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Development and optimization of plaque assays for rat coronaviruses

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Summary

Plaque assays under Sephadex or agarose overlays are described for rat coronaviruses (RCVs) grown in L2 mouse fibroblasts. A plaque assay using Sephadex was simple; however, viable plaques could not be collected for propagation, and fixation was necessary before evaluation. Plaque formation under agarose was optimized using diethylaminoethyl-dextran (DEAE-D) in the pre-treatment and absorption media and trypsin added to the absorption media and agarose overlay. The use of DEAE-D alone, trypsin alone or trypsin combined with DEAE-D significantly increased plaque numbers and visibility. Plaque numbers were highest when pre-treatment media contained DEAE-D, absorption media contained DEAE-D and trypsin, and the agarose overlay contained trypsin. The assay was useful for plaque isolation and quantification of sialodacryoadenitis virus (SDA), Parker's rat coronavirus (PRCV) and other coronavirus isolates from rats and its specificity was demonstrated by plaque-reduction neutralization testing. These methods will facilitate production of cloned virus stocks for study of RCV biology and virus quantification for in vitro and in vivo studies of RCVs.

Sialodacryoadenitis virus; Rat coronavirus; Plaque assay; DEAE-D; Trypsin; Agarose; Sephadex

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Introduction

Rat coronaviruses (RCVs) infect laboratory rats at high prevalence and interfere with research using rats (Jacoby, 1986). Relatively little is known about the basic biology and biochemistry of these viruses, largely due to their fastidious growth requirements in vitro. Recent improvements in methods for cultivation of RCVs, including the use of the L2 mouse fibroblast cell line (Percy, 1989; Percy, 1990) supplemented with trypsin and diethylaminoethyl-dextran (DEAE-D) (Gaertner, 1991), allow growth of virus to high titer and will facilitate study of RCV biology. Ideally, studies of RCV biology should be undertaken using plaque purified virus stocks and virus should be enumerated using a precise method such as plaque assay. However, plaque assays for RCVs have only been reported in LBC cells (Hirano, 1990) which grow slowly and support only limited replication of North American RCV isolates (Gaertner, 1991; Percy, 1989).

Plaque assays using the L2 mouse fibroblast cell line with Sephadex or agarose overlays were developed for RCVs and are useful for RCV isolates including sialodacryoadenitis virus (SDAV), Parker's rat coronavirus (PRCV) and 'field' isolates. Concentrations of DEAE-D and trypsin in media and overlays were optimized to increase plaque counts while minimizing toxicity to the cell sheet. The specificity of the plaque assay was demonstrated by plaque-reduction neutralization testing (PRNT) of SDAV strain 681.

Materials and Methods

Cells and media

A subline of L2 cells obtained from Dean Percy (Department of Pathology, Ontario Veterinary College, Guelph, Canada) was grown on 25 or 75 cm^2 polystyrene flasks and 6 or 24 well polystyrene plates (Corning Glassworks, Corning, NY, USA) at 37°C in a humidified 5% CO_2 atmosphere. L2 medium contained 50% Eagle's minimal essential medium, 45% Hanks' minimal essential medium, 5% fetal bovine serum (FBS), 100 μg/ml streptomycin and 100 U/ml penicillin. All media and supplements were purchased from Gibco BRL (Gaithersburg, MD, USA), unless otherwise noted. Cells were transferred when confluent using 0.12% porcine parvovirus-free, Mycoplasma-free trypsin (Gibco BRL) and 0.27 mM EDTA. L2 cells were tested for Mycoplasma spp. and were culture-negative.

Virus

Stocks of SDAV, strain 681 (SDAV-681), and Parker's rat coronavirus (PRCV) were prepared in infant mouse brain (Bhatt, 1977A; Bhatt, 1977B). Titration of SDAV-681 and PRCV stocks in primary rat kidney cells (Bhatt,

1977A; Bhatt, 1977B) yielded titers of $10^{6.5}$ and $10^{7.0}$ $TCID_{50}$ per ml, respectively.

A field isolate, designated SDAV-WT, was obtained by co-housing two athymic rnu/rnu rats with outbred SD rats from an endemically RCV-infected facility immediately after arrival of the SD rats (Paturzo, 1987). The athymic rats were euthanatized 15 days after co-housing and lung and salivary glands were collected and frozen at $-70°C$. A 10% submaxillary gland homogenate was prepared from frozen salivary gland and was used to inoculate both pathogen-free SD rats (oronasal inoculation) and 2-day-old Sencar mouse pups (IC inoculation). The concentration of virus in the tissue homogenates was not determined. Mouse brain stocks and lung and salivary gland homogenates from inoculated SD rats were used in the studies described here.

Animals

Cr:ORL Sencar mouse dams with 1-day-old litters, pathogen-free Sprague-Dawley (SD) rats and athymic rats (rnu/rnu) were purchased (Animal Genetics and Production Branch, National Institutes of Health, Bethesda, MD, USA) and housed under conditions which met or exceeded standards of the U.S. Public Health Service. Four-week-old SD rats were purchased from a specified vendor area (Charles River Wilmington, Area A-41).

Plaque assays

Sephadex overlay Sephadex was used to plaque RCVs following published methods (Smith, 1977). Dry powdered Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) was heated to 180°C in a sterile glass bottle for 60 min. A 2% weight/volume Sephadex suspension was prepared in L2 medium without FBS and was held overnight at 4°C to permit the Sephadex to swell. The virus stock to be inoculated was diluted in L2 medium and held on ice. For each virus dilution, three or more replicate 2.0 cm^2 wells of L2 cells were exposed to 0.3 ml of the inoculum per well for one hour at 37°C. The inoculum was aspirated and the monolayer was washed with sterile saline. Trypsin (2.5 $\mu g/ml$) was added to the Sephadex slurry. The slurry was agitated and one ml was added per well. Cultures were incubated undisturbed at 37°C with 5% CO_2 until fixed 1–5 days after inoculation. For fixation, 1 ml of 5% glutaraldehyde was added per well at 20°C for 1 h. The Sephadex-medium slurry was removed and the cells were stained with Giemsa (J.T. Baker, Phillipsburg, NJ, USA). Plates were rinsed and air-dried, plaques were counted and titers were expressed as plaque-forming units (PFU)/ml (Lorenz and Bogel, 1973).

Agarose overlay A 1% suspension of Sea Plaque agarose (American Bioanalytical, Natick, MA, USA) in sterile demineralized distilled water (Tissue culture water, Sigma Chemical Co., St. Louis, MO, USA) was autoclaved for 15 min at 121°C and allowed to solidify. L2 cells were seeded

onto 6 well plates (6 × 10^5 cells/9.6 cm^2 well) and the outer 16 wells of 24-well plates (2.5 × 10^5 cells/2.0 cm^2 well) 3 days prior to virus inoculation. Virus in mouse brain stock or isolated RCV plaques was diluted and inoculated as described above for Sephadex overlays unless noted otherwise below. Trypsin and/or DEAE-D (Pharmacia) were added to pre-absorption media, absorption media and agarose overlays as described below.

To prepare the overlay, agarose was microwaved until boiling and held in a 37°C waterbath until used. The nutrient mixture was prepared on the day of use and each 100 ml of nutrient mixture contained: 20 ml Eagle basal medium (10 ×), 2 ml l-glutamine 200 mM, 4.5 ml NaHCO$_3$ 7.5%, 40 000 units penicillin-streptomycin, 1.5 ml Hepes buffer (1 M) (ICN Biomedicals, Inc, Costa Mesa, CA, USA) and 68 ml sterile demineralized distilled water (Sigma). Nutrient mix at 37°C was combined with an equal volume of 1% agarose. The pH was adjusted to 7.5 by adding Hepes buffer or NaHCO$_3$, if needed, and trypsin was added immediately before cells were overlayed.

After absorption, cells were rinsed three times with phosphate buffered saline (PBS). Three ml (6-well plates) or 0.90 ml (24-well plates) of agarose-nutrient mixture were added to inoculated cells and the agarose was allowed to solidify undisturbed at 20°C. Plates were incubated at 37°C in a 5% CO$_2$ atmosphere. In order to maximize diffusion of CO$_2$ and minimize changes in pH, only the outer wells of 24-well plates were used and plates were not stacked in the incubator. L2 cells under agarose were observed daily and plaques were collected or cells were fixed when plaque formation was maximal. For fixation, 10% buffered formalin, equal in volume to the agarose, was added to each well for 24 h at 20°C. Formalin was replaced with an equal volume of PBS for 24 h, the PBS was removed and the agarose was melted in a microwave set to 30% power for 1 min. The cell sheet was rinsed once with PBS and stained with Giemsa. Plaques were counted and titers were expressed in PFU per ml, as described for Sephadex overlays.

Optimization of the agarose plaque assay

Overlay composition Final agarose concentrations from 0.3% to 1% were tested for maximal plaque formation and visibility. Since acidification of the overlay in the 5% CO$_2$ atmosphere killed the cell monolayer, two buffers, Hepes (1.2 mg/ml to 2.98 mg/ml final concentration in agarose) and TES (3.4 mg/ml to 8.6 mg/ml) (Sigma) were tested to find a buffer concentration tolerated by L2 cells. FBS (0.06–5%) and a synthetic cell supplement (0.06–1.0% Nutridoma SR, Boehringer Mannheim, Indianapolis, IN, USA) were added to overlays in an attempt to increase cell viability.

Trypsin concentration and inclusion in absorption medium and/or overlay
Trypsin was added to the absorption medium and/or the agarose overlay for incubation. Trypsin concentrations from 0.016 μg/ml to 40 μg/ml were added to the absorption medium and/or the agarose overlay to determine what

concentration of trypsin maximized formation and visualization of plaques without toxicity to the cells. After inoculation with equal concentrations of virus in diluent with or without trypsin, L2 cells were overlayed with agarose with or without trypsin.

DEAE-D pre-treatment of cells and inclusion of DEAE-D during absorption DEAE-D was added to pre-treatment media and absorption media. Concentrations of 50 to 200 μg/ml of DEAE-D were added to cultures inoculated with equal concentrations of virus. The ideal duration of DEAE-D pre-treatment was tested by treating L2 cells with 100 μg/ml of DEAE-D for 1–4 hours prior to absorption.

Methods for plaque visualization Concentrations of neutral red from 2–10% added to a second agarose overlay were tested for enhancement of plaque visualization. Three days after virus inoculation, a volume of neutral red-containing agarose overlay equal to the initial overlay volume was added and allowed to cool. Cell monolayers were also fixed and stained with Giemsa as described above, for comparison.

Propagation of individual plaques

To collect individual plaques for propagation, diluted virus stocks were inoculated into L2 cells grown in 24 well plates and cells were overlayed with agarose containing trypsin (1.25 μg/ml). Three days after inoculation of L2 cells, wells containing single discrete plaques were identified under a dissecting microscope and the plaque location was marked on the bottom of the plastic well using an indelible marker. Each plaque was aspirated into 0.5 ml of FBS using a Pasteur pipette and placed in a 1 ml plastic screw-top tube. Plaques were frozen at −70°C until used.

Plaque reduction neutralization testing (PRNT)

Individual sera from naturally infected, otherwise pathogen-free SD rats (Camm, Wayne, NJ, USA) were diluted 1:10, tested for RCV antibody by indirect immunofluorescence (IFA) (Smith, 1983). High titered sera were pooled, filtered (0.45 μm filter, Millipore, Bedford, MA, USA) and heat-treated for 20 min at 60°C. Pooled antibody had an IFA titer of 1:320.

Normal rat serum (NRS) was collected from specific pathogen-free rats, treated as described above, and did not react with coronavirus antigen by IFA.

The PRNT method was modified from a published method (Schmidt, 1979). For PRNT testing, two-fold dilutions (1:10 to 1:640) of RCV antiserum were made in L2 medium without FBS and held at 4°C until use. Stock SDAV-681 was diluted in L2 medium to contain approximately 100 PFU in 0.2 ml after 1 h at 37°C. RCV antiserum dilutions or NRS (1:10) were mixed with equal volumes of diluted virus and incubated in a 37°C waterbath for 1 h at 37°C. L2

TABLE 1

Comparative titration of mouse brain stocks of rat coronaviruses (per ml of stock).

Method	SDAV-681[b]	PRCV[c]
Infant mouse inoculation (IC)	$10^{6.4}$ LD_{50}	$10^{7.0}$ LD_{50}
Primary rat kidney cells	$10^{6.5}$ $TCID_{50}$	$10^{7.0}$ $TCID_{50}$
L2 cells with trypsin absorption and incubation (no overlay)	$10^{4.5}$ $TCID_{50}$	$10^{6.3}$ $TCID_{50}$
Sephadex overlay with trypsin absorption and incubation[a]	$10^{3.6}$ PFU	$10^{4.2}$ PFU
Agarose overlay trypsin in absorption and overlay	$10^{4.4}$ PFU	$10^{4.2}$ PFU
Agarose overlay DEAE-D pre-treatment DEAE-D and trypsin absorption trypsin in overlay	$10^{6.6}$ PFU	$10^{6.9}$ PFU

[a]Sephadex plates read at 96 h, all other plates read at 72 h.
[b]Sialodacryoadenitis virus, strain 681.
[c]Parker's rat coronavirus.

cells grown in 6-well plates were rinsed with PBS and inoculated with 0.4 ml/ well of serum-virus mixtures. Trypsin was included in the absorption media (1.25 μg/ml). Virus was absorbed for 1 h at 37°C. L2 cells were washed three times with PBS and overlayed with agarose containing trypsin (1.25 μg/ml). Cells were observed daily, fixed 48 h after inoculation, stained with Giemsa and plaques were counted.
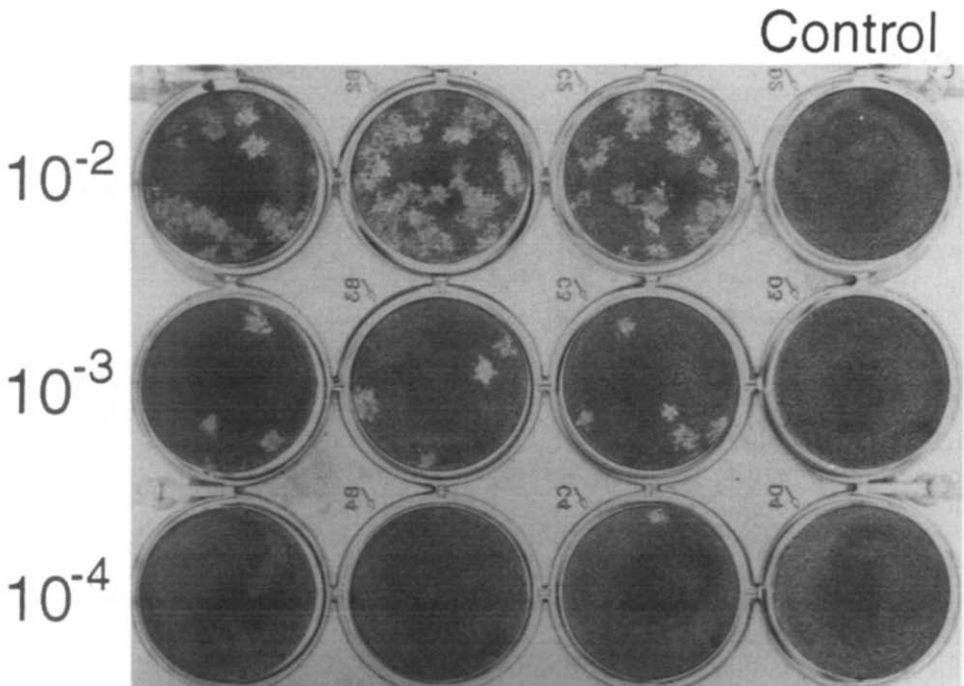

Fig. 1. Three replicates of L2 cells inoculated with 10^{-2} to 10^{-4} dilutions of SDAV-681 in trypsin-supplemented absorption media (1.25 μg/ml) and overlaid with Sephadex (1.25 μg/ml). 96 h after inoculation, stained with Giemsa and dried.

Results

Sephadex overlays yielded plaque formation by SDAV-681 and PRCV (Table 1). Plaques were most visible when trypsin (1.25–2.50 μg/ml) was included in the absorption media and cells were fixed 3–4 days after virus inoculation (Fig. 1). This technique was easy, readily reproducible, and provided a permanent record; however, cells had to be fixed prior to counting plaques and the method was not suitable for plaque cloning.

Using agarose overlays, optimally visible plaques formed using a final agarose concentration of 0.5% (Fig. 2). Lower agarose concentrations did not solidify and higher agarose concentrations resulted in the formation of syncytia which remained on the plastic and did not peel back to form a clear area. Concentrations of trypsin higher than 6.25 μg/ml in the absorption media or 2.52 μg/ml in the agarose overlay caused L2 cells to detach from the plastic during incubation. Plaques were much smaller and were less numerous when agarose overlays did not contain trypsin (Table 1 and Fig. 3). Trypsin was not used in pre-treatment media. Recently confluent L2 cells yielded best results, as
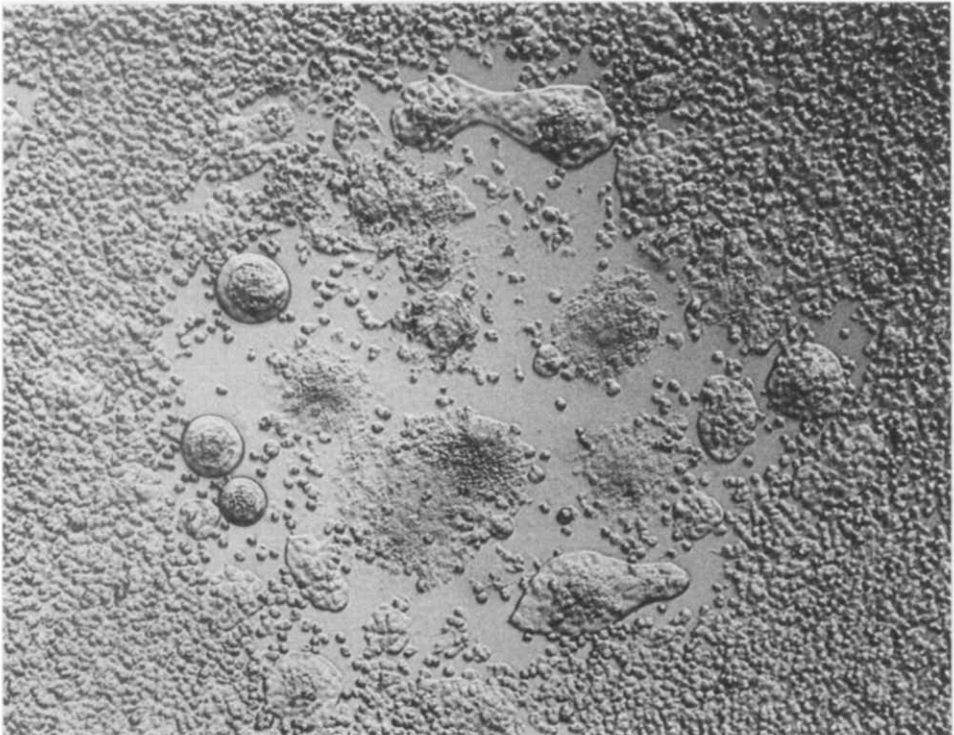

Fig. 2. Photomicrograph of a focus of cytopathic effect due to SDAV-681 forming in a monolayer of L2 cells. Trypsin was added to the absorption media (1.25 μg/ml) and agarose overlay (1.25 μg/ml). 48 h after inoculation, unstained.
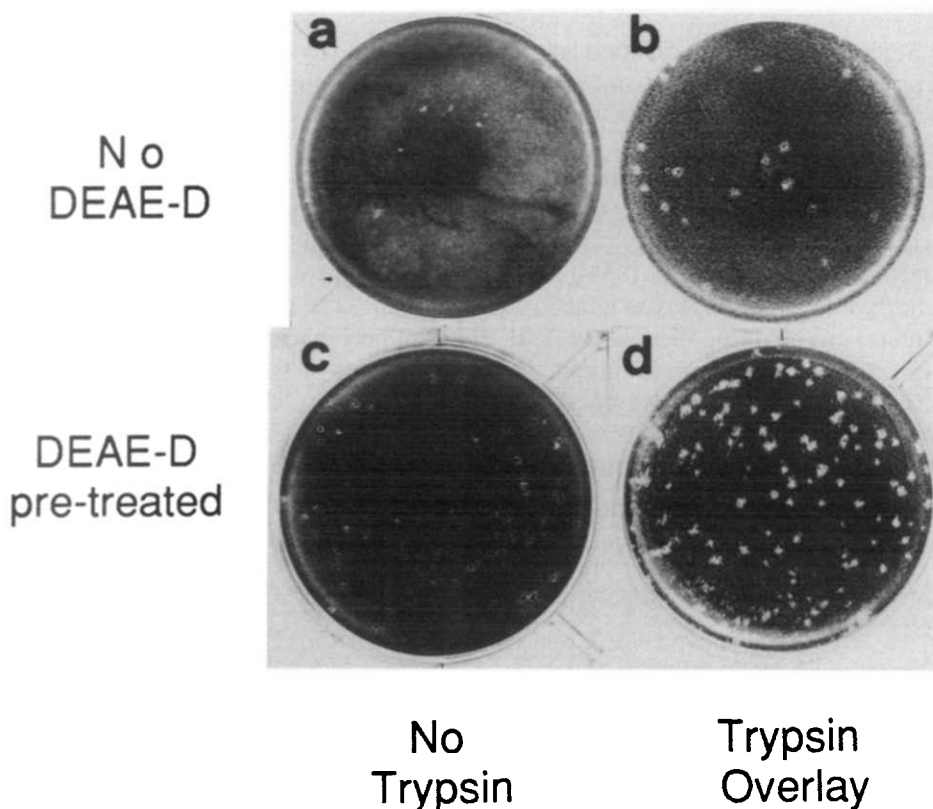
Fig. 3. Effect of DEAE-D pre-treatment (75 μg/ml) (c,d) and trypsin supplementation (1.25 μg/ml) of the agarose overlay (b,d) on plaque size and number. SDAV-inoculated L2 cells were overlayed with agarose. All inocula contained trypsin (6.25 μg/ml) during absorption. L2 cells were fixed 72 h after inoculation and stained with Giemsa.

heavily confluent cells tended to detach from the plastic and sub-confluent cells yielded indistinct plaques.

Hepes buffer (1.8 mg/ml) was well tolerated by L2 cells and reduced acidification of the agarose overlay during incubation. TES buffer was more toxic than Hepes and was not used.

The addition of FBS (0.06–5%) to the overlay reduced plaque size and visibility. Fusion of adjacent cells appeared reduced and cells on the periphery grew in to fill defects in the monolayer, rapidly obliterating plaques. Since serum contains trypsin inhibitors (Graham and Estes, 1980), higher trypsin concentrations (>1.25 μg/ml) were added to the overlay in an attempt to eliminate this effect. However, the addition of more trypsin increased cell detachment from the plastic and did not restore plaque visibility.

Nutridoma, a synthetic cell supplement, was added to the overlay in an attempt to improve cell viability without neutralizing trypsin. Concentrations

TABLE 2

Number of plaques (PFU/ml) detected by different methods in L2 cells inoculated with equal amounts of SDAV-681 (6 replicates per group).

	Overlay – no trypsin		Trypsin[b] overlay (0.41 μg/ml)	
	Absorption media		Absorption media	
No DEAE-D:	no trypsin 10.5 $\pm$ 3.7[e]	trypsin[b] (6.25 μg/ml) 12.5 $\pm$ 4.0	no trypsin 16.5 $\pm$ 3.3	trypsin (6.25 μg/ml) 17.2 $\pm$ 4.1
DEAE-D[a,b]: (75 μg/ml)	no trypsin 47.5 $\pm$ 11.2	trypsin (6.25 μg/ml) 79.7 $\pm$ 18.3	no trypsin 100.3 $\pm$ 24.4	trypsin (6.25 μg/ml) 139.5 $\pm$ 22.7

[a]pre-absorption for 3 h.
[b]effects of all three factors were statistically significant ($P \leqslant 0.0001$) when analyzed by 3-way analysis of variance (Systat, V 4.0, 1988, Systat, Inc. Evanston, Illinois).

of Nutridoma from 0.06% to 1% did not improve plaque visibility or cell viability.

Inclusion of DEAE-D pre-treatment, trypsin in the absorption media or trypsin in the overlay increased the number of plaques formed ($P < 0.0001$) (Table 2). Inclusion of trypsin in the absorption media or the overlay, but not DEAE-D (in the pre-absorption media and the absorption media) increased plaque size and visibility (Fig. 3). A trypsin concentration of 1.25 μg/ml in the overlay was optimal when cells were not treated with DEAE-D. Likewise, a trypsin concentration of 2.5 μg/ml in absorption media was well tolerated in combination with 1.25 μg/ml trypsin in the overlay when DEAE-D was not used. Trypsin concentration in the absorption media and the overlay was reduced (0.63 μg/ml) when used with DEAE-D pre-treatment. If used without trypsin, 100 mg/ml of DEAE-D was well tolerated. The concentration of DEAE-D was reduced (75 mg/ml) when used with a trypsin-containing overlay. A 3-h pre-absorption period with DEAE-D maximized the number of plaques seen (data not shown).

A 5% neutral red-containing second overlay allowed plaque visualization 12 h later while retaining virus viability in the plaque. A neutral red-containing second overlay allowed rapid enumeration of plaque numbers without waiting for cells to fix or stain. Concentrations of neutral red higher than 5% were rapidly toxic. However, neutral red was not needed to locate and aspirate viable plaques. Formalin fixation followed by Giemsa staining (Fig. 3) was simple and yielded a permanent record.

First, second and third passage plaques were successfully isolated from mouse brain stocks of SDAV-681 and PRCV and tissue homogenates of SDAV-WT. Individual plaques routinely yielded 50-125 new plaques when passaged using trypsin-containing agarose overlays without DEAE-D pre-treatment.

As demonstrated by PRNT results (Fig. 4), the plaque assay was specific with more plaques observed at higher dilutions of antiserum. Plaques were not
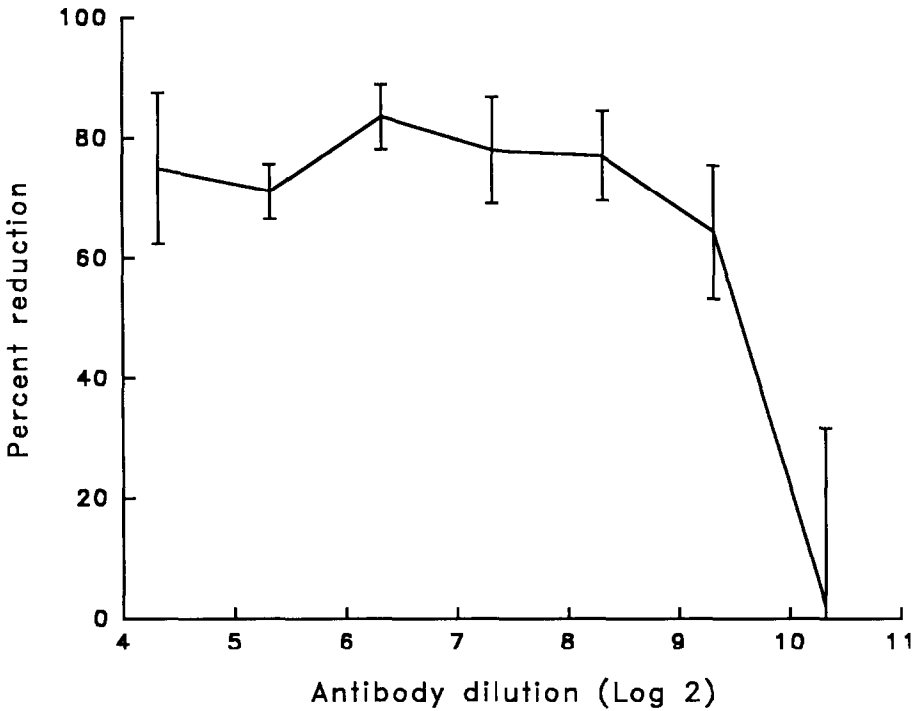
Fig. 4. Reduction of plaque numbers (PFU/ml of stock SDAV-681) by dilutions of SDAV-681 antibody. Each well was inoculated with a volume and dilution calculated to produce 100 PFU after incubation for 1 h at 37°C.

seen in the absence of inoculated virus. Anti-SDAV-681 serum dilutions as high as 1:320 neutralized more than 70% of plaques induced by stock SDAV-681.

Discussion

The use of DEAE-D and trypsin amplified growth of RCVs and increased the sensitivity of plaque assays using Sephadex and agarose. When both treatments (DEAE-D and trypsin) were combined, supplement concentrations had to be reduced because both DEAE-D and trypsin were somewhat toxic to L2 cells. DEAE-D had its most dramatic effect on plaque numbers, increasing the detectable number of RCV-infected foci by approximately five-fold. Used alone, DEAE-D pre-treatment for 3 hours followed by 30 min of absorption was optimal. When used with a trypsin-containing overlay, DEAE-D concentration had to be reduced. Treatment with DEAE-D increases viral penetration of cells via mechanisms not related to receptors (Bailey, 1984). Although viral titers obtained by different methods are not directly comparable, the use of DEAE-D and trypsin supplementation of L2 cells

resulted in titers equivalent to infant mouse inoculation.

Trypsin in the overlay had its most dramatic effect on plaque visibility, although plaque numbers were also increased. Used alone, a trypsin concentration of 2.5 μg/ml in the absorption media for 30 min and 1.25 μg/ml in the overlay was optimal. When L2 cells were pre-treated and absorbed with DEAE-D-containing medium, the trypsin concentration had to be reduced to 0.41 μg/ml. Because FBS inactivates trypsin, inocula containing more than 10% FBS were absorbed using a higher trypsin concentration (2.50–6.25 μg/ml). Reduced concentrations of trypsin (1.25 μg/ml) were used in the overlay after the FBS-containing inoculum was removed and the cell sheet was rinsed (data not shown). Trypsin treatment yielded larger, clearer, more easily countable plaques, probably by facilitating cell fusion through trypsin-cleavage of the viral S-glycoprotein (Storz, 1981; Sturman, 1985).

Critical factors in the success of the agarose plaque assay included pH control and a low agarose concentration. Control of pH within a narrow range (approximately 7.3 to 7.8) during incubation was crucial, since L2 cells died if pH fell outside this range. A constant-pH water source, such as commercially available deionized double distilled water, allowed consistency in the overlay pH between experiments.

Plaque assays for RCVs grown in LBC cells have been reported by others (Hirano, 1990); however, we have had little success growing North American RCV isolates in LBC cells. The RCV isolates used in the earlier study had been LBC-adapted by 20-30 passages in LBC cells (Hirano, 1990). Adaptation of RCV isolates may account for the differences in the successful use of LBC cells, including the large plaque sizes reported without the use of trypsin. L2 cells can be used to grow RCV isolates without adaptation by passage, as illustrated by their use for isolation (Gaertner, 1991) and for plaque assay of the field isolate, SDAV-WT, shown in this paper.

The development of plaque techniques for RCVs will facilitate plaque purification and production of plaque cloned stocks for future studies and will allow more exact enumeration for in vitro and in vivo studies of RCVs.

Acknowledgement

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