# EXPERIMENTAL TRANSMISSION OF INFLUENZA VIRUS INFECTION IN MICE

## IV. Relationship of Transmissibility of Different Strains of Virus and Recovery of Airborne Virus in the Environment of Infector Mice*

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Experiments in this laboratory with an experimental model designed to study transmission of influenza virus infection in mice provided evidence that transmission of infection was primarily, if not exclusively, due to the inhalation of infective airborne droplet nuclei (1). In a series of experiments in which infected and uninfected mice were housed together for 24 hr in a closed chamber through which the ventilation could be regulated, it was found that as the ventilation rate increased the rate at which infection was transmitted decreased. Furthermore, contact mice physically separated from infectors were infected as readily as contact mice allowed to mingle freely with infectors (1). It was reasoned that spread of infection by direct contact or by large droplets would not be influenced by changes in ventilation and would be appreciably influenced by physical separation of infected and uninfected animals. This evidence supporting the hypothesis of true airborne transmission by droplet nuclei was largely inferential and the actual recovery of airborne virus remained to be accomplished.

In other experiments it was found that the relative transmissibility of a particular strain of influenza virus was not clearly related to other indications of virulence for mice (2). The present report concerns a series of experiments designed to recover a highly transmissible, mouse-adapted Jap. 305 strain of influenza $A_2$ virus from air in the environment of infector mice, during the period of their maximum infectiousness (2), and to compare quantitatively the recovery of $A_2$ virus with that achieved with a mouse virulent but poorly transmitted strain of influenza $A_0$ (NWS) virus.

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## *Materials and Methods*

*Mice.*—Manor Farms (MF-1) specific pathogen-free male mice 10-16 wk of age were employed in all experiments. Mice were housed in stainless steel boxes in groups of 10.

Lungs were removed aseptically at designated periods and ground in glass tubes with techniques previously described (3).

*Viruses.*—The Stuart-Harris neurovirulent variant of WS virus (NWS) and PR8 virus were employed as strains of influenza $A_0$ virus; the S-15 strain of swine influenza virus, the Lee strain of influenza B virus and two strains of influenza $A_2$ virus, a mouse-adapted strain of Jap. 305 virus and an inhibitor-sensitive strain of virus isolated at the Rockefeller University, N. Y., ($RI/5^+$) (4) were also used.

*Viral Titrations.*—Virus content of nebulizer and impinger fluids was determined by making serial 4-fold dilutions of specimens in phosphate-buffered saline (PBS) (penicillin 500 units/cc, streptomycin 5 mg/cc). Diluted specimens then were inoculated into the allantoic cavity of 10 to 11-day-old embryonated eggs. After a 48 hr incubation period the eggs were chilled and the allantoic fluids harvested and tested for hemagglutinin in a 1,4 dilution against human "O" red blood cells. In some instances negative allantoic fluids upon subinoculation at a $10^{-1}$ dilution induced hemagglutinin production. These fluids were considered to have been positive for infectious virus in determining dilution end points.

*Aerosol Procedure.*—Serial dilutions of allantoic fluid seed virus were nebulized with a Vaponefrin No. 40 nebulizer into a closed chamber under conditions previously described (2).

*Contact Procedure.*—In transmission experiments, mice infected 24 hr earlier were housed together in small cages with previously uninfected mice under conditions previously described (2). Two infected and two previously uninfected mice were placed in each cage and a 24 hr period of contact was permitted, after which the contact animals were removed and separated for 48 hr. Lungs of previously uninfected mice then were removed and tested for the presence of infective virus by inoculation of ten 10-fold dilutions of ground lung suspensions into chick embryos.

*Air Sampling Procedure.*—During those periods when air inside the chamber was being tested for infective virus, air flow through the chamber was adjusted to 10 liters/min, with all air leaving the chamber initially passing through a Shipe impinger. This limiting orifice type is designed to recover efficiently airborne particulate matter in a 1-10 $\mu$ particle size range (5). The impinger flask contained 10 ml of PBS (penicillin 500 units/cc, streptomycin 5 mg/cc, gelatin 0.1%). Air sampling was performed under two kinds of experimental conditions: In the first, serial dilutions of virus were nebulized into the chamber containing previously uninfected mice, and during the last 2 min of each nebulization period an air sample totaling 20 liters was obtained. In the second procedure mice infected previously were placed in the chamber. During a 12 hr sampling period air was drawn through the chamber and sampled at a rate of 10 liters/min; a fresh impinger flask was substituted every 20 min. The impinger fluids were pooled, and concentrated in the ultracentrifuge (95, 540 *g* for 30 min). The sediment was resuspended in 2.0/cc of antibiotic PBS prior to dilution and inoculation into eggs. In one such experiment of Gelman air filter with a pore size of 10 $\mu$ was inserted in the air exhaust line between the chamber and the impinger.

## RESULTS

*Comparison of Transmissibility of Various Strains of Influenza Virus.*—Mice were infected by aerosol with 100 $MID_{50}$ of one of the following viruses: $A_0$ (NWS), $A_0$ (PR8), Swine (S-15), B (Lee), $A_2$ ($RI/5^+$), $A_2$ (Jap. 305). 1 day

later a 24 hr period of contact with previously uninfected mice was begun. Contact mice then were removed and 48 hr later their lungs were tested for the presence of infective virus. Infector mice were autopsied 48 hr after initiation of their infection (at the end of the contact period) and pulmonary virus titers of infective virus were determined by separate titration of each lung in eggs. Pulmonary lesions were scored in infector mice after 7 days of infection. The results can be seen in Table I which summarize three experiments. The Jap. 305 strain of influenza $A_2$ virus was transmitted far more readily than any of the other viruses tested although it was no more pathogenic for mice as judged by pulmonary virus titers or lung lesions than the $A_0$ viruses or swine virus. The RI/5$^+$ strain of influenza $A_2$ virus though notably less virulent for mice, was more readily transmitted than any of the other viruses except for Jap. 305 virus.

TABLE I

*Comparison of Transmissibility of Different Strains of Influenza Virus*

| Virus | Infector mice | | Contact mice |
|---|---|---|---|
| | Pulmonary virus titer 48 hr* | Lung lesions day 7‡ | No. infected |
| | | | % |
| Swine (S-15) | 7.8 | 45 | 2/20 (10) |
| $A_0$ (PR8) | 7.5 | 42.5 | 1/20 (5) |
| $A_0$ (NWS) | 7.6 | 65 | 3/40 (7.5) |
| $A_2$ (RI/5$^+$) | 6.8 | 2.5§ | 6/20 (30) |
| $A_2$ (Jap. 305) | 7.6 | 60 | 25/40 (62.5) |
| B (Lee) | 6.9 | 20 | 1/20 (10) |

* Mean of individual titrations—five animals in each group $\log_{10}$, $EID_{50}$.

‡ Extent of lung lesions (per cent)—five animals in each group.

§ Lesion (<25% of lung) in one of five animals.

Based on the assumption that virtually all transmission of infection in this experimental model was airborne, several theoretical explanations existed for the increased transmissibility of the Jap. 305 virus: this strain of virus might be biologically better adapted to the physical stresses inherent in entering the airborne state or to surviving better once it is airborne; less of this virus might be required to initiate infection in mice; and finally, the Jap. 305 strain of virus might be more readily shed from the respiratory tract of infector mice than the other virus strains tested. Each of these possibilities was examined, comparing two viruses of contrasting transmissibility: the Jap. 305 strain of $A_2$ and the NWS strain of $A_0$ influenza viruses.

*Comparison of the Recovery of Airborne Virus After Nebulization of Influenza $A_0$ (NWS) and Influenza $A_2$ (Jap. 305) Virus.*—Serial dilutions of NWS and Jap. 305 allantoic seed viruses were nebulized for 20 min into the aerosol

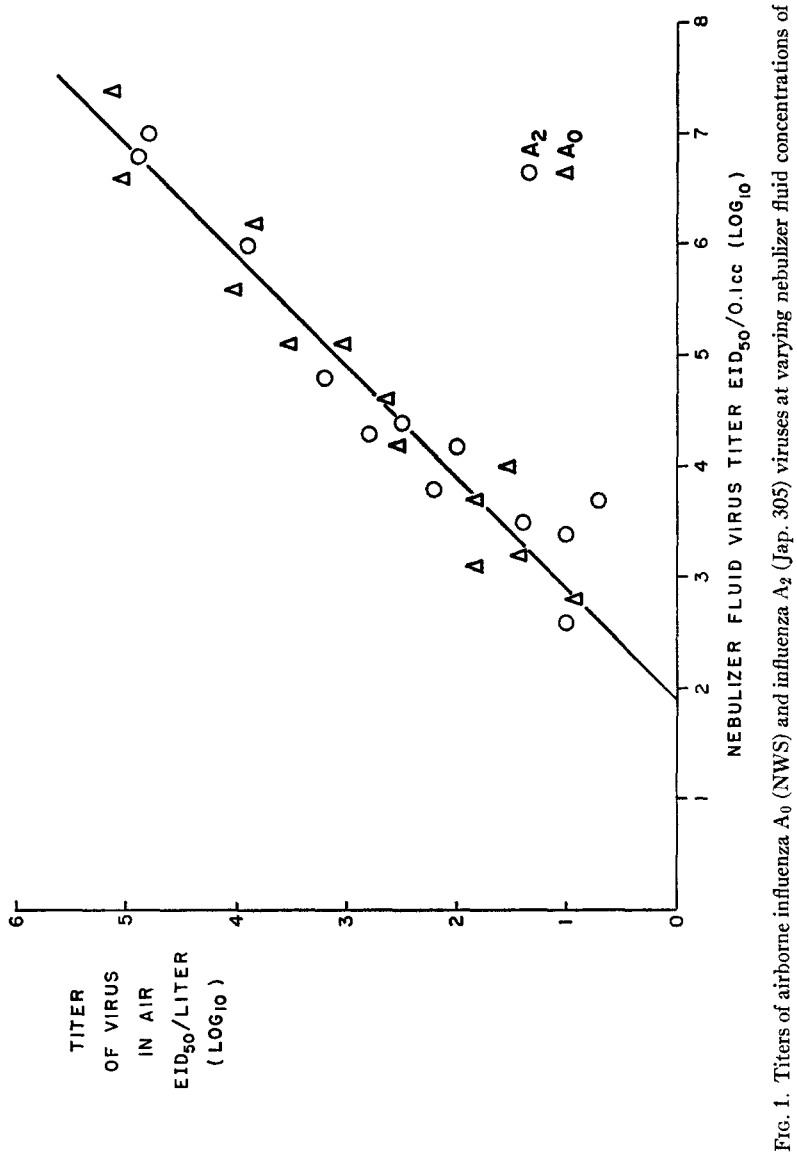


FIG. 1. Titers of airborne influenza $A_0$ (NWS) and influenza $A_2$ (Jap. 305) viruses at varying nebulizer fluid concentrations of virus.

chamber. During the last 2 min of each nebulization period air inside the chamber was sampled through a Shipe impinger. Virus titers of nebulizer fluids and impinger fluids then were determined in eggs. The results are shown in Fig. 1, in which nebulizer fluid virus titers are plotted against airborne virus titers. In general, a fairly reproducible, predictable relationship was found in that a straight line with a slope of 1 was obtained. Furthermore, no evidence was obtained suggesting that at equivalent nebulizer fluid concentrations more Jap. 305 virus survived entry into the airborne state.

Similarly, the survival of NWS virus *after* nebulization was comparable to that observed with Jap. 305 virus as seen in Fig. 2. In these experiments each of the viruses was aerosolized for 15 min and an air sample was taken immediately.

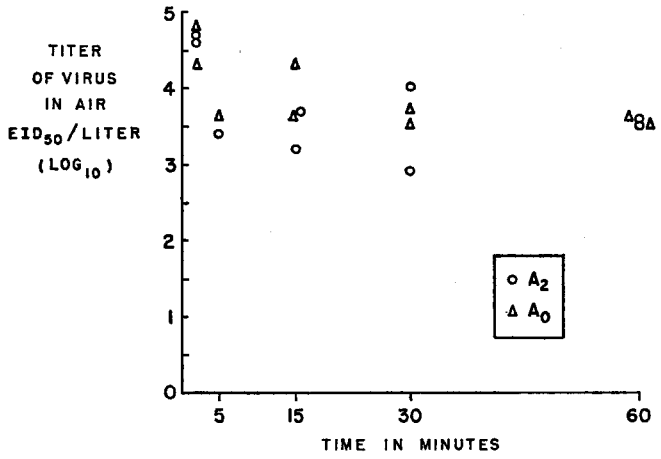


FIG. 2. Survival of airborne influenza $A_0$ (NWS) and influenza $A_2$ (Jap. 305) viruses at varying periods after nebulization.

The procedure then was repeated successively and air samples were taken at varying intervals following the end of the nebulization period. The declining titer of airborne virus was probably a consequence of the combined effects of physical loss due to gravity and to biologic inactivation of virus (6). The decay rates for the two viruses were identical.

*Comparison of the Quantities of Airborne Virus Required to Initiate Infection in Mice with Influenza $A_0$ and Influenza $A_2$ Viruses.*—In the previous experiments in which serial dilutions of NWS and Jap. 305 virus were aerosolized, 10 mice were present in the aerosol chamber during each nebulization period with each virus. 2 days later the mice were autopsied and the per cent infected during each exposure period was determined. The results are shown in Fig. 3 in which for both viruses the per cent of mice infected at each measured concentration

of airborne virus is plotted. It can be seen that the results for the 2 viruses are virtually identical, in that similar proportions of exposed mice were infected at equivalent titers of airborne virus. The slope of the line in Fig. 3 is quite steep so that all of the mice are infected within a 30-fold change in airborne virus titers. For both viruses 1 $MID_{50}$ was achieved at a titer of $10^{1.7}$ $EID_{50}$/liter of

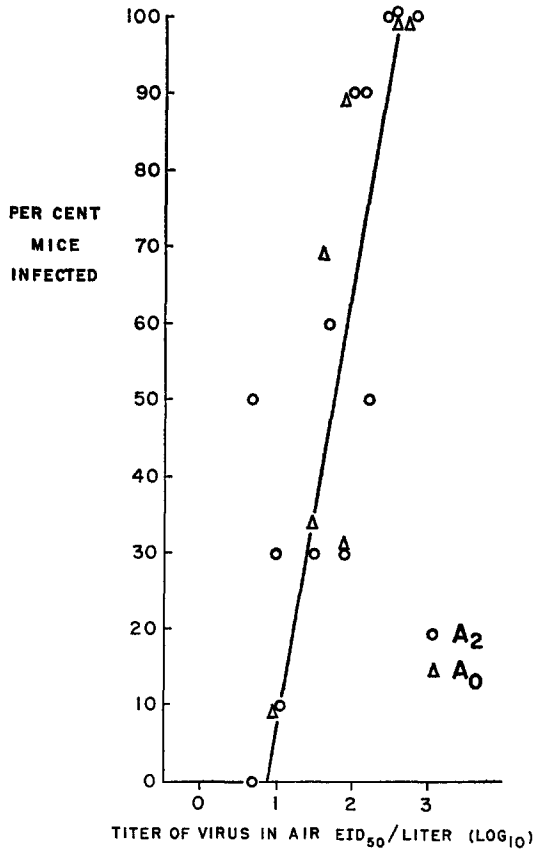


FIG. 3. Per cent of mice infected when exposed to varying airborne titers of influenza $A_0$ (NWS) and influenza $A_2$ (Jap. 305) viruses.

air. Calculating the minute volume of respiration of the mice to have been 1 cc/g of wt/min (7), each animal was exposed to only 500 cc. Furthermore, it is assumed that retention of inhaled particles in the lungs was less than 100% efficient (8), further reducing the estimated quantity of retained airborne virus which was required to initiate infection.

*Recovery of Airborne Mouse-Adapted Jap. 305 and NWS Virus from the*

*Environment of Infector Mice.*—30 mice infected 24 hr previously by exposure to an estimated 100 $MID_{50}$ of nebulized $A_2$ (Jap. 305) virus were placed in the closed aerosol chamber. Ventilation was adjusted to a volume of 10 liters/min, all air leaving the chamber passing through a Shipe impinger. Air sampling was conducted continuously for 12 hr; fresh impinger flasks were substituted every 20 min. Impinger fluids then were pooled and concentrated as described above. The following day, a second group of 30 mice infected 36 hr previously was placed in the chamber and the 12 hr sampling procedure was repeated. Thus in the two parts of the experiment the shedding of airborne virus during the

TABLE II

*Recovery of Infectious Airborne Virus from Environment of Infector Mice 24–48 hr after Infection*

| *Time, hr* | 0 | 12 | 24 | 36 | 48 | 60 |
|---|---|---|---|---|---|---|
| Group A infectors | | | | | | |
| $A_2$ | Infected | | Sampling | | | |
| $A_0$ | " | | of | | | |
| | | | air | | | |
| Group B infectors | | | | | | |
| $A_2$ | | Infected | | | Sampling | |
| $A_0$ | | " | | | of | |
| | | | | | air | |

| | Virus recovered in impinger fluids | |
|---|---|---|
| | $A_1$ | $A_0$ |
| | *$EID_{50}$* | *$EID_{50}$* |
| Group A | 40 | 0 |
| Group B | 20 | 0 |
| Total | 60 | 0 |

period from 24 to 48 hr after infection was measured. The results are presented in Table II. A total of 60 $EID_{50}$ was recovered. In a second experiment in which a Gelman air filter with a pore size of 10 $\mu$ was inserted in the exhaust line between the chamber and the impinger, 70 $EID_{50}$ were recovered. When the same procedure was repeated using mice infected with influenza $A_0$ (NWS) virus, no virus was recovered in the pooled impinger fluids. It was apparent therefore, that the greater transmissibility of the Jap. 305 strain of virus was associated with greater shedding of infective airborne virus by mice infected with the Jap. 305 strain than by mice infected with the NWS strain of influenza virus.

In a separate experiment, mice were infected with Jap. 305 virus and the

amounts of airborne virus shed were determined during the periods from 24 to 36 and 48 to 60 hr after infection. A total of 20 $EID_{50}$ was recovered during the period 24–36 hr after infection, whereas no airborne virus was recoverable from the environment of the same mice during the interval from 48 to 60 hr after infection. Thus, the shedding of infective airborne Jap. 305 virus was demonstrable only during the same period after infection in which infector mice most readily transmit infection to contact animals (2).

## DISCUSSION

The present studies confirm previous observations (9, 2), that infection with some strains of influenza virus is more readily transmitted from one mouse to another than infection with other strains equally pathogenic or more pathogenic for mice, and lends support to the hypothesis that transmissibility is at least in part independent of the viral factors which are responsible for other attributes of virulence.

Furthermore, the data indicate that transmissibility is not simply related to the quantity of virus required to initiate infection. In earlier studies, Ginsberg (10), demonstrated that with unadapted strains of influenza virus 100-fold more virus was required to initiate infection intranasally in mice than was required to infect chick embryos by allantoic inoculation. Following adaptation to mice, the relative quantities required to infect the mouse and the chick embryo were approximately the same. In the present studies, although infection with the NWS strain of virus was far less readily transmitted than infection with the Jap. 305 strain, no evidence was found indicating any difference in the relative amounts of virus necessary to infect mice. Similarly, the difference in transmissibility of the two viruses is not related to physical or biological factors affecting maintenance of infectivity upon entering the airborne state. At equivalent nebulizer fluid concentrations, identical concentrations of airborne virus were achieved with the NWS strain of virus as with the highly transmissible Jap. 305 strain, and for a period of 1 hr following nebulization the decay rates of the two viruses were indistinguishable.

The two strains of virus differed appreciably however, in the quantity of virus shed by infector mice into the environment as airborne droplet nuclei. The highly transmissible Jap. 305 strain of influenza $A_2$ virus could be recovered readily from the air surrounding infector mice during the period of maximum transmission. In contrast, there was no recoverable airborne virus in the environment of mice infected with the poorly transmitted NWS strain of influenza $A_0$ virus.

These observations provide further evidence in support of the hypothesis that transmission of infection in this experimental model is primarily airborne (1), in that the recovery of airborne virus was correlated with the relative transmissibility of two different strains of virus and was limited to the identical

period after infection during which virtually all transmission has been observed (2).

In addition it should be noted that the RI/5$^+$ strain of influenza $A_2$ virus, though unadapted for mice, was more readily transmitted than the mouse-virulent Swine and $A_0$ strains which were tested. Unpublished experiments in this laboratory with other nonadapted strains of influenza $A_2$ virus have demonstrated transmissibility similar to RI/5$^+$.

These observations suggest that the $A_2$ strains of influenza virus, particularly the mouse-adapted Jap. 305 strain of virus employed in the present studies, possess some property that facilitates release from the respiratory tissues of mice and enhances the shedding of virus into the environment in the airborne state. One hypothesis presently under investigation in this laboratory is that the $A_2$ strains of virus are more readily transmitted because of their high neuraminidase activity (11). Neuraminidase might facilitate the release of infective virus from mucoid secretions within the respiratory tract and thus increase the quantity of virus available for expulsion into the environment. Moreover, one may speculate further that the human pandemic associated with the introduction of the $A_2$ subtype was not simply a consequence of the antigenic distinctiveness of the new subtype but was also in part related to a greater potential for transmissibility in strains of the $A_2$ subtype.

The narrow range of concentrations of airborne virus within which all of the mice were infected either with the Jap. 305 strain or the NWS strain seen in Fig. 3, is of interest. These observations suggest great uniformity in susceptibility to infection among mice of the same strain, age, and sex. Furthermore, the steep slope of the line in Fig. 3 suggests that even small changes in host susceptibility, as measured by the quantity of virus required to initiate infection, would result in a considerable decrease in the number of contacts infected at threshold concentrations of airborne virus. Other experiments in this laboratory studying the susceptibility to transmitted infection of old and young (12), partially immunized (13), Newcastle disease virus (NDV)-inoculated, and male and female mice (unpublished results), have supported the premise that small changes in host susceptibility may profoundly affect the transmission rate. These observations may have significant epidemiologic implications.

## SUMMARY

A mouse-adapted Jap. 305 strain of influenza $A_2$ virus was found to be much more readily transmitted from one mouse to another than the NWS strain of influenza $A_0$ virus although the two viruses were equally pathogenic for mice as judged by pulmonary virus titers and lung lesions. The survival of artificially created aerosols of virus and the quantity of airborne virus required to initiate infection in mice were identical for the two viruses. The difference in transmissibility was associated with the recovery of infectious airborne virus in the

environment of mice infected with the Jap. 305 strain during the period of their maximum infectiousness, but not in the environment of mice infected with the NWS strain.

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