Aerosol Stability and Respiratory Infectivity of Japanese B Encephalitis Virus

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Experiments were conducted to examine the aerosol stability and respiratory infectivity of Japanese B encephalitis virus. At 75°F (about 24°C), survival of the virus as aerosol was inversely related to relative humidity. After correction for physical decay, the mean virus half-lives of the virus were 28, 38, and 62 min at relative humidities of 80, 55, and 30%, respectively. Virus recoveries as aerosol at 4 min after dissemination generally exceeded the theoretical limit of 100%, based on the amount disseminated, to suggest that the process of dissemination operated to deagglomerate or release bound virus from the tissue cells in suspension. Swiss-ICR mice and golden Syrian hamsters were highly susceptible to lethal infections after respiratory challenge. Hartley strain guinea pigs and Fisher-Dunning rats, although infected, based on seroconversion observations, survived the infections. Deaths occurred in squirrel monkeys only after exposure to a high aerosol dose of virus ($10^{6.0}$ plaque-forming units). Studies of the virus concentration dynamics and histopathological findings in mouse tissues after aerosol challenge supported a hypothesis for direct transport of virus across the foramina of the cribriform plate to the tissues of the central nervous system to produce primary encephalitis.

Japanese encephalitis, caused by a flavivirus, is endemic and enzootic throughout much of Southeast Asia. The virus was the apparent cause of a widespread epidemic in Japan as early as 1871 (4). A severe epidemic in Japan in 1924 resulted in 3,800 deaths among 6,000 clinical cases (11). Also, during epidemics in Japan and Korea in 1958 there were 1,879 deaths among 7,500 clinical cases. More recently, in late 1978 an outbreak of viral encephalitis in India, shown by serological testing and viral isolation to have been caused by Japanese encephalitis virus (JEV), resulted in at least 475 deaths (3).

The virus is found mainly in rural areas and is mosquito borne, with good evidence that swine play an important role as amplifiers of the virus. Epidemiological studies suggest that airborne transmission of infection does not regularly occur in nature, either from animal to human or from human to human, but the studies were not primarily designed to investigate such a potential (4).

JEV is used extensively in arbovirus research. Hence, there must be frequent opportunity for researchers as well as clinical medical personnel to be at risk of exposure to JEV aerosols. Comprehensive surveys of laboratory-acquired infections have shown that as many as 65 to 84% of these infections may result from the inhalation of spurious aerosols created by microorganism manipulation in laboratory environments (12, 13).

The objectives of the experiments reported here were: (i) to determine the aerosol stability of JEV as a function of environmental relative humidity (RH), (ii) to examine the infectivity of the virus for laboratory animals challenged by the aerosol route, and (iii) to obtain information on the pathogenesis of the respiratory infections in terms of the histopathological findings and primary sites of virus replication within the host.

MATERIALS AND METHODS

Virus suspension. Stock virus was prepared as a seventh suckling mouse brain passage of the Peking strain of JEV (6). Three- to 4-day-old suckling mice were inoculated intracerebrally (i.c.) with $10^{4.0}$ plaque-forming units (PFU) of sixth-passage suckling mouse brain virus contained in 0.02 ml of inoculum. Infected mouse brains were harvested 72 h after inoculation. The mouse brain material was suspended in a diluting medium consisting of equal parts by volume of brain heart infusion broth (BHI) and inactivated fetal calf serum (IFCS) plus gentamicin sulfate (125 µg/ml) and was homogenized in Ten-Broeck tissue grinders; remaining clumps of brain tissue were removed by low-speed centrifugation ($1,750 \times g$) for two periods of 10 min each. The final products, a 20% (wt/vol) suspension containing approximately $10^{8.5}$ PFU/ml, was stored at -70°C in 2.5-ml quantities.

Aerosol generation. Aerosols of JEV were created in either static or dynamic aerosol facilities, depending on the experimental objectives. Studies to measure airborne stability were conducted in an upright, cylindrical, gas-tight, environmentally controlled static aerosol chamber (approximately 6 feet [183 cm] in di-

ameter by 7 feet [213 cm] high with convex top and bottom) having a total volume of 6,200 liters. The design characteristics and operating principles of static aerosol chambers have been described previously (15). An FK-8 atomizer was operated with nitrogen gas at 50 lb/in² (gauge) to disseminate 1 ml of virus suspension in each aerosol trial (9). The FK-8 atomizer disseminates 1 ml in approximately 3.5 s and produces aerosols with particle size distributions characterized by a mass median diameter of approximately 4.0 μ m and geometric standard deviation of approximately 2.0 (10).

Animal exposures were performed in dynamic aerosol equipment. A Collison nebulizer (12) was operated at 26 lb/in² (gauge) to disseminate virus into a Henderson-type (8) aerosol transit tube modified by the incorporation of an animal exposure box appropriate to each animal species. Volume flow in the system was maintained at 28.0 liters/min. Dissemination was continuous throughout the period of animal exposure (10 min) in each trial. Graded exposure doses were achieved by dilution of the virus in the spray suspension. Total inhaled doses were computed in accordance with Guyton's formula (5) for determining respiratory minute-volumes and were based on measurements of the aerosol concentrations in the exposure box during exposure. Under the conditions used, the mass median diameter of virus-containing particles to which the animals were exposed would be expected to approximate 2.3 μ m, with 90% of the virus in particles of <4.5 μ m (1). These particles would be expected to penetrate the deep pulmonary spaces, with retention of approximately 50% of the virus in the lungs of exposed animals (7).

Aerosol sampling. Standard all-glass impinger aerosol samplers with the tip of the critical orifice raised 30 mm (AGI-30) from the bottom of the collection flask and calibrated to sample at 12.5 liters/min were used (2). The samplers were operated with 20 ml of collecting medium consisting of equal parts by volume of BHI and IFCS to which were added gentamicin (75 μ g/ml), mycostatin (40 U/ml), and 0.2 ml (total) of a 1:10 dilution of antifoam (Dow Corning Antifoam Y-30 Emulsion). Samples of 1-min duration were collected at period midpoints of 4, 32, and 60 min after dissemination in trials to measure aerosol stability. Similar 1-min samples were collected at period midpoints of 1.5 and 9.5 min during dissemination in the animal exposure trials; the contents of these samples were pooled before assay to provide a composite estimate of average aerosol concentration.

Virus assays. Plaque assay procedures were used to quantitate virus in suspensions, aerosol samples, and animal tissue specimens. Plaques were enumerated on monkey kidney (LLC-MK₂) tissue cell monolayers in six-well (9.6 cm²/well) plastic trays (Linbro Scientific Co., Inc., New Haven, Conn.). Confluent monolayers were inoculated with 0.2 ml of serial 10-fold dilutions of virus samples in Hanks balanced salt solution with IFCS, gentamicin, and mycostatin. The virus was permitted to adsorb for 1 h at 36°C in a humidified, 5% CO₂ environment before overlaying with 3 ml of 1% agarose in Hanks balanced salt solution containing 5% IFCS, 0.5% lactalbumin, and 40 U of mycostatin per ml (final pH = 7.4). After 3 days

of incubation at 36°C in 5% CO₂, a second overlay (1.5 ml) of the same medium with a 1:5,000 (wt/vol) dilution of neutral red was added. Plaques were counted at 24 h after the second overlay.

Animals. Swiss-ICR mice, procured from Flow Laboratories, Rockville, Md., were the primary animals used in this research. Adult mice at 7 weeks of age weighing approximately 30 g were usually used, although, for comparative purposes, selected experiments were conducted with weanling mice at 3 weeks of age (approximately 12 g). Four additional species were screened for susceptibility to lethal infection after aerosol challenge. These included golden Syrian hamsters (Charles River Breeding Laboratories, Wilmington, Mass.) weighing approximately 125 g, Hartley strain guinea pigs (from the colonies of the Walter Reed Army Institute of Research) weighing approximately 275 g, Fisher-Dunning rats (Charles River) weighing approximately 175 g, and squirrel monkeys (750 to 1,000 g). All aerosol challenges, animal care after exposure, and necropsies were performed within the confines of total containment, gas-tight biological safety cabinets fitted with arm-length rubber gloves. Animals in appropriate pans or cages were given water and ration pellets ad libitum during the observation periods after exposure.

RESULTS

Aerosol stability. One experiment was conducted in which four replicate aerosol trials were performed at each of three RH, 30, 55, and 80%. Aerosol temperature was maintained at 75°F (about 24°C) throughout the trials. One milliliter of a 1:1 dilution of the working pool of virus in BHI and IFCS was disseminated in each trial.

The aerosol sampling results, expressed in terms of both aerosol concentration (PFU/liter) and percentage recovery of the total virus subjected to dissemination, as a function of aerosol age were fitted to a simple exponential equation of the form:

$$C_t = C_0 e^{-kt}$$

where C represents virus concentration (or percentage recovery) as aerosol, t represents the aerosol age in minutes, and k represents the rate at which the aerosol concentration decreased with time. Since this function is log linear with k representing a measure of linear slope, the results may be summarized as shown in Fig. 1.

Even with the limited data, an analysis of variance suggested a nearly significant ($P < 0.10 > 0.05$) inverse relationship between aerosol stability and RH. The aerosol decay rates, with $100k$ defined as percentage decay/minute, were 2.6, 3.3, and 4.0%/min for RH of 30, 55, and 80%, respectively. These may be translated in terms of the virus half-life in aerosol as 26.5 min at 30% RH, 20.9 min at 55% RH, and 17.3 min at 80% RH.

The initial recoveries of virus were high at all

FIG. 1. Effect of RH at 75°F (about 24°C) on the aerosol concentrations of JEV when 1 ml of virus ($8.5 \log_{10}$ PFU/ml) was disseminated in a 6,200-liter aerosol chamber and sampled at 4, 32, and 60 min after dissemination.

three RH. In fact, the mean percentage recovery among all RH at 4 min after dissemination was 112%, indicating that the process of dissemination operated to deagglomerate or release bound virus from the tissue cells in suspension.

Respiratory dose responses. Graded respiratory dose-response titrations were performed in both adult and weanling mice. Table 1 summarizes these dose-response titrations and presents times to death as a function of challenge dose. By probit analysis, the median lethal dose for the adult mice was $10^{3.1}$ PFU with 95% confidence limits between $10^{2.6}$ and $10^{3.7}$. At this challenge dose, the mean time to death was approximately 13 days. Clearly, the mean time to death in the adult mice was inversely related to the challenge dose. The younger (lighter) weanling mice were more susceptible to lethal infection than were the older mice. Mortalities exceeded 50% even at the lowest dose of $10^{1.2}$ PFU. Again the trend for decreased time to death with increased challenge dose was observed. In both the adult and weanling groups, the mice that died showed few signs of illness until about 2 days before death, when rough coats, weakness, ataxia, and paralysis typical of encephalitis were observed.

Table 2 summarizes the results of screening studies conducted to examine the susceptibility of additional animal species to lethal infection after aerosol challenge. Hamsters proved susceptible, with five of six animals succumbing to the lowest dose of $10^{3.4}$ PFU. Deaths occurred in squirrel monkeys only at the highest dose of $10^{6.0}$ PFU. Neither guinea pigs nor rats died when exposed to doses as high as $10^{5.5}$ or $10^{5.8}$, respectively. All were infected, however, based on the presence of JEV-specific hemagglutination inhibition antibodies in sera collected 21 days after exposure. Reciprocal hemagglutination inhibition titers in guinea pig sera were higher (geometric mean = 320) than those in rat sera (geometric mean = 20).

Pathogenesis. Experiments were conducted to determine the primary sites of virus proliferation in mice after aerosol exposure. Groups of three mice were killed and necropsied periodically after challenge. Selected tissues were collected, homogenized (except blood) in tissue homogenizers (Ten-Broek), and assayed for virus concentrations.

Table 3 presents the combined results from two similar studies in which the lungs, upper respiratory tract tissues (URT), and brain were collected from each mouse. The URT specimens represented a composite of the mainstem bronchi, trachea, nasal turbinates, and pharynx.

Virus was recovered from the lungs of mice throughout the period from the 1st day after challenge through day 9. Virus concentrations were generally less than $10^{3.0}$ PFU/whole lung, and virus was recovered from the lungs of only one-third to two-thirds of the mice in any period. These results suggested that the lung was not a primary focus of viral replication; more likely the virus found in the lung represented deposits from the initial challenge or resulted from aspiration from the URT.

TABLE 1. Mortality and time-to-death responses in adult and weanling mice after aerosol challenge with graded doses of JEV

Mouse group	Determination		
	Dose ($\log_{10}$ PFU)	Dead/total	MDTD ^a (range)
Adult	4.7	17/20	8.5 (7-15)
	4.3	14/20	10.7 (7-16)
	3.0	10/20	13.5 (8-18)
	2.3	6/20	15.0 (10-18)
Weanling	4.6	14/15	8.0 (7-10)
	3.8	10/11	10.5 (8-14)
	2.5	11/15	14.5 (12-19)
	1.2	13/15	14.8 (9-20)

^a Median lethal doses were $10^{3.1}$ PFU for adult mice and $10^{1.2}$ for weanling mice. MDTD, Geometric mean days to death.

TABLE 2. Mortality and time-to-death responses of hamsters, guinea pigs, rats, and squirrel monkeys to aerosol challenge with JEV

Species	Dose ($\log_{10}$ PFU)	Dead/total	MDTD ^a (range)
Hamster	5.5	13/14	10.3 (7-21)
	4.5	6/6	18.2 (9-23)
	3.4	5/6	15.2 (7-22)
Guinea pig	5.8	0/8	
Rat	5.5	0/8	
Squirrel monkey	6.0	3/4	12.6 (12-14)
	4.9	0/4	
	2.8	0/4	

^a Geometric mean days to death.

TABLE 3. *Whole tissue concentrations of JEV in mouse respiratory tract and brain after aerosol challenge with 4.6 log₁₀ PFU of virus*

Days post-challenge	Mean log ₁₀ PFU/whole tissue ^a (positive/total)		
	Lung	URT	Brain
1	2.81 (2/3)	0 (0/3)	0 (0/3)
3	2.77 (4/6)	2.98 (4/6)	4.69 (1/6)
4	2.60 (5/6)	4.15 (5/6)	4.13 (6/6)
5	2.59 (2/3)	5.29 (3/3)	8.53 (3/3)
6	2.43 (1/3)	6.13 (3/3)	8.48 (3/3)
7	3.10 (1/6)	6.21 (5/6)	8.75 (6/6)
8	1.73 (1/3)	4.50 (3/3)	8.41 (3/3)
9	2.89 (2/3)	5.28 (3/3)	8.91 (3/3)
10	0 (0/3)	5.23 (3/3)	8.11 (3/3)
11	0 (0/6)	3.99 (2/6)	6.54 (3/6)

^a Virus concentrations are means of positive mice only.

Virus was first recovered from the URT sample 3 days after challenge and peaked to a moderately high level of 10^{6.0} PFU by 6 and 7 days after challenge; virus was recovered from nearly all mice. Highest virus concentrations were measured in the whole brain specimens. Only one of six mice was positive for virus by day 3, but all mice had virus in the brain by 4 days. Peak concentrations in the brain approached 10^{9.0} PFU over an extended period from 5 through 10 days after exposure.

No virus was isolated from blood samples, suggesting that the concentrations of virus, if any, were below the assay sensitivity and that virus transport to the brain was not hematogenous. Comprehensive histological examinations of infected mice revealed that viral lesions developed first in the olfactory bulb, the frontal lobe, and olfactory portions of the cerebrum at 5 days. Necrotic lesions of the olfactory bulb, cerebrum, brain stem, and spinal cord were seen in all of the mice examined at 7 and 10 days after challenge. Lesions were not seen in the olfactory epithelium of the nasal cavity until 10 days, suggesting that any infection of the nasal cavity developed late; also, early recoveries of virus from URT samples may have resulted from the presence of olfactory bulb tissue in the samples. Examination of visceral organs failed to reveal significant lesions suggestive of virus invasion.

In a subsequent study, nasal turbinate tissues were dissected out and assayed for virus. At the same time, samples of the cribriform plate were collected. The brain was also assayed for virus. Blood samples were again collected, but the blood was inoculated i.c. into weanling mice in an attempt to improve assay sensitivity. Low-level viremias, early in the infections, were detected by this method of inoculation (Table 4).

Virus was recovered sporadically from the nasal turbinates at moderately low levels. Higher virus concentrations were measured in the samples of the cribriform plate, but not uniformly from all mice. The highest virus concentrations were found in the brain and revealed patterns of recovery similar to those seen in the cribriform plate.

DISCUSSION

To our knowledge, the experiments reported here are the first which have been conducted to examine the airborne stability and respiratory infectivity of airborne JEV. These studies indicate that an SMB harvest of the virus can be disseminated efficiently as an aerosol in particles of a size range capable of remaining airborne and, when inhaled, being deposited in the respiratory tract of animals and humans. Moreover, the virus is relatively stable in aerosol after dissemination over a wide range of RH.

The measured total decay rates, including both the physical and biological components of decay, were within the range of 2.6 to 4.0%/min, depending on the RH used. Extensive testing with the FK-8 atomizer in our 6,200-liter aerosol chambers has indicated a physical decay rate of approximately 1.5%/min (10), suggesting that the virus inactivation rates (biological decay) in the studies reported here ranged from 1.1 to 2.5%/min. On this basis, virus half-lives after correction for physical losses ranged between 28 and 62 min. With this degree of stability, we must conclude that there exists a potential hazard of airborne infection with this virus in laboratory environments and possibly in nature.

Our studies have demonstrated also that the virus is infectious for several species of experimental animals when administered by the airborne route. Moreover, the infections are lethal for both mice and hamsters at relatively low

TABLE 4. *JEV concentrations in mouse tissues after aerosol challenge with 4.4 log₁₀ PFU of virus*

Days post-challenge	Mean log ₁₀ PFU/sample ^a			Blood inoculation (positive/6) ^b
	Brain	Cribriform plate	Turbinates	
1	0	0	0	0, 2, 3
2	0	0	0	5, 6, 6
3	0	3.0 (1)	2.5 (1)	6, 6, 1
4	4.0 (2)	4.0	2.7 (2)	0, 1, 1
7	7.4	7.2	4.4 (2)	0, 0, 0
9	7.7 (1)	6.8 (1)	0	0, 0, 0
11	7.6 (2)	6.6 (2)	3.7 (2)	0, 0, 0
14	7.8 (1)	6.1 (1)	2.5 (1)	0, 0, 0

^a *n* = 3, except as indicated in parentheses.

^b Number of weanling mice dead of six inoculated i.c. with 0.02 ml of blood from aerosol-challenged mice; three aerosol-challenged mice per point.

challenge doses and are lethal for squirrel monkeys at a relatively high dose. Others have reported infectivity and lethality in additional sub-human primate species after intranasal challenge with JEV (6).

Our results on the histopathological findings and virus population dynamics in mice provide some insight into possible mechanisms of virus transport and infectious processes within the host after respiratory exposure. The potential for hematogenous transport of virus to central nervous system tissues cannot be ruled out, but a stronger case can be made for virus transport from the nasopharynx directly across the foramina of the cribriform plate to the olfactory bulb. Viremia was not present except as an early transient phenomenon, detectable only by the highly sensitive assay involving i.c. inoculation of weanling mice. Virus concentrations in lungs were consistently minimal by comparison with those which developed in the URT and brain. Finally, the temporal development of lesions in central nervous system tissues, seen first in the olfactory bulb and cerebrum and later in the brain stem and spinal cord, supports a direct-transport hypothesis.

Particularly important to the issue of airborne transmission potential in nature is the observation that high virus concentrations were recovered from the URT of mice, including the nasal turbinates. If similar concentrations develop in humans and intermediate animal hosts during natural outbreaks of the disease, then the risks could be relatively high that virus would be shed in saliva and nasal discharges, for example, and these could serve as sources of airborne virus.

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