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Regulation of viral persistence in human glioblastoma and rhabdomyosarcoma cells infected with coronavirus OC43

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Cultures of human rhabdomyosarcoma (RD) and human glioblastoma (U87-MG) were compared for their ability to sustain a persistent infection with coronavirus OC43. Within 28 days, infectious virus and hemagglutinin were being produced at high levels in both types of cells. Temperature sensitive plaque variants were recovered at 31°C. In both cell types, the virus caused increased antigen synthesis and cell death, if the temperature was lowered to 31°C. Infectious virus was lost if cells were treated with antiserum to whole virus or if the temperature was raised to 39.5°C. Probing the cured cells with OC43-specific ^{32}P-cDNA showed that cured cells contained no detectable viral RNA. The relative ease of establishment and cure of these persistent infectious makes them attractive as models to study coronavirus regulatory processes.

Key words: coronaviruses; persistence; cDNA probes; neurotropism.

Introduction

OC43 is a human respiratory virus of the family *Coronaviridae*. It was originally isolated in human tracheal organ cultures[1] and then adapted to grow in suckling mice. Although clinical isolation from patient material requires the use of organ cultures, a number of human cell lines can be productively infected with OC43 virus and a plaque assay has been developed.[2] In infected mice age-related susceptibility to the virus is displayed and there is a selective vulnerability of neuronal cells.[3] Infected mouse neuronal cells in culture display differential susceptibility, a characteristic feature for all coronavirus infections. Neurons derived from dorsal root ganglia produced viral antigen and infectious viruses. Astrocytes and fibroblasts derived from mouse embryo brain produced viral antigen but not infectious virus. Oligodendrocytes derived from *corpus collosum* of 3–4 day old mice produced neither viral antigen nor infectious virus. Preliminary work on the susceptibility of human neuronal cells to OC43 virus showed that infected human embryo brain cells produced viral antigen but no infectious virus.[4]

Since cells cultured from human tumors of differentiated cell origin retain some of the characteristics of their untransformed counterparts, tumor cells of neural origin showing neuronal cell properties offered an easily accessible avenue to the study of the neurotropism of OC43 virus in human cells. Such studies are of interest in view of a still unconfirmed report of isolation of coronaviruses from brain cells of two multiple sclerosis patients.[5] Using a strain of OC43 virus which was adapted to give high yields of infectious virus, we undertook a comparative study of the replication and persistence

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of OC43 virus in two cell lines; U87-MG cells of human glioblastoma origin which were relatively insusceptible to virus infection and RD cells from a human rhabdomyosarcoma which were highly susceptible to virus infection. Initially, we set out to establish persistent infections in these cells, observe how virus replication is regulated and attempt to cure the cells of their virus infection. Another aim was to probe the cured cells to detect residual copies of viral genome and mRNA using an OC43 complementary DNA probe.

Results

Initiation of persistent infections

Susceptibility of RD and U87-MG cells to infection with OC43 virus was determined by measuring a single-cycle yield of virus. RD cells infected at a multiplicity of infection (m.o.i.) of four produced approximately 30 plaque forming units (PFU) per cell at 12 hours while U87-MG cells produced only one PFU per cell under the same conditions. In order to initiate a persistent infection, RD and U87-MG were infected at an m.o.i. of eight and maintained at 37°C. Samples for assay of infectious virus and viral hemagglutinin were taken immediately before each passage which was usually after 7–10 days. Microscopically, no syncytia were evident in these cultures. Infected cells were found to detach from the surface and float in the culture medium to be replaced by dividing cells in the culture. Figure 1 shows the PFU and hemagglutinating units (HAU) titers of virus recovered from the persistently infected cultures. In persistently infected RD cultures (PI-RD), after an initial half-log drop in PFU, a rise to 6×10^8 PFU was observed at 28 days and virus production continued at this level throughout the period of observation. Hemagglutinin was detected sporadically but its appearance did not correlate with a sharp drop in infectious virus production such as occurs when defective interfering virus particles are produced. Persistently infected U87-MG cells (PI-U87-MG) initially produced tenfold less virus than RD cells but at

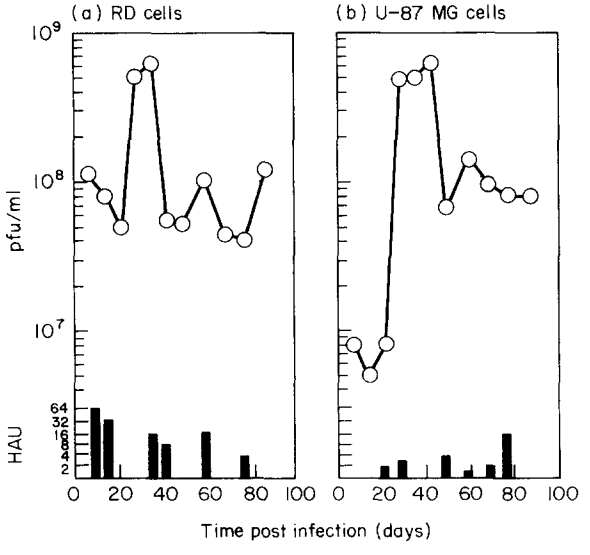
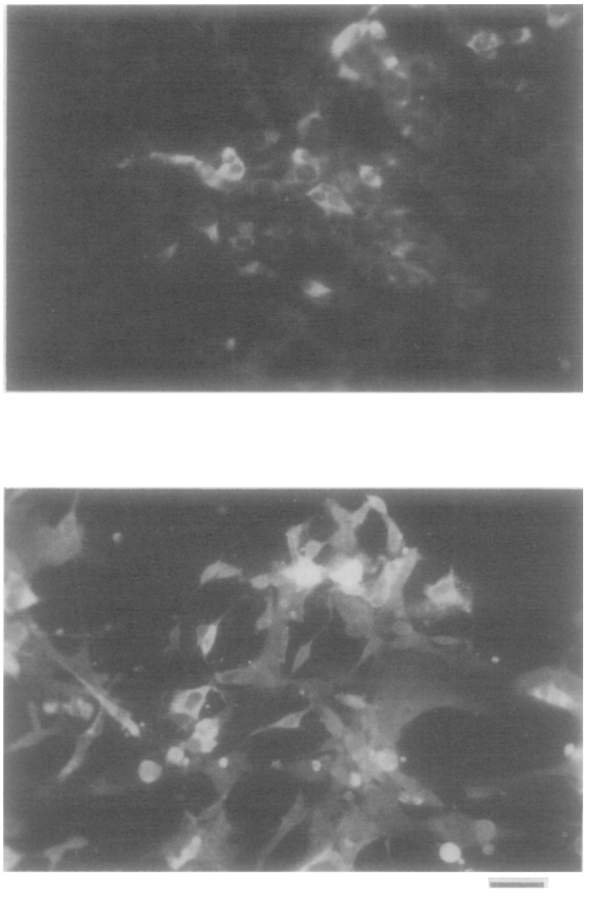


Fig. 1. Samples of supernatant medium were taken every 7–10 days and stored at −70°C until assayed for infections virus and hemagglutinin. Lower bar graph indicates hemagglutinating units (HAU) per 0.025 ml supernatant medium using human O erythrocytes. Open circles, O——O, indicate PFU/ml of OC43 virus recovered from the supernatant medium when assayed on RD cell monolayers at 37°C.

28 days an abrupt rise occurred and subsequent PFU titers equal to those of PI-RD cells were observed. Hemagglutinin was also detected sporadically in the culture medium as observed with PI-RD cells.

Characterisation of the persistent infections

Several experiments were conducted to characterise the persistently infected cultures. Examination of the adherent cell population of the persistently infected cultures by immunofluorescence showed that 10–20% of PI-RD cells and 1–10% of PI-U87-MG cells contained viral antigens (Fig. 2 (a), (b)). When persistently infected cultures were shifted down to 31C for incubation, the cells became rounded and rapidly degenerated. After 3–5 days, all the remaining cells contained viral antigens. Total cellular RNA was extracted from persistently infected cultures and was found to react by dot-blot hybridisation with OC43-specific ^{32}P-cDNA probe prepared from infected mouse brain. Superinfection of persistently infected cultures with vesicular stomatitis virus (VSV) resulted in the production of high titers of VSV. Both PI-U87-MG and PI-RD cultures produced interferon (IFN) in response to Sendai virus infection, whereas, no endogenous IFN was detected in the culture supernatant medium sampled over an 87 day period (Table 1).



(a)

(b)

Fig. 2. Immunofluorescent staining of persistently infected cells with rabbit antiserum to OC43 virus. (a) persistently infected RD cells; (b) persistently infected U87-MG cells. Size bar = 10 μm.

Table 1 Summary of properties of cells persistently infected with OC43 virus

Property	Cells	
	PI-RD	PI-U87-MG
Percent of cells with virus antigen[a]	10–20	1–10
Relative amount of viral RNA[b]	+++	++
PFU/ml of supernatant[c]	0.8–1.0×10^8	0.5–1.0×10^8
VSV-yield[d] superinfected cultures/control cultures	1×10^7/1.9×10^8	1.5×10^8/2.5×10^7
Interferon units produced[e]		
endogenous	<4	<4
induced	2560	160

[a] Intracellular viral antigens were detected by immunofluorescence on acetone-fixed cells. The primary antiserum was rabbit antiserum to OC43 virus, followed by biotinyllated goat anti-rabbit serum, then avidin FITC (Vector Laboratories, Burlingame, CA).

[b] Viral RNA was detected by dot-blot hybridisation with OC43 cDNA. The positive control was scored as 4+ indicating the amount of viral RNA in RD cells after 36 h of infection.

[c] Average PFU/ml in samples from 50–90 days in culture.

[d] Cultures were infected with VSV at an m.o.i. of 5 for 18 h. VSV yield was measured by plaque assay in L cells.

[e] One IF unit = 50% plaque reduction of VSV on human foreskin cells when HuIFN gave the standardised titer. For endogenous IFN, culture medium taken on days 7, 21, 35, 49, 67 and 87 was treated at pH 2 for 7 days. For induced interferon, cultures were stimulated with 20 HAU of Sendai virus for 18 h prior to treatment at pH 2.

Recovery of temperature-sensitive (ts) plaque variants

In an attempt to account for the sporadic appearance of hemaggultinin titers and the rapid cell degeneration occurring in cultures incubated at 31°C, the presence of temperature-sensitive virus was sought by incubating the plaque assays of PI culture supernatants at 31°C. In these assays two populations of plaques were observed, small plaques and larger hazy plaques. The large plaques were considered to be ts plaque variants. The plaque variants were more easily seen in HRT-18 cell monolayers as shown in Fig. 3. The titer of the variant population was ten-fold less than that of the small plaque forming virus and was quite constant when samples were tested over an interval of several months (Table 2). Therefore, it did not account for the fluctuations in hemagglutinin activity, a phenomenon for which we have no explanation at present.

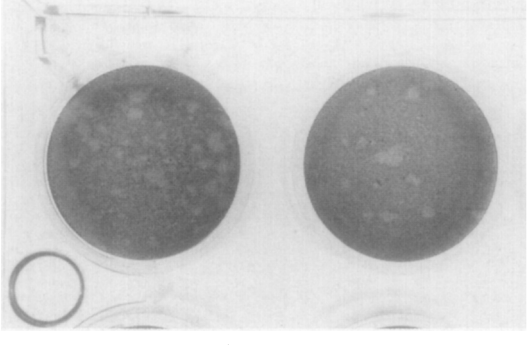


Fig. 3. Large and small plaques formed by OC43 virus from the supernatant medium of PI-RD cells. Ten-fold dilutions of supernatant medium were plated onto duplicate HRT-18 cell monolayers in 24 well trays at 0.2 ml per well, overlayed with agarose medium and incubated at 33°C. Plaques were visible after 9 days by neutral red stain. Left, 10^{-3} dilution; right, 10^{-4} dilution.

Table 2 Yield at two temperatures of OC43 virus from persistently infected cells

	RD Cells					U87-MG Cells				
	HAU[a]	PFU/ml				HAU[b]	PFU/ml			
		37°C		31°C[c]			37°C		31°C	
Days in culture		Large plaques	Small plaques	Large plaques	Small plaques		Large plaques	Small plaques	Large plaques	Small plaques
1	1	—	2.4×10^6	1.3×10^6	1×10^5	<1	—	2.5×10^6	2.3×10^4	—
28	1	—	4.8×10^8	6.25×10^7	2.1×10^8	4	—	4.8×10^8	5×10^7	2.3×10^8
42	8	—	2.5×10^8	8×10^7	3×10^8	1	—	6.5×10^8	3×10^7	2.7×10^8
59	16	—	9.8×10^7	3×10^7	1.3×10^8	1	—	1.4×10^8	3.5×10^7	1.75×10^8
77	4	—	4×10^7	3.5×10^6	1.5×10^7	16	—	8×10^7	6×10^7	2.25×10^8

[a] PFU/HAU RANGE during the period from day 28 to day 77: 4.5×10^6–5×10^7.
[b] PFU/HAU RANGE during the period from day 28 to day 77: 5×10^6–2.1×10^8.
[c] RD cells were used and incubated at 31°C in plaque assays to detect the large plaque variant.

Cure of persistently infected cells by antibody

It became apparent that OC43 virus most likely persisted in RD and U87-MG cells by continuous reinfection of susceptible cells by virus in the culture medium. If so, then the cultures could be cured of virus by introducing antiserum into the culture medium. Consequently, 25 HI units of rabbit immune serum was introduced into the culture medium of subconfluent monolayers of persistently infected cells. The same immune serum treatment was repeated at each passage and continued for 28 days. The treated cells were then passaged without immune serum two times and then tested for viral antigens by immunofluorescence. No viral antigens were detected (data not shown). To look for viral RNA, total RNA was extracted from cured cells at 3 weeks and 3 months after curing and probed with OC43-specific ^{32}P-cDNA by dot-blot hybridisation. No viral RNA was detected in cured PI-RD or PI-U87 cells at the times of sampling.

Cure of persistently infected cells by incubation at 39.5°C

The ability of elevated temperatures to cure the persistent infections was tested by shifting the PI cultures to 39.5°C and taking samples for assay infectious virus and viral RNA at the time the cells were to be subcultured. Both supernatant infectivity and viral RNA became undetectable in the PI cultures. Table 3 shows the results obtained with PI-RD cultures. Infectious virus was detected on day 23 but not on day 45 in PI-RD cells at 39.5°C. When total RNA extracted from 2×10^7 cells on day 48 was dot blotted onto nitrocellulose, no positive signal for OC43 RNA was obtained with the OC43 specific ^{32}P-cDNA probe. In PI-U87-MG, infectious virus was more rapidly lost and could not be detected at 20 days (results not shown). No viral RNA was detected with OC43 cDNA probe on dot blots.

Discussion

Persistent infectious with OC43 virus can be readily established in rhabdomyosarcoma (RD) cells and also in glioma (U87-MG) cells after a brief period of adaptation. There appear to be only minor variations in these two cell lines with respect to recovery of infectious virus and viral hemagglutinin, superinfection with vesicular stomatitis virus, interferon production and ability to be cured of the virus. Our strain of RD-adapted

Table 3 Effect of temperature elevation on virus expression in PI-RD cultures

Means of OC43 detection	Days of incubation	Incubation temperature	
		37°C	39.5°C
Infectivity in supernatant (PFU/ml)[a]	23	1.5×10^4	8×10^2
	45	2.7×10^3	<1
Viral cDNA probe[b]	48	++	neg.

[a] Duplicate cultures of PI-RD cells were incubated at 36.5°C and 39.5°C. Supernatant medium was removed on days 23 and 45 of the experiment and immediately transferred to HRT-18 cell monolayers for plaque assay.

[b] Total RNA was extracted from 2×10^7 cells after 48 days at 37°C or 39.5°C. RNA samples of 50 μl, 5 μl and 0.5 μl representing 3×10^4 cells per 50 μl were dot blotted onto nitrocellulose and probed with OC43-specific ^{32}PcDNA. ++ = positive ^{32}P signal in 50 and 5 μl samples. neg = no ^{32}P signal in the 50 μl sample.

OC43 virus apparently is able to develop a tropism for human tumor cells of neural origin such as U87-MG cells. However, it is difficult to extrapolate the results obtained with these two tumor cell lines to CNS infections in humans. A tropism of OC43 virus for cultured mouse neurons but lack of virus growth in astrocytes has been described and may reflect the selection of variants by passage in sucking mouse brain. In this regard the neural cytotropism of primary isolates and U87-MG adapted strains of OC43 virus has yet to be examined.

Both PI cells cultures produce ts variants which were detected by incubation of plaque assays at 31°C. Enhanced growth of these variants at 31°C was most likely responsible for the cellular degeneration observed when the PI cultures are shifted to incubation at 31°C. The recovery of ts plaque variants from PI cultures suggests a mechanism of persistence that is regulated by ts mutants which grow poorly at 37°C and not at all at 39.5°C. When assayed at 31°C, the titers of large and small plaque mutants recovered from the supernatant medium were faily constant over a 48-day period indicating that a stable mutant population was selected by the cells. A similar situation was observed in DBT cells infected with mouse coronavirus JHM[6] and in 17 Cl-1 cells infected with mouse coronavirus A59.[7] The mouse coronavirus variants selected from these cultures possessed different amounts and molecular weight forms of the virion peplomer E2; which is responsible for virus attachment.[8] Another human coronavirus, 229E, was found to establish persistent infection readily at 37°C but at 33°C the L132 infected cells did not survive. Plaque variants were isolated from these cells which were more cytocidal at 33°C than the parent virus.[9]

Variability in hemagglutinin production during persistent infection was observed. Particles with HA activity may be defective virus particles or empty particles. However, in these cells there is no drop in infectious virus when hemagglutinin is produced, which indicates that these hemagglutinating particles do not interfere. Synthesis of defective interfering particles was described for mouse coronavirus JHM.[10] Empty particles have been associated with infectious bronchitis virus (IBV).[11] Studies are in progress to characterise the particles produced by OC43 in RD cells.

Introduction of polyclonal rabbit antiserum to OC43 virus into the supernatant medium apparently cured PI cultures of virus antigens and viral RNA as indicated by the negative dot blots. In our experiment, 43 PRD_{50} of serum was used to effectively cure the cells at 37°C within 30 days. These results are in contrast to a similar experiment reported by Stohlman *et al.*[8] which indicated that 50 PRD_{50} units of polyclonal mouse antiserum to mouse coronavirus JHM was ineffective in curing persistently infected neuroblastoma cells.[8] Since unlike JHM, OC43 antibody is effective *in vitro* in elimination of the virus, it is important to understand what viral epitopes are involved and whether this mechanism works *in vivo*. Human respiratory coronaviruses apparently undergo a cyclic pattern of infection in nature with reinfections reportedly occurring quite frequently.[12,13]

Elevating the temperature to 39.5°C resulted in a loss of infectious virus and OC43 specific RNA from PI cultures. The amount of RNA sampled in the dot-blot represented the total RNA exactable from about 3×10^4 cells. This amount of RNA from PI-U87-MG cells gave a positive signal where only one in ten cells expressed viral antigen. It is likely, therefore, that OC43 virus was absent or unexpressed in these cultures at 39.5°C. Furthermore, analysis for OC43 RNA is predicated on the amplyfying effect of viral mRNAs and so the presence of only unexpressed genomic RNA in infected cells might escape detection by dot blotting.

The relative ease of establishment and cure of persistent infections with OC43 human coronavirus implies that other mechanisms must be involved if this virus is to be responsible for chronic infections in man. However, it is difficult to extrapolate the

results obtained with these two tumor cell lines to infections in humans. Nevertheless the ease with which the persistent infection may be regulated makes it an attractive model to study.

Materials and methods

Cells

Human rhabdomyosarcoma cells (RD)[14] were obtained from R. L. Crowell, Hahnemann Medical College and Hospital, Philadelphia, PA. These are cells derived from an embryonal rhabdomyosarcoma which do not contain myofibrils. U87-MG cells are a differentiated tumor cell line derived from a malignant glioma. They were obtained from the American Type Culture Collection Human Tumor Bank, Rockville, MD. U87-MG cultures contain process-bearing cells which associated in a glial pattern of small bundles and stain positive with anti-GFAP serum. HRT-18 cells, a human adenocarcinoma cell line[15] were obtained from P. Talbot, Institut Armand-Frappier, Laval, Quebec, Canada. All cell lines were maintained in EMEM supplemented with non-essential amino acids and 10% fetal bovine serum (heat inactivated).

Virus

OC43 virus was obtained from G. Gerna, Institute of Infectious Diseases, Pavia, Italy.[16] Upon receipt, the virus was passaged three times in M-7 cells of human embryonic lung origin (Microbiological Associates, Walkersville, MD) and subsequently adapted to RD cells. Uncloned virus stocks were prepared by infecting confluent monolayers of RD cells at a multiplicity of 0.1 and incubating the infected cells at 37°C. Plaque assays were usually performed in RD cells at 37°C. Neutral red stain was added after 7 days. Plaques were visible on day nine. Viral titers ranged from 10^7–10^8 PFU/ml. HRT-18 cells were used in some plaque experiments as indicated in the text.

Antisera

Rabbit antiserum was prepared to gradient purified virus propagated in suckling mouse brain. The antiserum had a 50% plaque neutralisation titer of 430 and a hemagglutination inhibition (HAI) titer of 256 using mouse erythrocytes. It was used at 1 : 20 dilution in immunofluorescence tests.

Immunofluorescence.

Antigens in infected cells were detected by indirect immunofluorescence on acetone fixed cells. Antiserum to glial fibrillary acidic protein was obtained from Accurate Chemical and Scientific Corporation, Westbury, NY 11590 and used at 1 : 20 dilution. Horse anti-mouse IgG and goat anti-rabbit IgG conjugated to biotin and avidin FITC were purchased from Vector Laboratories, Burlingame, CA. Biotin conjugated reagents were used at 1 : 20 dilution and avidin FITC was used at 1 : 50 dilution.

Interferon (IFN), induction and assay

To induce IFN 20 hemagglutination units of Sendai virus were added to a 75 cm^2 flask of cells. After 18 h at 37°C supernatant was collected and left at pH 2 for 7 days, then centrifuged at 75 000 ***g*** for 60 min. To assay for IFN, 1.0 ml of dilutions of treated supernatants and human IFN (1×10^6 units IFN activity) were inoculated onto human foreskin cells in 24 well tissue culture trays. After 18 h at 37°C; 100 PFU of plaque purified VSV, Indiana strain was added and the test was incubated until the virus controls showed 90–100% cell destruction.

Preparation of OC43 complementary DNA

Template mRNA was prepared from RNA extracted with 19 : 1 guanidine-HCl (8 M): potassium acetate (2 M, pH 5.0) from infected mouse CNS tissue. The poly (A)-tailed RNA was selected by oligo (dT)-cellulose column chromatography. Cellular RNAs were removed by twice hybridisation with nitrocellulose-bound uninfected mouse CNS DNA. Using the OC43 mRNA as template, complementary DNA (^{32}P-cDNA) was synthesised and isolated as described by Sorensen *et al.*[17] for JHM mouse coronavirus template.

Dot-blot hybridisation

Total RNA from infected, persistently infected, and control RD and U-87-MG cells (2×10^7 cells per sample) and from uninfected and OC43-infected mouse CNS (100 mg tissue containing in the order of 10^8 cells) was extracted with guanidine-HCl, precipitated with ethanol, sedimented and fixed in formalin. RNA samples were applied to nitrocellulose undiluted and at 1 : 10 and 1 : 100 The blots were probed at high stringency with ^{32}PcDNA (5×10^5 cpm/blot) as previously described.[18]

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