

Human Malignant Melanoma Cell Line (HMV-II) for Isolation of Influenza C and Parainfluenza Viruses

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HMV-II, a human malignant melanoma cell line, was compared with other cell lines (MDCK, Vero, and LLC-MK2) and primary cultures of monkey kidney (PMK) cells for the isolation and quantification of influenza and parainfluenza viruses. HMV-II cells were superior to MDCK and LLC-MK2 cells in quantification of the influenza C virus and were used successfully in the isolation of the virus from clinical specimens. The HMV-II cell line was also more sensitive for isolating parainfluenza viruses from clinical specimens than were Vero and PMK cells; there was, however, no significant difference in the quantification of the viruses among these cultures. As far as influenza A and B viruses were concerned, the HMV-II cell line was significantly less sensitive than MDCK cells, and no virus was isolated from clinical specimens with HMV-II cells. Thus, HMV-II cells are useful for the isolation of influenza C and parainfluenza viruses as an alternative to embryonated hen's eggs and PMK cells.

Recently, for the isolation of influenza A and B viruses, MDCK cells have been generally used instead of embryonated hen's eggs (3, 7, 19). However, there has been no established cell line for the isolation of influenza C virus, although some cell cultures such as primary monkey kidney (PMK) (1, 8, 18), primary chicken embryo kidney (16), LLC-MK2 (12), and MDCK (11) have been reported to be susceptible to the virus. For the isolation of parainfluenza viruses, PMK cells have been widely used because of their high sensitivity (2), and some established cell lines such as Vero (5, 9, 15) or LLC-MK2 (3, 17) have been used for the cultivation of the viruses.

We previously reported that HMV-II cells, a human malignant melanoma cell line, were highly susceptible to the influenza C virus (13). In the present study, our investigations have concerned the isolation of the viruses from clinical specimens obtained from children with acute respiratory infections, using HMV-II cells. We describe here the utility of HMV-II cells in the isolation of influenza C as well as of parainfluenza viruses, HMV-II cells being not only sensitive to the influenza C virus but also more sensitive to parainfluenza viruses than are Vero or PMK cells.

MATERIALS AND METHODS

Cells. HMV-II cells obtained from T. Kasuga (Tokyo Medical and Dental University) (10) were grown in RPMI 1640 medium containing 10% fetal calf serum and antibiotics (penicillin, 50 U/ml; streptomycin, 50 µg/ml) (13). Vero, LLC-MK2, and MDCK cells were grown in Eagle minimum essential medium (MEM) with 10% fetal calf serum and antibiotics. Primary cultures from cynomolgus monkey kidney (CPMK) cells were prepared in our laboratory and grown in MEM with 10% calf serum and antibiotics.

Viruses. The following viruses were used for infectivity titration. Influenza A/Sendai/790/85 (H3N2), A/Sendai/1004/86 (H1N1), and B/Sendai/27/85 were isolated and passaged twice in MDCK cells. Influenza C/JJ/50 was grown in the

amniotic cavity of embryonated hen's eggs. The prototype strains of parainfluenza viruses grown in PMK were finally passaged four to eight times in Vero cells. New isolates of parainfluenza virus types 1 and 2 were recovered and passaged three times in Vero cells. Type 3 virus was isolated and passaged once in CPMK cells.

Infectivity titration. The confluent monolayers of the cell cultures were prepared in 96-well tissue culture microplates (Toyoshima, Tokyo, Japan). Three wells of the cultures were inoculated with 25 µl of each decimal dilution of virus pools. The maintenance medium (MM) for MDCK, Vero, LLC-MK2, and CPMK cells consisted of MEM with 5% MEM vitamin solution (100× concentrate; GIBCO Laboratories), 2 mg of glucose per ml, antibiotics, and an appropriate concentration of crystal trypsin (type 1; Sigma Chemical Co.) by the cell culture because of the difference in the tolerance to trypsin (3.5 µg/ml for MDCK cells and 2 µg/ml for Vero, LLC-MK2, and CPMK cells). The MM for HMV-II cells consisted of RPMI 1640 medium with antibiotics and a high concentration (20 µg/ml) of crystal trypsin for influenza C virus (13). On day 7, hemadsorption (HAd) tests were performed as described below to determine the infectivity. The titers were calculated by the Reed-Muench method.

HAd test. For Vero, LLC-MK2, and CPMK plates, chicken or guinea pig erythrocytes (0.1%) were added to wells and the plates were covered with film. After incubation at 4°C for 30 min, the plates were gently rinsed to float the erythrocytes and examined for HAd under a microscope. For HMV-II, 25 µl of the culture fluid including detached cells was mixed with an equal volume of erythrocytes (0.1%) on a glass slide and examined under a microscope for HAd of the detached cells because HAd with the detached single cell could be easily demonstrated (Fig. 1), and there was no difference in the sensitivity of HAd with detached or fixed cells.

Clinical specimens. Throat swab specimens were obtained from 6,005 pediatric patients with acute respiratory infections who visited the outpatient clinic or were hospitalized in Yamagata City Hospital Saiseikan from June 1988 to June

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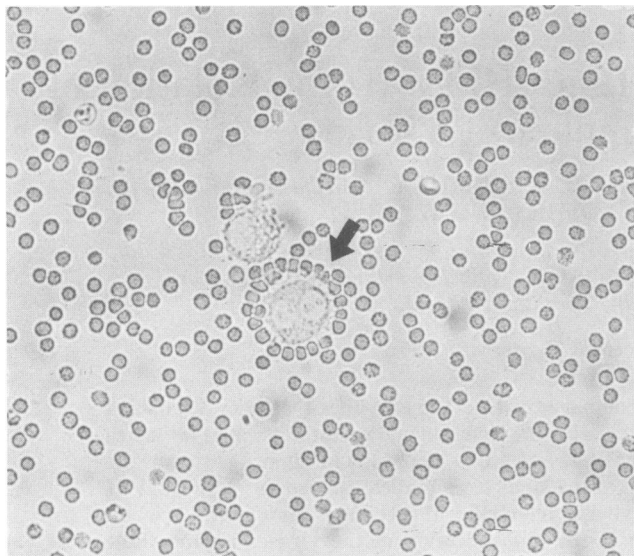


FIG. 1. HAd of detached HMV-II cell with guinea pig erythrocytes.

1989. Throat swabs were placed in tubes containing 3 ml of transport medium consisting of MEM with 0.5% gelatin, 0.2% bovine serum albumin, 500 U of penicillin, and 500 µg of streptomycin per ml. The collected specimens were kept for 1 to 4 days at 4°C and then transported twice a week to the Virus Center, Sendai National Hospital, for virus isolation.

Virus isolation. For virus isolation, the microplate method using 96-well microplate cultures (14) was employed. After centrifugation at 3,000 rpm (KS-5000P centrifuge with an RS-4 rotor; Kubota Manufactory, Tokyo, Japan) for 15 min, the supernatant of the specimens was inoculated directly into the two microplate cultures described below, and the remaining specimens were stored at -80°C for reisolation. The microplates containing Vero and MDCK cells were washed twice with phosphate-buffered saline without calcium and magnesium, and then 0.1 ml of the same MM as that described above for the titration was added to each well of the plates. The HMV-II plates were prepared by adding 0.1 ml of the cell suspension (10⁶/ml) in MM to each well of the plates just before the clinical specimens were inoculated. A throat swab specimen (0.1 ml) was then inoculated into each of three wells of the cultures. The inoculated plates were incubated at 33°C in a CO₂ incubator. On day 10, HAd tests with both chicken and guinea pig erythrocytes were performed.

Identification. Influenza A and B viruses were cytopathic effect or HAd positive in MDCK cells. Influenza C viruses were HAd positive with chicken erythrocytes in HMV-II cells but not with those of guinea pig, while parainfluenza viruses were HAd positive with guinea pig erythrocytes. Finally, the isolates were identified by means of a hemagglutination inhibition test (6), using the following antisera: influenza A/Yamagata/120/86 (H1N1), A/Sichuan/2/87 (H3N2), and B/Victoria/2/87 received from the National Institute of Health of Japan; influenza C/JJ/50 prepared in Yamagata University; and parainfluenza types 1 to 3 purchased from Denka Co. (Tokyo, Japan).

TABLE 1. Results of titrations of influenza and parainfluenza viruses in different tissue cultures

Virus	Infectivity titer (log ₁₀ /25 µl) ^a in cell line:				
	HMV-II	MDCK	LLC-MK2	Vero	CPMK
Influenza A (H1N1)	1.75 ^b	6.25 ^c	ND	ND	ND
Influenza A (H3N2)	1.25 ^b	4.75 ^c	ND	ND	ND
Influenza B	2.5 ^b	7.0 ^c	ND	ND	ND
Influenza C	6.5 ^b	4.75 ^b	3.75 ^b	ND	ND
Parainfluenza 1 ^d	6.5 ^b	ND	6.0 ^b	6.0 ^b	6.25 ^c
Parainfluenza 1 88-6936 ^e	2.75 ^b	ND	1.75 ^b	2.0 ^b	2.75 ^c
Parainfluenza 2 ^d	5.5 ^b	ND	4.75 ^b	5.5 ^b	4.75 ^c
Parainfluenza 2 89-2499 ^e	2.75 ^b	ND	3.0 ^b	2.5 ^b	2.75 ^c
Parainfluenza 3 ^d	6.5 ^b	ND	5.75 ^b	6.0 ^b	5.5 ^c
Parainfluenza 3 86S-470 ^e	2.75 ^b	ND	2.75 ^b	2.75 ^b	2.75 ^c

^a Geometric mean. ND, Not done.

^b Each experiment was repeated three times.

^c Each experiment was repeated two times.

^d Prototype.

^e Isolate.

RESULTS

Sensitivities of influenza and parainfluenza viruses to various tissue cultures. The infectivities of influenza and parainfluenza viruses were comparably titrated, using HMV-II, MDCK, LLC-MK2, Vero, and CPMK cells. The results of titrations of the viruses with different tissue cultures are shown in Table 1. Influenza A and B viruses were susceptible to both HMV-II and MDCK cells, but titers were significantly higher in MDCK than in HMV-II cells, with 10^{3.5} to 10^{4.5} 50% tissue culture infective dose differences. Although the influenza C virus was cultivated in HMV-II, MDCK, and LLC-MK2 cells, the titer was highest in HMV-II cells, there being a difference in excess of 10 50% tissue culture infective doses from the others. The prototype strains and new isolates of parainfluenza viruses types 1 to 3 were grown almost equally in all four cultures.

Isolation of influenza and parainfluenza viruses from clinical specimens by using microplate cultures of HMV-II, MDCK, and Vero cells. The 6,005 throat swab specimens were simultaneously inoculated into HMV-II, Vero, and MDCK cultures. The numbers of influenza and parainfluenza viruses isolated with each culture are shown in Table 2.

The influenza A (H1N1 and H3N2) and B viruses were isolated only with MDCK cells. The influenza A (H1N1) viruses were isolated from 412 samples during the influenza epidemic from December 1988 to March 1989, and influenza A (H3N2) and B viruses were sporadically isolated from each of four cases after the epidemic. The influenza C

TABLE 2. Isolation of influenza and parainfluenza viruses from clinical specimens

Virus	No. (%) isolated in cell line:		
	HMV-II	Vero	MDCK
Influenza A (H1N1)	0	0	412 (100)
Influenza A (H3N2)	0	0	4 (100)
Influenza B	0	0	4 (100)
Influenza C	17 (100)	0	7 (41) ^a
Parainfluenza 1	24 (92)	3 (12)	0
Parainfluenza 2	19 (68)	17 (61)	0
Parainfluenza 3	97 (100)	1 (1)	0
Mumps	11 (12)	91 (100)	0

^a Disappeared in the second passage.

TABLE 3. Reisolation of parainfluenza viruses from stored clinical specimens

Parainfluenza virus	No. (%) reisolated in cell line:		
	HMV-II	CPMK	
		With trypsin	Without trypsin
1	11 (92)	8 (67)	7 (58)
2	13 (93)	11 (79)	11 (79)
3	67 (99)	39 (57)	39 (57)

viruses were isolated from 17 samples with HMV-II cells and from 7 samples with MDCK cells. The seven isolates recovered with MDCK cells disappeared in the second passage, while these viruses could be passaged to hen's eggs. The parainfluenza viruses types 1 to 3 were isolated from 157 specimens with HMV-II cells and from 21 specimens with Vero cells. Among the three viruses, types 1 and 3 were mostly recovered with HMV-II but not with Vero cells, although there was no difference in the isolation rate of type 2 virus between HMV-II and Vero cells. However, of the 28 isolates of type 2, 11 were recovered only with HMV-II cells, 9 were recovered only in Vero cells, and the remaining 8 were recovered in both HMV-II and Vero cells. On the other hand, mumps viruses were isolated 100% in Vero cells and 12% in HMV-II cells.

Reisolation of parainfluenza viruses from clinical specimens, using CPMK and HMV-II cells. To compare the sensitivities of HMV-II and PMK cells for the parainfluenza viruses, 110 stored specimens which were positive in the test described above were inoculated simultaneously to both HMV-II and CPMK cells with and without trypsin (2 µg/ml) (Table 3). All of the parainfluenza viruses were isolated more highly in HMV-II than in CPMK cells, although there was no difference in the CPMK isolation rate according to the presence or absence of trypsin in the MM.

DISCUSSION

In the present paper, we have reported for the first time the isolation of influenza C virus with an established cell line, HMV-II, from pediatric patients with acute respiratory infections throughout a year. Although the influenza C viruses were isolated with both HMV-II and MDCK cells, the viruses were recovered 100% with HMV-II cells but only 40% with MDCK cells. In addition, all of the viruses recovered with MDCK cells disappeared in the second MDCK passage.

It may be an important fact that influenza C virus was isolated from only 17 of 6,005 samples tested over a year, although the HMV-II cell line is as sensitive to the virus as embryonated hen's eggs are (12). From seroepidemiological findings in Yamagata that most young children were seropositive to the virus (4), it may be concluded that the influenza C virus is prevalent in infants and young children. Therefore, the results of the study indicate the two possibilities that influenza C infections are usually inapparent and rarely included in the pediatric patients examined or that the specimens obtained from patients with influenza C infection are inadequate for virus isolation. It might be disadvantageous for the isolation of influenza C viruses that only throat swabs were used in this study and were kept at 4°C for 1 to 4 days before inoculation to the tissue cultures, although large numbers of influenza A (H1N1) and parainfluenza viruses were isolated from the same specimens.

Nevertheless, HMV-II cells are useful for the isolation of the virus to clarify the epidemiology and clinical features of influenza C infections.

The results described here also indicate that HMV-II cells are significantly more sensitive for the isolation of parainfluenza viruses than are other cell lines (Vero or LLC-MK2 and possibly even PMK). In the comparative isolation from clinical samples between HMV-II and Vero cells, the overwhelming majority of the viruses of types 1 and 3 were recovered with HMV-II cells, although there was no significant difference in the recovery of type 2 virus. In the reisolation results, it was also evident that HMV-II cells were more sensitive to all types of parainfluenza viruses than were CPMK cultures. Though we did not compare rhesus PMK cells in the study, it is also known that there is no difference in sensitivity to the parainfluenza viruses between CPMK and rhesus PMK cells (3). Therefore, it is emphasized that the HMV-II cells are the most sensitive for the isolation of the parainfluenza viruses among tissue cultures used to date, although there was no difference in the infectivity titrations of the viruses among various cells.

It is noticed that HAD is clearer in detached cells of HMV-II on a glass slide than in monolayers of Vero or CPMK cultures. Thus, HMV-II may have an advantage for detecting a small number of infected cells, which would result in a high rate of isolation.

In the present study, we applied the microplate method reported by Numazaki et al. (14) for virus isolation from pediatric patients, modifying the concentration of trypsin in the MM. By using this method, 6,005 clinical specimens were easily handled for isolation.

In summary, the studies described in this report indicate that the HMV-II cell line is useful for the isolation of influenza C as well as of parainfluenza viruses from clinical specimens.

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