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Infection of Hamsters with Para Influenza 3 Virus. (25816)

JOHN E. CRAIGHEAD,* M. KATHERINE COOK AND ROBERT M. CHANOCK
(Introduced by R. J. Huebner)
Dept. of HEW, Nat. Inst. of Allergy and Infectious Diseases, Lab. of Infect. Dis., Bethesda, Md.

A property shared by many myxoviruses is their capacity for multiplication in lungs of certain rodents(1,2). Many of the genetic factors and quantitative aspects of experimentally induced infections with influenza viruses have been described(3). With extensive use of tissue culture for virus isolation, a group of myxoviruses with common properties has recently been recognized(4,5). These viruses were designated as para influenza viruses 1, 2, and 3 to indicate their distinctness from influenza viruses. Because of their importance in the etiology of childhood respiratory illness, and of their potential importance in adult respiratory disease, experimental animal hosts were sought in which *in vivo* virus-host relationships could be studied(6). The present study is concerned with occurrence and quantitative aspects of pulmonary infection in hamsters with the type 3 para influenza virus.

Methods and materials. Virus. The C 243 strain of para influenza 3 virus, isolated in 1957 from a child with pneumonia(5), was passaged 7 times in rhesus monkey kidney cultures and twice in Salk monkey heart cultures. A stock pool of virus, prepared by combining equal volumes of infected monkey heart culture fluid and skimmed milk, was stored in glass sealed ampules in a CO_2 chest. *Inoculation of hamsters.* One-month-old, recently weaned, golden hamsters of both sexes, weighing 60 to 70 g were used. One-tenth ml of varying dilutions of tissue culture fluid or lung suspension was inoculated intranasally under ether anaesthesia. Inoculated animals were housed in groups of 2 or 3 in shoebox-sized containers in semi dark room. Control animals were inoculated with sterile normal lung suspension and housed similarly. At various intervals following inoculation, hamsters were lightly anaesthetized with ether and exsanguinated. Lungs were removed aseptically, washed twice in Hanks' balanced salt solution, and a 10% suspension prepared in 50% skimmed milk in Hanks' solution by grinding in mortar with alundum. Lung suspensions were stored at -60°C until titered. *Titration.* Decimal dilutions were prepared in Hanks' solution and 0.2 ml was inoculated into each of 2 rhesus monkey kidney cultures. These cultures were maintained in Eagle's basal medium without serum. Seventy-two hours following inoculation, cultures were examined for evidence of infection by the hemadsorption technic. Cultures were washed twice with Hanks' solution and 1 ml of 0.1% suspension of guinea pig red cells was added. Following 20 minutes incubation at 4°C they were examined for presence of erythrocytes adsorbed to the tissue culture sheet. TCD_{50} (50% tissue culture dose) was calculated by the method of Reed and Muench(7). *Serology.* Complement fixation tests were performed by modification of the method of Bengston(5). Complement fixing antigens for various myxoviruses were prepared as described previously (8).

Results. Para influenza 3 virus was carried through 15 consecutive intranasal passages in hamsters. The inoculum of tissue culture virus which initiated the passage series con-

* Present address: Middle America Research Unit, Balboa Heights, Canal Zone.

TABLE I. Multiplication of Para Influenza 3 Virus in Hamster Lung.

Hr after intranasal inoculation	Quantity of virus recovered from lungs at indicated time after inoculation—$\log_{10}$			
	Exp. 1		Exp. 2	
	Tissue culture virus* Inoculum = 4.7	Hamster lung passage 6 virus† Inoculum = 4.2	Tissue culture virus Inoculum = 4.7	Hamster lung passage 8 virus‡ Inoculum = 4.2
1	<2.4	<2.4	<2.4	<2.4
6	<2.4	<2.4	<2.4	<2.4
12	4.1§	2.8	2.4	3.1
24	5.4	4.8	4.9	4.9
36	5.9	5.2	6.1	6.1
48	5.8	5.9	6.4	6.1
72	6.2	5.8	——Not done——	

* 7 passages in tissue cultures derived from monkey kidney followed by 2 passages in monkey heart culture.

† 6 *in vivo* hamster passages initiated with tissue culture virus.

‡ 8 *Idem*

§ Mean of values obtained from 2 hamsters.

tained $10^{5.4}$ TCD_{50}. Hamsters were sacrificed after 48 hours and 10% suspension of lung tissue served as inoculum for the next passage. A 48-hour interval was selected for the passage series since growth curve study indicated that near maximal levels of virus were present at that time (Table I). Virus content of lungs ranged between $10^{5.9}$ and $10^{6.9}$ TCD_{50} and the quantity present during early passages did not differ from that during later passages. No respiratory symptoms were noted, no deaths occurred, and no gross lung lesions were observed.

Para influenza 3 virus was not detected in lungs 1 or 6 hours following inoculation of $10^{4.7}$ TCD_{50} of tissue culture grown virus. At 12 hours virus was present at a low level and increased in titer over the next 24 hours to reach a high level at 36 hours (Table I). These findings, together with results of 15 serial hamster passages, indicate that para influenza 3 virus possesses capacity for growth in the hamster lung.

Not shown in Table I is the finding that levels of virus comparable to those recovered at 48 and 72 hours were present 96 hours after intranasal instillation. At 120 hours, small quantities of virus averaging 10^3 TCD_{50} were present in the lung, while at 144 hours virus could not be detected. Lung lesions were not observed 6 days following virus inoculation.

The pattern of growth of 6th and 8th hamster passage virus in hamster lung was compared with that of tissue culture virus from which it was derived. This comparison (Table I), was performed to determine if variants with a higher level of growth or a more rapid pattern of replication were selected during animal passage. No significant difference was evident in the growth curves, suggesting that tissue culture virus population contained virus particles possessing the properties for optimum replication in the hamster lung.

Quantity of virus required to initiate infection in the hamster lung was determined (Table II) for virus grown only in tissue culture and for virus which had undergone 5 subsequent passages in hamster lung. Approximately 1 tissue culture infectious dose of tissue culture virus was sufficient to infect hamsters by intranasal route. After 5 hamster lung passages, the quantity of virus required to initiate infection of the hamster did not decrease. Tissue culture virus was thus as capable of infecting hamsters as virus which had been passaged in this animal. These findings suggest that selection of variants with increased infectivity for hamsters did not occur during hamster passage. It appears that virus from human source which had been grown only in tissue culture possessed both optimum growth characteristics and infectivity for the hamster lung.

Six hamsters were inoculated intranasally

TABLE II. Quantity of Para Influenza 3 Virus Required to Infect Hamsters by Intranasal Route.

Virus propagated in					
Tissue culture*			Hamster lung†		
Inoculum TCD_{50}	No. hamsters infected/No. inoculated	TCD_{50} recovered from lungs,‡ $\log_{10}$	Inoculum TCD_{50}	No. hamsters infected/No. inoculated	TCD_{50} recovered from lungs,‡ $\log_{10}$
320	3/3	5.4, 5.9, 5.9	500	3/3	4.9, 5.4, 5.9
32	3/3	3.9, 4.9, 5.9	50	2/3	2.4, 2.4
3.2	2/3	2.4, 3.4	5	0/3	
.3	1/3	3.4	.5	0/3	
.03	0/3				

* Monkey kidney culture passage 7, monkey heart culture passage 2.
† 5 *in vivo* hamster lung passages initiated with above described tissue culture virus.
‡ Lungs tested 48 hr after intranasal inoculation of virus.

with $10^{4.7}$ TCD_{50} of virus and bled 3 weeks later to study their serologic response to para influenza 3 infection. When tested by complement fixation, pooled serum from these animals had a titer of 1:1280 with para influenza 3 tissue culture antigen and 1:20 with para influenza 3 soluble antigen. This serum failed to react at a 1:10 dilution with the tissue culture or soluble antigens of para influenza 1 (hemadsorption type 2 and Sendai), para influenza 2, or mumps.

Discussion. Previous studies on replication of myxoviruses in the rodent lung have dealt mostly with influenza in the mouse. Egg propagated and mouse adapted influenza viruses differed in their pattern of growth in the mouse lung. Strains of influenza A or B not adapted to the mouse were characterized by a lag phase during which multiplication did not occur and by production of a smaller quantity of virus in the lung compared to the adapted strains which did not exhibit a lag phase(3). Adaptation following mouse passage has been interpreted as selection and overgrowth of virus mutants which replicate to a high titer without a preliminary lag phase.

The present findings which describe properties of tissue culture propagated para influenza 3 virus in hamster lung, differ considerably from those just summarized for influenza virus in mouse lung. Without prior multiplication in hamster lung, para influenza 3 virus appears to possess the genetic properties for hamster infectivity and replication which characterize an "adapted" strain. Thus, tissue culture para influenza 3 virus resembled virus which had been passaged in hamster lung as regards rate of multiplication in lung, quantity of virus produced in lung, and quantity of virus required to initiate infection. In fact, one infectious dose of tissue culture virus as measured in that system was sufficient to infect hamsters by the intranasal route.

The finding that para influenza 3 virus multiplies to high titer in hamster lung may have practical implications in standardization and evaluation of vaccines. In addition to the usual antibody production test, vaccines could be assayed for potency by their effect upon virus multiplication in the lung when hamsters are challenged subsequent to vaccination.

Previous studies have established that para influenza viruses share common antigens(5,8). Of some importance is the significance of heterotypic antibody in resistance to, or modification of infection. A study of hamsters infected with one para influenza virus and challenged with a heterotypic virus may yield information pertinent to this problem. An investigation of this nature is currently in progress.

Summary. Hamsters were infected by the intranasal route with para influenza 3 virus. One tissue culture infectious dose was sufficient to infect hamsters. Quantity of virus produced in the lung was the same, $10^{5.9}$ to $10^{6.9}$, during infection with virus grown only in tissue culture or virus passaged in hamsters. Infected hamsters developed high levels of CF antibody specific for para influenza 3 virus.

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Method for Detecting Antiviral Agents on Paper Chromatograms. (25817)

ERNEST C. HERRMANN, JR. AND JEAN-PIERRE ROSSELET
(Introduced by Preston L. Perlman)
Dept. of Biochemistry, Schering Corp., Bloomfield, N. J.

The procedure of placing paper chromatograms on the surface of agar seeded with bacteria to locate zones of antibiotic activity was first used to distinguish various components of a penicillin mixture(1,2). This method, now universally used in identification and purification of naturally occurring antibacterial agents, can also be used in a similar manner for characterization of antiviral agents. The following discussion describes an adaptation of the Dulbecco virus-plaque technic(3) to this purpose.

Materials and methods. Preparation of agar overlay, chick embryo cell cultures in Pyrex baking dishes, infection of these cultures with West Nile and vaccinia viruses, subsequent staining of cells to observe plaques, and detection of zones of virus inhibition have been described(4). *Antiviral test materials* used in our experiments include antibiotic W-112, extracted with ether from an unclassified streptomyces species culture and isatin-monothiosemicarbazone synthesized in our laboratories by Dr. Frank Villani. Antibiotic W-112 at concentrations as low as 0.1 μg produced significant inhibition of plaques formed by W. Nile, vaccinia, herpes simplex and Newcastle disease viruses. Isatin-monothiosemicarbazone inhibited only vaccinia virus plaque formation. Since this compound is also active in preventing death of mice infected with many lethal doses of vaccinia virus(5), it represents a model of an antiviral agent active both *in vitro* and *in vivo*. *Paper chromatographic* migration of antibiotic W-112 was obtained with partition systems using aqueous methanol as a stationary phase(6) on No. 1 Whatman paper at 20°C. Development time ranged from 1.1 to 3.2 hours. Solvent systems included system 6, a mixture of 300 ml toluene, 100 ml light petroleum, 300 ml methanol and 700 ml of water. Solvent system 9 was a mixture of 200 ml toluene, 800 ml light petroleum, 200 ml methanol and 800 ml of water. Analysis of isatin-monothiosemicarbazone was carried out on No. 1 Whatman paper at 20°C, using solvent system 16, a mixture of 120 ml n-butanol, 30 ml acetic acid and 50 ml of water, and solvent system 17 made up of 100 ml n-butanol, 100 ml pyridine and 100 ml water. After drying, the ethylene oxide sterilized paper chromatograms (10 x 34 cm) were cut in half and top and bottom portions placed for 5 min on separate agar overlays of virus infected cultures. This time period was adequate for diffusion of the antiviral agent into the agar. Longer time periods, however, frequently resulted in the cell sheet being destroyed, apparently by toxic residues left by chromatographic solvent systems. Position and orientation of the paper were indicated on bottom of baking dish. After removal of paper, baking dishes were sealed with Saran Wrap (Dow Chemical Co.), then incubated for 4 days at 36°C. The cell sheet was then