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# Age-Dependent Resistance of Mice to Sindbis Virus Infection: Viral Replication as a Function of Host Age

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The development of resistance of outbred white mice to two plaque size variants of Sindbis virus was studied as a function of host age. A sudden drop in mortality occurred over a 3-day period. As little as 1 plaque-forming unit (PFU) per mouse was lethal in 1-day-old animals, whereas inocula of $10^9$ PFU per mouse failed to produce high mortality in weanlings. A comparison of viral growth kinetics shows that early replication was similar in newborns and weanlings but that after 6 to 8 hr the production rate and, therefore, the maximal viral titer were suppressed in weanlings. The data demonstrate a lethal viral threshold. Titers in excess of this level lead to mortality, whereas those below it allow survival.

During the short span of time in which a mouse develops from neonate to weanling, the physiological changes accompanying maturation decrease susceptibility to primary viral infections. Early studies reviewed by Sigel (16) have documented an age-associated increase in resistance with a number of viruses. More recently, the defenses of the immature host have been studied with Sendai virus (15), variola virus (10), coxsackie virus B1 (7), and poxvirus (17). The complexity of the mammalian host has led to many possible explanations of this phenomenon. Increased interferon content in the tissues of older mice has been implicated in resistance to some infections (7, 9). Other investigators either have found interferon content to be essentially the same in mice of different ages (17) or have reported lower interferon levels in the older host (18). Host resistance to viral infection has also been shown to depend on sensitivity to interferon, and genetic control of this factor has been established (6). In addition, different resistance patterns for a given virus have been thought variously to reflect the developing immune response (2, 13), the decreased availability of cellular receptor sites (B. Postic, Ph.D. Thesis, Univ. of Pittsburgh, 1965), an alteration in the capability of susceptible tissues to withstand viral invasion (17), or changes in efficiency of the reticuloendothelial system (12).

The objectives of this and subsequent studies were to describe the pattern of developing resistance of outbred mice to Sindbis virus and to evaluate the factors which influence the host-parasite balance.

## MATERIALS AND METHODS

**Viruses.** Sindbis virus (American Type Culture Collection strain A339) was cloned by plaque purification into large (LP) and small (SP) plaque-forming types. Virus stocks were prepared and titrated on monolayers of chick embryo fibroblasts and frozen at −70 C until used.

MM virus was obtained from David J. Giron (School of Aerospace Medicine, Brooks A.F.B., Tex.). A stock was grown and titrated on L-cell monolayers.

**Mice.** Outbred conventional white mice obtained from Euer's Farms (Austin, Tex.) and Manor North (Staatsburg, N.Y.) were used to establish a breeding colony which provided all of the animals for these studies. Pregnant females were caged individually, and the date of birth was recorded for each litter. Litter size was adjusted to 7 to 10 to assure proper nourishment for each newborn mouse.

Mice were inoculated intracerebrally (ic) without anaesthesia or intravenously (iv) into the basal tail vein by using 0.05 ml of the appropriate viral dilution in Hanks balanced salts (5) containing 1% newborn calf serum (HBSC). Animals were weighed at the start of each experiment and every week thereafter. Mortality was recorded daily.

**Virus titrations.** All visible brain tissue, with the exception of the olfactory tract and optic chiasma, was removed aseptically from decapitated mice. The tissue was homogenized with a mortar and pestle with the addition of fine glass beads. Blood samples were collected and refrigerated for 24 hr, at which time clots

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were removed by centrifugation and the serum samples were stored. With modified Eagle's medium (3) containing Hanks base (5), 500,000 units of penicillin per liter, and 0.25 g of streptomycin per liter, 10% (w/v) or 10% (v/v) suspensions of organs or serum were prepared, centrifuged at low speed to remove cellular debris, and frozen at −70 C until titration. Organ and serum suspensions were titrated on chick embryo fibroblast monolayers. The overlay medium employed was modified Eagle's containing Hanks base, supplemented with 3% newborn calf serum, and 1% Colab Ionagar. Plaques were counted by staining with 2% neutral red after 48 hr of incubation at 37 C in a 5% $CO_2$ atmosphere.

A study compared the values obtained with individually assayed animals with assays of pooled mouse brains after inoculation of equal amounts of virus. Twenty mice were inoculated ic with $10^4$ plaque-forming units (PFU) of LP, and each brain was harvested separately after 24 hr. Each brain was homogenized and the sample was divided; part of the sample was titrated for each individual mouse, and a part of the sample in proportion to the weight of the individual brain was added to a pool. Individual and pooled samples were titrated on the same day. The mean viral content of the individual mouse brains was $1.2 \times 10^8 \pm 7.2 \times 10^7$ PFU per g of brain, demonstrating that each animal was infected. The titer of the pooled sample was $1.3 \times 10^8$ PFU per g of brain. It was concluded that experimental groups of 20 mice were a valid sampling number.

**Interferon assay.** A 10% homogenate of brain tissue was acidified to *p*H 2 for 5 days, neutralized, and centrifuged at 15,000 × *g* for 30 min. The samples were diluted twofold in Eagle's medium containing twice the usual level of amino acids plus 5% fetal calf serum. A 2-ml amount of test sample was added to four 60-mm tissue culture plates (Falcon Plastic) containing L-cell monolayers. After 18 hr, the samples were aspirated and the plates were washed with HBSC. Each plate was challenged with 40 PFU of MM virus, adsorbed for 1 hr, and overlaid with Eagle's medium containing twice the usual level of amino acids, 10% fetal calf serum, 0.08% protamine sulfate, and 1% Colab Ionagar. After 24 hr, the plates were stained with 2% neutral red (4). One unit of interferon was defined as equal to the reciprocal of the test dilution which reduced the control plaque number 50%.

**HI tests.** Hemagglutination-inhibition (HI) tests were done following the procedure of Clarke and Casals (1). The HI titer was taken as the highest dilution of serum which caused complete HI by 4 to 8 units of antigen prepared from suckling mouse brain (1).

## RESULTS

**Determination of lethal levels for 1-day-old mice.** Groups of 1-day-old mice were inoculated ic with 10.0, 3.0, 1.0, 0.3, 0.03, and 0.01 PFU per mouse contained in 0.05 ml. Control mice were inoculated with the same volume of diluent. All animals were observed for 21 days. Inoculations of 10.0 or 3.0 PFU per mouse gave 100% mortality. Groups

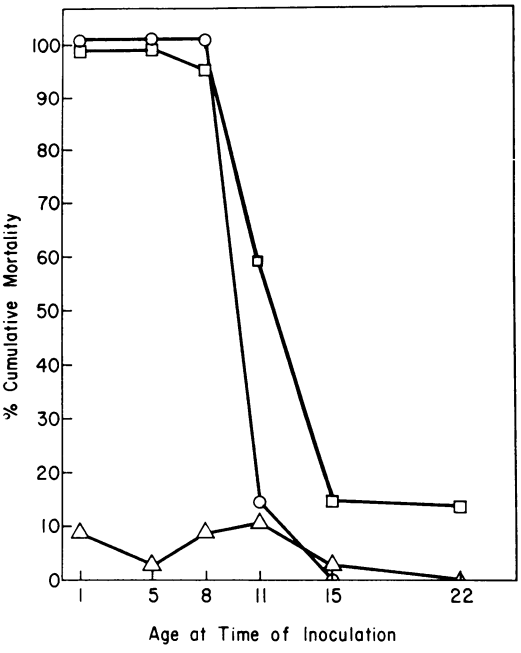


FIG. 1. *Total cumulative mortality of test animals inoculated at different ages with $10^4$ PFU of large plaque (□) or small plaque (○) or an equal volume of diluent only (△). The animals were observed for 21 days after infection.*

inoculated with 1.0 PFU per mouse showed 60 and 75% mortality with SP and LP, respectively. Doses of less than 1.0 PFU per mouse gave increasing numbers of survivors. After 6 weeks, survivors were bled and their sera were tested for HI antibodies, but none were found. Since survivors showed no evidence of infection, any mice infected failed to survive. It was concluded that infective level and lethal level were equivalent in 1-day-old mice for both LP and SP and approximated 1.0 PFU as measured in tissue culture.

**Lethality of LP and SP as a function of age.** Groups of 19 to 21 mice aged 1 to 22 days were inoculated ic with Sindbis LP or SP in HBSC. Cumulative mortalities over the next 21 days were determined. The results of experiments with inocula of $10^4$ PFU of either LP or SP types of Sindbis are summarized in Fig. 1. The development of resistance by age 8 to 11 days with SP and by age 11 to 15 days with LP was quite sudden and dramatic. Statistical analysis of the mean survival time obtained among mice 8 or 11 days of age at the time of inoculation showed that the difference in LP or SP inoculum was significant. The probable error (14) was calculated for each of these age groups after inoculation of $10^3$ or $10^4$ PFU of LP or SP. It was determined that the

TABLE 1. *Recovery of Sindbis large plaque and small plaque variants from brain tissue after 24 hr*

| Age at inoculation (days) | Large plaque | | Small plaque | |
|---|---|---|---|---|
| | PFU per g of brain | PFU per mouse brain | PFU per g of brain | PFU per mouse brain |
| 1 | $1.2 \times 10^9$ | $1.2 \times 10^8$ | $5.8 \times 10^9$ | $7.0 \times 10^8$ |
| 5 | $1.4 \times 10^8$ | $2.2 \times 10^7$ | $1.4 \times 10^9$ | $3.5 \times 10^8$ |
| 8 | $1.2 \times 10^8$ | $3.3 \times 10^7$ | $5.2 \times 10^8$ | $1.6 \times 10^8$ |
| 11 | $6.7 \times 10^7$ | $1.9 \times 10^7$ | $8.8 \times 10^7$ | $3.4 \times 10^7$ |
| 15 | $7.5 \times 10^7$ | $2.7 \times 10^7$ | $1.3 \times 10^8$ | $5.5 \times 10^7$ |
| 22 | $9.5 \times 10^6$ | $3.6 \times 10^6$ | $9.8 \times 10^7$ | $4.4 \times 10^7$ |

difference in the mean number of survival days with LP or SP was greater than three times the probable error. Since the duration of observation for mortalities was 21 days, the animals which survived this time period were included in the calculation as having survived 22 days.

The mean number of days postinoculation on which fatally infected animals died was noted. It was found that the decreased number of mortalities observed with increased host age was accompanied by longer survival times of those animals which eventually succumb. For example, animals which were 8 days old at the time of inoculation with LP and were infected fatally had lived an average of 7.0 days until death. Mice aged 11 and 15 days at inoculation and fatally infected with LP lived an average of 10.5 and 13.0 days, respectively. Animals aged 8, 11, and 15 days at the time of inoculation with the SP variant lived an average of 5.4, 10.3, and 13.0 days, respectively.

**Growth of LP or SP in brain tissue.** Groups of three litters of 7 to 10 mice each were inoculated ic with $10^4$ PFU of LP or SP. Brain tissue was recovered at 24 hr post inoculation (a time representating the peak viral titer after ic inoculation) and titrated. The results, summarized in Table 1, show that increased age and resistance of the host are accompanied by decreased viral titer in the brain. Johnson (8) demonstrated by immunofluorescence methods that the mouse brain is the site of maximal replication of arthropod-borne encephalitogenic viruses including Sindbis. Calculations of the number of PFU of LP or SP recovered from the whole mouse brain show that total viral yield per brain tends to decrease as a function of age.

**Kinetics of virus growth as a function of host age.** Groups of 1-day-old (susceptible) and 22-day-old (resistant) mice were inoculated ic with $10^4$ PFU of Sindbis LP to determine the time during infection at which weanling defense mechanisms altered the pattern of virus growth. Brain tissue was recovered from three litters of 7 to 10 animals at different times after infection. Viral multiplication began at the same time and followed the same pattern early in infection in both newborns and weanlings (Fig. 2). By 6 to 8 hr after infection, however, the viral growth rate was depressed and the peak titer was lower in weanlings as compared to newborns.

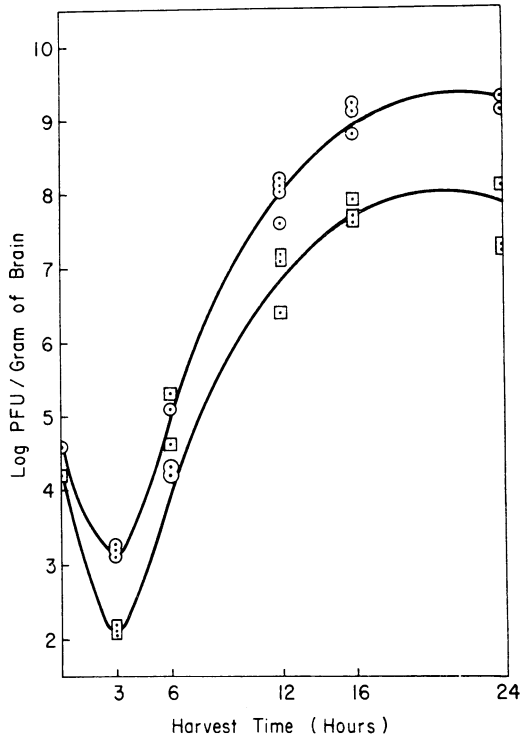


FIG. 2. *Viral growth kinetics of Sindbis large-plaque variant inoculated intracerebrally into 1-day-old (⊙) and 22-day-old (⊡) mice. Homogenates of mouse brains harvested at various times after infection were assayed for viral content.*

TABLE 2. *Resistance of weanling animals measured by survival after intracerebral inoculation of large doses of Sindbis large plaque*

| Inoculum per mouse (PFU) | No. of mice | Avg wt (g) | Cumulative mortality (%) |
|---|---|---|---|
| $10^4$ | 19 | 9.4 | 0 |
| $10^5$ | 18 | 9.4 | 22 |
| $10^6$ | 17 | 10.2 | 17 |
| $10^7$ | 18 | 8.4 | 39 |
| $10^8$ | 20 | 9.6 | 45 |
| $10^9$ | 20 | 10.9 | 35 |
| Diluent only | 20 | 9.4 | 0 |

**Resistance threshold of weanlings.** Having demonstrated that older animals show both a de-

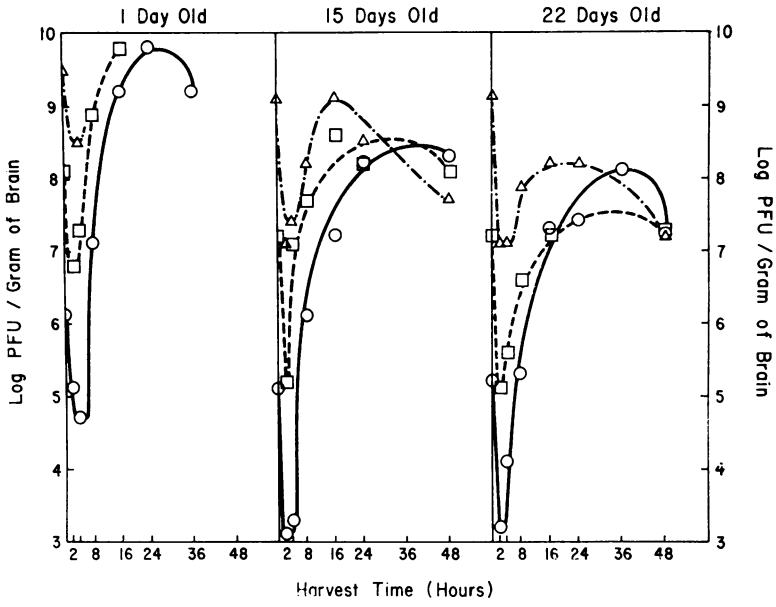


FIG. 3. *Mice aged 1, 15, and 22 days were inoculated intracerebrally with $10^5$ PFU (○), $10^7$ PFU (□), or $5 \times 10^8$ PFU (△). Viral content in the brain was measured in homogenates of tissue harvested at various times after infection. Measurement of viral content at later times was not possible in 1-day-old animals inoculated at the highest levels because of death of the test animals.*

creased rate of viral synthesis and a lower final titer, higher viral inoculum levels of LP were used to determine whether physiological resistance could be overcome. Groups of 17 to 20 mice were separated by sex, inoculated ic, and observed for mortalities over a period of 21 days. The results are summarized in Table 2 and indicate that the host defense mechanism(s) is operational and able to retard viral infection very soon after inoculation. This defense cannot be overcome totally simply by increasing the input multiplicity of virus particles per cell. No consistent difference in survival could be observed between mice of different sexes.

**Replication of LP as a function of age and inoculum.** To explore the apparent mortality threshold observed, replication of LP was measured in 1-, 15-, and 22-day-old mice as a function of inoculum level. Groups of 60 mice were inoculated ic with $10^5$, $10^7$, or $5 \times 10^8$ PFU of Sindbis LP, and the viral titer of brain tissue was measured at several times after infection. The data summarized in Fig. 3 show clearly that in weanling mice maximal titers of replicating virus never exceed $10^8$ to $3 \times 10^8$ PFU per g of brain even with an inoculum of $5 \times 10^8$ PFU per mouse (equivalent to $10^9$ PFU per g of brain). These findings contrast with those observed in newborn animals. Also shown in Fig. 3, 1-day-old animals inoculated with $10^5$ PFU per mouse ($10^6$ PFU per g of brain) showed a titer 50 times greater than 22-day-old animals inoculated with $5 \times 10^8$ PFU per mouse ($10^9$ PFU per g of brain), although the newborns were infected with a 1,000-fold lower level of virus.

Titers were also determined for 15-day-old animals, the age at which resistance develops to the LP variant. The values for all three levels of inoculum in 15-day-old mice were intermediate between newborn and weanling animals.

**Interferon production.** After ic inoculation of 1-, 15-, and 22-day-old animals with $10^5$, $10^7$, or $5 \times 10^8$ PFU of LP per mouse, brain tissue was recovered, and interferon content was measured by a plaque reduction assay. Weanling animals produced only low levels of interferon in brain tissue, whereas substantially higher levels were obtained from the brain tissue of newborns after inoculation of $10^5$ PFU per mouse (Fig. 4). Similar interferon titers were obtained from animals of the same ages inoculated with $10^7$ and $5 \times 10^8$ PFU per mouse.

**Production of antibody by weanlings.** Mice were inoculated iv or ic with $10^4$ PFU of LP or SP per mouse, and the sera were tested for HI antibody at various times after inoculation. The titers obtained are summarized in Table 3. These data show that weanlings inoculated ic with $10^4$ PFU

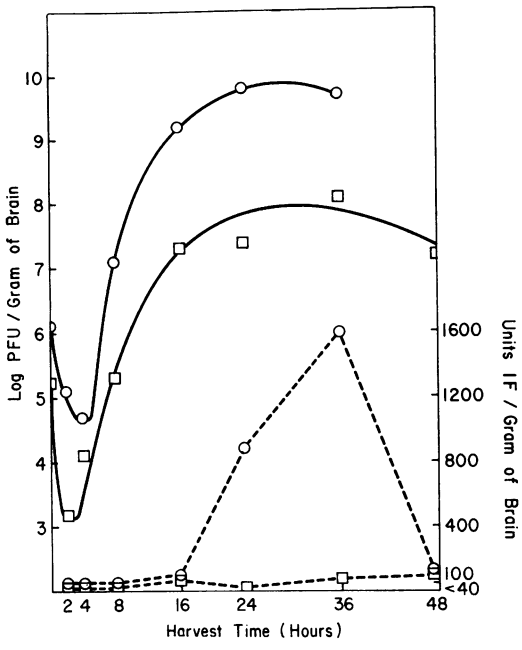


FIG. 4. *Viral replication (solid lines) and interferon production (dashed lines) measured in mouse brain homogenates at various times after intracerebral injection of $10^5$ PFU of large-plaque Sindbis into 1-day-old (○) and 22-day-old (□) animals.*

TABLE 3. *Hemagglutination inhibition titers of weanling mouse sera*

| Time after inoculation (days) | Intracerebral | | Intravenous | |
|---|---|---|---|---|
| | LP[a] | SP | LP | SP |
| 1 | <10 | <10 | <10 | <10 |
| 2 | <10 | <10 | <10 | <10 |
| 3 | 10 | 10 | <10 | 10 |
| 4 | 10 | 20 | 20 | 20 |
| 5 | 40 | 40 | 40 | 40 |
| 7 | 80 | 40 | 80 | 80 |
| 11 | 80 | 80 | 80 | 80 |

[a] LP, large plaque; SP, small plaque.

of LP produced antibody detectable in vitro after 3 days. Inoculation by the iv route with LP or SP also shows no measurable antibody earlier than 3 days after infection. These data may not, however, reflect virus-neutralizing capacity.

## DISCUSSION

Sindbis virus replication in the mouse brain will eventually lead to host death unless checked early. Newborn mice fail to halt viral replication after inoculation of as little as 1 PFU and die. Weanling mice, however, withstand infection with large inocula, in part because viral titers do not surpass a lethal threshold. Although the maximal viral titer per whole brain of fatally infected newborn animals is comparable to the larger amounts ($10^8$ and $10^9$) inoculated into each weanling, comparable mortalities do not result. It is probable that the cellular damage suffered through repeated cycles of replication after a smaller inoculum is much greater than that achieved simply by exposing weanlings to an equal amount of virus.

Calculations of PFU recovered per whole mouse brain (Table 1) generally demonstrate lower viral titers for each successive age group tested with both LP and SP. The change is not linear; it is rapid initially, reaching a plateau between 5 and 15 days of age, a period coincident with the change in survival patterns (Fig. 1). The calculations of PFU recovered per whole mouse brain demonstrate that the increasing size of the mouse with age does not account for the decreased final yield after inoculation of equal amounts of virus to all animals. It is possible that the viral inoculum may be more effectively cleared by the reticuloendothelial system in weanlings, since phagocytosis by polymorphonuclear leukocytes has been demonstrated to increase with increasing age of the host (11). By reducing the viral challenge to the brain in older animals, the reticuloendothelial system may effectively restrict the final titer. Although this phenomenon has not been quantitated, it is suggested by observations of early viral replication in the brain of newborns and weanlings (Fig. 3).

The studies which compare the rates of viral replication in the brains of newborns and weanlings (Fig. 2) show that after 3 hr postinfection increases in titer are parallel. After 8 hr, however, the rate slows in weanlings. These data suggest that the factor(s) limiting viral replication and influencing eventual survival is manifested very soon after infection.

Since the alteration of replication pattern is observed by 8 hr after infection, an early host defense mechanism such as interferon would be suggested as one responsible factor. However, measurement of interferon production both as a function of age and inoculum level does not support this suggestion. During the span of time after infection in which viral replication is significant, only minimal levels of interferon were detected in weanlings after ic inoculation of $10^5$, $10^7$, or $5 \times 10^8$ PFU. Conversely, the newborn animals which succumbed to infection produced high titers of interferon in the brain by 24 to 36 hr. Similar results have been reported by Vilček (18) and

suggest that interferon production is not responsible for diminished viral replication in weanlings but may be a host response to the higher viral titers in the newborn mouse brain. Further, Hanson (*personal communication*) has noted that these two plaque variants are equally sensitive to interferon and induce similar levels in mouse embryo fibroblast cultures. Differing interferon sensitivities cannot, therefore, be invoked to explain the different host ages at which resistance to these plaque variants develops.

Use of the standard in vitro HI tests to determine the presence of antibody to Sindbis virus shows that its appearance is too late to affect early viral replication. It is possible that an immune response of the host may suppress viral replication but simply may not be measurable by the HI method. Studies to be reported later use the ability to differentiate LP and SP variants in vivo as a device to measure early responses of neutralizing antibody based on the cross-neutralization observed between the variants.

Our data indicate that viral replication is diminished in resistant weanling animals very soon after infection and before a lethal threshold level is attained. Interferon production is much lower in weanlings than in newborn animals and probably is not available to depress viral synthesis in the brain. Antibody production is not measurable, at least by in vitro methods, at the significant times during the infection. It is possible, however, that an in vivo neutralization test may be a more sensitive measure of protection afforded by early antibody. In addition, viral clearance rates must be determined for susceptible and resistant hosts as a function of age.

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