Growth characteristics and protein profiles of prototype and wild-type rat coronavirus isolates grown in a cloned subline of mouse fibroblasts (L2p.176 cells)

Diane J. Gaertner*, Susan R. Compton, Deborah F. Winograd, Abigail L. Smith

Section of Comparative Medicine, Yale University School of Medicine, P.O. Box 208016, New Haven, CT 06520-8016, USA

Abstract

Rat coronaviruses (RCVs) infect laboratory rats and confound biomedical research results. In vitro systems developed so far have limited the growth in knowledge about RCVs by not permitting generation of plaque-cloned virus stocks, reliable isolation of RCVs from rat tissues, or growth of high titered stocks of all isolates. Due to the fact that less than 20% of L2(Percy) cells were becoming infected, sublines were produced and selected for maximal growth of RCVs. Screening of 238 cell sublines yielded L2p.176 cells which were highly susceptible to all RCVs tested; however, susceptibility declined after 30 passages in vitro. Low-passaged L2p.176 cells were used to isolate virus from natural outbreaks and to propagate individual RCV plaques into high titered stocks. Proteins from six RCV isolates were immunoblotted using polyclonal rat and mouse antibodies to sialodacryoadenitis virus and polyclonal monospecific rabbit and goat antibodies against the peplomer (S) and nucleocapsid (N) proteins of mouse hepatitis virus (MHV). Proteins of two prototype, one Japanese and three wild type RCVs were examined and found to be similar to those of MHV, although the exact sizes and ratios of protein forms were unique for most RCV isolates. This study reports the development of a continuous cell line which reliably supports RCVs opening an opportunity for further in vivo studies of the biology of these agents. As a first step in the characterization of RCVs, we have shown that RCV proteins are very similar to those of MHV.

Keywords: Rat coronavirus; Rat; Cell line; Cell culture; Virus strains; Sialodacryoadenitis virus

1. Introduction

Rat coronaviruses (RCVs) are agents which commonly infect laboratory rats. RCV infections may be either subclinical or clinically apparent and can confound research results (Jacoby, 1986; Boschert et al., 1988; Huang et al., 1989; Utsumi et al., 1991). Although isolation of several RCV strains which differ in their tissue tropisms have been reported, it is not clear how much RCV strains differ in biology and pathogenesis. Recent advances in the in vitro growth of rat coronaviruses have included growth of high titer

* Corresponding author. Institute for Animal Studies, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461. Tel.: +1 718 4308551; fax: +1 718 4308556.

stocks of prototype RCVs in the Percy subline of mouse fibroblast L2 cells (Percy et al., 1989, 1990) and improved virus quantification and yields in L2(Percy) cells treated with dietheylaminoethyl dextran (DEAE-D) and trypsin (Gaertner et al., 1991, 1993). These improvements in culture techniques have facilitated the production of high titered stocks of previously cultivated RCV isolates and production of individual RCV plaques for further study, but when individual plaques were put back into L2(Percy) cells, the resulting stocks had only low titers (Gaertner, unpublished). Alternative methods, including intracranial inoculation of infant mice and oronasal inoculation of athymic nude rats, also failed to yield high titered stocks from individual plaques. Additionally, RCVs could not be reliably isolated from rat tissues using L2(Percy) cells. Because less than 20% of L2(Percy) cells became infected when inoculated with high titered mouse brain suspensions of sialodacryoadenitis virus strain 681 (RCV-SDA-681, Bhatt and Jacoby, 1977) or with Parker's rat coronavirus (RCV-P, Parker et al., 1970), cell sublines were produced and selected to maximize growth of wild-type and prototype isolates. We report the generation of an L2(Percy) cell subline, L2p.176 cells, which exhibits marked CPE and yields high titered virus stocks when inoculated with all RCV strains. This subline was used to isolate RCVs from naturally infected rats and to propagate RCV stocks from plaques. Immunoblots of RCVs grown in L2p.176 cells revealed typical coronaviral proteins that were antigenically related to mouse hepatitis virus (MHV). Diversity in molecular weights and ratios of S and N proteins was detected among two prototypes, one Japanese and three wild-type isolates; suggesting that biological differences in the behavior of isolates may be related to differences in proteins.

2. Materials and methods

2.1. Cells and media

A subline of mouse fibroblast cells (referred to as L2(Percy) cells) was obtained from D. Percy

(Guelph, Ontario, Canada). Two L2(Percy) sublines, L2 41.a and L2 29.a were obtained from K. Holmes (Bethesda, MD, USA). Cells were maintained as previously described (Gaertner et al., 1993). Cells were tested for *Mycoplasma* spp. prior to use and were culture-negative.

Growth and maintenance medium for L2(Percy) cells contained 50% Eagle's minimal essential medium, 45% Hanks' minimal essential medium, 5% fetal bovine serum (FBS), 2 mM l-glutamine, 100 μg/ml streptomycin and 100 units/ml penicillin. To produce conditioned medium for cell cloning, the medium from L2 cells was replaced one day after cell transfer, the cells were permitted to grow for two additional days and the conditioned medium was removed, centrifuged to eliminate cell debris and filtered through a 0.22 μm filter. Cell cloning medium contained 15% L-15, 40% Dulbecco's minimal essential medium (DMEM), 30% L2-conditioned medium, 15% FBS, 7.5 mM HEPES buffer, 2 mM l-glutamine, 100 μg/ml streptomycin and 100 units/ml penicillin. Individual cloned cell lines were frozen in liquid nitrogen in cloning medium with 20% DMSO added. Medium containing 80% DMEM, 15% L-15, 5% FBS, 2 mM l-glutamine plus antibiotics was used for growth and maintenance of cloned cell lines. All media and supplements were purchased from GIBCO BRL (Gaithersburg, MD, USA) unless otherwise noted.

2.2. Virus

Because the prototype rat coronavirus isolates, sialodacryoadenitis virus, strain 681 (SDA), and Parker's rat coronavirus, are closely related antigenically (Jacoby, 1986) and probably represent strains of the same virus, we will refer to these isolates as RCV-SDA-681 and RCV-P, as recently suggested (K. Holmes, personal communication). Wild-type isolates of RCVs were named using the same system. Stocks of RCV-SDA-681, and RCV-P were prepared in infant mouse brains (Bhatt and Jacoby, 1977; Bhatt et al., 1977). Titration of RCV-SDA-681 and RCV-P stocks in primary rat kidney (PRK) cells (Bhatt and Jacoby, 1977; Bhatt et al., 1977) yielded titers of

$10^{7.5}$ and $10^{8.0}$ TCID$_{50}$ per gm, respectively. An L2-adapted isolate of RCV-SDA-681 (called RCV-SDA-L2), a gift of D. Percy (Percy and Scott, 1991), was supplied as an infected L2 cell lysate and was used at the 11th L2 cell passage without being passaged in our laboratory.

An additional rat coronavirus isolate supplied in two forms, RCV-V230 and RCV-V20, was obtained from W. Shek (Charles River Laboratories, Wilmington, MA) (Guberski et al., 1991). This isolate was derived from the salivary gland of a rat with clinical signs of sialodacryoadenitis. RCV-V230 had been passaged once in PRK cells and was supplied as infected PRK cell lysate. RCV-V20 had been passaged 19 additional times in intracranially-inoculated infant mice and was supplied as mouse brain suspension.

A Japanese isolate of rat coronavirus, causative agent of rat sialoadenitis (CARS), was a gift from M. Maru (Maru and Sato, 1982). Virus passaged 95 times in 3T3 cells was supplied lyophilized, rehydrated and passaged twice by intracranial inoculation into suckling outbred mice to yield a mouse brain stock. CARS did not cause CPE in PRK cells, it caused only sporadic seroconversion to RCV after inoculation of adult mice or rats, caused mild rhinitis in adult rats and only encephalitic lesions after oronasal, intracranial or intraperitoneal inoculation of suckling mice (Gaertner and Barthold, unpublished).

Three wild-type isolates of RCV were studied. RCV-WT was isolated from outbred Sprague Dawley (SD) rats immediately after receipt from a commercial facility housing rats endemically infected with RCV (Paturzo, 1987; Gaertner et al., 1993). Lung homogenates were inoculated into mouse pups to produce mouse brain suspensions and into L2(Percy) cells to produce the plaques used in these studies. Isolation and propagation of RCV-BCMM and RCV-W are described in Section 3. To confirm coronavirus isolation, cells were observed for CPE or plaque formation and cells were stained by immunofluorescence (IFA) to detect RCV antigens (Smith, 1983).

2.3. Animals

Cr:ORL Sencar mouse dams with one day old litters and pathogen-free SD rats were purchased (Animal Genetics and Production Branch, National Institutes of Health, Bethesda, MD) and housed under conditions which met or exceeded standards of the U.S. Public Health Service.

2.4. Plaque assays and propagation of plaque-purified virus isolates

Cells were inoculated when fully confluent to maximize plaque formation. DEAE-D and trypsin were used to increase CPE and plaque size and number except as specifically noted. DEAE-D (Pharmacia, 75 μg/ml) was added to pre-absorption medium, trypsin (2.50 μg/ml) and DEAE-D (75 μg/ml) were added to absorption media, and trypsin (2.50 μg/ml) was added to agarose overlay or medium for incubation. For virus titrations, three or more replicate 2.0 cm^2 wells of cells were exposed to 0.3 ml of inoculum per well for each virus dilution. Agarose overlays were prepared as previously described (Gaertner et al., 1993). Plates were fixed at 48 h with 10% buffered formalin and were stained with Giemsa, plaques were counted and titers were expressed as PFU per ml (Lorenz and Bogel, 1973). Individual plaques were collected, stored as previously described (Gaertner et al., 1993) and were inoculated into cells growing in 25 cm^2 flasks in attempts to produce plaque-cloned stocks. Flasks were observed daily, harvested 48 or 72 h after inoculation and virus in infected cell lysates was quantified.

2.5. Augmentation of RCV growth by DEAE-D and trypsin

To confirm that growth of RCV isolates in L2p.176 cells, like RCV growth in L2(Percy) cells, was augmented by media supplementation with DEAE-D and trypsin (Gaertner et al., 1993), L2p.176 cells grown on glass chamber slides (Nunc, Inc., Naperville, IL) were inoculated with or without DEAE-D and trypsin. Individual cultures inoculated with virus doses ranging from 0.01–0.0012 PFU/cell were observed twice daily

and were acetone-fixed 36 h after inoculation. Slides were stained by IFA and observed as previously described (Gaertner et al., 1991). Relative proportions of cells containing RCV antigens were estimated.

2.6. Production and selection of L2(Percy) cell line subclones

To produce L2(Percy) sublines by limiting dilution, a single cell suspension was plated in 96-well plates at a theoretical concentration of $10^{0.5}$ cells/ml, and only wells with clones observed to have originated from single cells were passaged (Freshney, 1994). Briefly, individual clones that grew to confluence in wells of 96-well plates were transferred to one well of a 24-well plate using trypsin and versene. Clones growing to confluency in 24-well plates were sequentially named L2p.1 to L2p.238 and were used to seed both a 25 cm^2 flask and multiple wells of a 96-well plate for screening by virus inoculation. Wells of each cell clone were initially tested for their ability to show CPE after inoculation of an m.o.i. of 0.1 PFU/cell of RCV-SDA-681, RCV-P, RCV-WT and RCV-BCMM using trypsin-supplemented medium without DEAE-D. CPE was scored subjectively. Cell lines that had visible CPE due to all four virus isolates were re-screened with a lower virus dose of the same isolates (0.02–0.06 PFU/cell) and were also inoculated with 0.1 PFU/cell of RCV-SDA-L2. The extent of CPE in each well was scored and cells were scraped into the media and the media was frozen from cell lines with CPE due to all five isolates. To make a final selection of a cell line for future use, this RCV titers of these materials were determined on the homologous cell lines with DEAE-D and trypsin.

2.7. Detection of RCV proteins by immunoblot

Cell lysates of RCV-infected L2p.176 cells or MHV-infected J774A.1 cells were prepared when CPE was moderate using a detergent-containing buffer (Springer, 1994). Cell debris was removed by centrifugation and lysates were frozen at − 20°C until use. Purified RCV-SDA-681 virus grown in L2p.176 cells and MHV-A59 grown in J774A.1 cells were prepared for use in immunoblots by centrifugation on 30/60% sucrose gradients in a SW 28 rotor at 24K for 4 h and were frozen at − 20°C until use.

Viral proteins were separated by SDS-polyacrylamide gel electrophoresis in 8 or 12% acrylamide running gels with 5% stacking gels (Towbin et al., 1979). Undiluted lysates of cells infected with RCVs were loaded into wells except for RCV-SDA-681, CARS and MHV-A59. Lysates of RCV-SDA-681 and CARS were diluted to two-thirds and one-third concentrations prior to loading, respectively, based on their stronger reactivity with polyclonal mouse antibody against RCV-SDA-681. Volumes of MHV-A59 lysate or virion were selected based on signal strength from previous blots (Kunita et al., 1995). Proteins were transferred to nitrocellulose (Hoeffer Scientific Instruments, MN). Blots were blocked with milk or bovine serum albumin. Proteins were detected using rat serum antibody produced by oronasal inoculation of rats with RCV-SDA-681 (Weir et al., 1990), mouse ascites antibody produced by intraperitoneal injection of RCV-SDA-681 (Smith, 1983), goat antiserum against the S protein of MHV-A59 (Sturman et al., 1980), rabbit antiserum produced against the N protein of MHV-A59 (Robbins et al., 1986) or rabbit antiserum produced against the M protein of MHV-A59 (Sturman et al., 1980). Bound antibodies were reacted with Staphylococcal protein A coupled to horseradish peroxidase (HRP-SPA, Kirkegaard and Perry, Inc. Gaithersburg, MD) except for rat antibody which was detected by goat anti-rat antibody (Kirkegaard and Perry). HRP-SPA was detected by an enhanced chemiluminescence (Amersham Life Science, Arlington Heights, IL) followed by radiography (Hyperfilm, Amersham). Goat anti-rat antibody was detected by HRP-induced color development (ProBlot System, Kirkegaard and Perry) (Towbin et al., 1979). Mouse, rat and rabbit antibodies were pre-absorbed against viable L2p.176 cells, L2p.176 lysate dot-blotted onto nitrocellulose and nitrocellulose strips coated with blocking agent to remove antibodies reactive against L2p.176 cells or blocking agents.

3. Results

3.1. Production of cloned L2 cell sublines and selection of L2p.176 cells

Initially in the search for a cell line to grow RCVs, we tested L2(Percy) cells and two L2(Percy) cell sublines which had been developed in the laboratory of K. Holmes (Bethesda, MD). When inoculated with mouse brain suspensions of RCV-SDA-681 and RCV-P both L2(Percy) cells and the cloned L2 sublines L2 41.a and L2 29.a exhibited CPE with virus titers at 48–72 h ranging from $10^{3.0}$ to $10^{5.8}$ PFU/ml. However, when individual isolated plaques of RCV-SDA-681, RCV-P and RCV-WT were grown in these cell lines, CPE was limited to less than 5% of cells and titers of stocks harvested at the peak of CPE (48–72 h) were less than $10^{3.0}$ PFU/ml. Because of the low titers of RCV stocks produced from initial passage of individual RCV plaques in these three cell lines, it was decided to subclone L2(Percy) cells in search of a more sensitive cell line.

All of the 238 cloned lines initially screened supported the growth of at least one RCV isolate and 35 clones had CPE due to growth of all 4 of the isolates (RCV-SDA-681, RCV-P, RCV-WT and RCV-BCMM). Prototype SDA-681 and wild-type SDA-WT grew in most clones and usually caused complete destruction of the cell sheet. Prototype RCV-P and wild-type RCV-BCMM consistently caused CPE in fewer clones and caused less widespread CPE. All cell clones that supported the growth of RCV-P and RCV-BCMM had complete CPE when infected with RCV-SDA-681 and RCV-WT. The 35 cell lines which had visible CPE due to each of the four virus isolates, were re-screened with five isolates, including RCV-SDA-L2, RCV-SDA-681, RCV-P, RCV-WT and RCV-BCMM. During the second screening the same pattern of virus growth was seen, with RCV-P and RCV-BCMM causing less frequent and less widespread CPE than RCV-SDA-L2, RCV-SDA-681, and RCV-WT. RCV-SDA-L2 and RCV-WT caused complete CPE in every cell clone re-tested. Growth of RCVs in the five cell lines with the most CPE due to RCV-P and RCV-BCMM was quantified by determina-

tion of the amount of virus produced in order to make a final selection of a cell line for future use (Fig. 1). For example, the titer of RCV-P produced from the first passage in L2p.176 cells was determined by counting the number of plaques produced when the stock was titrated in L2p.176 cells. L2p.176 cells were chosen for future studies based on their ability to grow all RCV isolates tested to high titer.

3.2. Comparison of RCV growth in L2p.176 cells with RCV growth in L2(Percy) cells

As detailed below, in order to determine if L2p.176 cells would be superior to L2(Percy) cells for growth of RCVs, we compared the sensitivity of cloned cells with the parental cell line, used L2p.176 cells to isolate 2 new wild-type RCVs and tested the ability of L2p.176 cells to grow a vari-
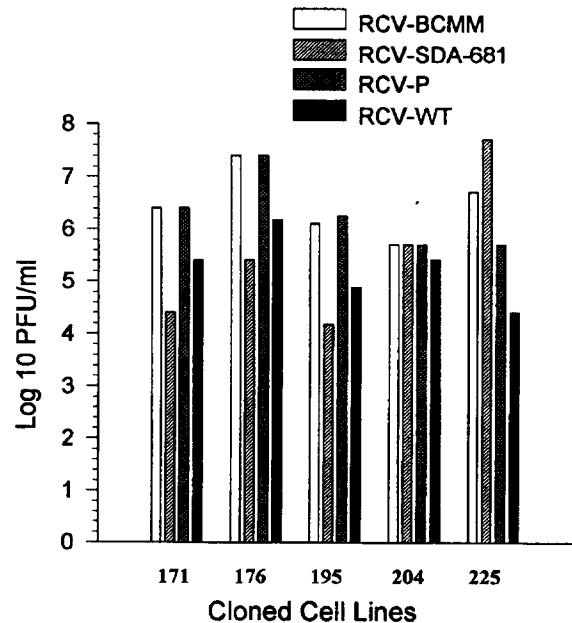

Fig. 1. Growth of RCV isolates in five L2 cell clones selected for maximal CPE due to RCV-P and RCV-BCMM. Virus growth at 72 h was quantified by determining the number of PFU/ml in the homologous cloned cell, i.e. virus grown in L2p.171 cells was detected in L2p.171 cells. Cells were pretreated for 3 h with DEAE-D, DEAE-D and trypsin were included in the inocula and trypsin was included in agarose overlays.

Table 1
Comparison of the sensitivity of in vitro and in vivo systems to detect RCVs

Isolate[a]	Infant mouse inoculation[b]	L2(Percy) cells[c]	L2p.176 cells[c]
RCV-SDA-681	$10^{6.4}$	$10^{5.5}$	$10^{6.9}$
RCV-P	$10^{7.0}$	$10^{6.4}$	$10^{7.0}$
RCV-WT	nd	$10^{4.7}$	$10^{7.1}$
RCV-BCMM	$10^{6.5}$	$10^{5.1}$	$10^{6.7}$

[a] 10% RCV-infected mouse brain.

[b] Intracranial inoculation, values expressed as LD_{50}/ml.

[c] Cells were pre-treated with DEAE-D, trypsin and DEAE-D were included in absorption mediumand trypsin was included in the incubation overlay, values expressed as PFU/ml.
nd, not done.

ety of previously cultivated RCVs. After documenting that L2p.176 cells showed more florid CPE and produced more virus than the parental cell line, we tested the growth kinetics of four RCV isolates in the two cell lines.

3.2.1. Testing the relative sensitivity of L2p.176 cells to RCVs

To determine if L2p.176 cells were more sensitive than L2(Percy) cells in their ability to detect RCVs, titers of mouse brain suspensions of RCV-SDA-681, RCV-WT, RCV-P and RCV-BCMM, previously measured by mouse inoculation, were measured by plaque assays in L2p.176 and L2(Percy) cells. Cells were fixed at 48 h, stained, plaques were counted and titers were calculated. For all isolates tested, L2p.176 cells consistently yielded higher titers of virus than parental cells (Table 1).

Because passage of RCVs in the L2p.176 cell line yielded stocks with high virus titers, L2p.176 cells were tested for their ability to produce plaques and to propagate individual plaques. Since they were developed, low-passaged L2p.176 cells have been used with consistent success to plaque purify RCVs and to grow tissue culture stocks from individual plaques. Stocks generated from individual plaques in L2(Percy) cells and the

subclones L2 41.a and L2 29.a contained 10^3 PFU/ml or less, whereas plaque cloned stocks containing $10^{6.0}$ PFU/ml of RCVs have been generated by first passage of individual plaques in L2p.176 cells. Individual RCV plaques from L2(Percy) cells yielded 50–250 plaques by a second plaqueing in L2(Percy) cells but yielded 100–1000-fold more plaques when the second plaqueing was in L2p.176 cells. Media supplementation with trypsin and DEAE-D augmented the growth of RCVs in L2p.176 cells, as had previously been observed for L2(Percy) cells (data not shown).

3.2.2. Use of L2p.176 cells to isolate wild-type RCVs

Two new wild-type RCVs, RCV-BCMM and RCV-W, have been isolated in L2p.176 cells, from rats originating in RCV-infected rat colonies. RCV-BCMM was isolated from the lung of a moribund 11 day old SD rat originating in a previously RCV-seronegative rat room where rats were showing clinical signs of sialodacryoadenitis. During this outbreak breeding rats were noted to have reproductive abnormalities and neonatal mortality (E. Weir, personal communication) as has been reported in RCV-infected rats in Japan (Utsumi et al., 1991). A coronavirus was isolated in L2p.176 cells from 3/3 lungs of sick rats (Fig. 2). The three lung tissues tested had $10^{4.6}$, $10^{2.7}$ and $10^{1.7}$ PFU/gm of RCV. The lung homogenate with the highest titer was used to isolate individual plaques. Plaques were used to produce passaged plaques and high titered ($> 10^{6.0}$ PFU/ml) tissue culture stocks. Lung homogenate was also used to make a mouse brain stock. This isolate has been named RCV-BCMM.

A second new isolate was obtained from 4 week-old Wistar rats immediately after their arrival from an endemically RCV-infected commercial breeding facility. Four of six rats in the shipment were RCV seropositive as determined by indirect immunofluorescence (IFA) (Smith, 1983) and a coronavirus was isolated in L2p.176 cells from nasopharyngeal washes of one seropositive and one seronegative rat (Fig. 2). Plaques grown from one nasopharyngeal wash cultivated in L2p.176 cells were used to produce plaques in

RCV-BCMM

RCV-WT

RCV-W

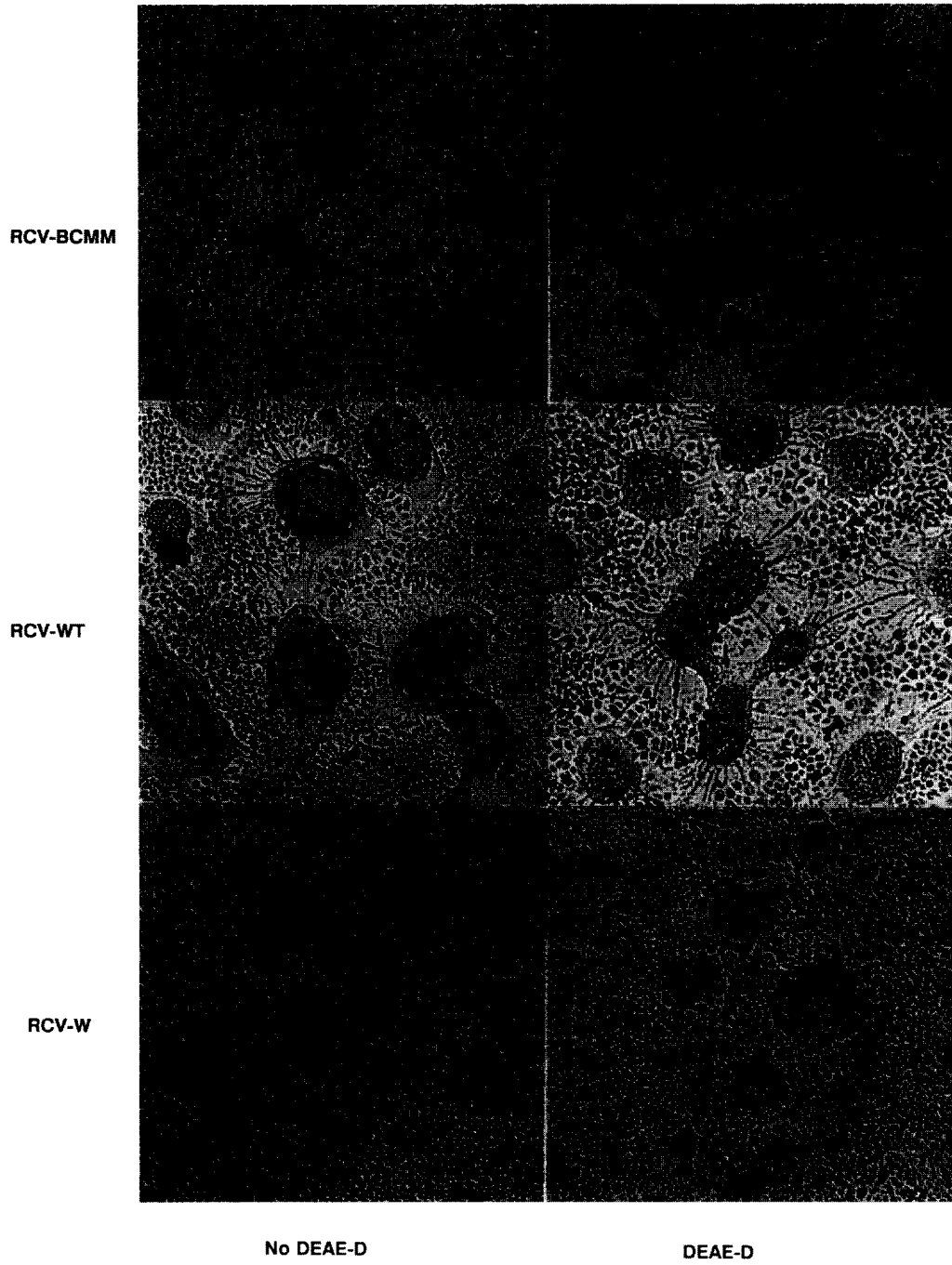No DEAE-D   DEAE-D

Fig. 2. Cytopathic effect (syncytia formation 48 h after inoculation) due to three wild-type rat coronavirus isolates in L2p.176 cells cultivated in medium with added trypsin, with and without DEAE-D treatment. × 144.
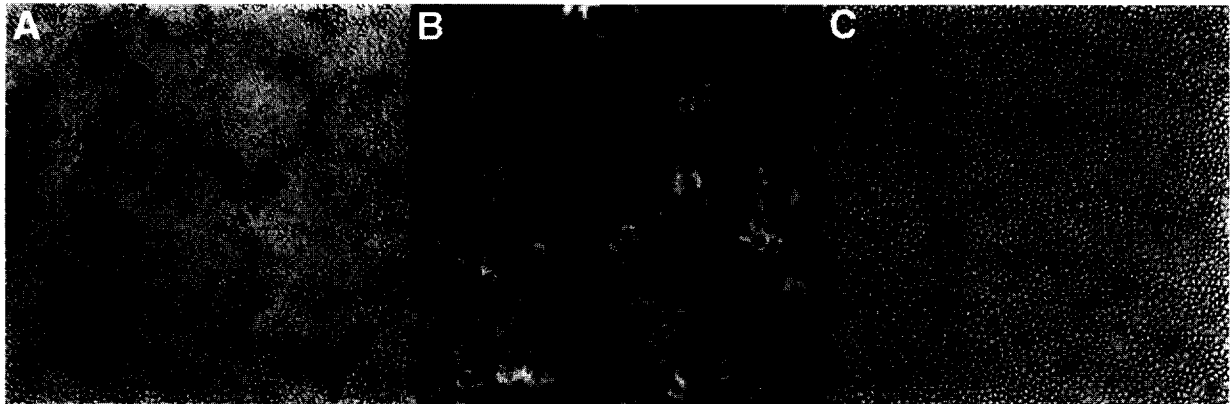
Fig. 3. Cytopathic effect (A) and coronavirus antigen (B) in L2p.176 cells 48 h after inoculation with the Japanese rat coronavirus isolate, CARS. Uninfected L2p.176 cells (C). Coronavirus antigen was detected by IFA using pooled rat anti-RCV-SDA-681 antibodies diluted 1:40. A and C × 144, B × 720.

L2p.176 cells and to produce high titered (> $10^{6.0}$ PFU/ml) tissue culture stocks. This isolate has been named RCV-W.

3.2.3. Use of L2p.176 cells to grow other RCVs

L2p.176 cells have also been useful in propagating additional RCV isolates including wild-type virus and isolates previously cultivated in L2(Percy) cells, primary rat kidney (PRK) cells and mice. An RCV wild-type virus previously cultivated only by mouse brain inoculation has been grown from mouse brain stock to a high-titered tissue culture stock ($10^{6.5}$ PFU/ml) in L2p.176 cells (Fig. 2). Two forms of an additional isolate, RCV-V230 and RCV-V20 also grew in L2p.176 cells. Both isolates produced typical CPE within 24–48 h and yielded titers of $10^{5.6}$ and $10^{4.4}$ PFU/ml on initial passage. CARS, a Japanese isolate of rat coronavirus propagated by mouse inoculation, produced a stock in L2p.176 cells containing $10^{5.3}$ PFU/ml and produced granular CPE consisting of small syncytia and less refractile (dark) areas of the cell sheet without forming the large syncytia typical of other RCVs (Fig. 3(A)). Plaques formed by CARS in L2p.176 cells under agarose were small, ragged and difficult to count. Although CPE due to CARS was subtle compared to other RCV isolates, more than 75% of L2p.176 cells inoculated with CARS contained RCV antigen when stained by IFA (Fig. 3(B)).

CARS also grew readily in cells lines which do not support growth of other RCVs, including the MHV-susceptible mouse fibroblast cell lines 3T3 and 17 Cl-1 (data not shown), confirming the earlier work of Maru and Sato (Maru and Sato, 1982)

3.2.4. Kinetics of RCV growth in L2(Percy) and L2p.176 cells

Growth kinetics of the two 'easy to grow' isolates, RCV-SDA-681 and RCV-WT in L2(Percy) cells supplemented with DEAE-D and trypsin did not differ from growth kinetics in L2p.176 cells supplemented with DEAE-D and trypsin (Fig. 4). Although growth rates of RCV-P and RCV-W in L2(Percy) cells were slower than growth of the same isolates in L2p.176 cells, final titers were similar. Cell-associated virus titers were higher than virus titers in the supernatant at early time points.

3.3. Proteins of RCV isolates

The proteins of RCV isolates grown in L2p.176 cells were similar to proteins of MHV A59 when rat polyclonal anti-RCV-SDA-681 and mouse polyclonal anti-RCV-SDA-681 antibodies were used in immunoblots (5(A) and 5(B)). Both rat and mouse antiserum primarily detected proteins of 40–55 kD, a size range likely to be forms of the

●● L2p.176 cell-associated
■■ L2p.176 supernate
⊙○ L2(Percy) cell-associated
▣▢ L2(Percy) supernate

RCV-P   **RCV-SDA-681**

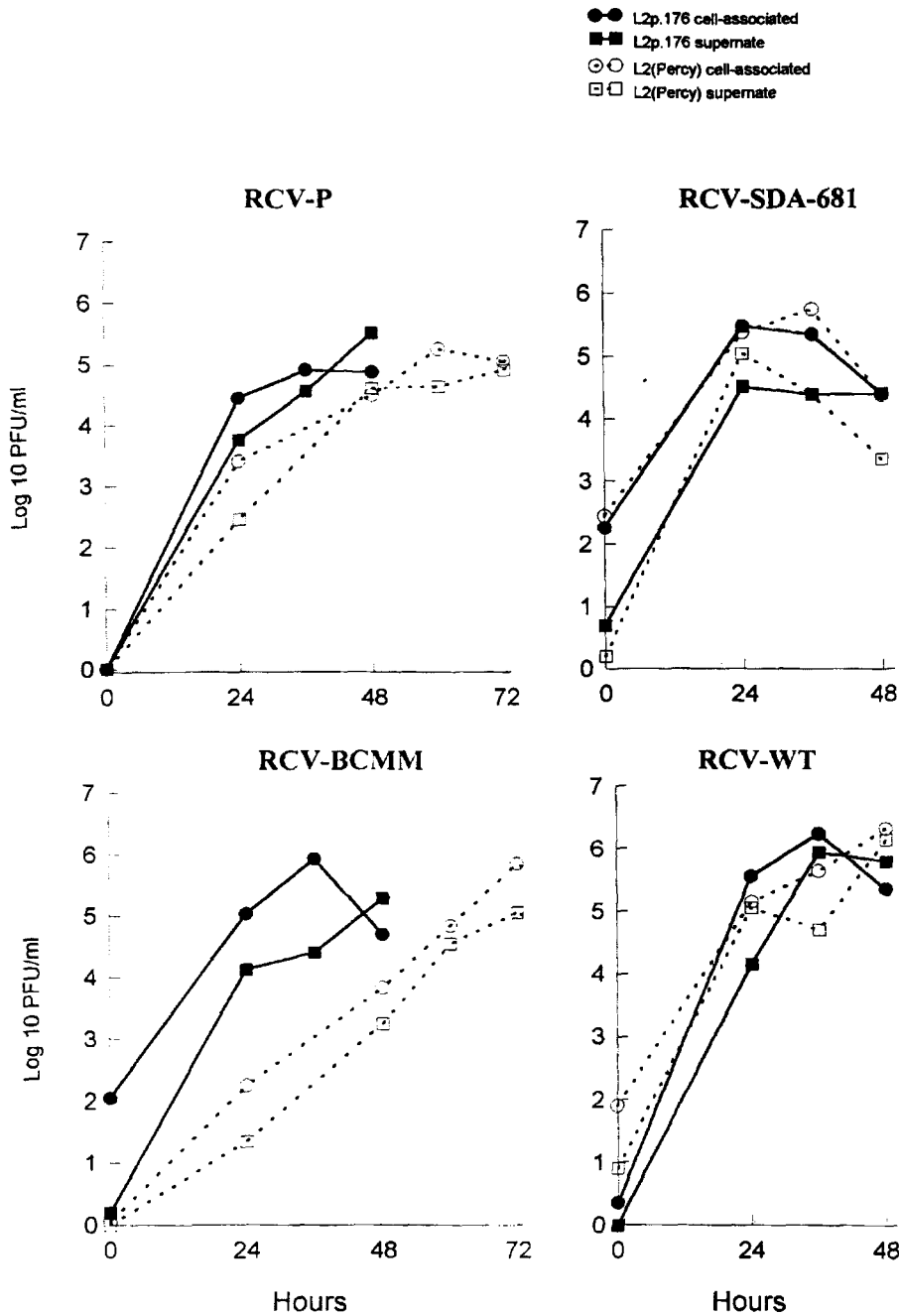**RCV-BCMM**   **RCV-WT**

Hours   Hours

Fig. 4. Growth of prototype (RCV-SDA-681 and RCV-P) and wild-type (RCV-WT and RCV-BCMM) rat coronaviruses in L2(Percy) and L2p.176 cells. Multiple wells of cells grown in a 24-well plate were inoculated with 0.01 PFU/cell of selected isolates in the form of infected mouse brain suspensions. Supernates and cells were harvested separately from two wells per virus isolate at 0, 12, 24, 36, 48, 60 and 72 h after inoculation or upon destruction of the cell sheet, whichever came first. Floating cells were removed from supernates by centrifugation and were combined with adherent cells scraped from the plastic. Samples were frozen at −70°C until titrated by plaque assay in L2p.176 cells treated with DEAE-D and trypsin.
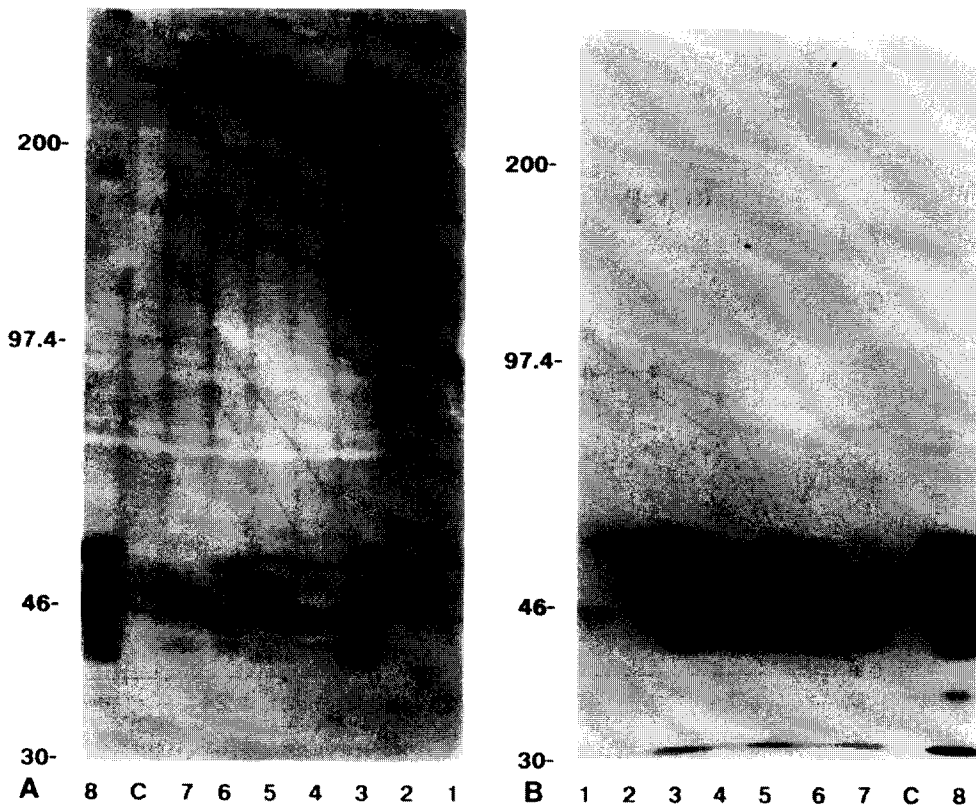
Fig. 5. Immunoblots of rat coronaviruses detected using (A) mouse anti-RCV-SDA-681 ascites antibody (B) rat anti-RCV-SDA-681 serum antibody. Viral proteins were separated by SDS-polyacrylamide gel electrophoresis in 8% acrylamide running gels with 5% stacking gels. Molecular weight markers (Rainbow markers, Amersham Life Science, Arlington Heights, IL) and uninfected L2p.176 cell lysate (lane C) were included on each gel. All samples were from infected L2p.176 cells except MHV-A59. 1 = MHV A59 virions grown in J7741.A cells; 2 = RCV-SDA-681 virions; 3 = RCV-SDA-681 lysate; 4 = RCV-P lysate; 5 = RCV-BCMM lysate; 6 = RCV-WT lysate; 7 = RCV-W lysate; 8 = CARS lysate.

N protein (Fig. 5(A) and 5(B)). The rat antiserum also detected a protein at approximately 180 kD in RCV-SDA-681 and CARS and a protein of greater than 200 kD in RCV-SDA-681, RCV-BCMM, RCV-WT, and RCV-W which are suspected to be uncleaved and multimeric S protein forms, respectively (Fig. 5(A)). Proteins greater than 55 kD were not detected using mouse anti-RCV-SDA-681.

Rabbit and goat polyclonal monospecific antibodies produced against gel-purified (denatured) proteins of MHV-A59 were used to confirm the identity of RCV proteins. The proteins, seen at 180 and > 200 kD using rat-anti-RCV-SDA-681 serum in RCV-SDA-681, RCV-BCMM, RCV-W

and CARS (lanes 3, 5, 6, 7 and 8, Fig. 5(A)) were visible using polyclonal rabbit anti-S MHV-A59 antiserum as uncleaved forms and multiples in all RCVs tested (lanes 2–8, Fig. 6(A)). Proteins visible at < 180 kD were suspected to be cleaved forms of S and were seen in varying proportions in RCV-SDA-681, RCV-BCMM, and RCV-W, as well as in MHV-A-59 (Fig. 6(A)). Each isolate had a different pattern of protein sizes and proportions recognized by anti-S antibody, with the exception of RCV-SDA-681 and RCV-WT which were similar. The cleaved S protein fragments of RCV-SDA-681, each approximately 90 kD in MHV A59, differ in size, resulting in two distinct bands (Fig. 6(A)). Thus the RCV S protein forms
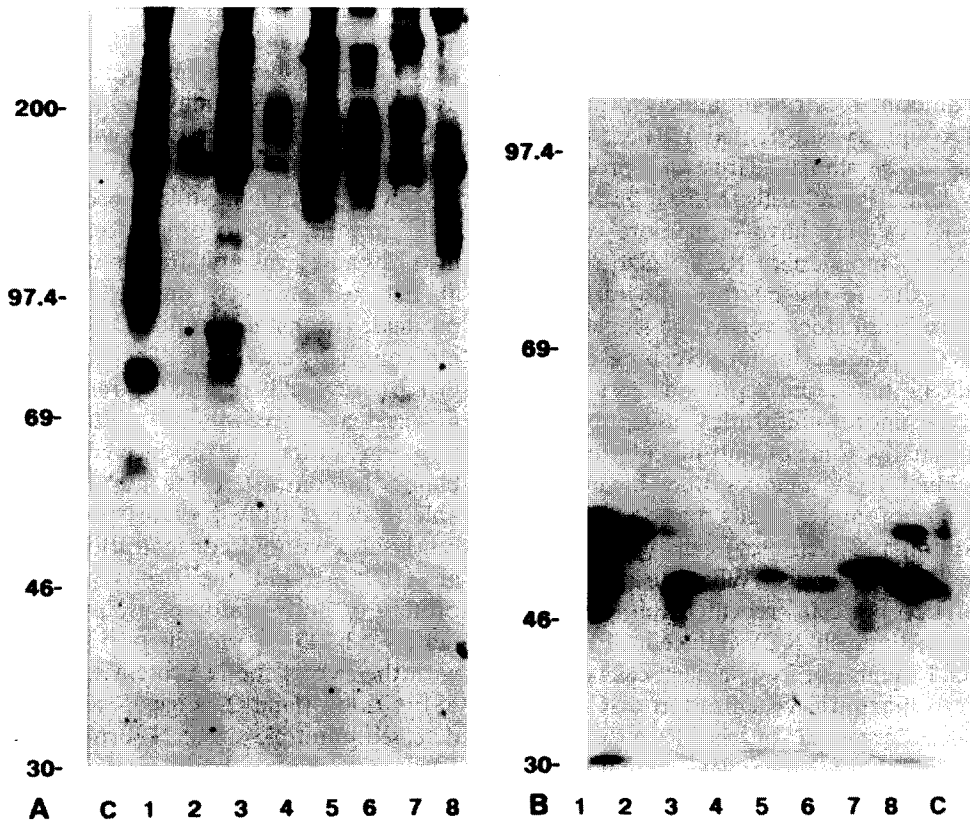
Fig. 6. Immunoblots or rat coronaviruses detected by antibodies specific for individual MHV proteins: (A) goat anti-MHV-A59 S protein serum antibody (B) rabbit anti-MHV-A59 N protein serum antibody. All lysates were from infected L2p.176 cells except MHV-A59. 1 = 1 = MHV A59 lysate grown in J774.A cells; 2 = RCV-SDA-681; 3 = RCV-SDA-681 lysate; 4 = RCV-P lysate; 5 = RCV-BCMM lysate; 6 = RCV-WT lysate; 7 = RCV-W lysate; 8 = CARS lysate.

include cleaved forms of 85–100 kD, uncleaved forms of 170–200 kD and multimers of > 200 kD, similar to the S forms seen in MHV-A59 lysate.

The protein bands ranging in size from 40–55 kD that were detected by polyclonal mouse and rat anti-RCV-SDA-681 antiserum (Fig. 5(A) and (B)) were confirmed to be multiple forms of the nucleocapsid (N) protein using rabbit anti-N MHV-A59 antiserum (Fig. 6(B)). The sizes and proportions of dominant N variants varied among RCV isolates, with the dominant N proteins of RCV-SDA-681, RCV-P and RCV-WT appearing identical in size. Thus the N protein of RCVs is the dominant protein against which antibody is generated in animals and ranges in size from 40–55 kD.

When the matrix (M) proteins were reacted with rabbit monospecific anti-M MHV A59 antibody, only MHV A59 matrix protein reacted strongly. Lanes from the six RCV isolates gave only faint bands (data not shown).

4. Discussion

Development of the L2p.176 cell line was initiated because the virus contained within individual plaques and RCV-P stocks produced minimal CPE and could not be grown to high titer in L2(Percy) cells. L2p.176 cells were shown to be superior to the parental cell line in their abilities to detect RCV stocks as higher in titer, to isolate

new RCVs from rats, and to grow all RCV isolates tested, including individual RCV plaques, to high titered stocks. Although growth curves showed the amount of virus ultimately produced by DEAE-D- and trypsin-treated L2(Percy) and L2p.176 cells to be similar, virus was produced more slowly in L2(Percy) cells. Mouse brain suspensions of RCVs may have been more readily cultivable than individual RCV plaques in L2(Percy) cells due to the mixture of viral types within a mouse brain stock versus the single viral type in an RCV plaque. In our screening of cell clones it is likely that we have propagated a clone of a cell type more uniformly susceptible to RCVs. Additionally, some of our difficulty propagating individual RCV plaques may have been due to the use of more highly passed cultures (> 80 generations) of L2(Percy) cells while using low passage L2p.176 cells. When using either L2(Percy) or L2p.176 cells, repeated cell line passage tended to diminish RCV-induced CPE and we recommend the use of low passage cells (< 30 generations) to maximize visualization of CPE.

L2p.176 cells grew all of the RCV isolates tested, including the Japanese RCV CARS, enabling us to demonstrate that CARS proteins are closely related to the proteins of other RCV isolates and to MHV A59. CARS, isolated in 1982 in Japan by Maru and Sato, is less well studied than the two American prototype isolates, RCV-SDAV-681 (sialodacryoadenitis virus) and RCV-P (Parker's rat coronavirus), and had been passed 95 times in cell culture prior to arrival in our laboratory. CARS differs from other RCVs studied in its cell tropism in vitro, its apparent inability to induce large syncytia formation and its failure to produce the typical pneumotropic infection associated with RCVs when inoculated parenterally into infant mice. In vitro cell tropism, syncytia formation and pneumotropism may have changed as a result of multiple cell culture passages. However, the cross reactivity of CARS with other RCVs by immunofluorescence and the similarity of CARS proteins to those of MHV and RCVs confirm that it is closely related to these coronaviruses. Furthermore, the recognition of CARS peplomeric S protein by rat anti-RCV antiserum confirms its likely identity as an RCV

strain rather than as an MHV strain. Because CARS forms ill-defined plaques on L2p.176 cells, generation of plaque-cloned CARS stocks and it's use in future studies may be hindered.

Our results are compatible with those of Barker et al. (1994) although the protein band patterns we saw are not identical to those seen in his study. This may be due to differences in viral passage histories and virus preparation methods. Barker et al. detected primarily the M and N proteins of purified virions of RCV-SDA-681 and RCV-P grown in L2(Percy) cells. Their use of virus grown in L2(Percy) cells resulted in relatively low-titered material and necessitated multiple concentration steps in order to produce detectable protein bands. In contrast, using L2p.176 cells, we were able to detect RCV proteins routinely in infected cell lysates without concentration steps. Barker et al. (1994) found the primary RCV virion N protein of both prototype isolates to be approximately 53 kD. A protein of 53 kD is consistent with the size of the N protein seen in RCV-SDA-681 virions (lane 2, Figs. 5 and 6). They also detected multiple-sized N-forms, similar to the forms we saw in both virion and infected-cell preparations and indicated that they are due to alternative phosphorylation sites. In Barker's studies, a band of 100 kD, detected with polyclonal antibody was postulated, but not confirmed to be monomeric RCV S protein. The authors attributed their difficultly in detecting the S protein to it's loss during purification steps. We readily detected the S protein using goat antiserum against the S protein of MHV-A59.

Both polyclonal RCV antibodies and monospecific MHV antibodies specific for the S and N proteins reacted strongly with all RCV isolates tested, illustrating that RCVs, like MHVs, have N proteins (40–55 kD) and monomers and multimers of the S protein (< 95, 180 and > 200 kD) which are closely related both to each other and to MHV. However, each RCV isolate tested had a unique pattern of S and N form sizes and proportions, with the exception of RCV-SDA-681 and RCV-WT which appear identical on all four blots, indicating that the RCV isolates studied are distinct. Using both polyclonal mouse antibody and monospecific rabbit anti-N antibody, RCV-SDA-

681 lysate and RCV-SDA-681 virions contained two dominant N forms migrating at approximately 55 and 46 kD. The proportions of these two forms are reversed in the infected-cell lysate compared to the virion preparation. Further investigations, including neutralization testing, RNA sequencing and pathogenetic studies in rats will be needed to determine if differences and similarities in proteins are related to differences in tissue tropisms and pathogenicity (Jacoby, 1986).

The development of the L2p.176 cell line should lead to a clearer consensus regarding the biology of RCV isolates, since RCVs can now be consistently isolated from rats, plaque-purified stocks can be generated and RCVs can easily be grown as high titer stocks to study RCV proteins and RNAs. Future studies using RCVs grown in L2p.176 cells should clarify the similarities and differences among RCV isolates.

Acknowledgements

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