Replication of Murine Paramyxoviruses in Hamster Tracheal Organ Culture and Comparison with Standard Tissue Culture Methods

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Replication of Sendai virus, pneumonia virus of mice, and SV5 was investigated in tracheal organ cultures from 2- to 4-day-old and 2- and 4-week-old hamsters, and viral infectivity in tracheal explants was compared with that in tissue culture monolayers. Explants from 2- to 4-day-old hamsters produced higher titers of the three paramyxoviruses, as detected by hemadsorption with guinea pig and murine erythrocytes in primary rhesus monkey kidney cells. Tracheal cultures from 2- and 4-week-old hamsters yielded 1.5 and 2.5 log₁₀ lower infectivity titers. Infected explants exhibited cytopathological changes that correlated well with cessation of ciliary activity. Viral titers in BHK-21, Vero, and BS-C-1 monolayer cells, the systems commonly used for isolation and propagation of murine paramyxoviruses, were lower than those in 2- to 4-day-old hamster tracheal explants. These observations suggest that hamster trachea organ culture could have practical application as an aid for primary isolation of paramyxoviruses from clinical specimens from rodents with respiratory ailments.

Murine paramyxoviruses are naturally occurring infectious agents of mice (4, 5, 7, 10-13), rats, and hamsters (14) (J. C. Parker, J. I. Hercules, and E. von Kaenel, *Abstr. Annu. Meet. Am. Soc. Microbiol.* 1967, V172, p. 163) which do not generally produce clinical signs or mortality in colony epizootics. Although most attention has been focused upon asymptomatic infections, natural outbreaks of these paramyxoviruses are responsible for pneumonia and bronchitis (2, 24). Experimental studies showed that these viruses replicate in the upper respiratory tract and lungs and produce a spectrum of clinical diseases that includes rhinitis, tracheitis, and bronchopneumonia (1, 3, 17, 21, 22). Despite major advances in laboratory animal medicine during recent years, paramyxoviruses exist within rodent breeder colonies as widespread enzootic infections and undermine the validity of many experiments.

Tracheal organ culture models derived from mice (25) and hamsters (19) have permitted studies on Sendai virus replication in differentiated respiratory tissue and observations on virus cytopathology independent of host defenses. Although organ cultures of ciliated epithelium have been used in human viral diagnostic work (6, 9, 18, 20), little attempt has been made to utilize them as a diagnostic aid for virus isolation in laboratory rodents. The present

study describes the use of hamster tracheal organ culture to investigate Sendai, pneumonia virus of mice (PVM), and simian virus 5 (SV5) virus replication and to compare the viral infectivity in tracheal explants with that in tissue culture monolayers.

MATERIALS AND METHODS

Preparation of tracheal organ culture. Tracheal explants were prepared by the technique described by Schiff and Shefner (19). Two- to four-day-old and 2- and 4-week-old outbred Syrian hamsters of the Cr:RGH(SYR) strain were used. The hamsters showed no evidence of previous infection with Sendai, PVM, or SV5 viruses, as indicated by undetectable levels of hemagglutination inhibition antibody.

Whole trachea structures were removed from 2- to 4-day-old hamsters under aseptic conditions and opened by a longitudinal incision through the cartilaginous dorsal wall. The explants were carefully arranged, lumen side up, on silk screens supported by stainless-steel grids in organ culture dishes (Falcon Plastics, Oxnard, Calif.), and medium was added until its surface was level with the epithelium of the tracheal explants. Tracheal ring organ cultures were prepared from 2- and 4-week-old hamsters by cutting transversely between each cartilage with a razor blade. Rings were placed on areas of crosshatch scratches on the floor of a petri dish (60 by 15 mm), and medium was added to a level that just covered the rings.

The medium used in this study was CMRL 1066 (GIBCO) supplemented with 0.2% bovine serum al-

bumin, 2 mM glutamine per ml, 250 U of penicillin per ml, 250 μ g of streptomycin per ml, and 100 U of mycostatin per ml and buffered with 0.025 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid). Medium was adjusted to pH 7.2, and cultures were incubated at 35°C in a humidified atmosphere of 5% CO₂ in air.

Cell culture. The BHK-21 cell line, originally derived from kidneys of 1-day-old Syrian hamsters, was grown in BHK-21 medium (GIBCO) supplemented with 10% tryptose phosphate broth (Difco), 10% mycoplasma- and virus-screened fetal bovine serum (Flow Laboratories) and 2 mM glutamine, plus 250 U of penicillin and 250 μ g of streptomycin per ml. The cell lines BS-C-1 and Vero, originating from African green monkey kidney, were grown on medium consisting of Eagle minimum essential medium (E-MEM; GIBCO) supplemented with fetal bovine serum, glutamine, penicillin, and streptomycin as described above. The cell lines were obtained from Flow Laboratories.

Stock viruses. Viruses used in this study were obtained from the American Type Culture Collection, Rockville, Md. The D/Sendai/52 strain of parainfluenza type 1 has been described (19). A stock of this virus was grown in the allantoic cavity of 10-day-old chicken embryos and had a titer of 10^{4.0}/ml. Sendai virus titers were determined by hemadsorption with guinea pig erythrocytes in BS-C-1 cell monolayers. PVM strain 15 was propagated and titrated in BHK-21 cell cultures; the titer of 10^{3.5}/ml was based upon the end point of cytopathogenic effect (CPE). The 21005-2WR strain of parainfluenza type 2-SV5 was grown in 10-day-old chicken embryos, and the amniotic fluid had a titer of 10^{4.3}/ml. SV5 virus titers were determined by hemadsorption with guinea pig erythrocytes in primary rhesus monkey kidney cells. All virus stocks were stored at -70°C in 1-ml volumes.

Inoculation of organ and cell cultures. Twenty-four hours after planting, the culture medium was removed, and explants were examined under an inverted microscope at 100 $\times$ magnification. Tracheal explants showing strong ciliary activity were used. The experiments were performed in duplicate, using 14 organ culture dishes containing two explants each from each of the three age groups.

Silk screens containing explants from 2- to 4-day-old hamsters were removed from the organ culture vessel and placed in a petri dish (35 by 10 mm). Individual explants were infected by inoculating 0.2 ml of tissue culture fluid containing 10¹, 10², or 10³ HAD₅₀ (dose at which 50% of cultures show hemadsorption) of Sendai, PVM, or SV5 viruses. After adsorption for 1 h at 35°C, the silk screens containing the explants were washed three times in Hanks salt solution and placed on a grid over the center well of an organ culture dish filled with enough medium to wet the explant, permitting the epithelial surface to rest at the medium-air interface.

In dishes containing ring explants from 2- and 4-week-old hamsters, the culture medium was withdrawn, and 0.2 ml of inoculum containing 10¹, 10², or 10³ HAD₅₀ was added. The cultures were incubated for 1 h at 35°C in a 5% CO₂ and air environment.

After adsorption, tissues were washed in Hanks salt solution and 2 ml of fresh medium was added. All cultures were incubated at 35°C in a humidified 5% CO₂-air atmosphere.

Confluent monolayers of BHK-21, BS-C-1, and Vero cells grown in duplicate 75-cm² plastic flasks were infected with 0.5 ml containing 10² HAD₅₀ of stock virus and allowed to absorb for 1 h at 37°C. Unadsorbed inoculum was aspirated from each flask, and BS-C-1 and Vero cells were refed with E-MEM containing 2% fetal bovine serum that had been heated to 56°C for 45 min. Cultures of BHK-21 cells were maintained in BHK-21 medium supplemented with 5% tryptose phosphate broth and 2% inactivated fetal bovine serum.

Titration of viruses. Each day throughout a 7-day period, culture medium was removed from two dishes of infected organ cultures and stored at -70°C until titrated. To assay viral replication in the cell lines, culture fluids harvested at 4 days after virus inoculation were frozen at -70°C. End point titrations of Sendai, PVM, and SV5 viruses were carried out in primary rhesus monkey kidney cells with 10-fold dilutions of virus made in E-MEM with 2% heat-inactivated fetal bovine serum, 2 mM glutamine per ml, penicillin, and streptomycin. In addition, assays for Sendai and PVM virus infectivity were performed in medium containing 0.1% SV5 hyperimmune serum. Before inoculation with the test dilution, the monolayers were washed twice in Hanks salt solution. The cultures were inoculated with 1 ml of the appropriate virus dilution (four tubes per dilution) and incubated at 36°C, and on day 5 after inoculation the culture medium was removed. Sendai and SV5 virus titers were determined by hemadsorption with 0.5 ml of 0.4% guinea pig erythrocytes; PVM titers were assayed by hemadsorption using murine erythrocytes. The 50% end point titration was determined by the method of Reed and Muench (16).

RESULTS

Viral replication in organ culture. Explants from animals in the three age groups supported the replication of the three paramyxoviruses for the 7-day experiment, as shown in Fig. 1. An inoculum of 10² HAD₅₀ of Sendai, SV5, and PVM viruses consistently produced the highest titers in tracheal organ cultures from 2- to 4-day-old hamsters.

Regardless of the concentration of the virus inoculum or the age of the hamster from which the explant was taken, the titer of Sendai virus increased slightly during the first 24 h and then rose 100- to 1000-fold between days 2 and 4. Sendai virus reached peak titers in tracheal explants from each of the three age groups at 4 days after initiation of infection, and then declined somewhat, but remained at high levels throughout the observation period of 7 days.

Tracheal organ cultures from 2- to 4-day-old hamsters inoculated with 10² HAD₅₀ of SV5

FIG. 1. Replication of Sendai, SV₅, and PVM viruses in tracheal organ cultures from hamsters of different ages. Each point represents mean titers from four culture dishes containing two explants each. Symbols: Organ cultures inoculated with 10¹ HAD₅₀ (○), 10² HAD₅₀ (●), and 10³ HAD₅₀ (△).

virus particles showed a significant increase in viral titer. Substantial amounts of virus were present in the medium by day 7. Cultures that received an inoculum of 10³ HAD₅₀ showed only a slight increase in titer during the first 48 h, and titers fell rapidly between days 2 and 7. When cultures were infected with 10¹ HAD₅₀ of SV₅ virus per explant, virus production reached a peak 4 days after infection and then decreased at a rate similar to that in the cultures infected with 10² HAD₅₀. In cultures from 2- and 4-week-old hamsters inoculated with SV₅ virus, no significant increase in viral titer was observed, but low levels of infectious virus were still present by day 7.

PVM virus replicated rapidly and to high titers in organ cultures of trachea from 2- to 4-day-old hamsters. An inoculum of 10² HAD₅₀ of PVM produced the highest titer (10^{5.75} HAD₅₀)

by day 4, dropping only slightly during the 7-day incubation period. A similar pattern of viral replication was observed when explants were inoculated with 10¹ HAD₅₀ of PVM virus. After inoculation of 10³ HAD₅₀, the titer of PVM virus in the medium rose 100-fold by day 2 and then fell slowly to 10²⁻³ HAD₅₀ by day 7. Virus titers in cultures from 2-week-old hamsters were maintained between 10² and 10⁴ HAD₅₀ through the 7-day cultivation period. Cultures from 4-week-old animals showed no significant increase in PVM titers.

Virus controls that did not contain tracheal explants and uninfected control cultures were examined daily during the 7-day period. In the explant-free virus control, all viruses were completely inactivated after 3 days of incubation. The uninfected control explants remained healthy; i.e., the epithelium remained intact

and smooth, and ciliary activity was strong throughout the observation period.

Comparison of viral susceptibility of cell lines with that of tracheal explants. Since, as shown in Fig. 1, maximum virus yields in explants from 2- to 4-day-old hamsters occurred at 4 days after inoculation with an inoculum of 10^2 HAD₅₀ of virus, these conditions were used to compare susceptibility of cell cultures and explants to the murine paramyxoviruses. The replication and CPE of Sendai, PVM, and SV5 viruses in established cell lines were compared with tracheal organ cultures from 2- to 4-day-old hamsters (Table 1).

Tracheal explants supported replication of Sendai virus and showed progressive damage to the superficial layers of the epithelium (19). A marked decrease in ciliary activity was noted 4 days after infection. The first signs of cytopathic change began to appear 4 days after inoculation; severe damage was observed by day 7. This CPE was characterized by vacuoles within the epithelium, in addition to rounded and loosely connected cells around the epithelial surface, which gradually detached, leaving the epithelium with a flattened appearance. Damage to the superficial epithelial cells correlated well with diminution or cessation of ciliary activity. Similar patterns of epithelial deterioration were seen in explants infected with PVM and SV5.

The viral titers in BHK-21, Vero, and BS-C-1 cells were lower than those in tracheal explants from 2- to 4-day-old hamsters. The CPE observed in stained preparations of infected BHK-21, Vero, and BS-C-1 cell monolayers exhibited small intracytoplasmic inclusions, as well as

extensive cellular degeneration as described by Reed et al. (15). The greatest difference between results in tracheal explants and cell lines were observed with PVM and SV5.

The cytopathic end point in both the explant and cell cultures corresponded with the production of virus; where the cultures showed no evidence of CPE, as with PVM and SV5 viruses in BS-C-1 cells, no virus was detected.

DISCUSSION

The value of whole hamster tracheal organ culture in laboratory rodent virology lies primarily in the fact that the cellular architecture remains morphologically and functionally similar to that in the intact host. Under the conditions of the present studies, and those previously described (19), the original anatomical features of the trachea epithelium can be maintained for periods of up to 27 days. The tracheal explants are small enough to be nourished by diffusion in the described organ culture system, making these cultures particularly easy to establish. Long-term maintenance of hamster trachea explants is of practical importance for routine diagnostic work, since it makes cultures available when clinical specimens are presented to the laboratory. Organ cultures have a further advantage over disaggregated cultured cells in that their preparation does not require enzymatic treatment that might induce cellular changes.

It has been shown that Sendai virus, an indigenous respiratory virus of rodents, replicates in organ culture of mouse (25) and hamster (19) trachea with destruction of the ciliated epithelium. In the present investigation it was shown that organ cultures of hamster trachea support the replication of PVM and SV5 viruses in addition to Sendai. The three paramyxoviruses multiplied to highest titers in tracheal explants from 2- to 4-day-old hamsters infected with 10^2 HAD₅₀ of virus, inducing a distinctive pattern of morphological changes. Explants inoculated with a higher inoculum (10^3 HAD₅₀) produced peak titers lower than those infected with 10^2 HAD₅₀, suggesting that an autointerference may be occurring, similar to that described by von Magnus (23). In this case, a high multiplicity could lead to the production of large amounts of incomplete, noninfectious virus. Huang and Baltimore (8) have shown that defective interfering virus particles are produced when cells are infected with a high multiplicity of viruses and that these particles interfere specifically with intracellular replication of nondefective homologous virus.

No significant differences in Sendai virus

TABLE 1. Titration of released infectious progeny and CPE of Sendai, PVM, and SV5 virus 4 days after inoculation of 10^2 HAD₅₀ of virus into cell lines and tracheal organ cultures from 2- to 4-day-old hamsters

Host system	Virus					
	Sendai		PVM		SV5	
	Titer	CPE	Titer	CPE	Titer	CPE
Tracheal explant	6.5 ^a	+ ^b	5.5	+	4.3	+
BHK-21	5.0	+	3.5	+	ND ^c	+
Vero	2.7	+	2.0	+	2.0	+
BS-C-1	4.5	+	— ^d	— ^e	—	—

^a Reciprocal titers expressed log₁₀ HAD₅₀ per milliliter.

^b +, Virus CPE noted.

^c ND, Not done.

^d —, No virus detected.

^e —, No virus CPE.

yields were noted between explants of trachea from 2- to 4-day-old versus 2- and 4-week-old hamsters. In tracheal tissue from 2- and 4-week-old hamsters, PVM and SV5 viruses consistently showed decreased infectivity titers or failed to multiply. No single mechanism can explain age-dependent resistance to viral diseases. With the absence of many host defense mechanisms in the organ culture system, replication factors may play an important role. There may be differences in cell susceptibility of respiratory epithelium from tissue of older animals for these two paramyxoviruses.

Taken together, these observations suggest that the whole tracheal organ cultures of 2- to 4-day-old hamsters may represent a more sensitive culture medium than standard monolayer tissue culture and may prove useful in the isolation of murine paramyxoviruses in subclinical and overt respiratory infections.

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