EXPERIMENTAL TRANSMISSION OF INFLUENZA VIRUS INFECTION IN MICE*

I. The Period of Transmissibility

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For 30 years it has been recognized that mice are susceptible to infection with the viruses of human and swine influenza (1, 2). When strains of virus that have been adapted by serial passage in mice are introduced intranasally or by aerosol, the virus multiplies to high titer, and the animals develop typical pulmonary lesions (1, 3).

Despite the susceptibility of mice to infection with influenza viruses, there have been few successful experimental attempts to induce transmission of infection from one mouse to another. In 1940, Eaton reported that mice infected intranasally with adapted strains of influenza virus transmitted infection to uninfected animals housed in the same jars (4); this was shown by the presence of lung lesions in the previously uninfected animals. However, other investigators have been unable to induce such transmission reproducibly, although they employed the same strain of mice and the same virus strains (5).

Attempts have been made in this laboratory to develop an experimental model for the study of transmission of influenza virus infection in mice, and to employ the model in the study of epidemiologic, virologic, and ecologic factors involved in the transmission of influenza virus infection. The present report will describe the experimental model that has been developed in this laboratory for the study of transmission of influenza virus infection in mice.

Materials and Methods

Mice.—CFW male mice were employed in all experiments. The weight of the animals varied from 18 to 30 gm in different experiments, a variation which was later found to alter susceptibility to transmitted infection.

Demonstration and Titration of Virus.—

Lungs: The presence of virus in the lungs of animals exposed to transmitted infection was demonstrated by inoculation of 10^{-2} dilutions of ground lung suspensions of individual mice

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into chick embryos. Lungs were removed aseptically and ground in glass tubes with teflon grinders as 10 per cent suspensions in 0.01 M phosphate buffered 0.85 per cent saline (PBS) (penicillin 500 U/ml; streptomycin 5 mg/ml). These suspensions were centrifuged at 6300 *G* at 4°C for 15 minutes. Supernatant fluids were diluted 10-fold in PBS and 0.1 ml inoculated into the allantoic cavity of fertile hens eggs, using 3 eggs for each lung suspension. The virus titers of lungs of infector animals were determined at designated intervals by inoculation of serial 10-fold dilutions of ground lung suspensions into chick embryos. Allantoic fluids were harvested after 40 to 48 hours of incubation and tested for viral hemagglutin in 1:4 dilution with 1 per cent human "O" RBC. Fifty per cent titration end points were calculated by the method of Reed and Muench.

Tracheas and noses: Noses were dissected by vertical incisions exposing the nasal septum and each nasal passage past the turbinates into the sinuses. These tissues after excision were ground as 10 per cent suspensions. Tracheas were removed by dissection from just below the pharynx to the bifurcation, and were ground as 1 per cent suspensions. The virus content of tracheas and noses was determined by the same method used to measure pulmonary virus.

Throat swabs: Throat swabs were taken at designated intervals from mice infected in the aerosol chamber. The swabs were made by wrapping wire strips in small bits of cotton. The technique consisted of dipping the autoclaved swab into a plastic tube containing 0.5 ml of buffered antibiotic saline, holding the mice firmly at the back of the head forcing the mouth open, and swabbing as far back in the pharynx as was possible. The swabs were then broken off inside the plastic tubes and the undiluted fluid within the tube inoculated into chick embryos.

Viruses: Initial experiments with the CAM strain of influenza A1 virus (passage history—eggs 71/mice 248/eggs 2/mice 1/eggs 1/mice 1/eggs 2/mice 1/eggs 1) and a strain of influenza A2 virus (Jap. 305) (eggs 4/mice 27/eggs 2), demonstrated that both of these viruses could be transmitted from mouse to mouse. For later experiments both of these viruses were adapted further by preparing allantoic fluid seed after the following procedure: mice were infected by exposure to aerosols of the original viruses; previously uninfected mice were housed in the same cages with infector mice; pooled lung suspensions from mice that had acquired transmitted infection were inoculated in serial 10-fold dilutions into eggs, and after 40 hours of incubation, the allantoic fluids from eggs infected with 100 EID_{50} were pooled as a new seed.

Eggs: 10 to 12-day-old white Leghorn chick embryos incubated at 37°C were employed in the testing and titration of tissues for virus.

Aerosol procedure: The chamber used to induce aerosol infection was a cylindrical autoclave, 13 inches in diameter and 20 inches long (Fig. 1). The chamber was modified so that the outlet of a No. 40 vaponephrin nebulizer could be introduced into the chamber through a hole bored in the front door. A fenestrated pipe along the top of the inside of the chamber was attached to a valve leading to a vacuum source. Another valve which opened at right angles to, and directly beneath the nebulizer outlet, was used as a room air source. A final valve opening was used to measure pressure within the chamber. The conditions under which the aerosol procedure was carried out were as follows:

1. A continuous vacuum was applied so that 25 liters of air/minute flowed through the chamber.

2. Compressed air flowed through the nebulizer under a pressure of 15 pounds/sq. inch, at a rate of 5 liters/minute. The remaining 20 liters of air/minute entering the chamber was room air.

3. Under these conditions, 0.3 cc/minute of nebulizer fluid was introduced into the chamber as an aerosol mist.

4. Particle size of the airborne droplet nuclei was measured by the Casella Cascade impactor. Over 90 per cent of the particles were in the range of 0.5 to 5 micra in diameter.

5. In a wire cage inside the chamber, mice were exposed to an estimated 100 mouse infective doses of virus.

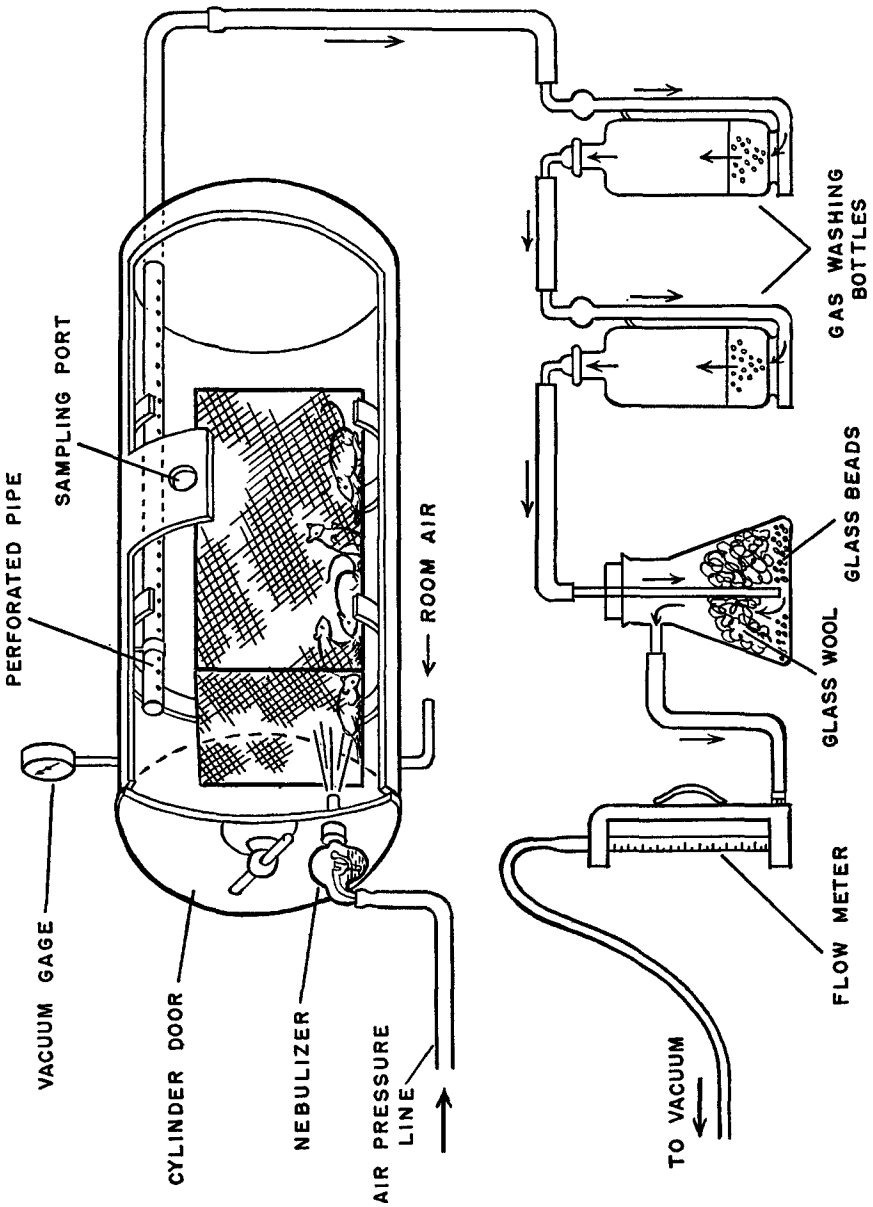

FIG. 1. Model of aerosol chamber for infection of mice with influenza virus.

Contact Procedure.—(See Fig. 2.)

"Incubation" period: Immediately after initiation of infection, mice were placed in small stainless steel cages, 2 mice per cage. The cages were 4 x 5 x 6 inches, solid in construction along both sides and the back wall. The front and bottom were wire mesh, and the open top of the cage fitted into the rack so that it was approximately ⅛ to ¼ of an inch below the solid shelf above. Individual cages were 1¾ inches apart. The infected mice remained in these cages for varying periods of time before two normal (previously uninfected) mice were added to each cage.

"Contact" period: Infected and uninfected mice were housed together in the same small crowded cage for a 24 hour period. During this time they drank from a common water supply and ate the same food pellets on the floor of the cage. Feces and urine passed through the wire mesh bottom on to sawdust pans beneath the cages.

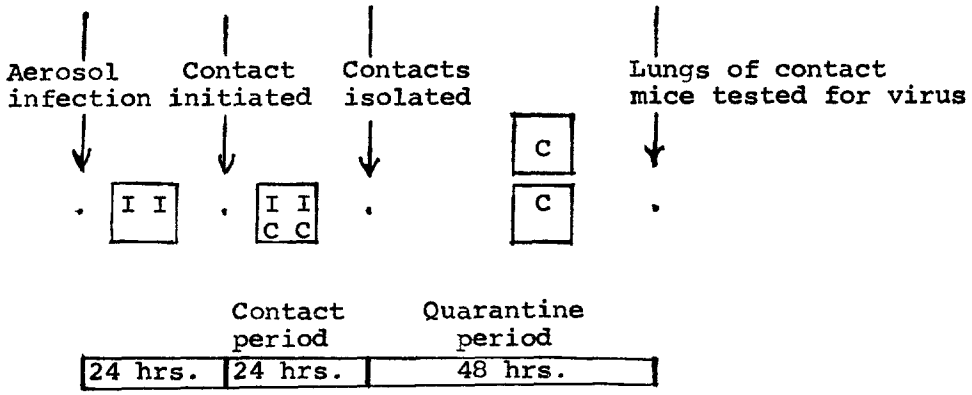

FIG. 2. Design of a typical contact experiment.

"Quarantine" period: At the end of the 24 hours of contact, the previously uninfected mice were removed, and housed in separate individual cages for a 48 hour period, after which their lungs were removed and tested for the presence of virus.

EXPERIMENTAL RESULTS

Initial experiments with the viruses that were used, and with crowded conditions during the contact period demonstrated that exposed uninfected animals acquired transmitted infection. Approximately 40 per cent of the infected contacts had small pulmonary lesions observable in the gross at the time of autopsy, 72 hours after initiation of contact with infector mice. This period of observation was too brief to make any observations of mortality among contact mice. Studies indicate that the virus titers in the lungs of infected contacts vary from $10^{-2.7}$ to $10^{-7.7}$ EID_{50}. Attempts at secondary transmission from infected contacts to new contacts have thus far been only occasionally successful.

Transmissibility in Mice of Two Different Strains of Influenza Virus—The CAM strain of influenza A1 virus and a mouse-adapted strain of Jap. 305 A2 virus were compared as to the frequency of transmitted infection. The CAM

virus seed had a titer of $10^{-7.7}$ EID_{50}/0.1 cc, and the Jap. 305 virus had a titer of 10^{-8} EID_{50}/0.1 cc. However, when serial 5-fold dilutions of the two preparations were administered by aerosol, (under conditions described in Materials and Methods) the MID_{50} was 10^{-4} for the CAM virus and 10^{-3} for the Jap. 305 virus. Therefore, to infect mice with the same number (100) of mouse infective doses with both viruses, it was necessary to employ a more dilute suspension (10^{-2}) of the CAM virus than of the Jap. 305 virus (10^{-1} dilution in the nebulizer fluid).

Mice were infected by aerosol with an estimated 100 MID_{50} of either CAM or Jap. 305 virus. Twenty-four hours later, two uninfected mice were placed in the same small cages with

TABLE I

Comparison of Transmissibility of Two Mouse-Pathogenic Strains of Influenza Virus

	CAM (AI*)	Jap. 305 (A2‡)
Mean pulmonary virus titers of infectors§ 72 hrs. after infection	7.8	7.1
Mean pulmonary lesion score‖ in infectors 7 days after infection	83	64
Number of contacts infected	15/60¶ (25 per cent)	31/60 (51.7 per cent)

* EID_{50} of seed $10^{7.7}$/0.1 cc; MID_{50} by aerosol 10^4/10 cc.

‡ EID_{50} of seed 10^8/0.1 cc; MID_{50} by aerosol 10^3/10 cc.

§ EID_{50} ($\log_{10}$), —mean, pulmonary virus titer of 30 animals in each group; mice in both groups infected with an estimated 100 MID_{50}.

‖ Mean extent of lung lesions (per cent)—30 animals in each group.

¶ P value of differences <0.05.

two previously infected animals. After a 24 hour period of contact, the previously uninfected mice were removed and quarantined for 48 hours in separate, individual cages. The lungs of the contact mice then were tested for virus by inoculation of 10^{-2} dilutions of ground lung suspensions into chick embryos. Seventy-two hours after initiation of infection, lungs of 5 infector mice from each group were removed and the virus content titrated in eggs. After 7 days of infection, the remaining infector mice were autopsied and the extent of pulmonary lesions recorded.

Table I summarizes the combined results of 6 such pairs of experiments. Infector mice infected with the CAM virus had higher pulmonary virus titers 72 hours after initiation of infection and more extensive pulmonary lesions 7 days after infection than mice infected with the Jap. 305 strain. Nevertheless, infector mice transmitted the Jap. 305 virus infection to previously uninfected contacts with greater frequency than did mice infected with the CAM virus.

Period of Optimal Transmission.—Experiments were designed to determine at what period during the course of influenza virus infection the infector mice most readily transmitted infection.

Four successive groups of uninfected mice were placed in contact for 24 hour periods with the same group of animals previously infected with Jap. 305 (A2) virus in the aerosol chamber. Two infector mice and two susceptible mice were placed in each small cage. The first susceptible group began its contact period immediately after the infector mice were removed from the aerosol chamber. Twenty-four hours later this group of susceptible mice was removed and a second group of susceptible mice placed in the same cages. The third and fourth susceptible groups began their 24 hour periods of contact 48 and 72 hours after initiation of infection in the infector mice. After each contact period, the previously uninfected mice were housed in individual cages for 48 hours prior to testing their lungs for the presence of virus.

Table II summarizes the results of 7 such experiments in which 80 susceptible animals were exposed during each time period. It is apparent that infector mice transmitted infection almost exclusively during the period from 24 to 48 hours after initiation of their infection.

TABLE II

*Transmission of Influenza Virus Infection in Mice during Successive 24-Hour Periods Following Initiation of Infection in Infector Mice**

Age of infection in infector mice, *hrs*.....	0 to 24	24 to 48	48 to 72	72 to 96
No. of susceptible mice that acquired transmitted infection‡.....	3	41	2	0

* Infector mice infected in aerosol chamber with an estimated 100 mouse infective doses of mouse-adapted Jap. 305 (A2) virus.

‡ Previously uninfected mice with demonstrable pulmonary virus 48 hours after the end of the contact period. 80 susceptible mice were exposed to transmitted infection during each time period.

Relationship of the Period of Optimal Transmission to the Course of Influenza Virus Infection in the Infector Mice.—After observing that mice transmitted infection almost entirely during the second 24 hours of their infection, an attempt was made to relate this observation to virus multiplication in various portions of the respiratory tract. Preliminary studies of the titers of infective virus in the lungs of infector mice at various intervals after the initiation of infection did not explain why transmission was optimal during a very limited period. Attention was then drawn to the possibility that peak titers of infective virus were reached sooner and declined more rapidly in the upper respiratory tract.

Twenty-five mice were infected in the aerosol chamber with an estimated 100 mouse infective doses of A2 virus. At 24, 48, 72, and 96 hours, 5 mice were autopsied for virus titrations of the nose, trachea, and lungs. Four groups of 10 uninfected mice were placed in contact for successive 24 hour periods with the same group of 10 infector mice, as described in the previous experiments. Throat swabs were obtained on all surviving infected mice at 24, 48, 72, and 96 hours.

During the period from 24 to 48 hours after initiation of their infection, the infector group transmitted influenza virus infection to 6 of the 10 exposed susceptible mice. None of the susceptible mice exposed during the three other time periods acquired transmitted infection (see Table III). These data demonstrate that peak titers of infective virus were not reached in the lungs until 48 to 72 hours after infection, and that at 96 hours the titers had not appreciably declined. Maximal virus titers in the trachea developed between 24 and 48 hours, but the titers at 72 and 96 hours were not significantly lower than at 48

TABLE III

*Period of Maximum Transmission of Influenza Infection as Related to Virus Titers in the Nose, Trachea, and Lungs, and to the Incidence of Positive Throat Swabs among Infector Mice**

	Duration of infection in infector mice, *hrs.*			
	24	48	72	96
	Virus titer‡			
Nose	<1.0	1.4	2.3	2.4
Trachea	4.8	5.4	5.1	5.0
Lungs	5.3	7.1	7.3	7.0
Per cent positive throat swabs.....	56	60	66	80
No. of susceptible mice with transmitted infection.....	0/10	6/10	0/10	0/10

* Log_{10} EID_{50}, —mean of tissue specimens from 5 mice individually titrated.

‡ Infected by aerosol exposure to 100 MID_{50} of Jap. 305 (A2) virus.

hours. Virus titers in the nose remained low throughout the 96 hour period, but were significantly higher at 96 hours than at 24 or 48 hours. There was no significant difference in the incidence of positive throat swabs taken from infectors at the four designated times. Thus, availability of virus in any of the four portions of the respiratory tract tested does not explain why transmission does not occur as readily in the 48 to 72 and 72 to 96 hour periods as in the 24 to 48 hour period after infection of the transmitters.

Minimal gross lung lesions among the infector mice generally began to appear between 48 and 72 hours after infection. By 96 hours the mice had developed pulmonary lesions involving an average of 30 per cent of the lung surface. The mortality rate among infector mice averaged 50 per cent, almost all of the deaths occurring on the 7th and 8th day.

DISCUSSION

These experiments confirm Eaton's observation (4), that under certain conditions, mice experimentally infected with influenza virus will transmit the infection to susceptible cage mates. Eaton also presented evidence that some strains of influenza virus were more readily transmitted than other strains equally pathogenic for mice. The present studies comparing the CAM strain of influenza A1 virus and the Jap. 305 strain of A2 virus suggest that transmissibility of influenza virus may be independent of other parameters of pathogenicity. Clearly, a critical degree of virus multiplication must occur before the infection can be transmitted, but the attainment of high titers of virus in the respiratory tract does not appear to be the only factor governing transmission. Thus, the Jap. 305 virus multiplied to a lower titer and produced less extensive pulmonary lesions but was more readily transmitted than the CAM strain of virus. Although no generalizations can be made from a comparison of 2 strains of virus, it is possible that the more mouse virulent strain as a consequence of inducing earlier or more extensive tissue reaction tends to inhibit the expulsion of virus from the lumen of the respiratory tract.

The limited period (24 to 48 hours) after initiation of their infection during which mice transmit infection permits at least one conclusion. Transmission of infection cannot be explained by the presence of residual virus on the fur of the infectors. If this were the source of transmitted infection, then the highest incidence of transmission should occur during the *first* 24 hour period following infection of the infector mice rather than the second. The fact that a 24 hour delay is required before infector mice begin to effectively transmit infection indicates that virus multiplication in the respiratory tract of the infector mice is necessary.

The relative inability of infector mice to transmit influenza virus infection when their infection is older than 48 hours is difficult to explain. Certainly it is not because of a decline in infective virus in any of the portions of the respiratory tract that have been studied. These observations suggest the same conclusion drawn from the studies comparing transmissibility of 2 strains of virus: that transmission of influenza virus infection is influenced by factors besides the peak concentrations of infective virus. Isaacs has suggested that the early decline of pulmonary virus titers in mice infected with influenza virus is due to the attainment of peak interferon concentrations at the time of peak virus titers (6). It is possible that interferon, or inactivated virus, transmitted along with the infective virus, tends to protect the susceptible mice. Another explanation is that after 48 hours of influenza virus infection, the inflammatory reaction in the infector mice tends to inhibit expulsion of virus-containing secretions from the lumen of the respiratory tract. Loosli has demonstrated (3) that a profound infiltrative reaction appears early in the lumen of bronchial and

bronchiolar passages in mice infected with influenza virus. Glover (7) showed that ferrets infected with influenza virus and streptococci developed thick encrusted lesions about the nose and were less able to transmit influenza virus infection than ferrets infected with influenza virus alone. A third hypothesis, that a change in the nature or extent of physical contact between infectors and susceptibles occurs as infection progresses in the infector mice, seems less plausible in view of evidence that influenza virus infection in mice is transmitted by small droplet nuclei and that direct physical contact is not required for transmission to occur (8).

It was of interest that virus could be detected in the membranes of the nasal passages. There is not sufficient evidence to conclude whether virus multiplied in these tissues or originated lower in the respiratory tract although gross edema and erythema of the turbinates were noted. However, Iida and Bang have demonstrated virus multiplication in the nasal epithelium of mice given small doses of influenza virus intranasally (9). Virus multiplication in the nasal passages does not appear to be the major factor involved in transmission of infection in the present model because virus titers in the nose were still increasing 24 hours after transmission ceased.

SUMMARY

An experimental model has been developed for the reproducible transmission of influenza virus infection from experimentally infected mice to uninfected cage mates. Infector mice transmit influenza virus infection most readily during the period 24 to 48 hours after initiation of their infection. This restricted period of transmission is not due to declining titers of infective virus in the nose, trachea, or lungs of infector mice after 48 hours of infection, since peak titers in these tissues are maintained for another 48 hours.

A mouse-adapted strain of A2 virus was found to be more readily transmitted than the mouse-adapted CAM strain of influenza A1 virus, although the CAM strain induced higher pulmonary virus titers and more extensive lung lesions.

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