STUDIES ON NEW VIRUS ISOLATES RECOVERED IN TRACHEAL ORGAN CULTURE

Kenneth McIntosh, M.D.

University of Colorado Medical School, Denver, Colo.

Marie Brůčková, Ph.D.*

Institute of Epidemiology and Microbiology, Prague, Czechoslovakia

Albert Z. Kapikian, M.D. and Robert J. Chanock, M.D.

Laboratory of Infectious Disease, National Institutes of Health, Bethesda, Md.

and

Horace Turner

Viral Carcinogenesis Branch, National Cancer Institute, National Institutes of Health, Bethesda, Md.

The failure of traditional tissue culture methods to recover viruses from a high proportion of adults and children with respiratory disease led to the testing of new diploid cell strains^{1,2} and to the application of organ culture techniques to virus recovery.^{3,5} To a large extent, these efforts made possible the isolation from the human respiratory tract of several virus strains that were of an unusual morphology when examined by negative staining and that have recently been grouped together under the name of "coronaviruses."⁶ On examination by electron microscopy, the coronaviruses are medium-sized particles of moderate pleomorphism, with widely spaced, club-shaped surface projections.^{4,7} The members of this group, which includes avian infectious bronchitis virus, many strains of mouse hepatitis virus (MHV), and several viruses of human origin, are RNA-containing and chloroform-labile. Twenty-three coronavirus strains have been recovered from the human respiratory tract.^{3,4,8,9} Of these, 14 strains were isolated in tissue culture and appeared to be serologically similar or identical to the prototype strain 229E.^{1,8} The nine remaining strains were isolated in human embryonic tracheal organ culture. Two of these, strains OC38 and OC43, were successfully adapted to growth in suckling mouse brain.¹⁰ These two strains were serologically indistinguishable and have been shown to bear a serologic relation to several strains of MHV by complement-fixation (CF) tests.

This communication outlines, first, recent epidemiologic studies of coronavirus infection and, second, attempts to adapt some of the more fastidious strains to growth in tissue culture monolayers.

Preliminary epidemiologic observations of human coronavirus infection suggested that they might be sporadically associated with upper respiratory tract infection in adults. We undertook to delineate in greater detail the seroepidemiology of coronavirus infection in adults with upper respiratory tract disease and in hospitalized children with lower respiratory tract disease (LRTD). The adults were employees of the National Institutes of Health with colds. The children were patients with croup, bronchitis, bronchiolitis, or pneumonia admitted to Children's Hospital, Washington, D.C.; the control pediatric group was made up of patients at the same hospital with nonrespiratory disease. Acute and convalescent serum samples were obtained at an interval of three weeks. The virus isolation procedures employed were those previously reported.¹⁴

* Visiting Scientist, 1968-1969, Laboratory of Infectious Disease, National Institutes of Health, Bethesda, Md.

The complement-fixing antigens used in this study represented three serologic groups.¹¹ Virus strain 229E, kindly supplied by Hamre, was grown in WI38 cells. Strains OC38 and OC43, originally recovered in human embryonic tracheal organ culture, were adapted to growth in suckling mouse brain; 10% brain suspensions served as CF antigens. MHV, strain A59, was grown in NCTC1469 cells. This virus strain was shown by Hartley and others to detect frequent antibody responses in man, although at the time a relationship to a human virus was only postulated.¹² Subsequently, strains OC38 and OC43 were shown to bear a one-way antigenic relation to this and several other strains of MHV. Appropriate control antigens were included in all tests. In the analyses that follow, serologic responses to strains OC38 and OC43 are grouped together. Moreover, because of frequent overlap between responses to OC38, OC43, and MHV (A-59),¹³ these three viruses are occasionally also considered as a group.

TABLE 1 shows the proportion of infants and children with and without LRTD showing fourfold or greater antibody responses to the three related coronavirus antigens, OC38, OC43, and MHV (strain A59). When all age groups were combined, a positive correlation between coronavirus infection and LRTD did not exist. However, in the youngest age group (under one year) patients with LRTD tended to have more coronavirus infection than those in the control group, but

TABLE 1
COMPLEMENT-FIXING ANTIBODY RESPONSES TO CORONAVIRUSES IN INFANTS AND CHILDREN
WITH AND WITHOUT LOWER RESPIRATORY TRACT DISEASE*

Age (months)	Non-Respiratory Tract Disease		Respiratory Tract Disease	
	Number Tested	Number with CF Antibody Rise†	Number Tested	Number with CF Antibody Rise†
0-12	238	10 (4%)	55	1 (2%)
12-24	120	3 (3%)	19	4 (21%)
25-36	65	4 (6%)	33	5 (15%)
37-48	36	0	26	3 (12%)
49-60	32	0	26	3 (12%)
61-84	32	1 (3%)	42	3 (7%)
>85	42	2 (5%)	44	1 (2%)
Total	565	20 (3.5%)	245	20 (8.2%)

* Results shown here include antibody responses to OC38, OC34, and/or MHV, strain A-59.

† Fourfold or greater.

TABLE 2
PREVALENCE OF CF ANTIBODY TO HUMAN CORONAVIRUSES (1965-1967)

Age	OC38 and/or OC43		229E	
	Number Tested	Number Positive*	Number Tested	Number Positive*
0-6 months†	188	11 (6%)	186	0
7-12 months†	104	14 (13%)	102	1
1 year†	137	37 (27%)	137	0
2 years†	95	48 (51%)	98	0
Over 2 years†	274	120 (44%)	277	4
Adults‡	242	161 (67%)	241	99 (41%)

* Number of individuals with complement fixing antibody level of 1:4 or greater in acute serum sample.

† Hospitalized children with and without lower respiratory tract disease.

‡ Employees of NIH with colds.

FIGURE 1. Coronavirus isolations and antibody responses in adults with upper respiratory tract disease.

FIGURE 2. Coronavirus isolations and antibody responses in children with lower respiratory tract disease.

the difference was not statistically significant ($p > 0.05$). In all age groups taken together, there was a significant negative correlation with LRTD ($X^2 = 7.4$, $p < 0.01$). This negative correlation was particularly striking in the children over one year of age ($X^2 = 10.6$, $p < 0.01$).

The prevalence in adults and children of CF antibody to the "human" coronaviruses in sera obtained during 1965–1967 is outlined in TABLE 2. Complement-fixing antibody to strains OC38 and OC43 appeared to be acquired early in life. The percentage of adults in the NIH population with measurable antibody (67%) was only slightly greater than that of hospitalized children aged two years (51%). On the other hand, CF antibody to strain 229E was rare in the children in this sample, but common in adults (41%).

A summary of the information obtained from serologic, tissue culture, and organ culture studies is shown in the Figures. Among adults (FIGURE 1), coronaviruses appeared to be rarely associated with upper respiratory tract disease during 1963 and 1964. The frozen nasopharyngeal washings of a small group of patients, who in preliminary studies had shown CF antibody responses to strain 229E, were examined in a diploid cell strain originally derived from human embryonic intestine (HEI).¹ Three of these yielded coronaviruses serologically similar to strain 229E. Sporadic frozen specimens were examined in tracheal organ culture, and one coronavirus was recovered. Apart from these isolations and a few scattered CF responses, there appeared to be little detectable coronavirus activity in the adult population.

During 1965–1967, however, two small "outbreaks" of coronavirus-associated colds occurred in adults. In the first of these, during the winter of 1965–1966, five coronaviruses were recovered in organ culture and numerous responses to OC38, OC43, and MVH CF antigens were measured. Infections with strain 229E were either absent or very rare. During the winter of 1966–1967, however, six strains

TABLE 3
ATTEMPTS TO ADAPT CORONAVIRUSES TO GROWTH IN TISSUE CULTURE

Tissue Culture Monolayer	Average Number 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	Suckling Mouse-Grown	Organ Culture-Grown	Suckling Mouse-Grown	Organ Culture-Grown
Rhesus Monkey Kidney	21	+ (3)*	+ (2)	+ (3)	+ (2)	— (7)
Vervet Monkey Kidney	21	Not done	+ (3)	Not done	+ (3)	Not done
Primary Human Embryonic Kidney	19	— (7)†	— (7)	— (7)	— (7)	— (5)
Diploid HEI	16	— (7)	— (7)	— (6)	— (7)	— (7)
Diploid WI38	26	— (5)	— (5)	— (5)	— (5)	— (5)
L-132	15	— (9)	— (8)	— (9)	— (8)	— (7)
BSC-1	19	— (3)	— (3)	— (3)	— (3)	— (3)
VERO	16	— (3)	— (3)	— (3)	— (3)	— (3)

* Earliest tissue culture passage at which CPE was detected.

† Highest tissue culture passage tested.

of 229E-like viruses were recovered in HEI cells, and CF antibody responses to 229E were frequent. In the second "outbreak," antibody responses to OC38, OC43, and MHV (strain A59) were rare, and no coronaviruses were recovered in organ culture.

In children, on the other hand, there appeared to be no meaningful temporal pattern of coronavirus CF antibody responses, and no strains were recovered in organ culture (FIGURE 2).

Research into the properties of the coronaviruses of human origin has been hampered by their fastidious growth requirements. Those members of the group that were isolated in or have been adapted to tissue culture monolayers have not been shown to infect laboratory animals. The two strains that were shown to infect mice could be cultivated *in vitro* only in organ culture. The studies reported in the following paragraphs describe the adaptation of these two strains, OC38 and OC43, to growth in cell monolayers of monkey origin.

TABLE 3 summarizes our attempts to adapt seven coronavirus strains, isolated originally in organ culture, to various types of tissue culture. Strains OC16, OC37, OC44, OC48, and B814³ did not produce cytopathic effect (CPE) in any of the tissue culture types selected for trial, even after multiple subpassages. Both strains OC38 and OC43, grown either in organ culture or in suckling mouse brain, produced CPE in primary kidney tissue cultures from rhesus or vervet monkeys after two or three blind passages. The CPE was focal at first, with a tendency to syncytium formation, and progressed slowly to involve the entire cell monolayer.

Further studies in primary monkey kidney tissue culture were hampered by contamination with adventitious viruses. Therefore an attempt was made to adapt the tissue culture-grown virus strains either to primary human tissue culture or to continuous cell lines. On first passage from primary monkey kidney cells into BS-C-1 cells, both virus strains produced a CPE that was again focal and progressive, with a less marked tendency to syncytium formation.

The BS-C-1-infected cells contained characteristic coronavirus particles when examined by the negative staining technique. Passage back into human embryonic tracheal organ culture was successfully accomplished. Suckling mice inoculated intraerally with tissue culture fluids died with limb paralysis four days after inoculation, and brain homogenates from affected animals fixed complement with anti-OC43 mouse serum. The agent was chloroform-labile and acid-labile and grew in the presence of 5 mM BUdR. Experiments now in progress and designed to identify the CPE-producing agent serologically suggest that it is identical to suckling-mouse-grown virus OC43.

References

1. KAPIKIAN, A. Z., H. D. JAMES, S. J. KELLY, J. H. DEES, H. C. TURNER, K. MCINTOSH, H. W. KIM, R. H. PARROTT, M. M. VINCENT & R. M. CHANOCK. 1969. Isolation from man of "avian infectious bronchitis virus-like" viruses (coronaviruses) similar to 229E virus, and some epidemiologic observations. *J. Infect. Dis.* 119: 282-290.
2. PHILLIPS, A. A., J. L. MELNICK & C. A. GRIM. 1965. Human aorta cells for isolation and propagation of rhinoviruses. *Proc. Soc. Exp. Biol. Med.* 119: 843-845.
3. TYRRELL, D. A. J. & M. L. BYNOE. 1965. Cultivation of a novel type of common-cold virus in organ culture. *Brit. Med. J.* 1: 1467-1470.
4. MCINTOSH, K., J. H. DEES, W. B. BECKER, A. Z. KAPIKIAN & R. M. CHANOCK. 1967. Recovery in tracheal organ cultures of novel viruses from patients with respiratory disease. *Proc. Nat. Acad. Sci. U.S.A.* 57: 933-940.
5. TYRRELL, D. A. J. & M. L. BYNOE. 1966. Cultivation of viruses from a high proportion of patients with colds. *The Lancet* 1: 76-77.
6. NATURE. 1968. Coronaviruses 220: 650.

7. ALMEIDA, J. D. & D. A. J. TYRRELL. 1967. The morphology of three previously uncharacterized human respiratory viruses that grow in organ culture. *J. Gen. Virol.* **1**: 175-178.
8. HAMRE, D. & J. J. PROCKNOW. 1966. A new virus isolated from the human respiratory tract. *Proc. Soc. Exp. Biol. Med.* **121**: 190-193.
9. BRADBURN, A. F. 1969. Sensitivity of L132 cells to some "new" respiratory viruses. *Nature* **221**: 85-86.
10. MCINTOSH, K., W. B. BECKER & R. M. CHANOCK. 1967. Growth in suckling-mouse brain of "IBV-like" viruses from patients with upper respiratory tract disease. *Proc. Nat. Acad. Sci. U.S.A.* **58**: 2268-2273.
11. MCINTOSH, K., A. Z. KAPIKIAN, K. H. HARDISON, J. W. HARTLEY & R. M. CHANOCK. 1969. Antigenic relationships among the coronaviruses of man and between human and animal coronaviruses. *J. Immunol.* **102**: 1109-1118.
12. HARTLEY, J. W., W. P. ROWE, H. H. BLOOM & H. C. TURNER. 1964. Antibodies to mouse hepatitis virus in human sera. *Proc. Soc. Exp. Biol. Med.* **115**: 414-418.
13. MCINTOSH, K., *et al.* Seroepidemiologic Studies of Coronavirus Infection in Adults and Children. In preparation.