EXPERIMENTAL TRANSMISSION OF INFLUENZA VIRUS INFECTION IN MICE*

II. Some Factors Affecting the Incidence of Transmitted Infection

By JEROME L. SCHULMAN, M.D., and EDWIN D. KILBOURNE, M.D.

(From the Division of Virus Research, Department of Public Health, Cornell University Medical College, New York)

(Received for publication, April 19, 1963)

The experimental model employed in this laboratory to study the transmission of influenza virus infection in mice has been described in a previous report (1). In these studies, evidence was obtained that different strains of influenza virus may vary in transmissibility independently of other parameters of mouse virulence (1). The present studies were designed to investigate the effect upon transmission of some variations in host factors and of environmental conditions.

Materials and Methods

The experimental procedures employed in these studies were identical with those indicated in the previous report (1).

Virus.—A mouse-adapted strain of influenza A2 virus (Jap. 305) was used in all experiments.

Mice.—Male CFW mice were used in most experiments. These mice varied in weight from 17 to 38 gm in different experiments. In some experiments NCS mice generously supplied by The Rockefeller Institute were used. These animals are Swiss mice which have been raised under conditions designed to maintain them free of the usual enteric pathogens of mice (2).

The techniques of aerosol infection, throat swabbing, virus isolation, and titration, and the procedures for establishing contact between infected and susceptible mice were identical with those previously reported (1).

EXPERIMENTAL RESULTS

Good and Poor Transmitters.—

Male CFW mice averaging 25 gm in weight were infected with influenza A2 virus in the aerosol chamber, with each mouse exposed to an estimated 100 mouse infective doses of virus. Twenty-four hours later these infected mice were placed in small stainless steel cages with uninfected susceptible animals, 2 infector mice and 2 susceptible mice in each cage. At the end of a 24 hour contact period the susceptible mice were removed, and placed in individual

* This work was carried out under the auspices of the Commission on Influenza of the Armed Forces Epidemiological Board and was supported in part by the Office of the Surgeon General and also aided by a grant from the National Tuberculosis Association-American Thoracic Society.

containers for a 48 hour period of quarantine prior to removing their lungs and testing for the presence of infective influenza virus. A record was kept of which susceptible mice had been paired in the same cages during the 24 hour period of exposure to transmitted infection.

In 28 such experiments, a total of 511 pairs or 1022 susceptible mice were exposed to transmitted infection; 433 mice became infected. Three combinations of results among the exposed pairs of susceptible mice were possible: infection transmitted to both animals; to one of the two; or to neither animal. Using binomial expansion, $(p + q)^2 = p^2 + 2pq + q^2 = 1$, the predicted frequency of each of the three possibilities was calculated individually for each experiment. This is similar to predicting that upon tossing two coins in the air, 25 per cent of the time both would come down "heads", 25 per cent "tails" and 50 per cent

TABLE I

*Expected and Observed Incidences of the Three Possible Combinations of Results among Paired Susceptible Mice Exposed to Transmitted Influenza Virus Infection**

Both mice infected				One of two mice infected				Neither mouse infected			
Expected		Observed		Expected		Observed		Expected		Observed	
No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent
116.2	22.7	156	30.6	210.9	41.3	131	25.6	184.1	36	224	43.8

* 1022 mice (511 pairs) exposed to influenza A2 virus infection; $P < 0.01$.

of the time one coin would show "heads" and the other "tails." Table I indicates the totals of the expected and actual incidences of the three possible combinations. More pairs in which both, or neither susceptible animal acquired infection were found than was predicted, and fewer pairs in which one animal acquired infection and the other did not were found than was predicted. This tendency for paired susceptible mice to share similar fates is interpreted as indicating that infector mice differ in their ability to transmit influenza virus infection. In cages where one or both infector mice transmitted infection, both exposed susceptible mice tended to become infected; in cages where neither infector was a good transmitter neither susceptible mouse acquired infection.

Course of Infection in Good and Poor Transmitters.—

In one of the above experiments throat swabs were obtained from infector mice 12, 24, and 48 hours after initiation of infection. These were inoculated into chick embryos to demonstrate the presence or absence of influenza virus. Forty-eight hours after infection, or at the end of the 24 hour period of contact with the exposed susceptible mice, all the infector mice were autopsied and ground suspensions of their noses, tracheas, and lungs were titrated in chick embryos for influenza virus. Infector mice that transmitted infection to one or both of the exposed susceptible mice were considered good transmitters, while infector mice that did not transmit infection to either susceptible were considered poor transmitters.

The proportion of positive throat swabs, and of 48 hour gross pulmonary lesions, as well as the mean 48 hour titers of infective virus in the nose, trachea, and lungs of both groups of infector mice are given in Table II. No differences were observed between good and poor transmitters in the number of mice with gross pulmonary lesions 48 hours after infection or in the proportion of mice with positive throat swabs 12, 24, or 48 hours after infection. Infector mice with positive throat swabs at both 24 and 48 hours after infection were found in the same frequency among poor transmitters as among good transmitters. The titers of infective virus in the nose, trachea, and lungs of poor transmitters 48 hours after infection were as high as in good transmitters.

TABLE II

Comparison of Good and Poor Transmitters

Incidence of positive throat swabs, pulmonary lesions, and the mean 48 hour titers of infective virus in the nose, trachea, and lungs.

	Incidence of positive throat swabs			Mean titer of virus at 48 hrs.*			Animals with gross pulmonary lesions
	12 hrs.	24 hrs.	48 hrs.	Nose	Trachea	Lung	
	per cent	*per cent*	*per cent*				*per cent*
Good transmitters‡	0	32	36	1.7	6.0	6.7	63
Poor transmitters§	0	25	42	1.6	6.1	6.8	60

* Log_{10} EID_{50}.

‡ Infector mice from cages where susceptible mice were infected, 28 mice.

§ Infector mice from cages where susceptible mice were not infected, 12 mice.

Differences in the Ability to Transmit Influenza Virus Infection by Different Strains of Mice.—Additional evidence that some mice transmit influenza virus infection more readily than others was obtained by comparing 2 strains of mice.

CFW and NCS mice of similar ages were infected in the aerosol chamber with influenza A2 virus. Twenty-four hours later four different contact situations were established as follows:

2 CFW infector mice with 2 CFW susceptible mice
2 CFW " " " 2 NCS " "
2 NCS " " " 2 NCS " "
2 NCS " " " 2 CFW " "

After a 24 hour period of exposure to transmitted infection, the susceptible mice were removed and placed in individual cages for 48 hours. Their lungs then were removed and individually tested for virus by inoculation into chick embryos.

Table III summarizes the results of 4 experiments in which 160 infector and 160 susceptible mice were used. NCS infector mice transmitted infection to 35 of 80 exposed susceptibles, (lines 3 and 4 Table III), whereas CFW infector mice transmitted infection to only 16 of 80 exposed susceptibles (lines 1 and 2

Table III). The P value for this difference is less than 0.05. Non-infected NCS mice were no more susceptible to transmitted infection than CFW mice. Eleven of 40, or 28 per cent, of exposed NCS susceptibles became infected as compared to 40 of 120, or 33 per cent, of CFW susceptibles. The mean 48 hour pulmonary virus titer of 20 NCS mice was not appreciably higher than the mean pulmonary virus titer of CFW mice. Preliminary studies have indicated that NCS mice have higher pulmonary virus titers 24 hours after infection than CFW mice, and that the mean titers of virus in the nose and trachea of both strains of infected mice are identical from 24 to 96 hours after infection.

TABLE III

Comparison of Two Strains of Mice in Their Ability to Transmit and Their Susceptibility to Transmitted Influenza Virus Infection

Infector strain	Susceptible strain	No. of susceptible mice infected	Mean 48 hr. pulmonary virus titer*
CFW	CFW	11/60	7.0
CFW	NCS	5/20	
NCS	NCS	6/20	7.3
NCS	CFW	29/60	

* EID_{50} $\log_{10}$; $P < 0.05$.

Age as a Factor in the Susceptibility to Transmitted Infection.—

CFW mice of two age groups,—4 to 7 weeks (17 to 24 gm) and mice over 14 weeks old (30 to 38 gm) were infected with influenza A2 virus in the aerosol chamber. Uninfected CFW mice from the same 2 age groups were placed in contact for 24 hours with the infector mice, four different contact situations being established as follows:

2 old infectors with 2 young susceptible mice
2 " " " 2 old " "
2 young " " 2 " " "
2 " " " 2 young " "

At the end of the contact period the susceptible mice were removed, placed in individual cages, and 48 hours later their lungs were tested for virus by inoculation of ground suspensions into chick embryos.

The incidence of transmitted infection in each of the four groups is shown in Table IV. Older susceptible mice acquired influenza virus infection more readily (34/108) than younger mice (14/108). Older infector mice transmitted infection to 26 of 108 exposed susceptibles and young infectors transmitted infection to 22/108 exposed susceptibles. These data indicate that although older mice are more susceptible to transmitted infection, older infector mice do not transmit influenza virus infection more readily. Also shown in Table IV are the mean titers of infective virus in the lungs of old and young infected mice 24, 48, and 72 hours after infection. Each figure is the mean virus titer of 15 lungs indi-

vidually tested. No appreciable difference in the titer of infective virus between old and young infector mice can be seen at any of the three periods. Other studies in this laboratory with the Lee strain of influenza B virus have shown that the MID_{50} by aerosol is 0.7 $\log_{10}$ lower for older animals than for young mice, and that with the same aerosol exposure to Lee virus, older animals develop higher pulmonary virus titers and more extensive lung lesions.

TABLE IV

Incidence of Transmitted Influenza Virus Infection in Mice as Related to Age

Infector mice	Susceptible mice	No. of susceptible mice infected	Mean pulmonary virus titer in infector mice*		
			24 hrs.	48 hrs.	72 hrs.
Old‡	Young§	8/54	6.0	6.8	7.2
Old	Old	18/54			
Young	Old	16/54	6.1	6.8	7.4
Young	Young	6/54			

* EID_{50} $\log_{10}$; $P < 0.01$.
‡ 30 to 38 gm (4 to 7 weeks).
§ 17 to 24 gm (>14 weeks).

TABLE V

Seasonal Differences in the Frequency of Transmission of Influenza A2 Virus Infection in Mice

Season	Mean pulmonary virus titer in infector mice 72 hrs. after infection	Mortality	No. of susceptible mice infected
		per cent	
July to Oct.	7.3*	72	1/120
Dec. to Jan.	7.1‡	76	48/216

* Mean EID_{50} $\log_{10}$ of 30 animals individually titrated.
‡ Mean EID_{50} $\log_{10}$ of 55 animals individually titrated.

Seasonal Factors.—Table V summarizes and compares the results of transmission experiments conducted at different times of the year.

The virus in all of these experiments was from a common seed of influenza A2 virus frozen in a CO_2 ethanol mixture and kept at −68°C until used. CFW male mice from 28 to 35 gm were used in all experiments, and the technique of aerosol infection was not changed. Infector and susceptible mice were placed in contact, 2 each, in small stainless steel cages. Contact was initiated 24 hours after the infector mice had been infected, and terminated 24 hours later. The lungs of exposed susceptible mice were tested for influenza virus 48 hours after the termination of contact by inoculation of ground lung suspensions into chick embryos.

Table V shows that although the course of infection in infector mice was not

appreciably different during the summer months, the frequency of transmitted infection was appreciably lower than the frequency of transmission observed during the winter.

Table VI summarizes a similar group of experiments conducted 1 year later in an environmentally controlled animal room which maintained a year round temperature of 72°F and 50 per cent relative humidity. In these experiments, 30 to 35 gm male NCS mice were used in all experiments, and the same frozen virus seed of A2 virus was again employed in all experiments. It is evident that when temperature and humidity were kept constant, seasonal differences in the rate of transmission were less striking than those seen in Table V, but that the rate of transmission from November to April was still appreciably higher than from May to October.

TABLE VI

*Seasonal Differences in the Frequency of Transmission of Influenza A2 Virus Infection in Mice Housed in Environmentally Controlled Quarters**

	May to Oct.	Nov. to Apr.
No. of susceptible mice infected.....	109/320 (34.1 per cent)	192/330 (58.2 per cent)

* Relative humidity, 50 per cent; temperature, 72°F.

DISCUSSION

These experiments confirm the work of Eaton (3) who found that older mice acquired transmitted infection more readily than younger mice.

Previous experimental studies in mice of the relationship of age and susceptibility to virus infections have demonstrated that results vary with different viruses, different strains of virus, route and dose of the inoculum, and with the parameter used to measure susceptibility (4). Most of these studies have compared newborn and young adult mice, and have shown that newborn mice are more susceptible to infection. Wagner, for example, (5) showed that infant mice are more susceptible to the intracerebral or intraperitoneal inoculation of neurotropic (NWS) influenza virus. Kalter (6), using intranasally administered influenza A (PR8) virus demonstrated that a 1000-fold greater concentration of virus was required to kill 50 per cent of older mice than to kill 50 per cent of 3-week-old mice, and Sawicki (7) showed that intranasally administered Sendai virus persisted in the lungs of newborn mice for longer periods than in the lungs of 4-week-old mice. In the present studies susceptibility was measured by the number of mice in the two adult age groups that acquired infection on exposure to low multiplicities of influenza virus. Furthermore, the use of ether anesthesia and the intranasal inoculation of fluid were not part of the experimental procedure. One possible explanation for the greater susceptibility of

older mice to transmitted infection is their greater minute volume of respiration. Minute volume increases linearly with increasing weight (8) and the heavier mice in these experiments inspired approximately twice as much air as the younger, lighter mice. However, 5 times as much Lee virus was required to infect 50 per cent of younger mice by the aerosol route as to infect 50 per cent of older mice. Differences in minute volume alone therefore do not seem adequate to explain the difference in susceptibility observed in these experiments. These data suggest that older mice have a greater susceptibility to low multiplicities of transmitted or nebulized influenza virus.

The tendency for both paired susceptible mice to become infected or to remain uninfected has been interpreted as showing that infector mice vary in their ability to transmit infection. If variations in the susceptibility to infection among uninfected mice were of primary importance, then assuming a randomized pairing of susceptibles, the distribution of newly infected susceptibles should also be random. The tendency for both of the paired animals to share a similar fate indicates that some pairs were in a more infectious environment during the contact period than others and therefore that some mice transmit influenza virus infection more readily than others. It is assumed that in these experiments susceptible mice did not transmit infection to one another. Such secondary spread of infection would obviously lead to a disproportionate number of pairs in which both animals were infected. There are several reasons why the assumption seems valid. Previously uninfected mice are housed together for only 24 hours and secondary transmission would require that transmission from infector to susceptible, multiplication of virus in the infected susceptible, and then secondary spread to the other susceptible all would occur within a 24 hour period. However, previous studies have shown that transmission rarely occurs during the first 24 hours of infection (1). Secondly, experimental attempts in the laboratory to induce secondary transmission have been successful only on rare occasions (1).

The concept of "dangerous transmitters" of respiratory infection is not a new one (9–11). The point of interest in the present studies is that good transmitters do not have greater concentrations of virus in any of the tissues studied than poor transmitters. As was noted in the studies of the period of optimal transmission (1), transmission of influenza virus infection in mice depends on factors other than the titers of influenza virus in the nose, throat, trachea, or lungs. One explanation is that good transmitters because of their social behavior establish more intimate physical contact with the exposed susceptibles. The NCS mice which were found to be better transmitters than CFW mice have been observed to be more active and more aggressive. Andrewes has shown that social patterns among chicks effect the transmission of Newcastle disease virus (12). However, in experiments in this laboratory it has been found that mice transmit influenza virus infection by the airborne route (13), an observation that tends to diminish the significance of direct physical contact.

Another explanation for difference in the ability to transmit infection is similar to one given for the limited period of optimal transmission (1). The ability to transmit influenza virus may be significantly affected by the nature and quantity of the bacterial flora of the respiratory tract and the type and extent of the inflammatory reaction within the bronchial passages following influenza virus infection. Experiments are currently in progress to discover whether changes in respiratory tract flora and of respiratory tract secretions significantly alter the frequency of transmission of influenza virus infection.

The "winter factor" in influenza has long been a subject of great interest. Explanations have been suggested on the basis of indoor crowding (14), activation of "masked" infection by wintertime stresses (15), the vulnerability of airborne influenza virus to high relative humidity (16), and seasonal changes in the character of respiratory secretions (17). Coburn showed (18) that mice were infected with epidemic strains of Group A streptococci more readily in the winter than during the summer, despite the fact that experiments were conducted in air-conditioned quarters and that aliquots of the same frozen stock culture were employed during both seasons. In Coburn's experiments, and in the present studies, crowding and stress due to exposure to cold are excluded as significant factors in explaining the seasonal variations.

Previous studies in this laboratory (12) demonstrated that transmission of influenza virus infection decreased as relative humidity increased from 47 to 70 per cent. However, seasonal differences in the rate of transmission were still evident in the experiments conducted in environmentally controlled rooms. Relative humidity during the period of contact is not therefore the only factor responsible for seasonal differences in transmission rates. The mice that are employed in these experiments are not bred in this laboratory and it is possible that seasonal differences in relative humidity during the first few weeks of life may be important.

Although evidence relating to seasonal changes in the bacterial flora of the mouse respiratory tract is limited, evidence that significant changes occur has been presented (19). Seasonal changes in the character of respiratory tract flora or of respiratory tract secretions might influence the transmitting ability of infector mice or the susceptibility to infection of the uninfected animals.

SUMMARY

Evidence has been presented that with the experimental model described, infected mice vary in their ability to transmit influenza virus infection. This variation is not explained by differences in titers of influenza virus in the nose, throat, trachea, or lungs of good transmitters. Older mice acquire transmitted influenza virus infection more readily than younger mice.

Seasonal variations in the incidence of transmitted influenza virus infection occur.

BIBLIOGRAPHY

1. Schulman, J. L., and Kilbourne, E. D., Experimental transmission of influenza virus infection in mice. I. Period of transmissibility, *J. Exp. Med.*, 1963, **118,** 257.
2. Nelson, J. B., and Collins, G. R., The establishment and maintenance of a specific pathogen-free colony of Swiss mice, *Proc. Animal Care Panel*, 1961, **11,** 65.
3. Eaton, M. D., Transmission of epidemic influenza virus in mice by contact, *J. of Bact.*, 1940, **39,** 229.
4. Sigel, M. M., Influence of age on susceptibility to virus infections with particular reference to laboratory animals, *Ann. Rev. Microbiol.*, 1952, **6,** 247.
5. Wagner, R. R., A pantropic strain of influenza virus: generalized infection and viremia in the infant mouse, *Virology*, 1955, **1,** 497.
6. Kalter, S. S., The effect of age upon susceptibility to infection with influenza virus, *J. Immunol.*, 149, **63,** 17.
7. Sawicki, L. S., Influence of age of mice on the recovery from experimental Sendai virus infection, *Nature*, 1961, **192,** 1258.
8. Guyton, A. C., Measurement of the respiratory volumes of laboratory animals, *Am. J. Physiol.*, 1947, **150,** 70.
9. Sultan, L., Nyka, W., Mills, C., O'Grady, F., Wells, W. F., and Riley, R. L., Tuberculosis disseminators: a study of the variability of aerial infectivity of tuberculosis patients, *Am. Rev. Resp. Dis.*, 1960, **82,** 358.
10. Langmuir, A. D., Public Health implications of airborne infection: medical aspects. Conference on airborne infection, (W. McDermott, editor), Baltimore, Williams & Wilkins Co., 1961, 356.
11. Hyge, T. V., Epidemic of tuberculosis in a state school, *Acta Tuberc. Scand.*, 1947, **21,** 1.
12. Andrewes, C. H., and Allison, A. C., Newcastle disease as a model for studies of experimental epidemiology, *J. Hyg.*, 1961, **59,** 285.
13. Schulman, J. L., and Kilbourne, E. D., Airborne transmission of influenza virus infection in mice, *Nature*, 1962, **195,** 1129.
14. Jordan, W. S., The mechanism of spread of Asian influenza, *Am. Rev. Resp. Dis.*, 1960, **83,** pt. 2, 29.
15. Shope, R. E., Influenza, history, epidemiology and speculation, *Public Health Rep.*, 1958, **73,** 165.
16. Harper, G. J., Airborne micro-organism: survival tests with four viruses, *J. Hyg.*, 1961, **59,** 479.
17. Lowry, R. H., The effect of environmental temperature and humidity on the spread of a virus infection in the respiratory tract of chicks, D.P.H., thesis, Johns Hopkins University School of Hygiene and Public Health, Baltimore, 1955, 1–97.
18. Coburn, A. F., Frank, P. F., and Nolan, J., Studies on the pathogenesis of streptococcus pyogenes. IV. The relationship between the capacity to induce fatal respiratory infections in mice and epidemic respiratory disease in man, *Brit. J. Exp. Path.*, 1957, **38,** 256.
19. Mayasi, S. A., The effect of temperature and humidity on virus infection, *Dissertation Abstracts*, 1959, **20,** 1963.