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RESPIRATORY INFECTIVITY OF A RECENTLY ISOLATED EGYPTIAN STRAIN --ETC(U)
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Respiratory Infectivity of a Recently Isolated Egyptian Strain
of Rift Valley Fever Virus

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Running Head: RESPIRATORY INFECTIVITY OF RVF VIRUS

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense.

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ABSTRACT

The respiratory infectivity of a strain of Rift Valley fever virus isolated in Egypt (ZH-501) was compared to an isolate from Uganda (Entebbe strain) and two isolates from South Africa (SA-51 and SA-75 strains). 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} PFU, 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 were infected with 3.1 \log_{10} PFU 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 histopathologic 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 (16). 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 (4, 17). Secondly, infections in humans were widespread and severe (1, 17). 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 developed which resulted in numerous deaths. Although the virus isolated in Egypt serologically was indistinguishable from other RVF virus (RVFV) isolates, health authorities were concerned that a new strain may have emerged that was more virulent for man (13).

Epizootic RVF in Egypt and elsewhere is thought to be transmitted primarily by mosquitoes (6). Livestock herdsman, veterinarians and abattoir workers, however, have acquired infection 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 to define 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 (6).

The primary objective of this study was to evaluate the potential for airborne transmission of a RVFV isolate from Egypt and to compare its infective properties to other isolates posited to be less

pathogenic for humans. In addition, 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 twice 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 twice in FRhL cells, (iii) SA-75, isolated in South Africa in 1975 from a human with 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 twice in FRhL cells (17). A working stock of each strain was prepared by inoculation of Vero cell monolayers maintained in Eagle's minimal essential medium containing Earle's balanced salt solution (EMEM), supplemented with glutamine and nonessential amino acids, 10% heat-inactivated fetal calf serum (IFCS), 100 units penicillin G/ml and 100 µg streptomycin/ml. The monolayers were incubated at 37°C for 48 h, at which time the maintenance medium contained 7.6 to 3.0 log₁₀ 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% IFCS, 2.4 mg/ml HEPES buffer (CalBiochem-Behring Corp., La Jolla, Calif.) and antibiotics. Vero cell monolayers established in 6-well (well area = 9.6 cm²) plastic culture plates (Flow Laboratories, 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% CO₂. Monolayers were then overlaid

with 2 ml of Eagle's basal medium with Earle's salts containing 0.5% agarose (Marine Colloids, Rockland, Me.), 4% IFCS, HEPES buffer (4 mg/ml) and antibiotics. Culture plates were then incubated for 96 h at 37°C in a humidified atmosphere containing 5% CO₂. A second 2 ml of agar overlay was added which contained 0.1 mg/ml of neutral red (Grand Island Biological Co., Grand Island, N.Y.) and the plates were incubated for an additional 24 h. Plaques were counted and titers were calculated as log₁₀ PFU/ml.

Mice. Male, 6-7-week-old Swiss Webster mice [Wrm: (ICR)BR] that weighed approximately 30 g were supplied by the Walter Reed Army Institute of Research, Washington, D.C. During and following 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 tenfold increments from approximately 0.5 to 4.5 log₁₀ PFU. Aerosols were generated from stock virus suspensions using 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. Prior to conducting aerosol exposure trials the particle size distribution of aerosols was determined. Aerosols were generated from the virus suspension medium in a routine manner, except that sodium fluorescein 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 sodium fluorescein 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% IFCS 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 of $3.5 \log_{10}$ PFU was selected because it represented an aerosol dose that might be attained under natural conditions and a dose 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% IFCS and antibiotics. Blood and tissue 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 histologic changes.

RESULTS

Dose-response experiments. Evaluation of 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 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 (MTD) was reduced at the higher dose levels for all RVFV strains. Calculated by probit transformation, the median lethal respiratory dose (LD_{50}) for ZH-501, Entebbe, SA-51 and SA-75 was 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 ZH-501 group exposed to 0.6 \log_{10} PFU, where three of 40 exposed mice died. Since antibody titers were not determined, it is not known if a sublethal infection occurred in any of the surviving mice. Although the range among the four strains was only 0.7 \log_{10} PFU, statistical evaluation showed that the differences were significant ($P < 0.01$). Only the LD_{50} values of the Entebbe and SA-51 strains were not significantly different, and both of these were two- to fivefold more virulent for mice than the other two strains. The different slopes of the 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 following exposure at time 0, virus was demonstrated in the lungs of three mice and the nasopharynx of one. During the first 48 h virus was demonstrated most consistently in the lungs. By 30 h the virus in the lungs had attained a titer of $2.4 \log_{10}$ PFU/g, which was significantly higher than the amounts detected in other tissues and blood. At 48 h the lung virus concentration remained high, while 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 evidenced in blood and liver. Virus titers in the brain consistently were lower than those in other tissues.

Gross pathologic 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 appeared pale due to 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 tissue also were associated with RVFV infection. "Histopathologic 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 (5). 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 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 (6, 18), which was determined to be less than 1.0 mouse i.p. LD_{50} (MIPLD₅₀). The highest reported dose was for puppies (11), where approximately 25 mouse intracerebral LD_{50} 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 respiratory LD_{50} for hamsters to be 0.525 MIPLD₅₀. Unfortunately, the values obtained for MIPLD₅₀ are not comparable with PFU; therefore, an equivalent comparison of mouse and hamster susceptibility cannot be made. It is our opinion, however, that

the LD₅₀ values obtained for mice indicate that the mouse is more resistant than the hamster to lethal respiratory infection with RVFV.

Comparison of the Egyptian isolate of RVFV (ZH-501) with three isolates from sub-Saharan Africa, using LD₅₀ and MTD values, yielded an interesting variance among the strains. The differences observed among the LD₅₀ values of three of the four strains were mathematically significant. Examined in perspective, however, the difference is minimal and we consider it of no practical significance in infections in mice. Significant differences were not observed among the MTD values. 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 (6). 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 evidenced by other tissues examined. 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 following intranasal inoculation of RVFV (7). Histologic 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 histologic 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 appears 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. Further, infections in man induced by aerosols may be complicated by the infectious processes originating within the pulmonary matrix, assuming that the pathogenesis of aerosol-induced RVF in man is analogous to that observed in the mouse model.

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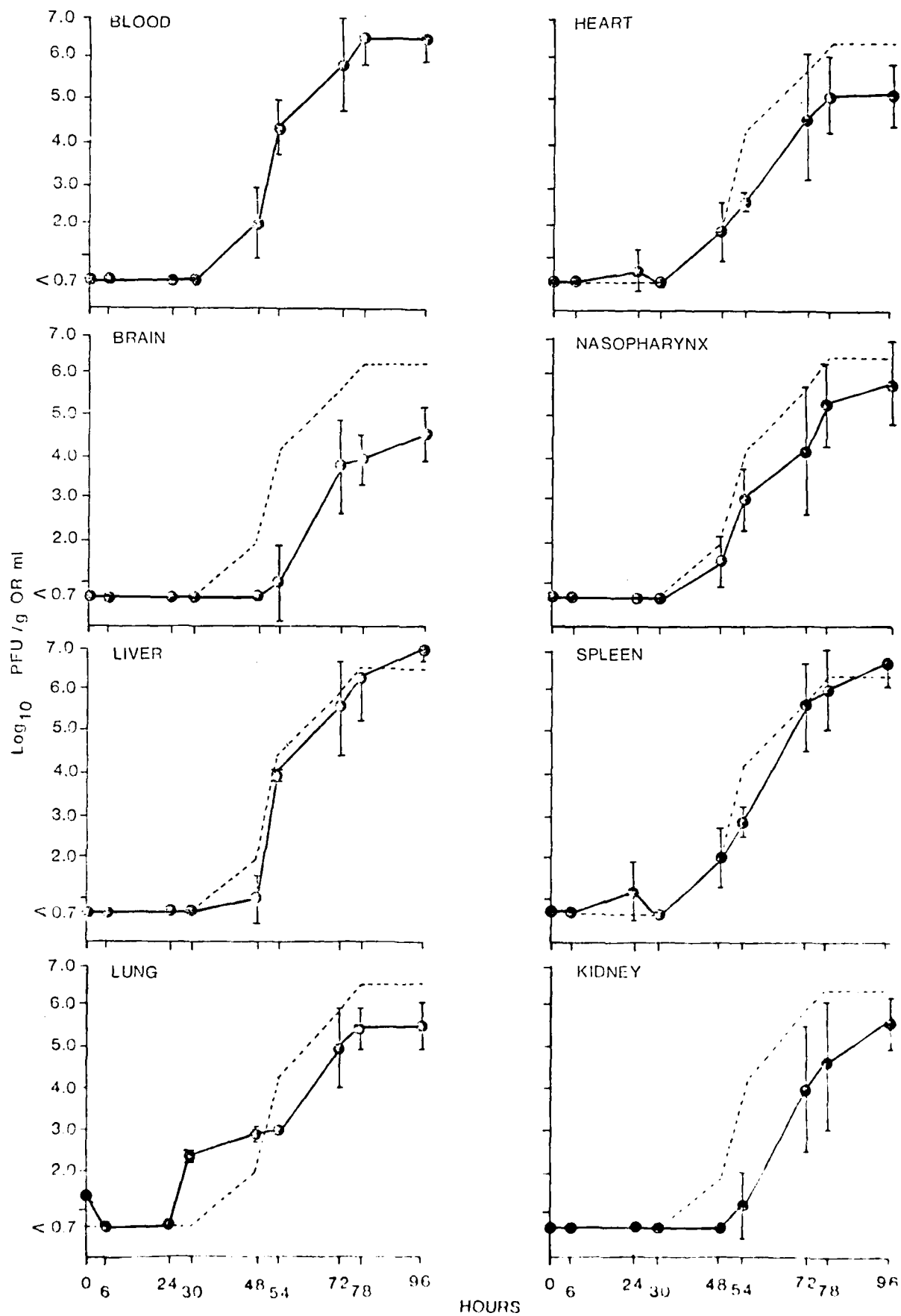
TABLE 1. Comparison of infectivity and LD₅₀ for four strains of RVFV

Dose (log ₁₀ PFU)	MTD ^b [days (range)]	Dead/Total	LD ₅₀	95% CI ^c	Slope
ZH-501					
0.6 ^d	4.0 (4-10)	3/40			
1.5 ^d	4.0 (3-8)	13/43	2.2	1.9-2.4	1.004
2.7 ^d	4.0 (3-9)	26/40			
3.8 ^d	4.0 (3-9)	39/40			
4.7 ^d	4.0 (3-6)	37/37			
LH-501a					
0.6	-	0/50			
1.6	3.5 (3-17)	12/50	1.9	1.6-2.1	1.959
2.9	3.0 (2-9)	19/20			
3.8	3.0 (2-3)	20/20			
4.8	3.0 (2-3)	20/20			
LH-501					
0.6	-	0/20			
1.6	4.0 (3-5)	1/20	1.9	1.3-2.8	1.030
2.9	3.0 (3-10)	10/20			
3.8	4.0 (3-10)	10/20			
4.8	4.0 (3-10)	20/20			
WA-501					
0.6	-	0/20			
1.6	4.0 (3-10)	0/20	1.6	1.2-2.1	1.007
2.9	4.0 (3-10)	16/20			
3.8	4.0 (3-10)	20/20			
4.8	4.0 (3-10)	20/20			

^aLog₁₀ titre at end of two readout days.^bMedian time to death.^cConfidence interval.

FIGURE LEGEND

FIG. 1. Virus concentration in mice (n = 4) following exposure to
3.1 log₁₀ PFU RVFV, ZH-501 strain, via the respiratory route. Tissue
from each mouse was assayed separately, in duplicate, and each point
represents the geometric mean + standard error of the mean. Viremia,
represented by broken lines, is superimposed on the plots for each
tissue.



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