STUDIES ON THE EXPERIMENTAL EPIDEMIOLOGY OF RESPIRATORY INFECTIONS

III. CERTAIN ASPECTS OF THE BEHAVIOR OF TYPE A INFLUENZA VIRUS AS AN AIR-BORNE CLOUD

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Previous reports in the literature on influenza virus have dealt with the successful infection of animals by the intranasal instillation of the virus from experimentally infected air.[1,2] This was followed by demonstrating air-borne infection of ferrets with the same agent.[3,4] The exposure of mice to influenza virus aerosol yielded a quantitative response to dosage of atomized virus.[5,6] Finally, contact transmission of the virus and the existence of an air-borne route of tion was established.[7–9] The further development of techniques to study air-borne infection has been reviewed in the first paper of this series, and reference for such information is therefore made to it.[10]

It is the purpose of this communication to present some of the results obtained in the study of the behavior of influenza virus aerosols. This investigation was conducted with the aid of the apparatus for the study of experimental respiratory infections described in detail by Leif and Krueger.[10] This instrument made possible the quantitative evaluation of the particle size and the viral content of the aerosols, and the determination of the relative efficiency of virus infection by the intranasal route and by inhalation of a virus aerosol.

MATERIALS AND METHODS

Virus.—The egg-adapted W. S. strain of type A influenza virus was used. This virus was obtained from Dr. M. D. Eaton, then direction of the California State Virus Laboratory. Three separate batches of 400 to 500 ml each of virus suspension were prepared by inoculation of 0.1 ml of 1×10^{-5} dilution of the preceding virus passage into the allantoic fluid of 11-day-old chick embryos. After incubation at 35 C for 40 to 44 hours, the eggs were chilled at 4 C for 4

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to 5 hours and the allantoic fluid harvested. The fluid was pooled and then subdivided into 20 ml aliquots. Each aliquot was tested for sterility, and the virus was stored in the dry ice chest (−72 C). Once a tube with virus was removed from the dry ice box, it was never returned.

Viral aerosols.—The viral aerosols were produced from dilutions of allantoic fluid virus suspensions prepared in 0.1 *M* Sorensen's phosphate buffer (pH 7.1) by means of a Wells atomizer[10] delivering uniformly small particles at the rate of approximately 0.2 ml per minute. Samples of these aerosols were collected with special capillary impingers using the Sorensen's phosphate buffer indicated above as the collecting medium, and were assayed for their viral content in the manner stated below.

Test animals.—Three- to four-week-old mice were used in all animal tests. Our own pen-bred colony, showing no indication of any infectious disease during the past 5 years, served as the only source of these animals. White Leghorn embryonated eggs were purchased from one local dealer and incubated to the desired age in our laboratory.

Assay of influenza virus.—Titrations were carried out in 11-day-old embryonated eggs and/or 21- to 23-day-old mice of approximately 10 g weight. Sorensen's 0.1 *M* phosphate buffer at pH 7.1 was used for all serial dilutions.

Five to six 11-day embryonated eggs were inoculated by the allantoic route with 0.1 ml of the test material, incubated at 35 C for 40 to 44 hours, subsequently chilled at 4 C for 4 to 5 hours, and then 0.5 ml of clear allantoic fluid was withdrawn from each egg for the determination of hemagglutinins.[11,12] Usually the results were "all-or-none" in nature. The maximum possible score for the red blood cell agglutination patterns was 4, while 0 represented the absence of any agglutination. Intermediate end points were determined by the pattern of the agglutinated erythrocytes. Fifty percent infectivity end points (ID_{50}) were calculated as suggested by Hirst's application of Reed and Muench's technique.[13,14]

When mice were used, 0.05 ml of the test material was instilled intranasally into each of 6 to 10 mice used for each tested virus dilution. Only the mice receiving an identical inoculum were confined to the same cage. All animals dying within 10 days after inoculation were autopsied for evidence of the degree of pulmonary involvement, and the 50% mortality end points (LIN_{50}) were computed according to the method of Reed and Muench.[14] Those surviving the 10-day holding period were sacrificed, the degree of lung consolidation noted[15] and the 50% maximum score end point (MS_{50}) computed.

The amount of viral aerosol inhaled and retained during a 3-minute exposure and lethal to 50% of the exposed animals, observed for a 10-day period, was symbolized as LRE_{50}.[16] In determination of LRE_{50}, groups of 6 to 12 mice were exposed seriatim for 3 minutes to 4 or 5 progressively increasing concentrations of the aerosol. Virus content of these aerosols was determined by titrating the impinger cloud samples intranasally in mice and/or eggs. Survivors of the inhalation exposure were sacrificed after the 10-day holding period, and inhalation LD_{50} was determined by the method of Reed and Muench. Prior to the exposure of mice to an aerosol or to a determination of the droplet diameter, every virus suspension was atomized for 15 minutes.

EXPERIMENTAL RESULTS

To determine the resistance of virus to atomization, aliquots of certain virus suspensions were titrated in eggs and in mice before and after the atomizing period. Representative results in table 1

TABLE 1.—*Titration of representative influenza virus suspensions in embryonated eggs and mice before and after atomization.*

Suspension no.	Concentration of suspension, titrated in: Eggs, virus units as ID_{50}/ml		Mice virus units as MS_{50}/ml	
	Before atomization	After atomization	Before atomization	After atomization
2	$10^{5.9}$	$10^{6.4}$	$10^{7.2}$	$10^{6.7}$
6	$10^{6.3}$	$10^{6.7}$	$10^{7.4}$	$10^{7.7}$
12	$10^{7.6}$	$10^{7.7}$	$10^{8.2}$	$10^{8.0}$
17	$10^{8.0}$	$10^{7.8}$		
20	$10^{7.5}$	$10^{6.5}$		
30	$10^{8.5}$	$10^{8.5}$	$10^{7.9}$	$10^{8.2}$

ID_{50} = quantity of virus infecting 50% of inoculated eggs.
MS_{50} = quantity of virus producing 50% of the maximum lung lesion score in mice.

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16. Young, G. A., Zelle, M. R. and Lincoln, R. E. 1946, J. Infect. Dis. **79**: 233–246.

indicate that the titers of these suspensions do not differ by more than may be expected from the variability inherent in the titration of viruses.[17] Since concentration of the suspension due to preferential fluid loss during atomization was not observed to be a factor with our refluxing atomizer, this agreement in titer was probably indicative of the resistance of this virus to atomization.

The percentage of virus recovery was computed with the aid of the following formulas:[18]

(1) The theoretical maximum cloud concentration (i.e., the concentration per liter of cloud sprayed) =

$$\frac{\text{(volume of suspension sprayed) (concentration of suspension)}}{\text{(air flow, in liters per minute) (period of spraying, in minutes)}}$$

(2) Concentration of cloud per liter, as recovered =

$$\frac{\text{(infective units per ml in sampler) (volume of sampling fluid)}}{\text{(volume of cloud sampled, in liters)}}$$

(3) Nominal percent recovery $= 100 \times [(2)/(1)]$

This method yields the "nominal percent recovery value" and is derived from the relation between the number of infective units recovered from the cloud and the theoretical maximum number present in the cloud. The volume of the virus suspension atomized per minute, the duration of spraying, the titer of the virus suspension before and after atomization, as well as the assay of the aerosol samples for their virus content, were determined for each experiment. It is evident that the formulas above do not attempt to explain the discrepancy between the theoretical maximum number of infective units in the aerosol and the actual number of infective units.

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The data presented in table 2 summarize the results of 26 experiments indicating the relation between the concentration of atomized virus suspension and the virus recovered from the resultant aerosol. Using mice for titration of these virus suspensions and the corresponding aerosols, the percentage of virus recovered was shown to be independent of the initial concentration of the atomized suspension. Within the range of the concentration studied, a constant nominal percent recovery was obtained, the observed differences being statistically not significant. Similarly, using the embryonated eggs for certain of these titrations, the average percent recovery, with its standard error, for 22 observations was 8.5 ± 1.7. Although there is no significant difference between the average percent recovery as determined using the two hosts above, variation within a particular experiment was occasionally pronounced.

The particle size of the aerosol, pro-

TABLE 2.—*Relation between the concentration of influenza virus in the atomized suspension and the resultant aerosols.*

Number of trials	Virus units as MS_{50}/ml of		Percent recovery
	Suspension	Aerosol	
8	$2.6 \pm 0.4 \times 10^7$	$3.0 \pm 0.9 \times 10^4$	6.4 ± 1.4
8	$1.9 \pm 0.1 \times 10^6$	$1.5 \pm 0.6 \times 10^3$	4.9 ± 1.6
10	$3.5 \pm 0.4 \times 10^5$	$7.2 \pm 0.7 \times 10^2$	8.5 ± 1.2

MS_{50} = quantity of virus producing 50% of the maximum lung lesion score in mice.

stant nominal recovery was obtained within the range of the investigated virus concentrations.

The mass mean diameter of influenza virus droplet was found to be about 0.5μ. Since the mass mean diameter of a distribution of droplets is greater than the mean diameter of the same droplet distribution, and since the diameter of the influenza virus is of the order of 0.1 μ,[23,24] there is a high probability that virus droplet nuclei of the indicated size range will produce a true pulmonary infection. "This does not, of course, preclude the possibility of absorption from the upper respiratory tract and subsequent transportation by body fluids to other areas."[25]

A comparison of the inhalatory method with the intranasal route of administration of India ink and radioactive chromic phosphate, as reported by the Personnel of the Naval Laboratory Research Unit #1 and W. R. Lyons,[26] definitely established the superiority of the inhalatory method with respect to the distribution and penetration of both materials. In their opinion, with which the writer concurs, the intranasal instillation of fluids results in an uneven distribution of the material. The results presented in table 4 indicate that the W. S. strain of influenza virus used in these experiments was apparently characterized by greater infectivity when introduced as an aerosol than when instilled intranasally in the form of a suspension of the same cloud. This difference in the LRE_{50} and LIN_{50} values may be explained in terms of a disaggregation of infective units in the air-borne form, and a reaggregation either later on, in the cloud, or in the sampling fluid.

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SUMMARY

Certain aspects of the behavior of the aerosol of the W. S. strain of type A influenza virus were investigated.

The mechanism of production of the virus aerosol did not affect the titer of the virus as measured in mice. The percent recovery of the virus aerosol was independent of the initial concentration of the atomized suspension.

The effect of the relative humidity on the recovery of the aerosol was investigated. High aerosol recovery was obtained in the extreme ranges of 32 and 68% relative humidity, while a minimum recovery was obtained at 60% relative humidity.

The mass mean diameter of the influenza virus aerosol was determined to be less than 0.5 μ.

This influenza virus is apparently characterized by greater infectivity when introduced by the air-borne rather than by the intranasal route.