Pneumonia Virus of Mice (PVM) in Cell Culture.* (27803)

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Although pneumonia virus of mice, first recognized by Horsfall and Hahn(1), has many properties amenable to laboratory investigation(2,3,4), previous attempts to detect its multiplication in an *in vitro* cell system have been unsuccessful (5). This report describes its propagation in primary hamster kidney cells and some characteristics of the virus multiplying in these cells.

Materials and methods. Virus. The strain of PVM was obtained from Dr. Wallace P. Rowe, NIH, Bethesda, Md.‡ The virus was passed 3 times, by intranasal instillation under light ether anesthesia, in 13-17 g Swiss albino mice. Five to 7 days following instillation the lungs of moribund mice were removed, homogenized in chilled phosphate buffered saline (pH 7.4), centrifuged at about 800 g for 10 minutes and the supernatant fluids stored as a 20% suspension at about −70°C

Cell cultures.§ Suckling hamster kidney cells were prepared by modification of the method of Diercks and Hammon(6). Cells were planted at a concentration of 2×10^5 per ml and grown to monolayers in a solution containing 94 parts Hanks' lactalbumin hydrolysate, 5 parts heated (56°C for ½ hour) calf or horse serum and one part of 200 mM glutamine. In some cases the cells were incubated in an atmosphere of 5% CO_2 for 24-48 hours after planting. After inoculation the cells were maintained with a medium of 99 parts Mixture 199 and one part horse or calf serum. Penicillin (200 units/ml) and streptomycin (200 mg/ml) were included in all media.

Cell monolayers in tubes or bottles were inoculated initially with a suitable volume of maintenance media containing the PVM mouse lung suspension at a 1:20 dilution of the stock material, equivalent to an activity of about 10^5 mouse ID_{50} doses per ml of tissue culture fluid. The inoculum was allowed to remain in contact with the cells for only one to 2 hours at room temperature or 37°C. A longer period of exposure caused cell toxicity. The inoculum was then removed and replaced by maintenance medium after one washing with Hanks' BSS. Maintenance medium was changed every 2 to 3 days thereafter and titrated for HA activity at those times.

Hemagglutination (HA) and hemagglutination-inhibition (HI) tests. Samples for HA or HI tests were heated at 70°C for one-half hour(2). Addition of an equal volume of 0.2% trypsin and incubation at 37°C for one hour prior to heating, proved desirable when titrating lung suspension in order to improve the agglutination patern(7). Two-fold serial dilutions with HA buffer‖ as diluent were performed in Microtiter plates¶(8). A 0.5% mouse red blood cell suspension containing 0.02% bovine serum albumin (BSA) (7) was added and the end point was taken as the highest dilution giving 50% agglutination after about one hour at room temperature. HI tests, employing 8 HA units of antigen, were performed in the Microtiter system.

Infectivity and neutralization tests. Infectivity titrations were carried out by instillation of the virus intranasally into CFW or Ha/ICR strain** mice or inoculation into cell cultures. MS_{50} values were calculated by the method of Horsfall(9) and ID_{50} or LD_{50} doses by the Reed-Muench method(10).

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§ Cell cultures and media were obtained from Microbiological Associates, Inc.

‖ Difco, Detroit, Mich.

¶ Cooke Engineering Co., Arlington, Va.

** Carworth Farms, New City, N. Y. and Millerton Farms, Millerton, N. Y.

TABLE I. Serial Propagation of PVM in HKCC.

Passage	Cumulative virus dilution	Days before HA production	Maximum HA titer	Day of maximum titer	Days of HA production
1	10^{-2}	7	1:64	9	> 8
2	$10^{-5.6}$	10	1:32	17	41
3	$10^{-17.9}$	7	1:32	10	>14
4	$10^{-21.9}$	9	1:16	12	>19
5		7	1:16	14	32
6		7	1:16	14	>21

Mouse neutralization tests were carried out with mixtures containing a final concentration of at least 20 mouse LD_{50} doses per ml of lung or cell culture-derived virus and $10^{-1.7}$ dilutions of heated (56°C for 30 minutes) serum. All mixtures were incubated at room temperature for 30 minutes prior to use.

Plasma lactic dehydrogenase. Mice were inoculated intraperitoneally with lung or cell culture samples. Plasma was obtained 48 hours later by the orbital bleeding technic. Lactic dehydrogenase levels were assayed colorimetrically(11) and the end point dilutions converted to standard units by the method of Rowe *et al.*(7).

Hemadsorption. Tests for the ability of infected cell cultures to hemadsorb(12) were carried out by incubating tube or bottle cultures with 0.5% mouse erythrocytes, with or without BSA, at room temperature for one-half hour. The cell sheets were washed 3 times with Hanks' BSS before and after incubation with red blood cells. Hemadsorption-inhibition tests were performed by incubating the cell cultures with dilutions of sera for one-half hour at 37°C or room temperature, washing 3 times with BSS and adding the red blood cells.

Results. 1. The multiplication of PVM in hamster kidney cell cultures (HKCC) was demonstrated by testing the culture fluid for hemagglutinins and examining the cells for cytopathic effect. These approaches were chosen because of technical ease compared to infectivity titrations and because previous observations(3) have indicated a close association of the hemagglutinin with the infectious particle. Hemagglutinins were first detected from 7 to 10 days following inoculation, usually reaching a maximum 2 to 7 days later. Some cultures produced hemagglutinins at low titers up to 41 days following inoculation before the cells degenerated. Table I presents representative virus passage data and cumulative dilutions of the original inoculum.

2. Cell culture fluid from first, third, sixth and eighth cell culture passages of the virus caused death in 5 to 7 days when instilled into mice. Other passages of the virus were not tested. Pathologic changes grossly similar to those caused by lung-derived PVM were seen in all cases. Histological examination of the lungs of mice receiving the third cell culture-passed PVM showed an interstitial pneumonitis consonant with lung-derived PVM(5). Table II presents a comparison of HA and infectivity titers of lung and cell culture-derived virus.

3. Generally, cytopathic effect (CPE) in HKCC started on the seventh day at about the time of hemagglutinin production, but was often variable in degree. CPE was characterized by focal destruction of cells and accumulation of debris. The progression of CPE appeared to be more marked in cells grown on cover slips, while in bottle cultures regrowth of the cell sheet occasionally took place. While CPE was rather uniform in occurrence, we did not find it suitable for use in end point titrations.

4. In addition to suckling hamster kidney cell cultures, *Cercopithecus* monkey kidney

TABLE II. Comparison of HA and Infectivity Titers of Lung and Cell Culture-Derived PVM.

Inoculum	Reciprocal of HA titer	End point log LD_{50}	End point log ID_{50}	End point log MS_{50}
Lung PVM	512	−2.3	>−5.0	−3.1
HKCC PVM (1)*	8	<−1.0	−2.4	<−1.0
" " (1)*	32	−1.5	−3.5	−2.1
" " (8)*	16	− .1	−1.7	− .6

* Passage in HKCC.

TABLE III. HI Comparison of Lung and Cell Culture-Derived PVM.

Antigen	Reciprocal of HI titer — Mouse antiserum — Anti-lung PVM	Anti-HKCC PVM	Anti-lung PVM*	Negative serum
Lung PVM	256	256	80	0
HKCC PVM	256	128	80	0
" "	512	256	160	0

* Obtained from Dr. Wallace P. Rowe.

(BSC-1) gave some evidence of low titer hemagglutinin production. The following cell lines have been screened for hemagglutinin production and CPE with negative results: adult mouse kidney, mouse lung (adult and embryonic), mouse embryo, human embryonic skin and muscle (MAF), human embryonic kidney, chick embryo, bovine embryonic kidney, mouse fibroblast (L-929) and HeLa.

5. Hyperimmune mouse serum was prepared against lung-propagated PVM which had not been in cell culture. This serum, heated and at a $10^{-1.7}$ dilution, neutralized at least 20 mouse LD_{50} doses per ml of both lung and cell culture-derived PVM in mouse neutralization tests. Hemagglutination of cell culture PVM was also inhibited to the same degree as mouse-lung PVM by the sera prepared in our laboratory and PVM hyperimmune mouse sera obtained from Dr. Wallace P. Rowe. Heated mouse serum selected for the absence of HI antibodies to lung virus failed to inhibit HA by cell culture virus or protect mice or cell cultures against cell culture virus. Table III presents the HI comparison of lung and cell culture-derived viruses.

6. PVM combines with a component or components, present in many normal and infected tissues, which inhibits hemagglutination by the virus(3). The complex is destroyed by heating (70°C for ½ hour), unmasking the hemagglutinin. The absence of any significant quantity of these substances in cell culture fluids is shown in Table IV. The sensitivity of unheated cell culture PVM to trypsin is also significant, in that the lung-derived hemagglutinin is sensitive to this amount of trypsin only after heating(7). Also, homogenates of lungs infected with cell culture PVM hemagglutinated only after heating.

7. Hemadsorption of mouse erythrocytes occurred on infected cultures which had begun to produce hemagglutinins but not on uninfected cultures. Immune but not negative serum inhibited the adsorption. This procedure appears to be applicable in end point titrations.

8. Lung passaged PVM (as received from Dr. Wallace P. Rowe) was contaminated with the Lactic Dehydrogenase Agent of Riley (13). Previous attempts to eliminate the contaminant had not been successful(7). This agent does not appear to propagate in HKCC as shown in the experiment presented in Table V. The control groups fall within the 2,000 unit limit established as normal(7).

TABLE IV. Comparison of Effect of Heat and Trypsin on Lung and Cell Culture-Derived Hemagglutinins.

Sample	Reciprocal of HA titer — Not heated	Heated	Trypsin treatment — Before heat	After heat
Lung A	0	128	512	0
" B	0	128	128	0
" C	0	32	32	0
HKCC A	32	16	0	0
" B	128	128	0	0
" C	128	128	0	0

Discussion. PVM exhibits several characteristics which make it a useful model system in the study of cell-virus relationships, including hemagglutination(2), combination with tissue components(3,4) and inhibition by certain bacterial polysaccharides(14). Its multiplication in an *in vitro* cell system, as here described, provides opportunity for new

TABLE V. Comparison of Plasma Lactic Dehydrogenase Levels in Mice with Various Inocula.

Inoculum	No. of mice tested	Units of plasma lactic dehydrogenase — Median	Range
Mouse lung PVM	8	7680	5760–12800
HKCC PVM	10	760	280– 1200
" control	5	640	260– 720
None	9	720	400– 1600

approaches to the study of its interaction with susceptible cells.

The following evidence indicates that PVM propagates in HKCC: (a) evidence of hemagglutinins and infectious virus, produced in a cyclic fashion, in cultures where the initial inoculum had been diluted beyond its extinction point; (b) neutralization of the virus and inhibition of HA by serum prepared against lung passage PVM; (c) hemagglutination by the virus, and hemadsorption by infected cultures, of mouse erythrocytes; (d) production in the mouse of pathologic lesions like those of lung-passaged PVM; (e) combination with tissue components when re-isolated from mouse lungs, and (f) trypsin sensitivity(15).

Virus multiplication was first detected after a period of 7 to 10 days after inoculation. Production of virus continued for a period of days, and was usually accompanied by increased CPE. In some cases there was hemagglutinin production for as long as 41 days before complete cell destruction. Significant quantities of tissue components, which combine with the virus *in vivo,* do not appear to be present in cell culture fluids. The possibility that small amounts of such substances are present in cell cultures has not been excluded. The failure of cell culture PVM to induce increased levels of plasma lactic dehydrogenase in mice suggests that it is separated from the Riley Agent present in mouse lung-passaged material.

Summary. Evidence has been presented for the multiplication of pneumonia virus of mice in suckling hamster kidney cell culture monolayers. Virus was produced in low titer and caused CPE. Infected cultures hemadsorbed mouse erythrocytes. Tissue components which combine with the virus *in vivo* were not detected in cell culture fluids. Propagation in cell culture appears to free PVM from the Riley Agent present as a contaminant of the animal passaged virus.

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