primary rat kidney cells [1, 9]. Continuous cell lines have now been identified that will support the growth of SDAV [6, 10], and PRC [5]. In this communication, we report the successful cultivation of PRC in L-2 and L-929 cells, and the results of our studies on the characterization of this coronavirus in L-2 cells.

Materials and methods

Cells

The L-2 cell line, a subline of L-929 [12], was acquired from Dr. V. L. Morris (University of Western Ontario, London, Ontario). L-929 (of mouse origin) were obtained from The American Type Culture Collection (Rockville, MD). Cells were grown in Eagle's minimal essential medium (Gibco/BRL Inc., Burlington, Ontario) containing 200 U/ml penicillin, 80 µg/ml streptomycin, 0.025 µg/ml of gentamycin supplemented with 5% fetal bovine serum. Cells were propagated on 100 × 15 mm Nunc polystyrene tissue culture dishes (Gibco/BRL Inc.) at 37 °C (unless otherwise stated) in a humidified atmosphere containing 5% CO_2.

Virus

Parker's rat coronavirus (ATCCVR-635, Strain 8190) was obtained from the American Type Culture Collection (Rockville, MD). The virus was thawed, and reconstituted with 10 ml of Eagle's MEM, then 1 ml was inoculated onto L-2 cells. Parker's rat coronavirus (PRC) was allowed to adsorb for 1 h, then monolayers were washed, and culture medium was replaced. Inoculated and control cultures were then incubated, and examined regularly for CPE.

Viral infection

Monolayers of L-2 and L-929 cells were grown until approximately 80% confluent, then inoculated with 1.0 ml of stock virus suspension or infected cell culture supernatant. Virus was allowed to adsorb for one hour, then monolayers were washed and the culture medium replaced. Mock infected and inoculated cell cultures were then incubated at 37 °C and 5% CO_2 for 48–72 h. Ten-fold serial dilutions of virus were made in cell culture medium and used to inoculate L-2 or L-929 cells. Replicate cultures were then fixed and examined by immunofluorescence microscopy as described below. The demonstration of viral antigen in 50% of inoculated cultures was interpreted to be the $TCID_{50}$.

In PRC-inoculated L-929 cells, serial passage was performed, using cell culture fluid from inoculated cultures collected at 48–72 h post inoculation. Fifteen passages were performed in this manner. In addition, L-929 cells were inoculated with culture fluid from the 14th and 20th passage of PRC replicated in L-2 cells. Inoculated and control cells were examined for CPE and viral antigen as described below.

Microscopy

For light microscopy, control and infected cell cultures were fixed in methanol, and stained by the Giemsa method. Tissues for immunofluorescence microscopy were fixed in methanol, and incubated with antiserum from SDAV-infected rats, and identified with fluorescein-labelled goat anti-rat IgG (Antibodies Incorporated, Davis, CA).

Kinetics of PRC replication

Using an incubation temperature of 37 °C, the time course for the production of infectious virus was determined. Replicate cell cultures were inoculated with approximately $10^{4.0}$ $TCID_{50}$ of the 10th pass of PRC in L-2 cells. Infected cells were collected at 12 h intervals for up to 84 h post inoculation. Viral titers at each time point were determined as described above.

Determination of optimum temperature and pH

For the evaluation of optimum pH for PRC replication in L-2 cells, following inoculation with virus, replicate cultures were maintained in culture medium adjusted with sterile sodium bicarbonate to pH 6.0, 7.0, 7.5, 8.0, and 9.0. For the determination of optimum temperature for viral replication, replicate cultures of L-2 cells inoculated with the 10th pass of PRC were incubated at 31, 33, 35, 37, and 39 °C for 48–72 h.

Results

Replication in L-2 or L-929 cells

Infectious virus and viral antigen were observed on the first passage of stock virus in L-2 cells. In virus-infected cells, viral antigen and early evidence of CPE were observed as early as 12 h post inoculation. In Giemsa-stained preparations of PRC-inoculated cells, degeneration of individuals cells, and scattered syncytial giant cells were observed, particularly in monolayers examined at 24
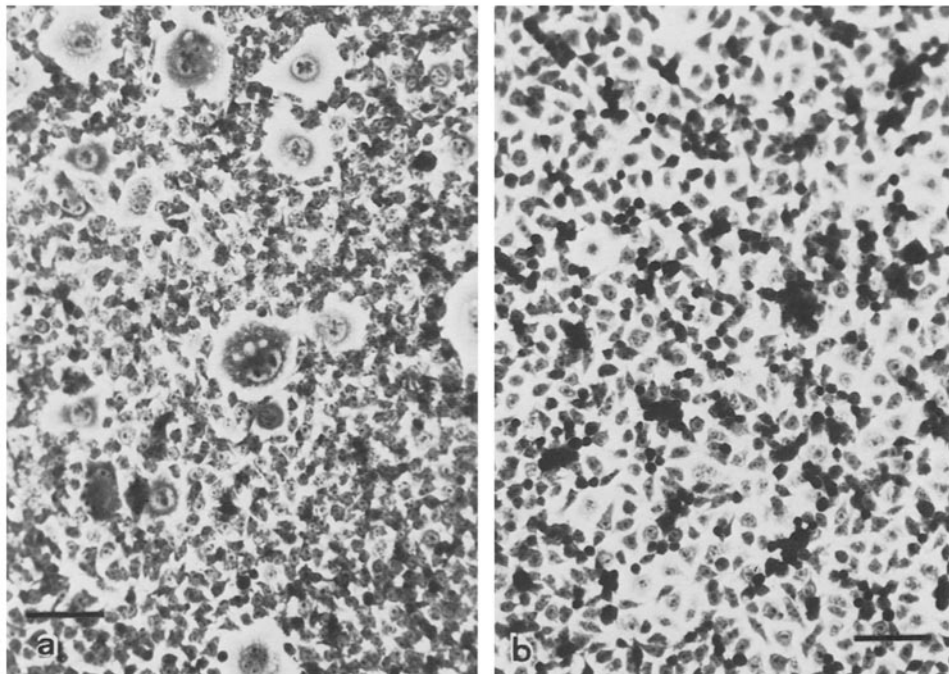

Fig. 1. a Control monolayer culture of non-inoculated L-2 cells. Giemsa stain. b Monolayer at 48 h after inoculation with the tenth pass of Parker's rat coronavirus (PRC) grown in L-2 cells. Note the pyknosis of individual cells, and the sparse numbers of syncytial cells. Giemsa stain. Bars: 20 µm
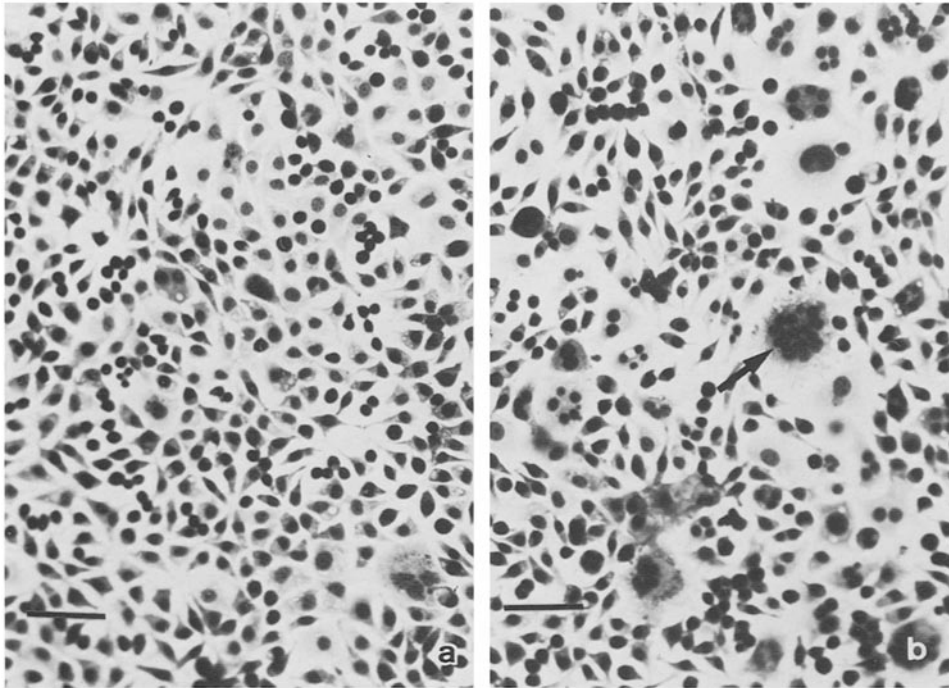
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Fig. 2. a Control monolayer culture of control L-929 cells. **b** PRC-inoculated monolayer of L-929 cells 48 h after inoculation with cell culture fluid from the 35th pass in L-2 cells. There are numerous densely-stained cells scattered in the infected cell sheet and a few syncytial giant cells (arrow). Giemsa stain. Bars: 20 μm
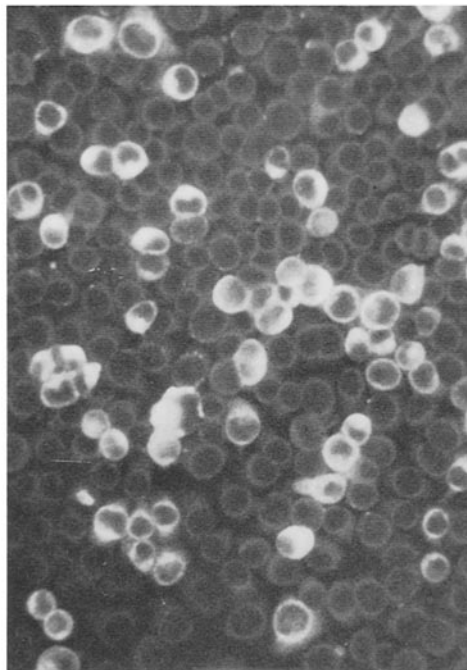

Fig. 3. Immunofluorescent microscopy preparation for the demonstration of viral antigen in PRC-infected L-2 cells examined at 48 h post-infection. There is marked cytoplasmic fluorescence primarily in individual cells. × 250
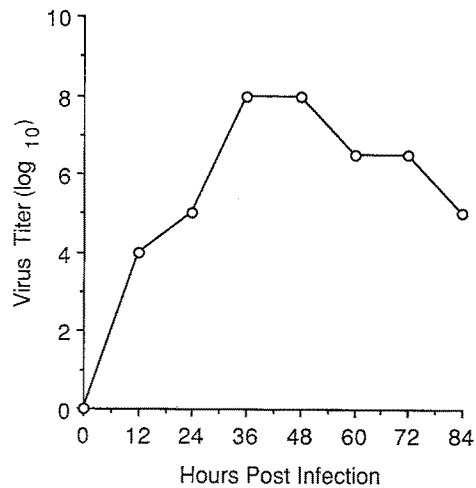
Fig. 4. Comparison of viral titers of PRC in L-2 cells when collected at 12 h intervals post inoculation. Virus titers are expressed in $TCID_{50}/1.0$ ml

or more h post inoculation. Densely-stained cells compatible with cell degeneration were the most consistent finding (Fig. 1). In monolayers with advanced CPE, there was destruction of the cell sheet, with separation of infected cells from the substrate. Following the inoculation of L-929 cells with stock virus, neither CPE nor viral antigen were detected after up to 15 serial passages. In attempts to replicate PRC in L-929 cells after replication in L-2 cells, viral antigen and CPE were not demonstrated following the 14th passage in L-2 cells, but were evident in L-929 cells after the 20th passage in L-2 cells. In Giemsa-stained L-929 cells infected with PRC with evidence of CPE, there were minimal numbers of syncytial cells, and relatively large numbers of densely-stained cells consistent with cellular degeneration (Fig. 2). In virus-infected L-2 or L-929 cells stained for immunofluorescence microscopy, there was marked intracytoplasmic fluorescence of individual cells, with relatively sparse numbers of syncytial giant cells containing viral antigen in the cell cytoplasm (Fig. 3).

Kinetics of PRC replication in L-2 cells

At 12 h post inoculation, titers of virus were approximately $10^{3.0}$ $TCID_{50}$/ml. Titers peaked at 36 h post inoculation ($10^{8.0}$ $TCID_{50}$), and fell to approximately $10^{4.0}$ $TCID_{50}$/ml at 84 h (Fig. 4).

Optimal growth conditions

Optimal titers of PRC were obtained in L-2 cells at a pH of 7.5. However, infectious virus was detected at a pH ranging from 6.0 to 9.0 (Fig. 5). In a study of the range of temperatures which would support viral replication, PRC was demonstrated at temperatures of 31–37 °C. The highest titer of virus was obtained in L-2 cells incubated at 33 °C (Fig. 6).
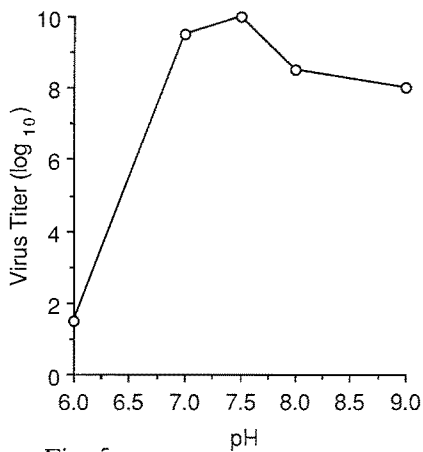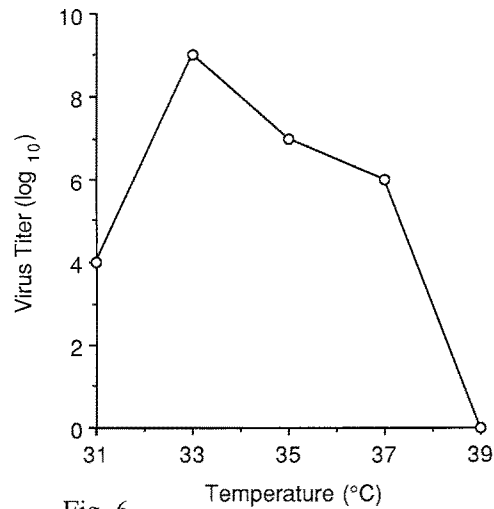
Fig. 5. Comparison of titers of PRC when virus-infected L-2 cells were incubated at 37 °C at different pH. Titers are expressed in TCID$_{50}$/ml

Fig. 6. Comparison of titers of PRC when virus-infected L-2 cells were incubated at different temperatures and tested after 48 h. Titers are expressed in TCID$_{50}$/ml

Discussion

Although previous studies have revealed that PRC and SDAV are closely related antigenically [1], it is evident that the properties of the two viruses differ in some respects in their in vitro cultural characteristics. For example, in PRC-infected L-2 cells, fewer syncytial cells were evident both in Giemsa-stained preparations and in positive cultures stained by immunofluorescence micros-copy. In SDAV-infected L-2 cells, syncytial giant cell formation was an im-portant feature of the virus-associated change [10]. In a comparison of the kinetics of viral replication in PRC and SDAV, with both strains, peak viral titers were reached at 36–48 h pi (Fig. 4) [10]. Although the optimal pH for viral replication was observed to be 7.5 in both viruses, PRC was shown to replicate to relatively high titers over a wider pH range (pH 7–9) than does SDAV [10]. Optimal temperatures for viral replication were different in the two strains of rat coronavirus. Whereas previous studies revealed that SDAV replicated best at 37 °C [10], the optimal temperature for the replication of PRC in vitro was 33 °C. Replication was more than 2 logs lower at 31 °C, and lower by one log at 37 °C (Fig. 6). This finding is consistent with previous observations that PRC is primarily a disease of the respiratory tract in the naturally-occurring and experimentally-induced disease [2, 9]. The lower op-timum temperature for PRC replication in vitro may reflect the ability of the virus to replicate readily at a cooler site in the body, the upper respiratory tract. However, lesions were observed in the salivary and lacrimal glands in Wistar rats inoculated with PRC replicated in L-2 cells at 37 °C [11].

Previous in vitro characterization studies of the known rat coronaviruses have been hampered by the lack of a readily available continuous cell line to replicate PRC or SDAV in vitro. The reports of the successful replication of PRC and SDAV in a continuous cell line of rat mammary gland origin, LBC [5, 6], represented an important contribution to facilitate the replication of these viruses in the laboratory. However, the LBC cell line is not readily available, and in our laboratory, the L-2 cell line has replicated higher titers of virus than LBC cells. The demonstrated ability of both L-2 and L-929 cells to support the replication of PRC should facilitate additional studies on the characterization of these viruses. L-2 cells are a subline of L-929 cells. This cell line was developed from a primary strain of fibroblasts obtained from an adult mouse of the C3H strain [4]. In a previous karyologic study of several sublines of L-929 cells, there were marked chromosomal variations between individual lines [12]. In addition, there is some evidence that the properties of L-2 cell lines maintained in different laboratories may vary in some respects. For example, although the line of L-2 cells used in our studies will readily replicate SDA virus in our facility and in other laboratories, not all L-2 cell lines have proven capable of replicating SDA virus (Smith, A. L., Yale School of Medicine, New Haven, CT, and Holmes, K.V., Uniformed Services, University of the Health Sciences, Bethesda, MD, pers. comm.).

The ability of PRC to replicate in Earl's clone L-929 cells after serial passage is another indication of the degree of adaptation of the virus which may take place over time in vitro. Cell-virus receptor sites may be a critical factor in this adaptation process, but the mechanisms involved in this change are currently unknown. This aspect warrants further investigation. It may be possible to identify specific changes in viral proteins and/or nucleic acid composition of PRC which permits adaptation and subsequent replication in L-929 cells. The identification and characterization of such changes should provide additional information on the adaptation process associated with the replication of certain coronavirus in vitro.

Acknowledgements

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