The Adaptation of Two Human Coronavirus Strains (OC38 and OC43) to Growth in Cell Monolayers (35068)

MARIE BRUCKOVÁ,[1] KENNETH MCINTOSH,[2] ALBERT Z. KAPIKIAN, AND ROBERT M. CHANOCK

Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014

Coronaviruses (1) of at least two, and probably more, serotypes (2) have been recovered from adults with upper respiratory disease occurring during the winter months (3–6). Serologic surveys have shown that coronavirus infection is moderately common during these periods of prevalence (6, 7), and the administration of coronaviruses to volunteers has established their pathogenicity for the human respiratory tract (4, 8). Research into their properties has, however, been hampered by their fastidious growth requirements.

Fourteen of the 23 reported coronavirus strains were isolated in tissue culture monolayers of human origin (3, 6). Attempts to adapt these 14 strains to growth in laboratory animals were unsuccessful. The remaining 9 strains were isolated in human embryonic tracheal organ culture (HETOC) where further study has proved to be cumbersome (4, 5). Two strains, OC38 and OC43, were successfully adapted to growth in suckling mice (9). In this system the strains were shown to be serologically identical, and a one-way serologic relationship with mouse hepatitis virus (MHV) was demonstrated. Both strains, however, failed, in previous attempts, to grow in tissue culture monolayers.

A coronavirus strain which grew both in tissue culture monolayers and in laboratory animals would be a valuable tool in further investigations of viral antigenic structure. This paper reports the successful adaptation of strains OC38 and OC43 to monolayers of monkey origin.

Materials and Methods. Viruses. Coronavirus strains with the prefix "OC" were recovered in HETOC in this laboratory (5). Strain B814, recovered in HETOC, was kindly provided by Dr. D. A. J. Tyrrell (4). All organ culture grown viruses were passaged more than four times in HETOC before use in these studies. Strains OC38 and OC43 were adapted to growth in suckling mouse brain as described (9).

Sera. Mouse serum hyperimmune to strain OC43 was prepared in weanling Charles River CD-1 Swiss mice (9). Polyvalent MHV mouse serum was kindly provided by Dr. John C. Parker of Microbiologic Associates. Guinea pig serum hyperimmune to strain 229E was prepared as previously described (3).

Tissue culture. Primary vervet and rhesus monkey kidney (VMK and RhMK) and primary human embryo kidney (HEK) tissue cultures were obtained from commercial sources. Diploid human embryonic intestine (HEI) cultures were prepared as previously reported (6). WI38, BSC-1, and VERO tissue cultures were prepared by standard methods. L132 cells were obtained from the American Type Culture Collection and propagated in monolayers by standard methods.

Passage techniques. Roller tubes containing inoculated tissue culture monolayers were incubated at 33° on roller drums. A mixture of 49% Eagles MEM, 49% medium 199, and 2% inactivated fetal calf serum with added glutamine, penicillin, and streptomycin was used. Passages were performed by inoculating

[1] Visiting scientist; present address: Institute of Microbiology and Epidemiology, Prague, Czechoslovakia.

[2] Present address: Department of Pediatrics, University of Colorado Medical School, Denver, Colorado 80220.

TABLE I. Attempts to Adapt Coronaviruses to Growth in Tissue Culture.

Tissue culture monolayer	Av no. of days in each passage	Development of CPE in tissue culture monolayers inoculated with indicated virus				
		OC38 virus		OC43 virus		OC16, OC37, OC44, OC48, B814 viruses; organ culture grown
		Organ culture grown	Suckling mouse grown	Organ culture grown	Suckling mouse grown	
Rhesus monkey kidney	21	+(3)[a]	+(2)	+(3)	+(2)	—(7)
Vervet monkey kidney	21	Not done	+(3)	Not done	+(3)	Not done
Primary human embryonic kidney	19	—(7)[b]	—(7)	—(7)	—(7)	—(5)
Diploid HEI	16	—(7)	—(7)	—(6)	—(7)	—(7)
Diploid WI38	26	—(5)	—(5)	—(5)	—(5)	—(5)
L132	15	—(9)	—(8)	—(9)	—(8)	—(7)
BSC-1	19	—(3)	—(3)	—(3)	—(3)	—(3)
VERO	16	—(3)	—(3)	—(3)	—(3)	—(3)

[a] Earliest tissue culture passage at which CPE was detected.
[b] Highest tissue culture passage tested.

0.2 ml of a mixture of cells and medium directly into a fresh tissue culture tube. The interval of incubation between passages varied from 15 to 26 days, depending on the condition of the tissue monolayer.

Electron microscopy. The techniques of negative staining and examining for characteristic coronavirus particles have been described (5).

Serologic methods. Neutralization tests were performed in tissue culture tubes by standard methods. Complement fixation (CF) tests were performed by the micromethod (9). Fluorescein conjugated antihuman globulin was obtained from Antibodies Incorporated, Davis, California, and tissue cultures were stained and examined by standard methods.

Results. Attempts to adapt coronavirus strains OC16, OC37, OC44, OC48, and B814 to monolayer tissue culture were unsuccessful in the tissues tested (Table I). Similarly, strains OC38 and OC43 did not produce either cytopathic effect (CPE) or particles detectable by electron microscopic examination after passage in HEK, HEI, WI38, L132, BSC-1, or VERO cells (Table I). However, in primary kidney tissue from either rhesus or vervet monkeys, a CPE appeared on the 2nd or 3rd passage of material originating from either infected suckling mouse brain or human embryonic tracheal organ culture (Table I). The CPE was focal, with a tendency to the formation of syncytia, and appeared at least 1 week after inoculation, progressing to involve the entire cell sheet.

When cells and media harvested from tubes showing this CPE were frozen and thawed, clarified, and concentrated for examination by electron microscopy characteristic coronavirus particles were seen. RhMK grown material was successfully passaged back into HETOC. Such virus suspensions also fixed complement with hyperimmune mouse sera prepared against strains OC43 and OC38, and, to a lower titer, with polyvalent hyperimmune mouse serum prepared against MHV. The CPE produced in RhMK tissue culture was neutralized by hyperimmune mouse serum prepared against the homologous virus, but not by mouse serum prepared against MHV, strain A-59. Moreover, a characteristic granular cytoplasmic fluorescence was seen when the cells were

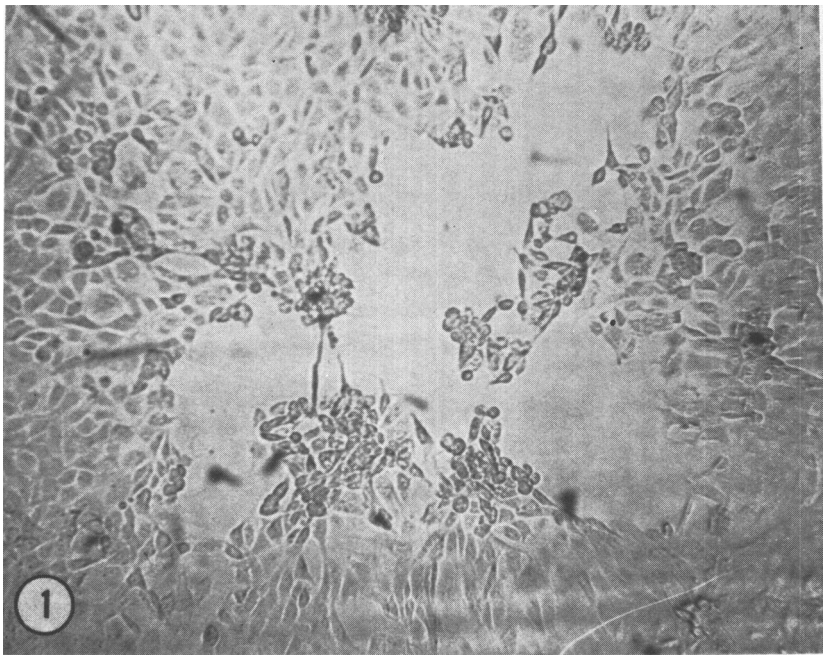

FIG. 1. Focal cytopathic effect of strain OC43 in BSC-1 cells, 14 days following inoculation.

stained with convalescent human serum by the indirect immunofluorescence technique. In the same test, no fluorescence was observed using the corresponding acute serum. The virus suspensions used in these experiments were tested for their capacity to fix complement with a variety of antisera and were found free of detectable contamination by extraneous viruses. However, later passages became contaminated with SV40, SV5, or measles virus. Because of this, we attempted to transfer the RhMK grown viruses to continuous, or primary human, cells.

Attempts to adapt the monkey kidney grown virus to cells of human origin (primary HEK, HEI, and WI38) were unsuccessful. Five blind passages were carried out in each tissue. However, when strain OC38 or strain OC43, passaged either 5 or 10 times in RhMK, was inoculated into BSC-1 cells, a CPE appeared on first passage. This CPE was similar to that seen in RhMK cells but the tendency to form syncytia was less (Fig. 1). Foci of bunched small round refractile cells appeared about 1 week after inoculation. The affected cells often retracted, leaving small holes in the monolayer. Irregular syncytia were occasionally seen in these foci. The CPE gradually spread to involve the whole cell monolayer. In later passages, using large inocula, CPE first appeared after 3–4 days.

The serologic and biologic properties of BSC-1-adapted strain OC43 were then examined. Passage back into HETOC was successfully accomplished. Suckling mice inoculated intracranially with tissue culture fluids died with limb paralysis 4 days after inoculation, and brain homogenates from affected animals fixed complement with anti-OC43 mouse serum. Tissue cultures showing CPE contained characteristic coronavirus particles when examined by electron microscopy. The agent was chloroform-labile and acid-labile, and grew in the presence of 5 m*M* bromodeoxyuridine. It was neutralized by anti-OC43 antiserum and by a standard convalescent human serum, but not by acute human serum, anti-229E guinea pig serum, or normal mouse serum, and only to a low titer by polyvalent anti-MHV serum (Table II). The agent fixed complement with homologous hyperimmune mouse serum but not with the lowest dilution tested of polyvalent MHV

TABLE II. Serological Identification of BSC-1-Adapted Coronavirus Strain OC43.

Serum or antiserum		Reciprocal of antibody titer measured against BSC-1-adapted OC43	
With antibody against	Originating in	Complement fixing	Neutralizing ($TCID_{50}$ used in test)
OC43	Mouse	160	2560 (100)
	Human (acute)	<4	<8 (3000)
	Human (conv.)	16	48 (3000)
MHV (polyvalent)	Mouse[a]	<10	20 (100)
229E	Guinea pig[b]	—	<10 (100)
	Human (acute)[c]	<4	Not done
	Human (conv.)[c]	<4	Not done

[a] Reciprocal of homologous antibody titer against MHV, strain A59, was ≥160 (complement fixing) and 1280 (neutralizing) (2).

[b] Reciprocal of homologous antibody titer against strain 229E was 320 (neutralizing) (2).

[c] This serum pair consistently showed a fourfold or greater rise in CF antibody to strain 229E.

mouse serum (Table II). Characteristic granular cytoplasmic fluorescence was seen in cells showing early CPE when stained with standard convalescent human serum by the indirect immunofluorescence technique (Fig. 2). Again, the corresponding acute serum failed to stain infected cells. The titer of infectious virus in BSC-1 grown virus suspensions was $10^{3.3}$– $10^{4.5}$/0.2 ml, when tested in BSC-1 roller tubes, and $10^{4.5}$ LD_{50} when inoculated intracranially into suckling mice.

Discussion. Previous efforts to adapt the

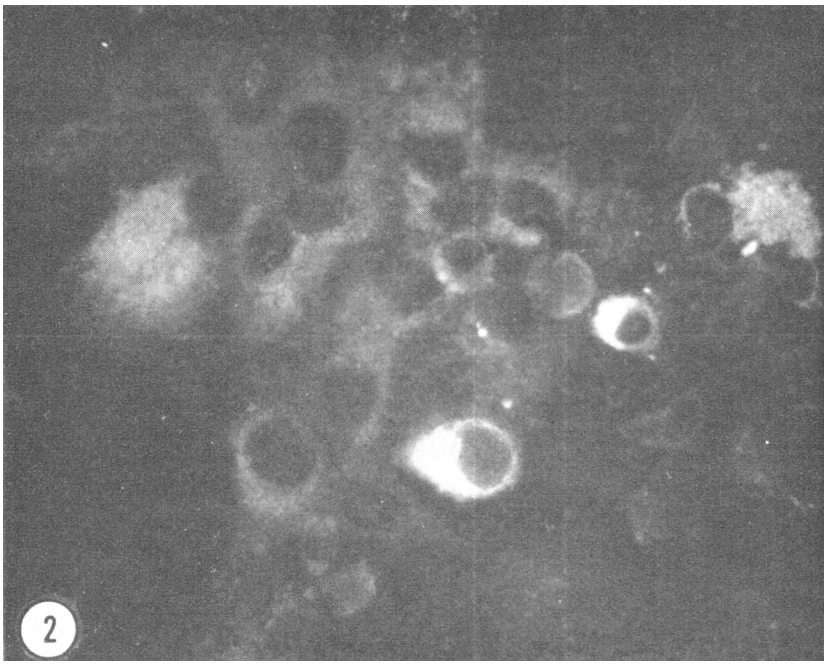

FIG. 2. Granular cytoplasmic immunofluorescence in BSC-1 cells infected with strain OC43 and stained with human convalescent serum by the indirect technique.

"OC" group of coronavirus strains to growth in monolayer tissue culture failed, and it is not clear what led to the success described in this report. Certain factors may have been of importance: infected monolayers were held as long as possible during each passage; and subpassages of cells and media were performed without freezing and thawing. Contrariwise, we were unable to grow organ culture propagated strain B814 in L132 cells, although this had been successfully performed previously by Bradburne and Tyrrell (10). The latter report described the growth of the virus in tissue culture directly from clinical specimens; it may be that virus particles incapable of tissue culture growth were selected by further passages in organ culture made in this laboratory. An alternative explanation for this failure would implicate a change in the cell line L132 in our or in Bradburne's laboratory. Although both groups obtained the cells originally from the American Type Culture Collection, the selection of a sensitive (or insensitive) line of cells might have occurred. Undetected contaminating mycoplasma might also have changed the sensitivity of the line.

The persistent failure of the organ culture grown coronavirus strains other than OC38 and OC43 to produce CPE in tissue culture monolayers remains unexplained. Propagation of virus without CPE was not tested for in these studies, although in previous studies (5) attempts to detect virus in monolayers by fluorescence, electron microscopy, and challenge with ECHO 11 were unsuccessful.

Mouse brain grown strain OC43 has consistently fixed complement at low levels with antisera hyperimmune to various strains of MHV (9). It is probable that the absence of such fixation by BSC-1 adapted OC43 is a reflection of the low titer of CF antigen produced in this cell line.

With the successful adaptation reported here, two "human" coronavirus strains have been found which grow both in tissue culture monolayers and in laboratory animals. In addition, a recent publication described the discovery of hemagglutinating activity in these same two coronavirus strains (11). The combined findings in these two reports make available serologic tools which will facilitate further investigation of the antigenic composition of human coronaviruses.

Summary. Two human coronaviruses, strains OC38 and OC43, were successfully adapted to growth in tissue culture monolayers. The adapted strains produced a cytopathic effect in primary rhesus or vervet monkey kidney and in BSC-1 cell monolayers. The adaptation described will facilitate further investigations of the structure of coronaviruses.

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