Assessment of Aerosol Mixtures of Different Viruses

CHARLES J. MAYHEW AND NICHOLAS HAHON

Aerobiology and Evaluation Laboratories, Fort Detrick, Frederick, Maryland 21701

Aerosol mixtures of the psittacosis agent, yellow fever virus, and variola virus were assayed by selective immunofluorescence in conjunction with fluorescent cell counting. The aerosol behavior of each agent could be readily delineated at test conditions of 80 F (26.67 C) and three relative humidities (30, 50, or 80%). Of the three agents, variola virus exhibited the lowest biological decay. The biological decay rates of the airborne agents were not significantly affected by humidity changes.

Recently, a rapid and highly specific procedure was developed for the assay of mixtures of viruses in suspension (6). The procedure is based on selective immunofluorescent staining in conjunction with fluorescent cell counting of cell monolayers infected by virus mixtures. Each virus could be assayed quantitatively in the presence of other viruses. Simultaneous introduction of mixtures containing three or more viruses did not interfere with the ability of each virus to infect cell monolayers. The applicability of this procedure to the assessment of aerosols containing a mixture of viruses was investigated. This report describes the aerosol survival of the psittacosis agent, yellow fever virus, and variola virus in mixture at different levels of relative humidity (RH).

MATERIALS AND METHODS

Viruses. The three agents used in this study in mixture were variola (Yamada) and yellow fever (Asibi) viruses, and the psittacosis (Borg) agent. Stock suspensions of each were prepared as follows: variola virus from infected McCoy cell cultures with harvest of virus at the onset of virus cytopathogenicity, yellow fever virus from infected rhesus monkeys by bleeding animals at the height of the febrile response for infectious plasma, and the psittacosis agent by harvesting infective embryos and yolk fluid from moribund chick embryonated eggs. Titers of these agents were 4.0×10^7 cell-infecting units (CIU) per ml for variola virus, 1.1×10^8 CIU per ml for yellow fever virus, and 4.4×10^8 CIU per ml for the psittacosis agent. Each virus preparation was stored in an electric freezer (-60 C).

Aerobiological procedures. Aerosol tests with virus mixtures were made in a 1,500-liter rotating drum similar in design to that described by Goldberg et al. (3). Humidity of the drum atmosphere was controlled by varying the moisture content of the secondary air supply with dryers or by atomizing small amounts of water. Relative humidity was measured directly in the drum by humidity elements, ML-379/AM (Viz Manufacturing Co., Philadelphia, Pa.) and a conduc-

tivity bridge, model RC 16B2 (Industrial Instruments Co., Cedar Grove, N.J.). The temperature in the drum was generally 80 ± 2 F (26.67 ± 1.12 C). Preparations were atomized and aerosols were sampled through a hollow axle in the drum.

Virus mixtures to be atomized consisted of 2 ml of yellow fever virus, 2 ml of psittacosis agent, 1.5 ml of variola virus, and 0.5 ml (0.44 mg) of a solution of uranine (the disodium salt of fluorescein), which was incorporated as a physical tracer. Preliminary tests indicated that the concentration of tracer used was not detrimental to the viruses. For each aerosol test, suspensions of viruses and tracer were mixed and immediately atomized through an FK-8 atomizer (Fort Detrick, Frederick, Md.). Complete dissemination of material was achieved within 20 sec with 50 psi of nitrogen. At designated intervals after dissemination, the cloud was sampled for 2 min with midget Shipe impingers (Fort Detrick, Frederick, Md.) at a flow rate of 6 liters per min (13). A 10-ml amount of impinger fluid consisting of medium 199 with 5% fetal calf serum and 2 drops of sterile olive oil as an antifoaming agent was used to sample the cloud. Simultaneously with biological sampling, impingers containing distilled water were used to sample the cloud for physical tracer.

Assay of physical tracer. Fluorometric analysis of collecting fluids used to sample the cloud for the tracer (uranine) were made with a Turner model 110 Fluorometer (G. K. Turner Associated, Palo Alto, Calif.). A standard curve was established for a particular lot of tracer that was then used to estimate the tracer content of samples.

Cell line and cultivation. The McCoy cell line was used in the assay of the three viruses. Nutrient medium for cells consisted of medium 199 containing 0.5% lactalbumin hydrolysate, 10% fetal calf serum, and 50 μg of streptomycin and 75 μg of kanamycin per ml. Cells were maintained in medium 199 and 5% fetal calf serum. For assay of virus mixtures, cells were cultivated on circular cover slips (15-mm diameter) inserted in flat-bottomed glass vials (18 by 100 mm or 19 by 65 mm). A 1-ml amount of cell suspension containing 10^5 to 3×10^5 cells was introduced onto cover slips, which were then incubated at 35 C

for 24 hr, or until a complete cell monolayer was formed.

Virus assay procedures. The immunofluorescent cell counting procedure was used to assay impinger fluids for all three agents. Generally, nine cover slip cell monolayers were inoculated for each impinger fluid used to sample the cloud. Inoculum in 0.5-ml volume was introduced onto eaeh cover slip cell culture; agents were attached to cell monolayers with the aid of centrifugal force in accord with techniques described previously for the assay of each agent (4, 5, 7). Cell monolayers were then incubated at 35 C from 16 to 24 hr depending on the agent to be assayed. Cover slip cell monolayers were rinsed twice with phosphate-buffered saline (PBS), fixed with cold (−60 C) acetone, and either prepared immediately for immunofluorescent staining or stored at −60 C. Fluorescence of viral antigens in fixed cell cultures was not diminished after storage for several weeks.

Virus antisera. Variola antiserum was prepared in rabbits, yellow fever antiserum in monkeys, and psittacosis antiserum in Rhode Island Red roosters. Details of the immunization schedules were reported previously (4, 5, 7). Antisera were individually conjugated with fluorescein isothiocyanate by the method of Riggs et al. (11). Conjugated globulin was passed through a column of Sephadex G-50 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) to remove unbound dye. To reduce nonspecific fluorescence, 5 ml of conjugated globulin was diluted with an equal volume of PBS and adsorbed twice with 200 mg of acetone-dried mouse liver powder (2).

Immunofluorescence staining, fluorescence microscopy, and infected cell counting. The direct fluorescent antibody method was used to demonstrate immunofluorescence of viral antigens in infected cells. Fixed cell monolayers were washed once with PBS and separated into three groups; each group was selectively stained with one of three conjugated globulins for 30 min at room temperature. Cover slip cell monolayers were rinsed in two changes of PBS and mounted in a semipermanent medium (12).

Cover slip cell monolayers were examined with an American Optical microscope equipped with a fluorolume illuminator (model 645), Corning no. 5840 and Schott BG-12 excited filters, and an E.K. no. 2A barrier filter. With this optical system at a magnification of 430 times, the number of microscopic fields contained in the area of a 15-mm cover slip was 1,064. For each cover slip cell monolayer, 50 microscopic fields were examined for infected cells. To calculate the number of CIU of virus per milliliter, the average number of infected cells per field was multiplied by the number of fields per cover slip, the reciprocal of the virus dilution, and a volume factor for conversion to milliliters.

Experimental design and analyses. Physical limitations of equipment restricted the investigation to one aerosol test per day. Tests were made on days when the ambient temperature was approximately 80 F (26.7 C). The drum atmosphere into which a virus mixture was aerosolized was 30, 50, or 80% RH. The RH condition for each test was randomly selected; four tests were made at each RH.

The basic data reported were the total number of CIU aerosolized, the number of CIU recovered per milliliter of impinger fiuid, the milligrams of fluorescein dye aerosolized, and the milligrams of fluorescein recovered per milliliter of impinger fluid.

Percentages of recoveries were computed as follows:

Percentage of viable recovery

$$= \frac{(\text{CIU/ml})\ (\text{impinger fluid volume})}{(\text{impinger flow rate in liters per min})}$$

$$\times \frac{(\text{drum volume})}{(\text{CIU aerosolized})} \times 100$$

Percentage of physical recovery

$$= \frac{(\text{mg/ml})\ (\text{impinger fluid volume})}{(\text{impinger flow rate in liters per min})}$$

$$\times \frac{(\text{drum volume})}{(\text{mg aerosolized})} \times 100$$

Percentage of biological recovery = viable recovery/physical recovery × 100

From the exponential decay model ($C_t = C_0 e^{-kt}$, where C_t = percentage of recovery at time t, C_0 = percentage of recovery at time zero, and k = the
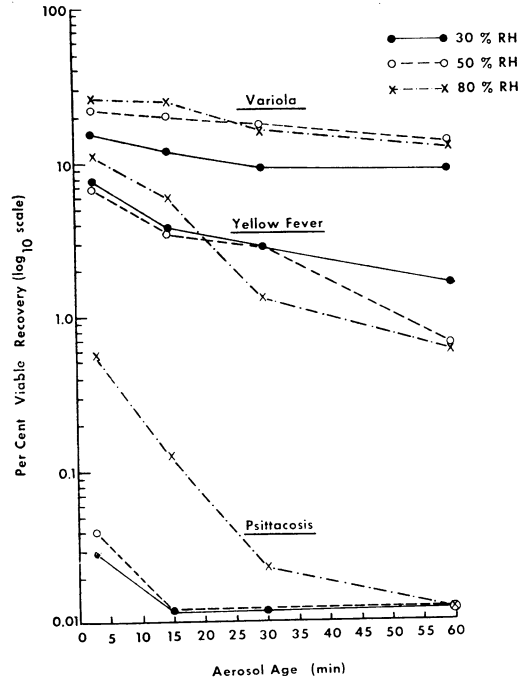

FIG. 1. *Mean per cent viable recoveries of viruses from aerosol mixtures as a function of cloud age for each of three relative humidities at 80 F (26.67 C). Recoveries of psittacosis agent were less than 0.1% with 30 and 50% RH at aerosol age of 15, 30, and 60 min, and with 80% RH at aerosol age of 60 min.*

exponential decay rate), linear regressions of the logarithms of percentage of recovery on time were computed for both the physical and biological per cent recoveries of each trial. Decay rates (per cent per minute) were estimated from a least squares fit of cloud per cent recovery with aerosol age.

RESULTS AND DISCUSSION

Results in Fig. 1 and 2 show that the aerosol survival of each agent in aerosol mixtures could be readily delineated. Mean per cent viable and biological recoveries of variola virus at the three levels of RH were higher than those of yellow fever virus. Recoveries of the latter virus, in turn, were higher than those of the psittacosis agent. The initial decline of the psittacosis agent, compared with that of variola and yellow fever viruses, was very marked. Although this could be attributed to the relatively large size of the psittacosis agent, size alone is not the determinative factor in aerosol stability. Higher recovery values were obtained with variola virus, which is approximately 10 times larger than yellow fever virus. It would appear that other factors are responsible for the initial inactivation of the psittacosis agent that may be related to the stress of aerosolization, i.e., dehydration, reagglomeration, denaturation. Viable and biological recoveries of yellow fever virus decreased signifi-

cantly ($P < 5\%$) at 30% RH and highly significantly ($P < 1\%$) at 50 and 80% RH as the aerosol age increased. There was no significant decrease in recoveries of either variola virus or the psittacosis agent at these levels of RH. Physical recoveries, however, were significantly decreased as the age of the aerosol increased at all RH conditions under consideration (Fig. 3).

Biological decay rates of each agent (Table 1) associated with the three relative humidities were not significantly different at the $P < 5\%$ level. This was also true of the decay rates of the physical tracer. On the basis of biological decay rates, the insensitivity of yellow fever virus to humidity changes under the temperature and RH conditions used in this study is in agreement with the findings of others (8, 10). The biological stability

FIG. 3. Mean per cent physical (uranine) recoveries from aerosol mixtures as a function of cloud age for each of three relative humidities at 80 F (26.67 C).

TABLE 1. Mean biological and physical decay rates of virus mixtures in aerosol[a]

Virus	Relative humidity		
	30%	50%	80%
Psittacosis.....	0.64[b] (±2.10)[c]	1.59 (±1.78)	6.73 (±5.12)
Yellow fever...	3.26 (±2.99)	7.04 (±9.20)	4.53 (±1.84)
Variola........	0.86 (±0.48)	0.81 (±0.67)	0.56 (±0.25)
Physical.......	0.57 (±0.25)	0.64 (±0.41)	0.59 (±0.95)

[a] Temperature condition 80 F (26.67 C).

[b] Decay rate, per cent per minute; mean of four replicate trials.

[c] Confidence factor computed by multiplying the standard error of the mean by 3.182, the 5% level of Student's t.
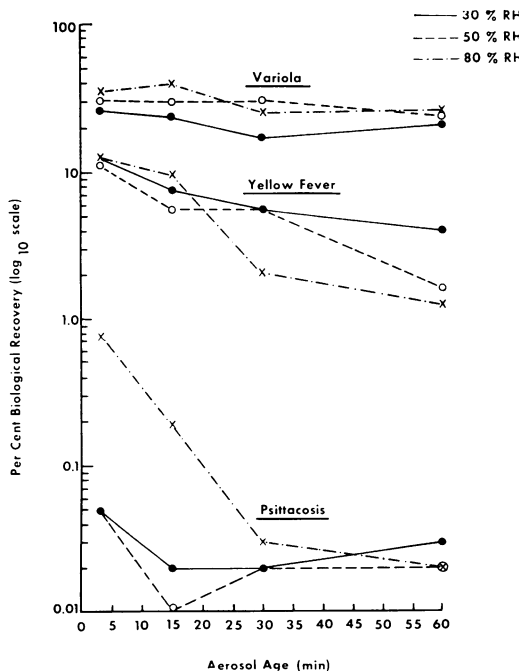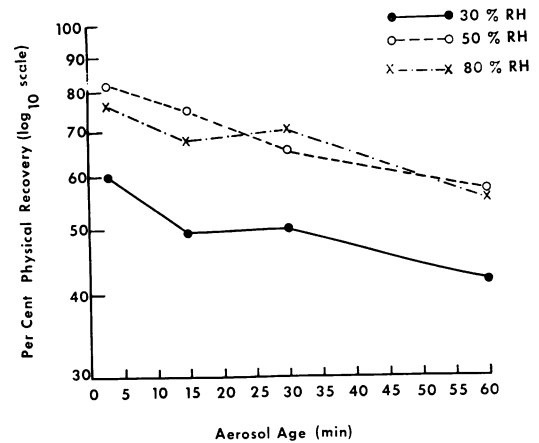

FIG. 2. Mean per cent biological recoveries of viruses from aerosol mixtures as a function of cloud age for each of three relative humidities at 80 F (26.67 C).

of airborne viruses as a function of RH, in the light of variable findings reported with other viruses (1, 9, 14), makes it difficult to generalize on the influence of this factor. The decay rate of variola virus was significantly lower, however, than that of yellow fever virus at each RH and of the psittacosis agent at the highest RH tested.

The feasibility of employing selective immunofluorescence procedures in conjunction with fluorescent cell counting assays for estimating the concentration of each virus in aerosol mixtures was established by this study. The broad applicability and advantages of this procedure have been cited earlier (6, 8). Additional considerations of the immunofluorescent assessment method relevant to characterizing the aerosol behavior of viruses are (i) that interpretation of results may be facilitated because the basis of assay for each virus in mixture is the same, (ii) that virus assessment of aerosol samples may be accomplished within 24 hr, (iii) that a more valid comparison of the aerosol behavior of viruses may be obtained because each virus in an aerosol mixture is subjected to identical stresses and test conditions, and (iv) that the aerosol behavior of several viruses may be studied simultaneously.

ACKNOWLEDGMENT

LITERATURE CITED

1. Akers, T. G., S. Bond, and L. J. Goldberg. 1966. Effect of temperature and relative humidity on survival of airborne Columbia SK group viruses. Appl. Microbiol. 14:361–364.

2. Coons, A. H., and M. H. Kaplan. 1950. Localization of antigen in tissue cells. II. Improvements in a method for the detection of antigen by means of fluorescent antibody. J. Exp. Med. 91:1–13.

3. Goldberg, L. J., H. M. S. Watkins, E. E. Boerke, and M. A. Chatigny. 1958. The use of a rotating drum for the study of aerosols over extended periods of time. Amer. J. Hyg. 68: 85–93.

4. Hahon, N. 1965. Assay of variola virus by the fluorescent cell-counting technique. Appl. Microbiol. 13:865–871.

5. Hahon, N. 1966. Fluorescent cell-counting assay of yellow fever virus. J. Infec. Dis. 116:33–40.

6. Hahon, N. 1967. Multiple infection of cell monolayers by virus mixtures. Appl. Microbiol. 15:458–459.

7. Hahon, N., and R. M. Nakamura. 1964. Quantitative assay of psittacosis virus by the fluorescent cell-counting technique. Virology 23:203–208.

8. Mayhew, C. J., W. D. Zimmerman, and N. Hahon. 1968. Assessment of aerosol stability of yellow fever virus by fluorescent-cell counting. Appl. Microbiol. 16:263–266.

9. Miller, W. S., and M. S. Artenstein. 1967. Aerosol stability of three acute respiratory disease viruses. Proc. Soc. Exp. Biol. Med. 125:222–227.

10. Miller, W. S., P. Demchak, C. R. Rosenberger, J. W. Dominik, and J. L. Bradshaw. 1963. Stability and infectivity of airborne yellow fever and Rift Valley fever viruses. Amer. J. Hyg. 77:114–121.

11. Riggs, J. L., R. J. Seiwald, J. H. Burckhalter, C. M. Downs, and T. G. Metcalf. 1958. Isothiocyanate compounds as fluorescent labeling agents for immune serum. Amer. J. Pathol. 34:1081–1097.

12. Rodriguez, J., and F. Deinhardt. 1960. Preparation of a semipermanent mounting medium for fluorescent antibody studies. Virology 12:316–317.

13. Shipe, E. L., M. E. Tyler, and D. N. Chapman. 1959. Bacterial aerosol samplers. II. Development and evaluation of the Shipe sampler. Appl. Microbiol. 7:349–354.

14. Webb, S. J., R. Bather, and R. W. Hodges. 1963. The effect of relative humidity and inositol on air-borne viruses. Can. J. Microbiol. 9:87–92.