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Canine Kidney Cell Line for Isolation of Respiratory Viruses

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By means of a continuous canine kidney cell line (MDCK), influenza viruses were rapidly isolated from specimens collected from patients with respiratory disease. The cell line proved more sensitive than either eggs or rhesus monkey cells for currently circulating influenza A and B strains. Influenza viruses caused a distinct cytopathology within 5 days of inoculation if trypsin-ethylenediaminetetraacetic acid was incorporated into the medium. Sufficient hemagglutinin was produced on the initial tissue culture passage to allow direct identification of isolates by hemagglutinin inhibition tests. A variety of other respiratory viruses replicated in MDCK, and over a 10-month period 211 of 600 specimens (35%) yielded viruses.

For reliable isolation of influenza viruses, inoculation of primary rhesus monkey kidney cells (RMK) and 10- to 11-day-old embryonated hen's eggs has been recommended in light of the finding that influenza A strains are most consistently isolated in eggs, whereas RMK is more sensitive for some influenza B strains. The development of a single continuous cell line highly sensitive to wild strains of both influenza A and B would be of practical importance. Recent reports indicated that MDCK (9), a canine kidney cell line, can efficiently support the growth of a variety of laboratory strains of influenza A and B viruses without adaptation (3, 11, 12). Trypsin has been incorporated into the medium because it hastens the appearance of cytopathic effect (CPE) of influenza A viruses by some as yet undetermined mechanism(s). Preliminary reports on the use of MDCK for influenza virus isolation from human specimens have appeared (11, 12).

In this paper, we report the use of MDCK for primary virus isolation from clinical respiratory specimens. Our data show that not only influenza but also other respiratory viruses can be isolated in MDCK. We also report the presence in some chicken sera of antibody that will inhibit the hemagglutination of MDCK-grown influenza viruses.

MATERIALS AND METHODS

Specimens. Respiratory secretions were collected from 600 patients with acute febrile respiratory illness at four facilities during a 10-month period ending 30 April 1977. These included the Vanderbilt Pediatric Vaccine Clinic (children under 3 years old), the Vanderbilt Children's Hospital Wards, the Pediatric Out-Patient Clinic of Nashville General Hospital, and Vanderbilt University Student Health Service (young adults). During epidemics of influenza A/USSR and A/Texas in the winter of 1977-1978, further comparisons of MDCK and egg inoculation were carried out. A nasal wash with 10 ml of normal saline was obtained from infants under 3 years old, whereas a throat swab was obtained from individuals over 3 years old and put into 2 ml of Hanks balanced salt solution supplemented with 0.5% gelatin and antibiotics. Specimens were kept on ice and inoculated immediately on receipt from the clinics, usually within 12 h of collection.

Cell cultures and media. MDCK was purchased from Flow Laboratories (Rockville, Md.) and passaged in Eagle minimal essential medium supplemented with 10% fetal bovine serum, antibiotics, and bicarbonate at weekly intervals. Monolayers 2 to 4 days old, prepared from passage levels 85 through 128, were used for inoculation. Maintenance medium used after inoculation of specimens was Eagle minimal essential medium supplemented with 0.1% bovine serum albumin, 0.1% glucose, antibiotics, and bicarbonate. Trypsin (2 μg/ml; Worthington Biochemicals Co., Freehold, N.J.; lyophilized) and 0.9 μg of ethylenediaminetetraacetic acid (EDTA) per ml were added to two of four tubes used for each specimen.

Virus isolation and identification. Specimens were inoculated within 12 h of collection into MDCK tubes that had been washed three times with Hanks balanced salt solution. The inoculum was 0.2 per tube. Adsorption was for 1 to 2 h; 2 ml of media, with or without trypsin was then added, and incubation was carried out in a stationary position at 34°C for up to 20 days. Hemadsorption with 0.1% guinea pig erythrocytes was done for 30 min at 4°C every 5 days. Blind passage was not done.

The majority of the samples, 420/600, were each inoculated on collection into two 10- to 11-day-old embryonated hen's eggs intraamnionically, 0.2 ml, and intraallantoically, 0.2 ml. Eggs were incubated at 34°C for 3 days. A blind passage of egg harvests was done.

One hundred fifty-eight specimens collected from the Pediatric Vaccine Clinic and the hospital wards were inoculated onto HEp-2, RMK, WI-38, and human embryonic kidney (HEK) cell cultures as well as MDCK.

MDCK and RMK cultures with 2+ to 4+ CPE or positive hemadsorption, and all egg harvests, were tested for hemagglutinin (HA) to guinea pig and chicken erythrocytes. When sufficient HA was present in the infected medium, identification by the hemagglutination inhibition (HAI) test was performed. Respiratory syncytial virus (RSV) and herpes simplex virus (HSV) isolates were identified by indirect immunofluorescence after passage in HEp-2 cells.

Antisera. Chicken anti-influenza A/Victoria/3/75 (H3)-equine/Prague/1/56 (Neq1), anti-A/New Jersey/8/76(Hsw1)-equine/Prague/1/56 (Neq1), anti-A/swine/76/31 (Hsw1 N1), and anti-B/HK/5/72 sera were obtained from the Center for Disease Control, Atlanta, Ga. Guinea pig anti-parainfluenza types 1, 2, 3, 4a, and 4b sera and negative control serum were obtained from the Reference Reagents Branch, National Institutes of Health, Bethesda, Md. The chicken and guinea pig sera were treated with receptor-destroying enzyme by a standard method (10) to inactivate nonspecific HA inhibitors. A guinea pig anti-RSV serum, a fluorescein-labeled goat anti-guinea pig globulin serum, rabbit anti-herpes simplex types 1 and 2 sera, a fluorescein-labeled goat anti-rabbit globulin serum, and a fluorescein-labeled rabbit anti-chicken immunoglobulin G serum were obtained from commercial sources and were shown to be specific for the homologous antigens.

Immunization of rabbits with MDCK. Two New Zealand white rabbits (male, 3 kg) were immunized with 2×10^7 MDCK cells that had been washed 10 times in normal saline. An initial intravenous injection was followed by two intramuscular injections at 4-week intervals. Freund complete adjuvant was used in the second immunization. Sera were collected before immunization and a week after the last injection. These sera were heat inactivated at 56°C for 30 min and were tested for anti-MDCK antibody by indirect membrane fluorescence of unfixed MDCK cells using fluorescein-labeled goat anti-rabbit globulin as the second antibody. The rabbit sera were also treated wtih receptor-destroying enzyme to inactivate the nonspecific HA inhibitors for HAI tests with influenza viruses.

Purification of influenza viruses. MDCK-grown influenza B/HK and A/Victoria isolates were purified by adsorption onto and elution from chicken erythrocytes (7). Allantoic fluids infected with these viruses were mixed with equal volumes of supernatant fluid from a sonically disrupted MDCK cell suspension and were purified by the same method.

Membrane fluorescence method. Chicken and rabbit antibody against MDCK cells was measured by a standard indirect membrane fluorescent antibody method using trypsinized MDCK cells as antigens (5).

RESULTS

Viruses isolated and their CPE. Of 600 specimens, 211 (35%) yielded viruses in MDCK over a 10-month period. These viruses included influenza A/Victoria (6), influenza B/HK (158), parainfluenza type 3 (24), parainfluenza type 4A (2), RSV (9), and HSV type 1 (14). Two specimens yielded both influenza B/HK virus and HSV in MDCK. There was no difference in the isolation rate of viruses in the MDCK cultures maintained with or without trypsin-EDTA. However, trypsin-EDTA hastened the appearance of CPE by approximately 1 day for influenza B/HK and by 2 to 3 days for influenza A/Victoria. The appearance of hemadsorption by parainfluenza type 3 isolates was also hastened by the addition of trypsin-EDTA. Trypsin-EDTA did not affect the CPE of RSV, HSV, and parainfluenza type 4a isolates.

All influenza virus isolates had distinct CPE within 5 days postinoculation when trypsin-EDTA was added to the medium. The CPE began with focal areas of rounding cells 1 to 4 days postinoculation. These cells were readily detachable. Once a focus developed, the entire monolayer became rapidly involved in the CPE. Therefore, hemadsorption with guinea pig erythrocytes was not necessary for the recognition of influenza isolates. Every medium harvested when the cells showed 2+ to 4+ CPE contained sufficient HA activity (1:8 to 1:1,024, mostly 1:64 to 1:256) to allow prompt identification by HAI. Fifty-seven percent of influenza isolates were identified by day 2, 90% by day 3, and 100% by day 5 of inoculation.

CPE of parainfluenza viruses was absent or minimal during the isolation passages in MDCK. If present, it was characterized by rounding and detachment of a few scattered cells. Hemadsorption was uniformly present, but the HA titer in the infected medium did not reach sufficient titer for HAI during the initial passage. Further passages or sonic disruption of the monolayer resulted in an increase of HA to levels that allowed specific identification.

RSV isolates showed focal areas of rounding cells of variable size, usually at the edge of the monolayer, but these foci extended only a little on further incubation. Syncytium formation was rarely seen. The CPE was first observed 2 to 11 days postinoculation (mean 4.3 days). RSV isolates could be passed in MDCK as well as in HEp-2, but the CPE did not progress on several passages in MDCK.

HSV isolates showed focal clusters of enlarged rounding cells 1 or 2 days postinoculation. The progression of CPE was slower than in more sensitive cell lines.

Comparison of the sensitivity of MDCK and other systems. Based upon testing 600 specimens over 10 consecutive months, MDCK was as sensitive as the conventional systems for influenza A/Victoria, influenza B/HK, parainfluenza type 3, and RS viruses (Table 1). Subsequently A/Texas and A/USSR viruses were epidemic in Nashville, and the ability to isolate these in eggs and MDCK was compared (Table

TABLE 1. *Comparison of MDCK and other systems for virus isolation*

Isolation system	Viruses isolated[a]							
	Influenza B/HK	Influenza A/Victoria	Influenza A/Texas	Influenza A/USSR	Parainfluenza 3	RSV	Adenovirus	Enterovirus
MDCK	126/127	6/6	36/38	27/27	8/8	4/4	0/12	0/3
Eggs	10/72	6/6	32/38	12/27	ND	ND	ND	ND
RMK	51/55	ND	ND	ND	6/8	ND	0/12	3/3
HEp-2	ND	ND	ND	ND	ND	4/4	4/12	ND
HEK	ND	ND	ND	ND	ND	ND	11/12	ND

[a] Successful isolations in indicated system/Positive isolation in any system. ND, Not done.

1). Of particular note, only 10 of 72 influenza B/HK isolates and 12 of 27 influenza A/USSR isolates that were positive in MDCK were detected in eggs. When frozen specimens known to be positive for influenza B virus in both MDCK and RMK were titrated simultaneously in MDCK and RMK, the titer was consistently 30-fold or more higher in MDCK than in RMK (Table 2). Even RMK-grown isolates showed higher titer in MDCK than in the original RMK system.

Presence of host antigen on the MDCK-grown viruses and of natural anti-MDCK antibody in sera. During identification of influenza B/HK and A/Victoria strains isolated in MDCK, we found nonspecific HAI with some chicken sera reagents (Table 3). The nonspecific inhibition occurred both with and without trypsin-EDTA in the media at titers ranging from 1:8 to 1:32. Influenza harvests obtained from RMK or egg allantoic fluids were inhibited only by the corresponding specific antiserum. The specificity of the antisera for egg-grown virus indicates that the usual influenza nonspecific inhibitors were appropriately inactivated.

Nonspecific inhibition was observed when eight clones from an influenza B/HK isolate (by three successive plaque-to-plaque clonings in MDCK) were tested, indicating that another agent was not present in the isolate.

Single passage of the MDCK-grown influenza B/HK and A/Victoria isolates in eggs or in RMK resulted in the disappearance of such nonspecific inhibition. On the other hand, a single passage of egg-grown influenza viruses (A/Victoria and B/HK/8/73) in MDCK was enough to acquire the "host antigen" and give nonspecific inhibition (Table 3). However, a simple admixture of the egg-grown virus with MDCK lysate did not result in the nonspecific inhibition. The data suggest that the chicken sera used had either a specific or a cross-reactive antibody to MDCK and that influenza viruses acquired the host antigen upon growth in MDCK.

Six of nine chicken sera (4/5 immune sera and 2/4 negative control sera) inhibited HA activity of MDCK-grown influenza viruses. Adsorption of sera with MDCK cells eliminated nonspecific inhibition (Table 3), but absorption with HEp-2 cells was ineffective. Chicken sera with nonspecific inhibitors had anti-MDCK antibody as detected by fluorescence.

TABLE 2. *Comparative titration of influenza B/HK in MDCK and in RMK*

Specimen	Titers in cell culture[a]:		
	MDCK	RMK (A)	RMK (B)
TS 1[b]	$10^{2.8}$	$10^{0.5}$	$10^{0.5}$
TS 2[b]	$10^{2.5}$	$10^{1.0}$	$10^{0.5}$
TS 3[b]	$10^{3.2}$	$10^{1.0}$	$10^{0.5}$
TS4/MDCK1[c]	$10^{7.0}$	$10^{5.5}$	$10^{5.5}$
TS5/MDCK1[c]	$10^{7.0}$	$10^{5.5}$	$10^{5.5}$
TS6/RMK1[c]	$10^{6.5}$	$10^{4.3}$	$10^{4.5}$

[a] Titers were calculated by the method of Reed and Muench and are expressed as 50% tissue culture infective dose per milliliter.

[b] Throat swab specimens that were initially positive in both systems and had been frozen until titration.

[c] Throat swab specimens that had been passed once in MDCK or RMK cultures and had been frozen until titration.

Rabbits immunized with MDCK developed anti-MDCK antibody detected by immunofluorescence with the simultaneous appearance of HAI activity against MDCK-grown influenza viruses (Table 4). One rabbit possessed low antibody activity in preimmune serum. The use of purified MDCK-grown influenza viruses made by adsorption onto and elution from chicken erythrocytes consistently increased the nonspecific HAI activity of these rabbit sera twofold, suggesting competitive inhibition of HAI by free host antigen when the crude harvests were used as the antigens. Absorption of these sera with MDCK cells again eliminated the HAI activity.

Five lots of guinea pig anti-parainfluenza virus sera and a normal guinea pig serum showed no such inhibition of MDCK-grown influenza HA activity.

DISCUSSION

The data presented here are in agreement with the reports of Tobita et al. in that MDCK

TABLE 3. *Representative results of HAI of influenza strains by chicken sera*

Chicken serum	Antigen						
	B/Hong Kong			A/Victoria		A/swine/2/26/75	
	MDCK2	MDCK2/RMK1	MDCK2/egg1	MDCK2	MDCK2/egg1	Egg	Egg/MDCK1
Anti-B/HK, lot 750216	1:16 (1:8)[a]	1:8	1:16	1:8	<8	<8	1:8
Anti-A/Victoria, lot 770050	1:8	<8	<8	1:256	1:512	ND	ND
Anti-A/New Jersey, lot 760047	1:16 (<8)	<8	<8	1:8	<8	1:128	1:128
Negative control lot 1	<8	<8	<8	<8	<8	<8	<8
Negative control lot 2	1:16 (<8)	<8	<8	1:16	<8	<8	1:8 (<8)
Negative control lot 3	1:8	<8	<8	ND	ND	ND	ND

[a] Figures in parentheses indicate the HAI titers after absorption with MDCK cells.

TABLE 4. *Effects of rabbit anti-MDCK sera on the HAI of egg- or MDCK-grown influenza viruses*

Antigen	Titers on rabbit 1 sera		Titers on rabbit 2 sera	
	Preimmune	Postimmune	Preimmune	Postimmune
A/Victoria/MDCK	<8	16	8	16
	<8[a]	*32*	*8*	*32*
A/Victoria/egg	<8	<8	<8	<8
	<8	*<8*	*<8*	*<8*
B/HK/MDCK	<8	16	16	32
	<8	*32*	*32*	*64*
B/HK/egg	<8	<8	<8	<8
	<8	*<8*	*<8*	*<8*
MDCK[b]	<4	256	4	64

[a] Italic figures indicate that HAI titers against purified viruses.

[b] Tested by the indirect membrane fluorescent antibody method.

is a suitable cell line for clinical isolation of influenza A and B viruses (11, 12). Several observations in this study extended our knowledge of the suitability of MDCK. First, MDCK was as sensitive as RMK for the current influenza B strains. However, when the infectivity of the specimens was compared by 50% tissue culture infective dose, MDCK was 100 time or more sensitive than RMK. Second, an advantage of MDCK over RMK or other cell cultures is that hemadsorption may not be necessary for the recognition of influenza A and B viruses, since all 164 isolates were recognized to be influenza viruses by the distinctive CPE and were subsequently identified directly by HAI. Third, MDCK is a system that permits rapid identification of influenza viruses; of 164 influenza isolates, 57% were identified by day 2, 90% by day 3, and 100% by day 5 after inoculation. RMK usually needed 1 to 2 days more than MDCK to show equivalent HA (data not shown). Last, the sensitivity of MDCK to several laboratory strains of influenza A and B was constant during a 10-month period with over 40 subcultures, as judged by plaque numbers in bimonthly titration. Therefore, MDCK is more suitable than primary cell lines, which vary in sensitivity, for the quantitative study of virus shedding in natural or live-vaccine infections.

MDCK was also sensitive to parainfluenza types 3 and 4a, RSV, and HSV. After the surveillance period, parainfluenza types 1 and 2 were also isolated in MDCK (unpublished data). However, MDCK is not a suitable system for the propagation of these viruses, since these viruses did not yield high infectivity or HA titers during several MDCK passages.

Fifteen specimens positive for adeno- or enteroviruses (six adeno-type 1, two adeno-type 35, three adeno-untyped, two coxsackie A 9, and one poliovirus type 1) failed to grow in MDCK. Gaush et al. also reported the ineffectiveness of MDCK for poliovirus cultivation (2), but adenovirus types 4 and 5 are reported to grow in MDCK (1).

It is known that influenza viruses mature by budding from infected cell membrane and incorporate some host components into the virions. However, host antigen has been detected immunologically only on influenza viruses grown in chicken and turkey embryos or in mouse lung (4, 6, 8). Influenza viruses grown in some cell cultures were reported to have no detectable host antigen (4, 8).

The present study has shown that MDCK-grown influenza viruses acquire host antigen(s) that can be detected immunologically. It has also shown the frequent presence of natural antibody to MDCK in chicken sera. Although the antibody level is usually low, attention must be

directed to the presence of nonspecific antibody when chicken sera are used for the identification of MDCK-grown influenza viruses. The nature and origin of this antibody are not clear at present. The presence of antibody against MDCK-grown influenza viruses in some diagnostic sera poses the one identifiable drawback to utilization of MDCK as a routine diagnostic system. As noted, appropriate adsorption of the sera with the cells or a single passage of the isolate in another system can allow specific identification.

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