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Recovery and Identification of Human Myxoviruses[1]

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INTRODUCTION

The myxoviruses which infect man can be divided into two major subgroups (Table 1): the influenza subgroup which includes influenza virus types A, B, and C and the noninfluenza subgroup which includes parainfluenza types 1 (HA-2), 2 (CA), 3 (HA-1), and 4, mumps, measles, and respiratory syncytial viruses.

Related but distinct antigenic variants or subtypes have been detected among types of both subgroups. In this manner, three subtypes are included among influenza virus type A: A, A_1, and A_2 (16, 17, 18). Influenza virus type B comprises three subtypes: B, B_1, and B_2 (16, 17, 23). Recently, two subtypes, A and B, were recognized for parainfluenza type 4 virus (4). In addition, at least two different antigenic variants have been detected among respiratory syncytial virus strains (7).

BIOPHYSICAL AND BIOLOGICAL PROPERTIES OF HUMAN MYXOVIRUSES

As determined by electron microscopy, with the use of the phosphotungstic acid-negative staining technique, human myxoviruses are medium-sized, that is, they measure from 80 to 120 mμ (influenza subgroup) or from 150 to 250 mμ (noninfluenza subgroup) (29). These viruses contain ribonucleic acid (RNA). The nucleoprotein or inner component possesses a helical symmetry and is surrounded by an outer envelope of lipoprotein with periodic projections which represent the hemagglutinin. The ribonucleoprotein helix of the parainfluenza viruses appears to be single-stranded (6).

Myxoviruses are inactivated by diethyl ether and by a low *p*H (i.e., 3.0 or less) (1, 14). They are also rapidly inactivated at temperatures between 20 and 37 C. Most types hemagglutinate and hemadsorb avian and/or mammalian erythrocytes, the exception being respiratory syncytial virus. They exhibit variable cytopathology during replication in tissue culture, ranging from minimal cell degeneration (as with parainfluenza types 1 and 4 virus) to marked syncytial formation (as with measles and respiratory syncytial viruses).

ISOLATION OF HUMAN MYXOVIRUSES

For successful isolation of these viruses, adequate collection and handling of clinical specimens is of the utmost importance.

Because of the instability of most myxoviruses, specimens should be collected in media containing protein stabilizers, i.e., bovine albumin or gelatin, and adjusted to *p*H 7.2. In addition, specimens should be inoculated into susceptible

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TABLE 1. *Myxoviruses of man*

Subgroup	Type	Subtype
Influenza	Influenza A	A, A_1, A_2
	Influenza B	B, (B_1, B_2?)
	Influenza C	—
Noninfluenza (Paramyxovirus)	Parainfluenza 1	—
	Parainfluenza 2	—
	Parainfluenza 3	—
	Parainfluenza 4	A, B
	Mumps	—
	Measles	—
	Respiratory syncytial	Number not known

tissue cultures shortly after collection without prior freezing and thawing (2, 3).

Furthermore, infectivity is enhanced when tissue cultures are maintained in fluid media free from specific or nonspecific inhibitors. Optimal growth of influenza A_2 virus occurs when inoculated cultures are incubated at 33 C rather than at 37 C (25). Other myxoviruses grow as well at 33 C as at 37 C. Incubation of cultures on a roller drum enhances growth of parainfluenza type 4 virus (4).

Most human myxoviruses, with the exception of influenza virus types A, B, and C, grow poorly or not at all in embryonated hen's eggs. However, most naturally occurring strains grow readily in primate kidney tissue culture cells. Since certain strains of influenza A_2 and B viruses and most of the parainfluenza virus types fail to produce a detectable cytopathic effect during isolation in tissue culture, other techniques are often required for their detection. The property most commonly used for this purpose is hemadsorption (5, 28). Hemadsorption occurs when erythrocytes added to a myxovirus-infected tissue culture are adsorbed to the host-cell surface.

The occurrence of the hemadsorption phenomenon is accounted for by certain factors involved in the replication of myxoviruses. Assembly or maturation of the infectious virus particle takes place at or near to the host-cell surface, and its release is carried out over a long period of time by a process of extrusion or by filament formation (22). Thus, the virus outercoat material is present at the host-cell surface, providing sites to which the erythrocytes can adsorb.

These characteristics make the hemadsorption phenomenon a reliable indicator system for detection of myxovirus strains in susceptible tissue culture systems. Consequently, it is a fundamental technique for the recognition of naturally occurring strains of parainfluenza viruses.

The use of primary monkey kidney culture combined with hemadsorption for isolation-passage of hemadsorbing myxoviruses provides certain advantages over the use of embryonated eggs. Monkey kidney cultures are readily available, are easily manipulated, and can be held in incubation for at least 4 weeks; embryonated eggs must be inoculated when they are of a certain age and cannot be held after 3 weeks of incubation. In addition, tissue culture favors the replication of some human myxoviruses which grow poorly, or not at all, in eggs, such as parainfluenza virus types 1, 2, 3, and 4, and respiratory syncytial virus.

Conflicting results have been reported concerning the sensitivity of monkey kidney cultures for recovery of influenza A2 strains (19, 20, 21, 24). Even though we have not compared the sensitivity of embryonated eggs and monkey kidney tissue culture for primary isolation of influenza A2 strains, our experience suggests that monkey kidney is an efficient system for recovery of these strains. Several comparative studies have indicated that monkey renal tissue culture is superior to eggs for recovery of influenza B viruses (13, 26).

The use of monkey kidney tissue culture in myxovirus isolation attempts, however, does involve a serious problem, and that is the frequent occurrence of two simian myxoviruses, SV5 and SV41, as indigenous contaminants of the cell culture system (5, 20*a*). Both simian myxoviruses share antigenic components with the prototype human parainfluenza 2 virus (CA) when tested by complement fixation (5, 20*a*). However, they are distinct among themselves and from the prototype human parainfluenza 2 virus (CA) when tested by tissue culture neutralization or hemadsorption inhibition with specific guinea pig sera (5). This disadvantage, however, should not preclude the use of monkey kidney tissue culture for isolation of human myxoviruses. Contamination by these simian agents, most often by SV5, can be controlled by adding 0.2% of hyperimmune rabbit serum to both growth and maintenance media of monkey kidney cultures. Although the growth of human parainfluenza type 2 virus (CA) is not inhibited by this concentration of anti-SV5 rabbit serum, there is cause for concern. Recently, evidence has been presented that SV5 can infect man (15). In addition, there may exist as yet undetected human myxoviruses which share major antigenic components with

these simian agents. If so, they will not be recovered in cultures containing simian virus immune serum. Furthermore, during isolation passage avid strains of influenza A2 virus are frequently nonspecifically inhibited by muco-proteins contained in the anti-SV5 rabbit serum used in the maintenance media.

HEMADSORPTION TECHNIQUE

As a standard method in myxovirus isolation studies we have employed guinea pig erythrocytes at a concentration of 0.4%. A 0.2-ml amount of such a suspension is added to a culture tube which contains 1 ml of fluid medium. Incubation of the cultures to which erythrocytes have been added is usually carried out for 30 min at 4 C. All of the myxovirus-infected cultures give a strong hemadsorption reaction with guinea pig erythrocytes at this temperature, except parainfluenza type 4 virus which requires 25 to 37 C for maximal patterns. Temperatures above 25 C cause a reversal of positive patterns in cultures infected with parainfluenza virus types 1 and 3.

Tissue cultures inoculated with specimens from individuals with illness are tested for hemadsorption at 5-day incubation intervals from 3 to 4 weeks, depending on the appearance and viability of the tissue culture cell monolayer. The quantity of free virus in the fluid medium is usually insufficient to interfere with the hemadsorption reaction.

During subsequent passage of the isolates, however, higher concentrations of free virus, sufficient to interfere with hemadsorption, are found in the fluid medium 3 to 5 days after subinoculation of the tissue cultures. Because of the formation of large aggregates of agglutinated erythrocytes in the medium, few such cells are available for hemadsorption. It is advisable, when large quantities of virus have been inoculated, to wash the cultures three times before attempting to test for hemadsorption. It is also desirable to use fresh or recently harvested guinea pig erythrocytes, since older cells often produce nonspecific hemadsorption patterns.

The time required for initial detection of human myxoviruses in primary rhesus monkey kidney by hemadsorption with guinea pig erythrocytes is shown in Table 2. In a 5-year period, 94% of 745 isolates were detected by hemadsorption within 2 weeks after inoculation of the cultures with throat-swab specimens. The majority of influenza virus types A2 and B and parainfluenza virus types 1 and 3 isolates were detected within 10 days, and mumps virus isolates were detected within 15 days after inoculation of the cultures. Parainfluenza virus

TABLE 2. *Time required for detection of myxovirus isolates in primary rhesus monkey kidney tissue culture by hemadsorption, January, 1958, through December, 1963*

Virus type	No. of isolates	Per cent of isolates detected at indicated time after inoculation (days)				
		1–5	6–10	11–15	16–20	21–25
Parainfluenza 1	197	47	42	8	3	0
Parainfluenza 2	73	4	38	38	14	6
Parainfluenza 3	274	40	46	12	22	0
Parainfluenza 4	29	4	4	57	14	21
Influenza A_2	67	33	39	21	4	3
Influenza B	77	40	59	1	0	0
Mumps	28	14	46	32	4	4
Total	745	35	43	16	4	2

TABLE 3. *Comparison of sensitivity of primary rhesus and cercopithecus monkey kidney tissue cultures for isolation of myxoviruses, January, 1962–March, 1963*

Virus type	No. of isolates	Tissue culture of isolation					
		Rhesus		Cercopithecus		Rhesus and cercopithecus	
		No.	Per cent	No.	Per cent	No.	Per cent
Parainfluenza 1	51	49	96	47	92	45	88
Parainfluenza 2	2	2	100	2	100	2	100
Parainfluenza 3	38	36	95	34	89	32	84
Parainfluenza 4	23	23	100	7	30	7	30
Influenza A_2	15	15	100	2	13	2	13
Influenza B	45	45	100	34	76	34	76
Mumps	9	8	89	7	78	6	67
Totals	183	178	97	133	73	128	70

TABLE 4. *Time required for detection of cytopathic effects produced by respiratory syncytial virus isolates in a human heteroploid cell line (HEp-2), March, 1960–May, 1963*

Days after inoculation of cultures	Isolates	
	No.	Per cent
1–3	8	2
4–6	88	19
7–9	170	36
10–12	120	26
13–15	43	9
16–18	29	6
19–	11	2
Total	469	100

type 2 and 4 isolates grew somewhat more slowly, and usually required from 2 to 3 weeks of incubation before hemadsorption could be detected.

Although naturally occurring strains of human myxoviruses can replicate in human heteroploid cell lines and in human diploid cell strains, primary monkey kidney has been found to be the most sensitive system for isolation of the hemadsorbing types.

As shown in Table 3, primary rhesus and cercopithecus monkey kidney tissues were equally sensitive for recovery of parainfluenza type 1, 2, or 3, or mumps viruses. However, primary rhesus monkey kidney culture was more sensitive than cercopithecus for isolation of parainfluenza type 4 and influenza types A2 and B viruses. Ninety-seven per cent of all the isolates were recoverable in rhesus monkey kidney tissue cultures.

RESPIRATORY SYNCYTIAL VIRUS

Among the human myxoviruses, respiratory syncytial virus alone fails to hemagglutinate or hemadsorb. In addition, it is extremely thermolabile, and therefore special precautions should be taken during isolation attempts. Specimens should be inoculated fresh into tissue culture without prior freezing and thawing (2).

During the isolation passage in human heteroploid cell lines, respiratory syncytial virus produces a characteristic syncytial change. Table 4 shows the time required for detection of cytopathic effects in HEp-2 cell cultures. Ninety-two per cent of the respiratory syncytial virus strains produced easily observable changes within 2 weeks after inoculation of tissue culture. In addition, 57% of the isolates could be detected by syncytium formation within 9 days after inoculation of HEp-2 cells with throat-swab specimens.

Table 5 presents a comparison of sensitivity of HEp-2 and primary rhesus and cercopithecus monkey kidney cultures for isolation of respiratory syncytial virus strains. During three consecutive outbreaks of respiratory syncytial virus infection, 340 strains were isolated; 99% of these strains were recovered in HEp-2, whereas 28% were detected in rhesus and only 14% in cercopithecus monkey kidney cultures. Thus, HEp-2 cell cultures proved to be the most sensitive culture system for isolation of naturally occurring strains of respiratory syncytial virus.

However, one difficulty is associated with the use of human heteroploid cell lines for recovery of respiratory syncytial virus; namely, differences in sensitivity of HEp-2 and HeLa cells to these strains have been found on a number of occasions (27).

IDENTIFICATION OF HUMAN MYXOVIRUS ISOLATES

Preparation of Specific Immune Sera

The preparation of specific immune sera for human myxoviruses deserves special consideration. Among the most common laboratory animals, guinea pigs, hamsters, and ferrets are susceptible to infection by human myxoviruses when inoculated by the intranasal route (8, 9, 10, 11, 12). Although rabbits and roosters are insensitive to infection, they can be used for preparation of myxovirus antisera. However, heterologous antibody responses may occur in these animals after repeated booster inoculations.

Animals used for preparation of immune sera should come from colonies kept in isolation and cared for by only one individual. In addition, they should be free from antibodies for known myxoviruses. Certain colonies of guinea pigs are found to have antibodies against human parainfluenza type 3 (HA-1) and less often against human parainfluenza types 1 or 2 or mumps virus.

After intranasal inoculation, the infected

TABLE 5. *Comparison of sensitivity of HEp-2 and primary rhesus and cercopithecus monkey kidney cultures for recovery of respiratory syncytial virus, October, 1960-March, 1963**

Tissue culture of isolation	No.	Per cent
HEp-2	338	99
Rhesus	94	28
Cercopithecus	48	14

* Specimens were from throat swabs; the number of isolates was 340.

animals should be kept in isolation rooms to avoid cross-infection, and then given an intramuscular or intraperitoneal booster inoculation, 2 or 3 weeks after initial infection.

Destruction of nonspecific inhibitors. Nonspecific inhibitors for the hemadsorbing myxoviruses can be destroyed by treatment of the immune sera with receptor-destroying enzyme (RDE), heating at 56 C for 30 min, trypsin, periodate, or CO_2. In our experience, pretreatment of the immune sera with receptor-destroying enzyme (RDE) plus heat-inactivation at 56 C for 30 min has proved to be a satisfactory method for destruction of nonspecific inhibitors for parainfluenza viruses.

Identification of Hemadsorbing Myxovirus Isolates

All human hemadsorbing myxoviruses can be readily identified by the hemadsorption-inhibition test.

Hemadsorption-inhibition test. After 2 or 3 days of incubation, the tissue cultures inoculated with virus isolates are washed twice with Hanks' balanced salt solution; then 0.6 ml of this solution and 0.2 ml of a 1:10 dilution of a potent RDE-treated, heat-inactivated specific immune serum is added. The serum is incubated with the culture for 30 min at room temperature, after which 0.2 ml of a 0.4% suspension of guinea pig erythrocytes is added. The cultures are examined after incubation for 30 min at 4 C. Isolates are identified by the immune serum which prevents hemadsorption.

Alternatively, the hemadsorbing isolates can be identified by the conventional hemagglutination-inhibition technique or by tissue culture neutralization. These procedures are used less often than the hemadsorption-inhibition test for routine identification purposes.

Complement fixation. Whenever a myxovirus isolate cannot be identified by these techniques, its identity should be sought by complement fixation with the use of specific guinea pig sera. This test is performed with 2 exact units of complement, 4 units of antigen, and overnight fixation at 4 C.

Complement-fixing antigens can be prepared from infected monkey kidney culture suspensions. Unfortunately, these complement-fixing antigens are often anticomplementary. When infected tissue culture complement-fixing antigens fail to fix complement, it is possible to prepare suitably potent antigens by concentration of the original material by centrifugation at 30,000

TABLE 6. *Relation of parainfluenza virus serotypes by complement fixation with specific guinea pig sera*

Immune guinea pig serum*	Reciprocal of antibody titer with 4 units of antigen							
	PARA 1	PARA 2	PARA 3	PARA 4-A	PARA 4-B			
				M-25	19503	19057	19241	19429
Parainfluenza 1 (HA-2)	*320*	<20	<20	<20	<20	<20	<20	<20
Parainfluenza 2 (CA)	<20	*320*	<20	<20	<20	<20	<20	<20
Parainfluenza 3 (HA-1)	<20	<20	*320*	<20	<20	<20	<20	<20
Parainfluenza 4 subtype A (M-25)	<20	<20	<20	*320*	160	160	160	160
Parainfluenza 4 subtype B (19503)	<20	<20	<20	160	*320*	320	320	160

* Guinea pigs infected intranasally and then given an intramuscular booster inoculation after 3 weeks.

TABLE 7. *Identification of isolates as parainfluenza type 4 virus by complement fixation with specific guinea pig sera*

Infected monkey kidney tissue culture suspension	No. of isolates tested	Fixation of complement with 8–16 units of indicated serum				
		Para 4 (M-25) 1:10	Para 1, 2, or 3 (Hominis), 1:20	Para 2 (SV5) 1:20	Mumps, 1:20	Influenza C, 1:20
Undiluted	19	3+ to 4+†	0	0	0	0
10 × concentrated*	6	4+	0	0	0	0

* Centrifugation at 30,000 rev/min for 1 hr (spinco model L).
† Indicates 75% (3+) to 100% (4+) fixation of complement.

TABLE 8. *Representative relationships among parainfluenza type 4 virus strains and other myxoviruses by tissue culture neutralization*

Immune guinea pig serum	Reciprocal of neutralizing antibody titer against 10 to 320 $TCID_{50}$ of indicated virus						
	Para 4 subtype A		Para 4 Subtype B		Para 1 (HA-2)	Para 3 (HA-1)	Mumps
	M-25	19899	19503	19057			
Parainfluenza 4 subtype A (M-25)	*320*	*160*	<20	20	<20	<20	<20
Parainfluenza 4 subtype B (19503)	20	<20	*160*	*160*	<20	<20	<20
Parainfluenza 1 (HA-2)	<20	<20	<20	<20	*640*	<20	<20
Parainfluenza 3 (HA-1)	<20	<20	<20	<20	<20	*640*	<20
Mumps	<20	<20	<20	<20	<20	<20	*80*

TABLE 9. *Patterns of hemadsorption-inhibition observed during identification of parainfluenza type 4 virus isolates*

No. of isolates	Hemadsorption-inhibition with indicated guinea pig serum*				Interpretation of hemadsorption-inhibition	Identity by tissue culture neutralization
	Para 4 subtype A	Para 4 subtype B	Para 3	Normal		
2	++	0	0	0	Parainfluenza 4 subtype A	Parainfluenza 4 subtype A
26	0	++	0	0	Parainfluenza 4 subtype B	Parainfluenza 4 subtype B†
1	0	+	0	0	Parainfluenza 4 subtype B	Parainfluenza 4 subtype B†

* No inhibition, 0; almost complete inhibition, +; complete inhibition, ++.
† Only 11 isolates tested by tissue culture neutralization.

rev/min for 1 hr in the model L Spinco ultracentrifuge.

Table 6 shows the specificity of the complement fixation test for differentiation of parainfluenza virus serotypes. Results presented in the table indicate the lack of relationship among parainfluenza types 1, 2, 3, and 4 viruses. However, parainfluenza 4 subtypes A and B are closely related when tested by complement fixation.

If, after exhausting all of these serological techniques, the hemadsorbing isolate still remains unidentified, it is likely that such an isolate may well represent a new antigenic variant or subtype. In these cases, specific antiserum against the isolate can be prepared by intranasal inoculation of guinea pigs or hamsters free from antibodies for known human myxoviruses. As soon as the specific immune serum is available, the unidentified strain must be fully characterized and cross-tested by tissue culture neutralization, hemadsorption-inhibition, and complement fixation against the known human myxovirus prototypes. This characterization is required to determine the extent and nature of the antigenic differences.

Identification of Nonhemadsorbing Myxovirus Isolates

Respiratory syncytial virus strains are routinely identified by complement fixation. After the isolation passage, the isolates are subinoculated into HEp-2 cell cultures and complement-fixing antigens are prepared from infected culture suspensions. Specific antiserum for respiratory syncytial virus can be produced by intranasal infection of ferrets (8). Characterization of antigenic variants is best accomplished by the tissue culture neutralization technique with the use of this specific serum.

PROBLEMS INVOLVED IN IDENTIFICATION OF HUMAN MYXOVIRUS ISOLATES

Antigenic Variation

Influenza A and B viruses. These myxoviruses show major periodic changes in antigenic composition (12, 16, 17, 18, 23). Antigenic subtypes emerge more frequently among Type A viruses. These subtypes share major antigenic components in variable proportion with subtypes prevalent in previous outbreaks of influenza infection.

Newer antigenic subtypes can be characterized by hemadsorption inhibition, complement fixation, or hemagglutination inhibition with specific immune sera prepared against past and current strains. However, the most sensitive technique for detection of antigenic differences is the tissue culture neutralization test, employing hemadsorption as the indicator system.

Respiratory syncytial virus. Antigenic variants have been detected among respiratory syncytial virus strains by tissue culture neutralization with specific ferret sera (7). At least two distinct antigenic variants are recognized at present. The extent and significance of these antigenic changes are as yet undetermined.

Parainfluenza type 4 virus. During 1962 and part of 1963, 29 myxovirus strains with the biological properties of parainfluenza type 4 virus were isolated from children with respiratory-tract infection. During the course of characterizing the strains isolated in 1962, it was found that the majority of isolates could not be identified by hemadsorption-inhibition with the use of antiserum prepared against the prototype M-25 parainfluenza type 4 virus.

Because of these failures, the isolates were subsequently examined by complement fixation. As shown in Table 7, these isolates reacted only with the specific guinea pig serum prepared against the parainfluenza type 4 virus (M-25 strain). Further complement fixation tests definitively established that these new strains were antigenically identical, and indistinguishable from the prototype strain.

However, the earlier results obtained by hemadsorption-inhibition suggested the possibility of antigenic differences between them. Therefore, evidence of specific antigenic differences was sought and demonstrated by tissue culture neutralization (Table 8). Only two isolates were identical to the prototype strain, whereas the remaining isolates were found related but distinct from it.

A more complete characterization of all the 1962 and 1963 strains was carried out by hemadsorption inhibition with the use of specific guinea pig sera pools, as summarized in Table 9. Results permitted the segregation of all of the isolates into two subtypes, according to their external antigenic structure; subtype A, containing strains indistinguishable from the prototype virus, and subtype B, containing those strains related but distinct from the prototype strain (4).

It is not known whether both subtypes have existed for a long time or whether this antigenic variation is discontinuous in nature, as is the case with influenza A and B viruses.

Other human myxoviruses. Influenza C, parainfluenza types 1, 2, and 3, mumps, and measles viruses appear to be antigenically stable and homogenous. Thus far, antigenic variation of these myxovirus serotypes has not been detected.

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