Replication of sialodacryoadenitis virus in mouse L-2 cells

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Summary. Sialodacryoadenitis (SDA) is a naturally-occurring infection of the laboratory rat raused by the coronavirus, sialodacryoadenitis virus (SDAV). The study of SDAV has been limited because there is no widely available continuous cell line for the propagation of high titers of the virus. The purpose of this study, therefore, was to compare the ability of SDAV to replicate in the permanent cell lines, LBC, of rat origin, and the mouse cell lines. L-929 and L-2. Following 2 to 6 repeated passages of SDAV in LBC cells, the virus could be readily propagated in LBC and L-2 cells, but not in L-929 cells. Similarly, SDAV adapted to replicate directly in L-2 cells could be readily propagated in LBC, but not L-929 cells. In LBC and L-2 cells, cytopathic effect (CPE), viral antigen, viral particles, and virus infectivity could be demonstrated. Titers of up to $10^{8.0}$ infectious viral particles/0.25 ml of culture fluid were obtained at 48 hours in L-2 cells. Titers in LBC cells were one to two logs lower. When susceptible rats were inoculated with eighth passage L-2 cell-adapted virus, they developed typical lesions of SDA. Virus could be recovered from infected tissues and propagated in L-2 cells on first passage. The ability to propagate SDAV to high titers in the widely available L-2 cell line should promote the study of this virus and facilitate its comparison with other murine coronaviruses.

Introduction

Sialodacryoadenitis virus (SDAV) infection is widespread in the laboratory rat. Based on reported serological surveys, the incidence of this coronavirus in rat colonies may be as high as 75% [15, 17]. Lesions associated with SDAV infection include sialoadenitis, dacryoadenitis, rhinitis [1, 13, 14, 19], reproductive disorders [7], and tracheobronchitis [25]. SDAV may also accelerate the progression of murine respiratory mycoplasmosis [23], and through damage to submandibular salivary glands, may cause depletion of epidermal growth factor [21]. Although the course of the disease is generally short, with rapid recovery [1], SDA can cause a variety of complications which may affect the results of behavioral, eye, reproductive, cancer and respiratory research [5, 7, 21, 25].

In an early study by Bhatt et al. [2], a variety of continuous cell lines including BHK-21, VERO, and Hep-2 cells were evaluated for their ability to support the replication of SDAV. A variety of primary monolayer cultures including primary rat kidney cells (PRK) were also tested. Bhatt et al. [2] also tried propagating SDAV in explant cultures of rat submaxillary (submandibular), parotid, Harderian and exorbital glands; and in trypsin-dispersed mouse brain cell cultures. Replication and cytopathic effect (CPE) were observed only in PRK cells. Maximum titers of $10^{2.7}$ TCID$_{50}$ were obtained PRK cells at 36 hours post-inoculation (pi) with strain #681 of SDAV [2]. All attempts to isolate virus from tissue culture systems other than PRK cells were negative. However, they found that SDAV could be propagated by intracerebral inoculation of suckling mice, and recorded titers of up to $10^{5.3}$ TCID$_{50}$ in PRK cells [2].

Recently Hirano et al. [11] reported the ability of rat coronaviruses to replicate in the LBC continuous cell line derived from a mammary tumor in a Lewis rat. In LBC cells inoculated with strain #681 of SDAV, CPE was first detected in infected cultures 48 hours pi, and syncytia were observed by 72 hours pi [11]. By the sixth passage of SDAV in LBC cells, CPE was extensive by 24 hours. Viral antigen and TCID$_{50}$ titers of $10^7/0.2$ ml could be detected in inoculated culture fluid at 24 hours pi by the tenth passage of SDAV in LBC cells [11]. The work by Hirano et al. [9–11] with the LBC cells thus confirmed that it was possible to propagate rat coronaviruses in a continuous cell line. LBC cells are not, however, widely available and they are relatively slow growing. Thus, we attempted to find another permanent cell line which would support the production of high titers of SDAV. In this report we compare the titers and the in vivo and in vitro infectivity of SDAV grown in LBC cells, L-2 cells, and in L-929 cells.

Materials and methods

Cells

The LBC cell line was kindly supplied by Dr. K. Kai (University of Yamaguchi, Yamaguchi City, Japan). Mouse L-929 cells were obtained from the American Type Culture Collection (Rockville, MD). The L-2 cell line, a subline of L-929 cells [22] was acquired from Dr. V. L. Morris (University of Western Ontario, London, Ontario). The characterization of the original cell line has been previously described [4]. Cell cultures were grown in Eagle's minimal essential medium (Gibco/BRL Inc., Burlington, Ontario) containing 200 U/ml penicillin, 80 µg/ml streptomycin and 0.05 µg/ml gentamicin supplemented with 5% (L-2; L-929) or 10% (LBC) fetal bovine serum. Cells were propagated on 100 X 15 mm Nunc polystyrene tissue culture dishes (Gibco/BRL Inc.) at 37 °C in a humidified atmosphere containing 5% CO_2.

Virus

Strain #681 of SDAV was obtained from Dr. P. N. Bhatt (Yale School of Medicine, New Haven, CT). Virus was passaged five times in specific pathogen-free (SPF) male Wistar rats which were serologically negative for antibodies to rat coronavirus (Charles River

Canada, St. Constant, Quebec). Infected parotid and submandibular salivary glands were harvested and homogenized in Tenbroeck tissue grinders to make a 10% (w/v) suspension in culture medium. Aliquots of this stock virus suspension were stored at — 70°C.

Viral infection

When approximately 80%-confluent, monolayers of LBC, L-929 or L-2 cells were inoculated with 0.25 ml of stock virus suspension or infected tissue culture supernatant. Following adsorption at 37 °C for one hour, the monolayers were washed and the culture medium was replaced. Inoculated and control cell cultures were incubated at 37 °C and 5% CO_2 for 48–72 hours. Tenfold serial dilutions of virus were made in cell culture medium and used to inoculate L-2 or LBC cells. Replicate cultures were then fixed and examined by immunofluorescence microscopy as described below. The $TCID_{50}$ was determined by the demonstration of viral antigen in 50% of inoculated cultures.

Microscopy

Cultures for light microscopy were fixed with methanol and stained with Giemsa stain. Tissues for immunofluorescence microscopy were fixed with methanol, incubated with antiserum from SDAV infected rats, then labelled with fluorescein-labelled goat anti-rat IgG (Antibodies Inc., Davis, CA) as previously described [24]. For electron microscopy, pelleted cells were fixed in 2.5% glutaraldehyde, and stained with 1% osmium tetroxide. Cells were then diluted 1 : 1 in 4% agar, embedded in Epon, processed, stained with uranyl acetate and lead citrate, and examined in a Jeol 100 S electron microscope operating at 60 kV. Supernatant fluid of infected cultures was negatively stained with 2% phospho-tungstic acid [3].

Evaluation of optimum temperature and pH

Two replicates of duplicate cultures were infected with 0.25 ml of stock eighth passage virus containing $10^{7.75}$ $TCID_{50}$/0.25 ml and incubated at 33, 35, 37, and 39 °C for 72 hours. Cells were fixed, stained and examined, and the titers calculated as previously described. In order to determine the optimal pH for replication of SDAV, cells were infected in the presence of culture medium adjusted to pH 7.0, 7.25, 7.5, 7.75, and 8.0 with sterile sodium bicarbonate. Cells were incubated at 37 °C for 72 hours.

Kinetics of SDAV production

Using optimal conditions, the time course for production of infectious SDAV in L-2 cells was determined. L-2 cells infected with $10^{7.75}$ $TCID_{50}$ of SDAV were harvested at 12 hour intervals for up to 84 hours. Virus was collected at each time point and titered as previously described under viral infection.

Animal inoculation studies

Specific pathogen free (SPF) male Wistar rats approximately four months of age were inoculated intranasally with approximately 0.1 ml of eighth or 25th passage L-2 cell adapted virus. Animals were killed at 6, 8, 14, or 21 days pi by the intraperitoneal administration of pentobarbitone sodium (MTC Pharmaceuticals, Mississauga, Ontario). In the 25th pass, four rats were used for each time point. Blood was collected by cardiac puncture for serology. Salivary glands, trachea, and lung were collected from inoculated and control rats for light microscopy. Tissues for light microscopy were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin. Serum samples from inoculated and

control rats were diluted 1 : 10, 1 : 20, and 1 : 40 in phosphate buffered saline (PBS) and evaluated for antibodies to SDAV. Binding of anti-SDAV antibodies to coronavirus-infected cells [24] was detected using fluorescein-labelled goat anti-rat globulin. Submandibular and parotid salivary glands from animals infected with the eighth pass of SDAV were homogenized to make a 10% (w/v) suspension in PBS, then 0.5 ml was inoculated onto L-2 or L-929 cells, incubated at 37 °C, and observed for evidence of viral antigen and CPE.

Results

LBC cells

Repeated attempts to demonstrate SDAV in LBC cells after initial infection with either purified #681 virus or SDAV-infected salivary gland material were unsuccessful. Following 5–6 serial passages of supernatant fluid from infected cell cultures, viral antigen, infectious virus, and CPE could be readily demonstrated. Microscopic findings were similar to those previously described [10, 11].

L-2 cells

In L-2 cell cultures infected with tissue culture fluid from SDAV-infected LBC cells, CPE was evident in the first passage at 48 hours pi. There was extensive
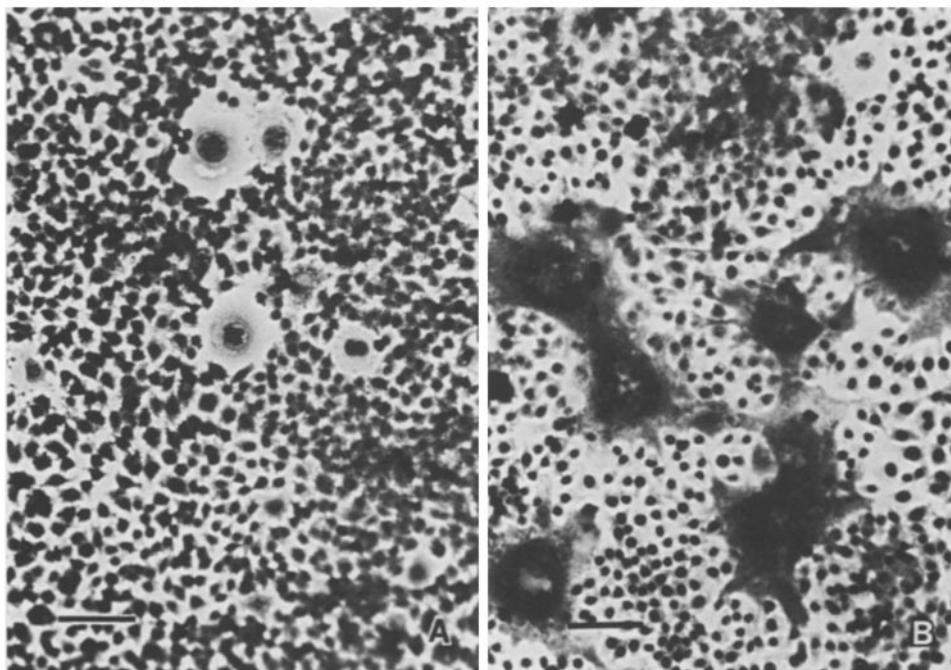

Fig. 1. A Monolayer culture of uninoculated L-2 cells. Giemsa stain. Bar = 20 μm. B L-2 cell monolayer at 48 hours after inoculation with cell culture fluid from the 6th passage of LBC adapted SDAV. Numerous irregular syncytial giant cells are present in this field. Giemsa stain. Bar = 20 μm
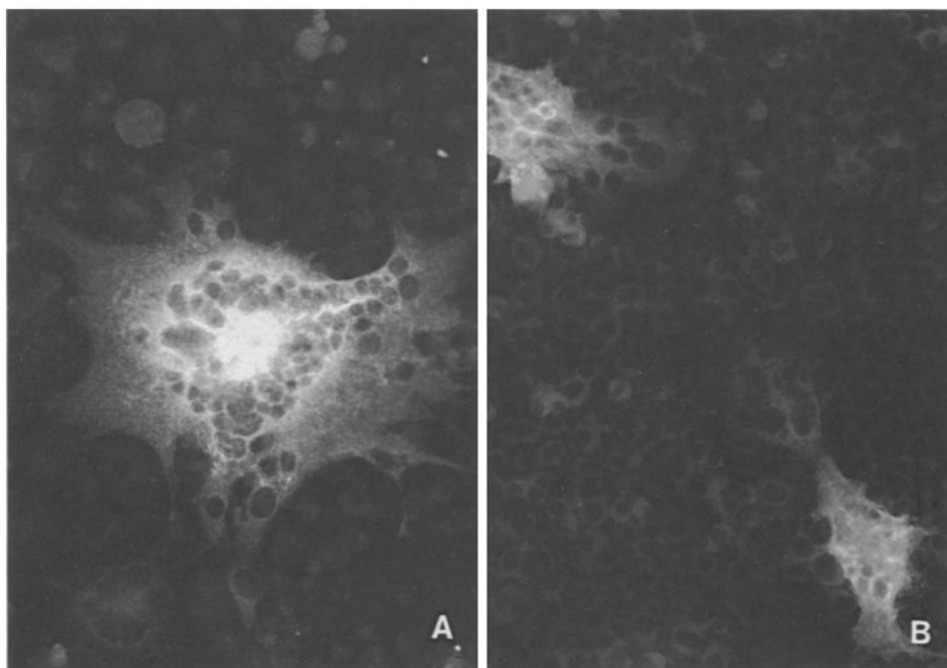
Fig. 2.A Immunofluorescence microscopy preparation of L-2 monolayer 48 hours after inoculation with LBC-adapted SDAV and stained for the detection of viral antigen. Note the intracytoplasmic fluorescence in the syncytial giant cell. Indirect immunofluorescence microscopoy. × 250. B Immunofluorescence microscopy preparation of L-2 monolayer after the ninth passage of SDA virus-infected salivary gland homogenate in these cells (48 hours pi). Viral antigen is present in the cytoplasm of syncyital cells. Indirect immunofluorescence microscopy. × 250

cell destruction, with fragmentation and separation of cells from the monolayer and the formation of syncytial giant cells by 48–72 hours pi (Fig. 1A, B). Viral antigen was evident in the cytoplasm of infected single and syncytial giant cells stained for immunofluorescence microscopy (Fig. 2A). We also attempted to infect L-2 cells directly with purified #681 stock virus and with SDAV-infected salivary gland material. In repeated experiments, 2 to 6 serial passages were required before virus could be detected by the indirect fluorescent antibody (IFA) technique at 48–72 hours pi. Once SDAV was adapted to replicate in L-2 cells, viral antigen could be detected by immunofluorescence microscopy at 24–48 hours pi in the cytoplasm of individual cells, and in syncytial giant cells (Fig. 2B). Uninoculated monolayers were consistently negative for viral antigen and CPE throughout the study.

Viral particles were also demonstrated in SDAV-infected L-2 cells by electron microscopy. In infected cells, reduced electron density, duplication of endoplasmic reticulum, cytoplasmic vacuolation, and fragmentation of cells were observed. Typical coronaviral particles 60 to 130 nm in diameter were demonstrated within cytoplasmic vesicles (Fig. 3).
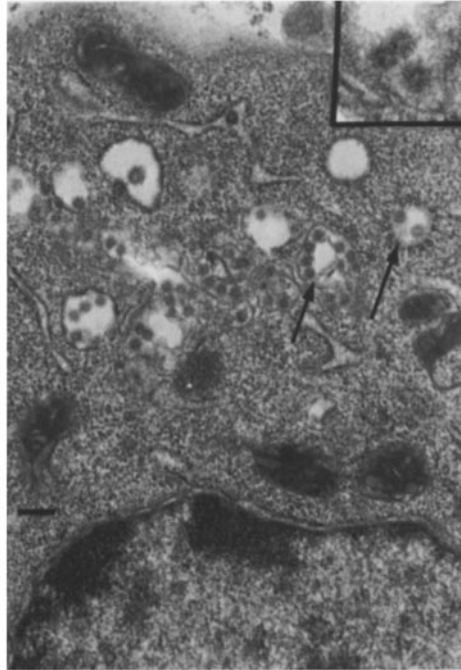
Fig. 3. Electron micrograph of SDAV-infected L-2 cells. Cells were examined 48 hours pi with SDA virus. Characteristic coronaviral particles (arrows) are present in the cytoplasmic vesicles. Uranyl acetate and lead citrate. Bar = 150 nm. **Inset** Higher magnification of viral particles

L-929 cells

In repeated trials of up to 15 serial passages of stock SDAV in L-929 cells, neither CPE nor viral antigen were demonstrated. In addition, we were consistently unable to demonstrate CPE or infectious virus in L-929 cells inoculated with cell culture fluid from SDAV-infected LBC cells or SDAV-infected L-2 cells.

Optimal growth conditions

Optimal titers were obtained in cultures at pH 7.5 (Fig. 4). Titers fell to $10^{5.0}$ $TCID_{50}$ at a pH of 7.25 and $10^{4.0}$ $TCID_{50}$ at pH 7.0 (Fig. 4). The drop in titre at a pH greater than 7.75 was less marked (Fig. 4). Temperature also had a marked effect on viral replication. Optimal titers were obtained at 37 °C. Titers fell by 4 logs at 39 °C and by 1.5 logs at 35 °C (Fig. 5).

Kinetics of SDAV production

Titers of up to $10^{2.5}$ $TCID_{50}$ were detected at 12 hours pi (Fig. 6). Viral titers rose rapidly within the first 36 hours, reaching a maximum of $10^{8.0}$ $TCID_{50}/$ 0.25 ml at 36 hours pi, with subsequent decline. Titers fell rapidly after 60 hours (Fig. 6). In our studies, the maximum titers obtained in LBC cells were approximately $10^{6.0}$ $TCID_{50}/0.25$ ml.
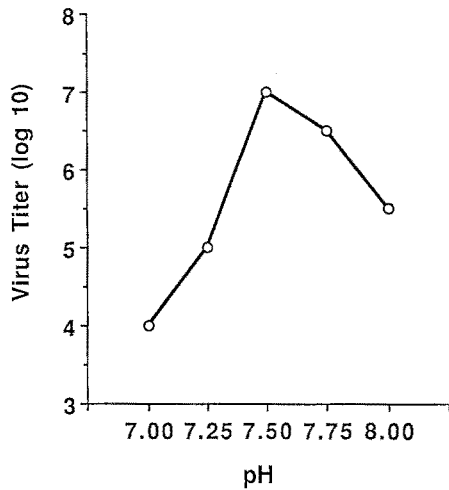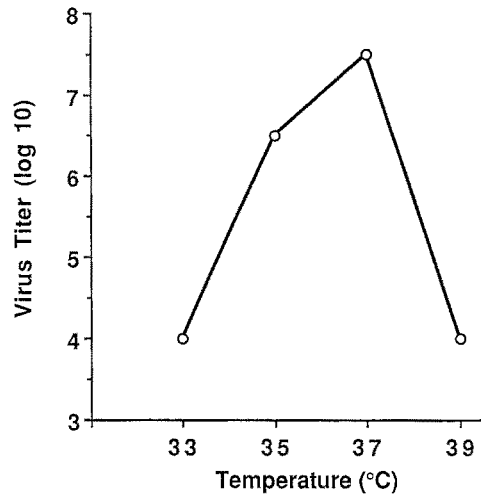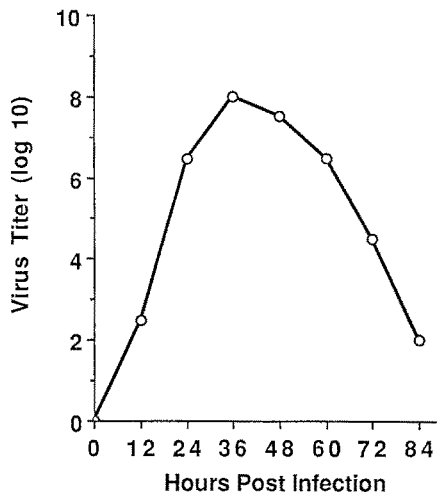
Fig. 4

Fig. 5

Fig. 4. Comparison of titers of SDA virus obtained when L-2 cultures were incubated at 37 °C, and at different pH. Virus titers expressed in $TCID_{50}/0.25$ ml

Fig. 5. Comparison of titers of SDA virus obtained when virus-infected L-2 cells were incubated at different temperatures. Virus titers expressed in $TCID_{50}/0.25$ ml

Fig. 6. Comparison of titers of SDA virus obtained when virus-infected L-2 cells were collected at 12 hour intervals pi. Virus titers expressed in $TCID_{50}/0.25$ ml

Animal inoculation studies

The eighth passage of L-2 cell adapted SDAV was used to infect Wistar rats. Animals inoculated intranasally with 0.1 ml of infected tissue culture supernatant developed the typical clinical signs of SDA within 3–6 days pi [1, 13]. Characteristic lesions were observed in the salivary and lacrimal glands on gross and microscopic examination (Fig. 7). In inoculated rats examined microscopically during the acute stages of the disease at 6 days pi, there was a destructive sdialoadenitis and dacryoadenitis typical of SDA [13, 19]. Typical lesions were
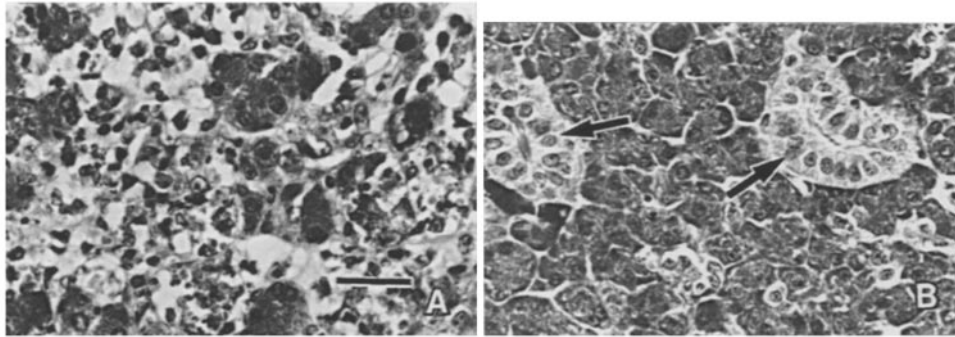
Fig. 7. A Parotid salivary gland from Wistar rat at 6 days pi with the eight pass of SDA virus in L-2 cells. Note the destructive sialoadenitis, with loss of normal architecture. Bar = 20 μm. B Control parotid salivary gland, same magnification. Note the normal glandular structures, and the prominent ducts (arrows)

also present in the trachea and bronchi in those rats examined at 6 days pi. No lesions were observed in the glands of control rats inoculated with supernatant from uninfected L-2 cells. Antibodies to SDAV at dilutions of 1:20–1:40 were readily demonstrated in rats inoculated with SDAV-infected L-2 cells and killed at 14 days pi, but not in control rats. Using submandibular gland homogenates prepared from infected animals killed at 6 days pi and inoculated onto L-2 cells, SDA viral antigen and typical syncytial cell formation were detected on the first passage. When virus from the 25th passage of SDAV in L-2 cells was used to infect SPF rats by intranasal inoculation, salivary and lacrimal glands were histologically normal at necropsy. Lesions were minimal in the respiratory tract in these animals. By 21 days pi, 4/4 of these rats had anti-SDAV antibody titers of up to 1:40. Non-inoculated control rats and rats killed at 6 days pi were serologically negative for detectable antibodies to SDAV.

Discussion

Although there have been numerous reports on the characterization of the various strains of the mouse coronaviruses [12, 16], there is little information available on coronaviruses in the rat. Until recently, the study and characterization of SDAV was hampered by the absence of a continuous cell line capable of supporting the replication of high titers of the virus. The report by Hirana et al. of the ability of SDAV to grow in LBC cells was an important advancement [11]. Maru and Sato [18] isolated a strain of coronavirus from the submandibular salivary glands of the rat with sialoadenitis, which replicated in mouse-derived A 31 (3 T 3) cell culture. However, strain #681, acquired from Bhatt's original isolate of SDAV, failed to replicate in 3 T 3 cells [18]. Although LBC cells do support the replication of SDAV, these cells are not widely available and have a doubling time of approximately twice as long as L-2 cells. In addition, it is not possible to make a direct comparison of SDAV grown in LBC cells with other murine coronaviruses cultivated in L-2 cells.

Using LBC cell-adapted SDAV, we were able to infect L-2 cells, and virus could be demonstrated in L-2 cells on the first passage. Titers up to $10^{8.0}$ TCID$_{50}$/ 0.25 ml were obtained at 36 hours pi. Bhatt's original strain #681 SDAV was also adapted directly to grow in L-2 cells. Optimal titers were obtained when that virus was propagated at 37 °C for 36 hours in medium at pH 7.5. In the case of both the L-2 and the LBC cells, 2 or more passages were required before infectious virus and/or viral antigen was detected. Serial passage presumably allowed for the selection of virions which could readily replicate in continuous cell cultures. The fact that LBC-adapted SDAV could be propagated in L-2 cells (and vice versa) on the first passage suggested that any alterations in the virion required for the replication in the two cell lines were similar if not identical. Adaptation also appears to occur in vivo. For example, SDAV has produced encephalitis in newborn rats only after prior intracerebral passage of the virus in suckling mice [20].

There have been several mechanisms proposed which could explain variations in virulence and adaptation in animal viruses; variation by mutation, host-induced variation, and genetic recombination are all possible explanations [6]. The failure to replicate SDAV in Earle's clone L-929 cells, while virus replicated in the L-2 subline, emphasizes the variations that may occur in cells originally derived from the same parent source. In a karyologic study of various sublines of L-929 cells, Rothfels et al. emphasized the marked chromosomal variations observed in these lines [22].

Despite the fact the L-2 cell-adapted SDAV presumably underwent some alteration(s) to allow high levels of expression in cell culture, the passage of the virus grown in vitro retained the ability to produce lesions typical of SDA in intranasally-inoculated rats. Furthermore, the virus, when recovered from infected animals, could again infect L-2 cells and viral antigen could be readily demonstrated on the first passage. These data indicate that although the virus was adapted to replicate in L-2 cells, this strain was still capable of producing lesions typical of SDA in susceptible rats. Following 25 passages in L-2 cells, SDAV produced in culture failed to produce the typical lesions in salivary and lacrimal glands, although lesions were evident in the respiratory tract. Animals infected with 25th pass L-2 cell virus did, however, have significant antibody titers against SDAV. Experiments are currently underway to see if the immune response induced by the high passage virus is protective. Although attenuation of virulence following extended passage outside the natural host is a common phenomenon, the precise mechanism is not known. Further in vitro and in vivo characterization studies of the high and low passage SDAV are currently in progress.

Acknowledgements

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