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Respiratory Infectivity of a Recently Isolated Egyptian Strain of Rift Valley Fever Virus

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The respiratory infectivity of a strain of Rift Valley fever virus isolated in Egypt (strain ZH-501) was compared with that of one isolate from Uganda (Entebbe strain) and two isolates from South Africa (strains SA-51 and SA-75). Studies were performed with ICR mice which were infected by exposure to infectious aerosols composed of particles with a mass median diameter of 0.96 μm. The respiratory median lethal doses for ZH-501, Entebbe, SA-51, and SA-75 were 2.2, 1.9, 2.6, and 1.9 $\log_{10}$ plaque-forming units, respectively. Although these values are statistically different, the biological implications of such differences seem unimportant. In an additional study of pathogenesis, a single group of mice was infected with 3.1 $\log_{10}$ plaque-forming units of ZH-501, and tissues were assayed sequentially through 96 h postinfection. Between 6 and 30 h, demonstration of an increasing virus concentration only in the lungs indicated that initial replication occurred there; however, determination of histopathological changes did not reveal evidence of pneumonia. Virus was isolated from the liver by 48 h, and the ultimate outcome of infection was a fulminating and fatal hepatic necrosis.

In 1977, a severe epizootic of Rift Valley fever (RVF) occurred in Egypt (17). The outbreak was especially noteworthy, as RVF had never been recognized before in Egypt. The disease was known to be epizootic in Africa since 1930, but it always had been confined to areas south of the Sahara Desert (4, 16). In addition, infections in humans were widespread and severe (1, 16). Human RVF previously had been described as a "dengue-like" disease in which complications occasionally were noted, but the patient was expected to recover. The human infections observed in Egypt differed in that complications which resulted in numerous deaths developed. Although the virus isolated in Egypt serologically was indistinguishable from other RVF virus (RVFV) isolates, health authorities were concerned that a new strain that was more virulent for humans may have emerged (13).

Epizootic RVF in Egypt and elsewhere is thought to be transmitted primarily by mosquitoes (5). Livestock herdsmen, veterinarians, and abattoir workers, however, have acquired infections merely from close association and direct contact with infected livestock or their tissues (10), and in some of these cases airborne transmission was thought to be the means of infection. Evidence that defines the frequency of airborne transmission under natural conditions is lacking. It is known that laboratory workers have acquired RVF after inhalation of infectious aerosols generated by careless handling of infected tissues. Also, several animal species have been shown experimentally to be highly susceptible to airborne RVFV (5).

The primary objective of this study was to evaluate the potential for airborne transmission of an RVFV isolate from Egypt and to compare its infective properties with those of other isolates posited to be less pathogenic for humans. In addition, the pathogenesis of airborne infection was studied by examining tissues at sequential time intervals for evidence of virus replication and pathology.

MATERIALS AND METHODS

Virus. The four isolates of RVFV used in this study (hereafter referred to as strains) are identified by name or code, origin, and passage history as follows: (i) Entebbe, isolated in 1944 from mice inoculated with a pool of *Eretmapodites* mosquitoes trapped in Uganda, passed 184 times in mice and 2 times in fetal rhesus lung (FRhL) cells (19); (ii) SA-51, isolated in South Africa in 1951 from a naturally infected lamb, passed three times in sheep and two times in FRhL cells; (iii) SA-75, isolated in South Africa in 1975 from a human with a benign infection, passed three times in FRhL cells; and (iv) ZH-501, isolated in Egypt in 1977 at the Zagazig Hospital from a patient with fatal hemorrhagic fever, passed two times in FRhL cells (16). A working stock of each strain was prepared by inoculation of Vero cell monolayers maintained in Eagle minimal essential medium containing Earle balanced salt so-

lution (EMEME), supplemented with glutamine and nonessential amino acids, 10% heat-inactivated fetal calf serum, 100 U of penicillin G per ml, and 100 μg of streptomycin per ml. The monolayers were incubated at 37°C for 48 h, at which time the maintenance medium contained 7.6 to 8.0 $\log_{10}$ plaque-forming units (PFU). The medium was then harvested, centrifuged to remove cellular debris, and stored at −70°C.

Virus assay. Assays were performed in duplicate by plaque enumeration. Tenfold dilutions of viral specimens were made in Hanks balanced salt solution containing 2% heat-inactivated fetal calf serum, 2.4 mg of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (CalBiochem-Behring Corp., La Jolla, Calif.) per ml, and antibiotics. Vero cell monolayers established in six-well (well area, 9.6 cm^2) plastic culture plates (Flow Laboratories, Inc., Hamden, Conn.) were inoculated with 0.2 ml of each viral dilution and adsorbed for 60 min at 37°C in a humidified atmosphere containing 5% CO_2. Monolayers were then overlaid with 2 ml of Eagle basal medium with Earle salts, containing 0.5% agarose (Marine Colloids, Rockland, Maine), 4% heat-inactivated fetal calf serum, HEPES buffer (4 mg/ml), and antibiotics. Culture plates were then incubated for 96 h at 37°C in a humidified atmosphere containing 5% CO_2. A second 2 ml of agar overlay which contained 0.1 mg of neutral red (GIBCO Laboratories, Grand Island, N.Y.) per ml was added, and the plates were incubated for an additional 24 h. Plaques were counted, and titers were calculated as $\log_{10}$ PFU per milliliter.

Mice. Male 6- to 7-week-old Swiss Webster mice [Wrm: (ICR)BR] weighing ca. 30 g were supplied by the Walter Reed Army Institute of Research, Washington, D.C. During and after infection, mice were maintained inside a class III biological safety cabinet in a maximum containment laboratory.

Aerosol exposure. Mice were infected by exposure to infectious aerosols in dynamic aerosol equipment. The virus dose of each of the four strains was increased in 10-fold increments from ca. 0.5 to 4.5 $\log_{10}$ PFU. Aerosols were generated from stock virus suspensions with a Collison atomizer and disseminated into a Henderson-type aerosol mixing tube, modified by attachment of the exposure tube to a plastic animal exposure box (2, 9, 15). Mice were exposed in groups of 20 for 10 min and held 21 days for observation. The formula of Guyton was used to calculate the respiratory minute-volume, and exposure doses were reported as the total inhaled virus dose per mouse (8).

Particle size distribution. Before aerosol exposure trials were conducted, the particle size distribution of aerosols was determined. Aerosols were generated from the virus suspension medium in a routine manner, except that fluorescein sodium was added as a tracer at a concentration of 0.1 mg/ml. Aerosol samples were then collected with a series of single-stage impactors (14), and the fluorescein sodium concentration was measured with a fluorophotometer.

Aerosol sampling. During each exposure trial, aerosol within the exposure box was collected with an all-glass impinger calibrated to sample at the rate of 12.5 liters/min (3). Each impinger contained 20 ml of collection fluid consisting of EMEME supplemented with 5% heat-inactivated fetal calf serum and antibiotics plus an additional 0.2 ml of a 1:10 dilution of antifoam Y-30 emulsion (Dow Corning Corp., Midland, Mich.). Samples were collected for either 1 min at midpoints of 1.5 and 9.5 min or for 5 min at a period midpoint of 5 min.

Pathogenesis study. Mice were exposed to an estimated respiratory dose of 3.5 $\log_{10}$ PFU of the ZH-501 strain. This relatively low dose was selected because it represented an aerosol dose that might be attained under natural conditions and that would be capable of causing greater than 95% mortality. Immediately after exposure and at 6- or 12-h intervals through 96 h, four mice were randomly selected for collection of blood and tissues. Selected mice were anesthetized with halothane, bled by cardiac puncture, and killed by cervical luxation. Brain, lungs, heart, nasopharynx, kidneys, spleen, and liver were removed and separately homogenized (SDT-Tissumizer; Tekmar Co., Cincinnati, Ohio) in EMEME containing 10% heat-inactivated fetal calf serum and antibiotics. Blood and tissues were stored at −70°C for subsequent assay. For a parallel study of microscopic lesions, two additional mice were killed at each time interval. Tissues were examined for gross lesions and fixed in 10% Formalin. The fixed tissues were embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined for histological changes.

RESULTS

Dose-response experiments. Evaluation of the size of the particles distributed in the infectious aerosols, based on eight replicate experiments, demonstrated that 98.5% of the particles were 5 μm or less in diameter and that the particle mass median diameter was 0.96 μm. The mortality responses of mice exposed to graduated doses of each of the four RVFV strains are shown in Table 1. Signs observed in infected mice were limited to listlessness and a rough hair coat. The infection was fulminant in those mice exhibiting clinical signs of disease. Median time to death was reduced at the higher dose levels for all RVFV strains. Calculated by probit transformation, the 50% lethal respiratory doses (LD_{50}s) for ZH-501, Entebbe, SA-51, and SA-75 were 2.2, 1.9, 2.6, and 1.9 $\log_{10}$ PFU, respectively. Of mice exposed to the lower dose levels, deaths occurred only in the group exposed to 0.6 $\log_{10}$ PFU of ZH-501 with 3 of 40 exposed mice dying. Because antibody titers were not determined, it is not known if a sublethal infection occurred in any of the surviving mice. Although the doses among the four strains differed by only 0.7 $\log_{10}$ PFU, statistical evaluation showed that the differences were significant ($P < 0.01$). Only the LD_{50}s of the Entebbe and SA-51 strains were not significantly different, and both of these strains were two- to fivefold more virulent for mice than the other two strains. The different

TABLE 1. *Comparison of infectivity and LD_{50}s for four strains of RVFV*

Strain	Dose (log_{10} PFU)	MTD[a] in days (range)	No. dead/ total	LD_{50}	95% CI[b]	Slope
ZH-501	0.6[c]	4.0 (4–10)	3/40	2.2	1.9–2.4	1.004
	1.7[c]	4.0 (3–8)	13/43			
	2.7[c]	5.0 (3–9)	26/40			
	3.8[c]	4.0 (3–9)	39/40			
	4.7[c]	4.0 (3–6)	37/37			
Entebbe	0.9		0/20	1.9	1.6–2.1	1.959
	1.9	4.5 (3–17)	12/20			
	2.9	3.0 (2–9)	19/20			
	3.8	3.0 (2–3)	20/29			
	4.8	3.0 (2–3)	20/20			
SA-51	0.5		0/20	2.6	2.3–2.8	1.530
	1.6	5.5 (5–6)	2/18			
	2.7	5.0 (4–13)	10/20			
	3.8	4.0 (3–5)	20/20			
	4.8	3.5 (3–4)	20/20			
SA-75	0.5		0/20	1.9	1.7–2.1	2.477
	1.5	4.5 (4–6)	4/20			
	2.2	5.5 (3–19)	16/20			
	3.1	4.0 (3–7)	20/20			
	4.3	3.0 (3–9)	20/20			

[a] MTD, Median time to death.
[b] CI, Confidence interval.
[c] Geometric mean of two replications.

slopes of the dose-response curves reflect a variance in death patterns among the strains; however, the biological importance of these differences remains undefined.

Pathogenesis study. After determination of the dose response, an additional study was performed in a single group of mice. Assay results of aerosol samples indicated that mice received an inhaled dose of 3.1 log_{10} PFU. Tissue assay results representing each time period are shown in Fig. 1. After 48 h, the virus titer in most tissues correlated closely with that in blood. Therefore, the blood values are superimposed on each of the plots for tissues to facilitate interpretation of data. Immediately after exposure at time 0, virus was demonstrated in the lungs of three mice and in the nasopharynx of one. During the first 48 h, virus was demonstrated most consistently in the lungs. By 30 h, virus in the lungs had attained a titer of 2.4 log_{10} PFU/g, a titer significantly higher than those detected in other tissues and blood. At 48 h, the virus concentration in the lungs remained high, whereas viremia and liver infection were not evident until 48 h. The liver contained high titers of virus by 54 h. Between 54 and 96 h, virus titers progressively increased in all tissues, with the highest concentrations being evident in the blood and liver. Virus titers in the brain consistently were lower than those in other tissues.

Gross pathological alterations varied widely among the mice. Lesions noted commonly involved the liver or lungs, with essentially no gross changes being observed in other organs. From 60 h after exposure onward, liver lesions were present. The liver usually was darkened and swollen, but in some mice it appeared pale because of the presence of tightly grouped, minute white foci throughout all lobes. By 96 h, the liver was extremely soft, and petechial hemorrhages covered serosal surfaces of the viscera. Lung pathology was not detectable until 72 h after exposure, and then it was quite inconsistent. The lungs either appeared bright red throughout or contained large, mottled, red areas.

Most microscopic lesions were noted in mice killed between 72 and 84 h after exposure. The most consistent lesion was midzonal to diffuse hepatocellular necrosis, with little or no inflammatory cell infiltration. Varying degrees of lipidosis, occasional eosinophilic intranuclear inclusions in hepatocytes, and occasional necrosis of spleen and lymphoid tissues were also associated with RVFV infection. Histopathological changes which could be directly related to the RVFV infection were not observed in the lungs, nasopharynx, or brain.

DISCUSSION

In 1963, Easterday and Murphy reported that mice were highly susceptible to infectious aerosols of a RVFV strain isolated in South Africa (6). The results reported here both corroborate and extend their findings. Mice were shown to be highly susceptible to aerosols prepared from four strains of RVFV isolated from disparate geographic regions over a time span of 30 years. On the basis of the deaths which occurred after exposure to the lowest dose of ZH-501, we conclude that a respiratory dose as low as 0.6 log_{10} PFU will initiate fatal infection in some mice. Similarly, low respiratory doses have been infectious for other animal species. The lowest reported infectious respiratory dose was for monkeys and hamsters (5, 18); it was determined to be less than 1.0 mouse intraperitoneal LD_{50}. The highest reported infectious respiratory dose was for puppies (11), for which ca. 25 mouse intracerebral LD_{50}s were required to induce infection.

This is the first time the respiratory LD_{50} has been established for mice. A comparable value is available for only one other animal species. Miller et al. (18), using procedures similar to those described in this study, reported the res-

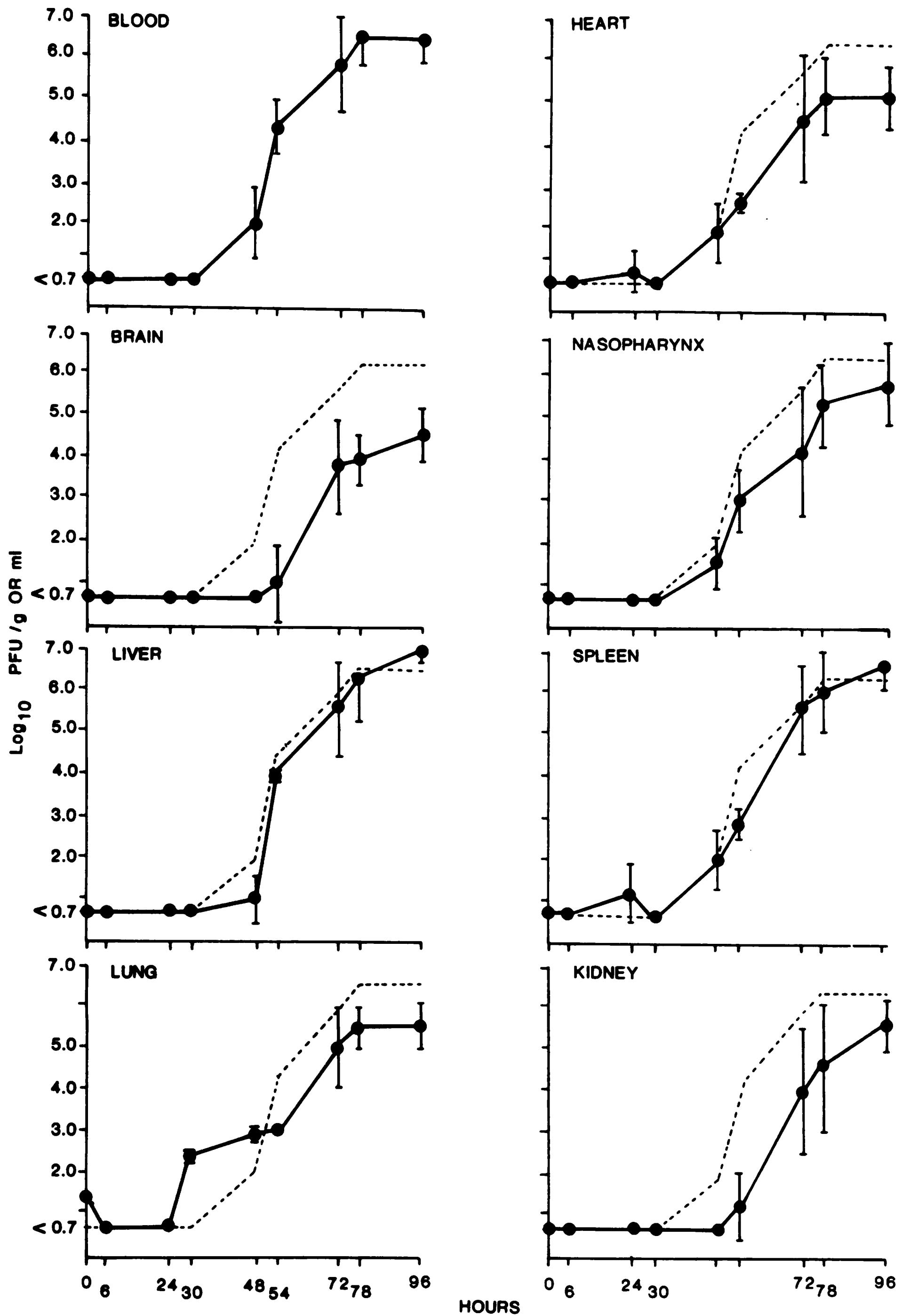

FIG. 1. *Virus concentrations in mice (n = 4) after exposure to 3.1 $\log_{10}$ PFU of RVFV strain ZH-501 via the respiratory route. Tissues from each mouse were assayed separately, in duplicate; each point represents the geometric mean, and the vertical bars indicate ± one standard error. Viremia, represented by broken lines, is superimposed on the plots for each tissue.*

piratory LD_{50} for hamsters to be 0.525 mouse intraperitoneal LD_{50}. Unfortunately, the mouse intraperitoneal LD_{50}s are not comparable with PFU; therefore, an equivalent comparison of mouse and hamster susceptibilities cannot be made. It is our opinion, however, that the LD_{50}s obtained for mice indicate that mice are more resistant than hamsters to lethal respiratory infection with RVFV.

Comparison of the Egyptian isolate of RVFV (ZH-501) with three isolates from sub-Saharan Africa, using LD_{50}s and median times to death, yielded an interesting variance among the strains. The differences observed among the LD_{50}s of three of the four strains were mathematically significant. Examined in perspective, however, the differences are minimal, and we consider them of no practical significance in infections in mice. Significant differences were not observed among the median times to death. Thus, these data indicated that the virulence of the Egyptian isolate of RVFV was no greater than the virulence expressed by previous isolates of the virus.

With some microorganisms, the pathogenesis of infection from inhalation of airborne agents differs appreciably from that expressed when introduction occurs by other routes. RVFV has a propensity for nervous tissue (5). Thus, the potentiality existed that airborne infection might permit, and possibly provoke, direct invasion of the central nervous system via the olfactory nerves. Supportive evidence for this hypothesis was not obtained. Virus infection of the brain remained undetectable until after significant virus concentrations had developed in the blood and liver. Furthermore, RVFV titers in the brain consistently were substantially lower than those in other examined tissues. Pneumonia, likewise, was not a dominant factor in the pathogenesis of RVFV infection in mice by aerosol exposure. Deposition of the virus directly into the pulmonary tract would seem to favor initiation of pneumonia, such as occurred in ferrets after intranasal inoculation of RVFV (7). Histological alterations compatible with pneumonia were not observed in any of the mice examined. The sporadic gross lung lesions exhibited may be attributable to a modification of blood clotting factors secondary to the severe hepatic necrosis.

An absence of visual histological modifications within the lung parenchyma does not diminish the pathogenic role of this organ in airborne RVF. On the contrary, initial virus replication and establishment of infection appear to occur within the lung matrix when the mode of entry is via inhalation. The sequence of virus events in the pathogenesis of RVF in mice exposed to infectious aerosols probably occurs as follows: (i) virus is deposited throughout the respiratory tract, where adsorption to and penetration of susceptible cells occurs; (ii) from 6 through 30 h, virus replicates in the lungs; (iii) after 30 h, infectious virions are released from the lungs, establish a primary viremia, and invade the liver; (iv) between 48 and 54 h, the liver becomes the principal site of virus replication, with concomitant establishment of infection in other organs; and (v) after 54 h, massive viremia and concentrations of virus in all tissues develop and persist until death.

These investigations substantiated the highly infectious characteristics of RVFV in aerosols. The markedly elevated virus titers detected in the blood and tissues of infected animals provide an excellent source for airborne transmission, particularly for abattoir workers, veterinarians, and laboratory workers. Furthermore, infections induced in humans by aerosols may be complicated by the infectious processes originating within the pulmonary matrix, assuming that the pathogenesis of aerosol-induced RVF in humans is analogous to that observed in the mouse model.

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