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Macrophages and Age-Dependent Resistance to Hepatitis Induced by Herpes Simplex Virus Type 2 in Mice

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An age-dependent increase in the resistance of BALB/c mice to induction of focal necrotic hepatitis by herpes simplex virus type 2 was demonstrated. In 3-week-old mice inoculated intraperitoneally with virus, numerous necrotic foci developed in the liver. As the mice matured, the number of lesions declined until the age of 8 weeks, when no further increase in resistance appeared. Corresponding to this, the virus titers of livers and spleens of 3-week-old mice were higher than in 8-week-old animals throughout the infection, and the infection was apparently terminated in these organs of the adult mice by day 5. In vitro infection of peritoneal macrophages from 3-week-old and 8-week-old mice showed that this age-related resistance was concomitant with an increased restriction of virus replication in peritoneal macrophages from adult mice. Since, furthermore, the resistance of adult mice could be abolished by intravenous inoculation of the macrophage-toxic agent silica before infection, and since adoptive transfer of 2×10^6 syngeneic macrophages from adult mice to young ones conferred to the latter a resistance comparable to that of the adult mice, it is concluded that macrophage maturation is responsible for the age-dependent resistance seen in this infection.

Macrophages are generally accepted as being of key importance in determining the susceptibility of animals to infection (9). The age-dependent increase in resistance to infection seen in many virus-host systems has thus been attributed to a "maturation" of this particular cell population as the animals reach adult age (4, 5, 17, 19, 20).

In previous studies (10-14), a murine model of the focal necrotic hepatitis seen in disseminated cases of herpes simplex virus type 2 (HSV-2) infections has been established. Young mice inoculated intraperitoneally (i.p.) with this virus regularly develop macroscopic foci of hepatic necrosis, which become visible after 2 or 3 days of infection and reach a size of about 1 mm in diameter on day 4 or 5 (14). However, the age of the host was found to be important to the outcome of the infection. When older mice were infected, fewer animals showed macroscopic liver lesions, and the number and size of individual foci diminished (11).

This study was undertaken to provide a more thorough examination of the age-dependent resistance to this infection and to evaluate the role of macrophages and macrophage maturation in limiting infection in adult mice.

MATERIALS AND METHODS

Mice. Inbred specific-pathogen-free mice of the BALB/c/A/BOM strain were originally obtained from the Gl. Bomholtgaard Laboratory Animal Breeding and Research Center, Ry, Denmark. Female mice for experimentation were locally bred behind specific-pathogen-free barriers. Mice aged less than 4 weeks were nursed by the mother during the experiment.

Virus. HSV-2 strain MS used in this study was described previously (14).

In vivo experiments. Groups of BALB/c mice aged 3, 4, 5, 6, 7, and 8 weeks and 6 months were inoculated i.p. with 5×10^4 plaque-forming units (PFU) of HSV-2 in 0.1 ml of diluent. After 4 days, the mice were killed by exsanguination in ether anesthesia, and their livers were examined for macroscopic lesions. The course of infection in young and adult mice was examined by inoculating groups of 3-week-old (young) and 8-week-old (adult) mice with 5×10^4 PFU of HSV-2. After infection, five mice in each group were killed each day, and the number of liver lesions was recorded. In both experiments, the liver and spleen (and, in the latter experiment, the brain) were removed aseptically and frozen at −70°C for subsequent virus assay. In this experiment, groups of 29 young and 20 adult mice were left for 3 weeks for observation of morbidity and mortality.

Silica dust (Dörentrup Quartz no. 12, <5 μm) originated from A. C. Allison, Clinical Research Center, Harrow, England. It was autoclaved, suspended in phosphate-buffered saline at a concentration of 15 mg/ml, and dispersed by brief exposure to ultrasonic vibration immediately before use. It was injected slowly in the tail vein of 8-week-old mice (3 mg in 0.2 ml). After 2 h, the mice were inoculated i.p. with 5×10^4 PFU of HSV-2 in 0.1 ml of diluent. On day 4 after the infection, the mice were killed and examined for

hepatic necrosis, and the livers were removed for virus titration.

For macrophage transfer studies, peritoneal resident cells from 8-week-old mice were harvested as described for the infectious center assay and grown in 1-liter Roux bottles with 100 ml of RPMI 1640 medium containing 20% fetal calf serum. After 24 h in culture, the flasks were washed three times with Eagle minimum essential medium supplemented with 5% fetal calf serum to remove nonadherent cells. About 99% of the remaining cells on the bottle were adherent, spindle-shaped, phase-dark macrophages as judged by phase-contrast microscopy. These cells were removed mechanically with a rubber policeman and suspended in Eagle minimum essential medium with 5% fetal calf serum at a concentration of 6×10^6 macrophages per ml. Three-week-old mice were inoculated in the tail vein with 0.3 ml of this suspension, and after 2 h they received 5×10^4 PFU of HSV-2 i.p. After 4 days, the mice were killed, and the livers were examined for lesions.

Scoring of liver lesions. The number of liver lesions was scored semiquantitatively from 0 to 4: 0, no lesions; 1, less than 5 lesions; 2, 5 to less than 20 lesions; 3, 20 to less than 100 lesions; 4, 100 or more lesions.

Assay of organs for virus. Livers, spleens, and brains were homogenized to a 10% suspension in Eagle minimum essential medium supplemented with 5% fetal calf serum and antibiotics. The suspensions were clarified by centrifugation at $4,000 \times g$ for 30 min at 4°C and were tested for virus in human embryonic lung cell cultures by a plaque method previously described (11).

Infectious center assay. Replication of HSV-2 in peritoneal macrophages from young and adult BALB/c mice was measured by an infectious center assay previously described (10). Briefly, unstimulated peritoneal resident cells were obtained by peritoneal lavage and differentially counted in a fluorescent microscope after cytoplasmic staining with acridine orange as the macrophage marker. The cells were plated on plastic petri dishes (35 mm; Falcon Plastics, Oxnard, Calif.) at a concentration of 5×10^5 macrophages in 2 ml of RPMI 1640 medium supplemented with 20% fetal calf serum and antibiotics. The next day, nonadherent cells were removed by washing, and the cultures were infected with 5×10^5 PFU of HSV-2 in 0.2 ml of diluent. After adsorption for 1 h at 37°C, nonadsorbed virus was removed by washing and HSV hyperimmune serum treatment, and finally the macrophage cultures were overlaid with mouse embryonic cells in methyl cellulose medium. The plaques appearing in this target cell monolayer were counted after 2 days of incubation.

Adsorption experiments. Adsorption of HSV-2 in macrophage cultures was assessed as previously described (10). Macrophage cultures prepared from BALB/c mice aged 3 and 8 weeks were prepared as described for the infectious center assay. The cultures were inoculated with 2×10^4 PFU of HSV-2, and, after 60 min of adsorption at 37°C, the inoculum was diluted in 20 ml of cold medium, which was then assayed for nonadsorbed virus. Adsorption was calculated as the percentage of virus lost from the inoculum in macrophage cultures compared with the amount lost from control petri dishes without macrophages.

RESULTS

Age-dependent resistance to HSV-2 hepatitis. Mice at various ages were infected by the i.p. route with 5×10^4 PFU of HSV-2. At 4 days after infection, an age-related decline in the number of necrotic foci in the liver was seen (Fig. 1). Furthermore, the size of individual lesions diminished as the mice grew older, and the few lesions seen in mice beyond the age of 5 weeks were mainly located on the margins of the liver, whereas lesions in younger mice were scattered all over the organ. This age-related resistance pattern was reflected in the amount of virus isolated from livers (Fig. 1) and from spleens (data not shown) of infected mice, which also showed a gradual decrease with increasing maturity.

Course of infection in young and adult mice. To further analyze the patterns of the age-dependent resistance to HSV-2 hepatitis, groups of 3- and 8-week-old mice were inoculated i.p. with 5×10^4 PFU of HSV-2, and the course of infection was followed for the next few days. In the young mice, many lesions were detectable in the liver from day 2 onward, increasing in size until the animals died. In the adult mice, on the other hand, only a few smaller lesions became apparent on the liver margins in about half of the mice on days 4 to 6 (Fig. 2). Corresponding to this, the liver and spleen virus titers of the young mice were higher than those of the adult mice throughout the infection (Fig. 3). Furthermore, none of the adult mice showed

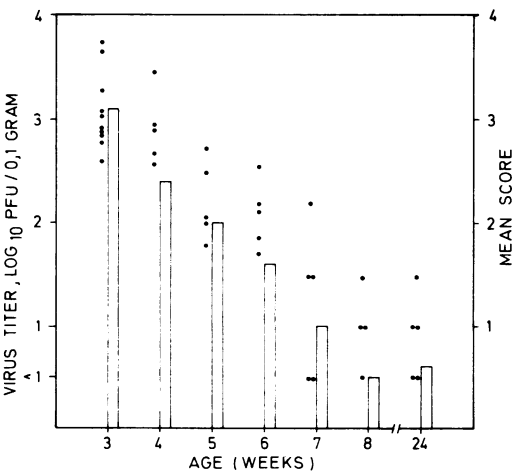

FIG. 1. *Virus titers (●) and mean score of liver lesions (□) in BALB/c mice of varying ages 4 days after i.p. inoculation of 5×10^4 PFU of HSV-2. The lesions were graded semiquantitatively from 0 to 4.*

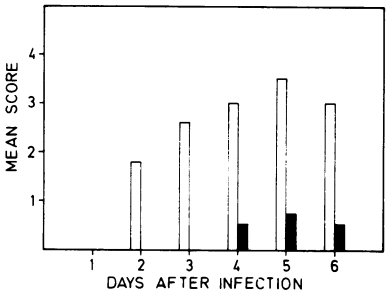

FIG. 2. *Mean score of liver lesions during infection in 3-week-old (□) and 8-week-old (■) BALB/c mice after i.p. inoculation of 5 × 10⁴ PFU of HSV-2.*

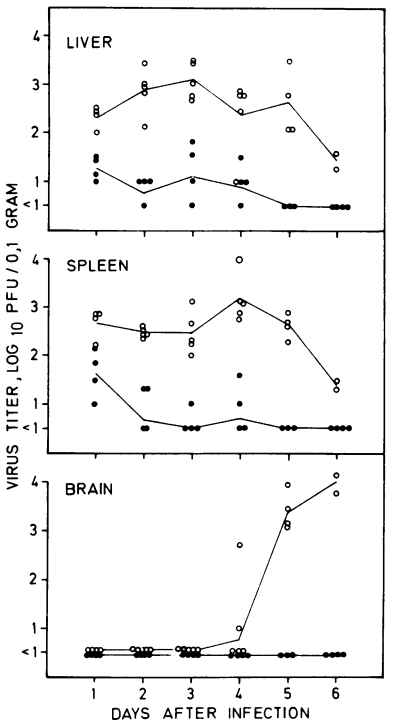

FIG. 3. *Titers of virus in organs from 3-week-old (○) and 8-week-old (●) BALB/c mice inoculated i.p. with 5 × 10⁴ PFU of HSV-2. Each point represents one mouse. Lines are drawn between the means of each group.*

any virus in the two organs on days 5 and 6, indicating that the infection had been terminated as far as these organs were concerned. In the brain, increasing amounts of virus were seen from day 4 onward in the young mice, whereas the old mice showed no virus in this final target organ for at least the first 6 days of infection. The comparison between young and adult mice of liver lesions and virus titers was not conducted beyond day 6 because of the high mortality in the young group. Adult mice examined later on showed no progression or delayed onset of liver infection, although virus was sometimes isolated from the brains of these mice.

In morbidity and mortality control groups, posterior paralysis and, later on, signs of encephalitis started on day 4 in the young mice, whereas symptoms in the adult mice were not detectable until day 6. Corresponding to this, the overall mortality was 100% in the young group and 60% in the adult group, which is highly statistically significant ($2P = 0.0006$ by Fisher's exact test). The mean survival time of the young mice was 4.9 days, whereas the mean survival time among the adult mice that died was 9.0 days. The variances of the survival times of the two groups are statistically significant ($F = 19.2$, $P < 0.01$).

Replication of HSV-2 in macrophages from young and adult mice. Since previous studies have indicated that restriction of virus growth in macrophages is responsible for the relative inability of HSV-1 to induce focal necrotic hepatitis in mice (10) and, furthermore, is the cellular basis of the difference in resistance of inbred mouse strains to HSV-2 hepatitis (12), it is likely that the age-dependent resistance described above was mediated by an augmented restriction of HSV-2 replication in macrophages from mature mice. Therefore, virus replication in peritoneal macrophages from young and adult mice was investigated in vitro. As seen from Fig. 4, showing two macrophage cultures infected with HSV-2 and overlaid with mouse embryonic cells, macrophages from young mice permitted a much more unrestricted replication of HSV-2

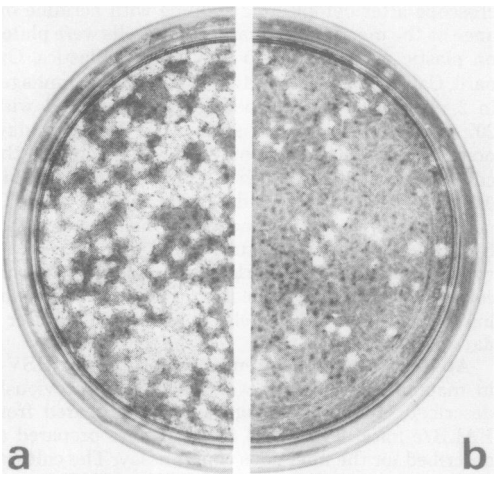

FIG. 4. *Infectious centers in macrophage cultures from (a) 3-week-old and (b) 8-week-old BALB/c mice. The cultures were infected with 5 × 10⁵ PFU of HSV-2 and stained after 2 days of incubation.*

than did macrophages from adult mice, as judged by the number of plaques appearing in the cell overlay. Adsorption experiments showed that this difference in replication was not due to differences in adsorption of HSV-2 to macrophages from young and adult mice, since the adsorption percentages were between 30 and 35%, irrespective of the age of the macrophage donors.

Abolition by silica of the age-dependent resistance to HSV-2 hepatitis. Since silica has previously been found to be effective in blocking the macrophage restriction of HSV infections in mice (13), the ability of this agent to break down the age-dependent resistance to HSV-2 hepatitis was investigated. Intravenous inoculation of 3 mg of silica 2 h before HSV-2 infection rendered 8-week-old mice just as susceptible to the induction of hepatitis by the virus as 3-week-old mice (Fig. 5).

Effect of macrophage transfer from adult to young mice. As seen from Fig. 5, intravenous transfer of 2×10^6 syngeneic macrophages obtained from the peritoneal cavity of adult donors to 3-week-old mice resulted in a decrease in the severity of liver involvement in the young mice that was comparable to the age-dependent natural decrease seen in 8-week-old mice.

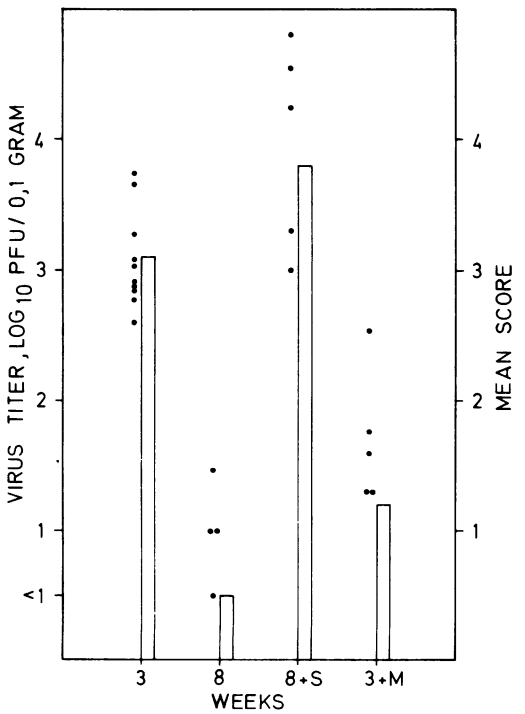

FIG. 5. *Virus titers (●) and mean score of liver lesions (□) in 8-week-old and 3-week-old BALB/c mice 4 days after receiving intravenous injections of 3 mg of silica (S) or 2×10^6 syngeneic adult macrophages (M), respectively, 2 h before 5×10^4 PFU of HSV-2.*

DISCUSSION

Age-dependent resistance to infection has long been recognized in many viral diseases (18). In HSV infections of mice, the development of resistance to herpetic encephalitis after peripheral inoculation of the virus was first demonstrated by Andervont in 1929 (3) and later confirmed by others (7, 8, 16). In 1964, Johnson's extensive immunofluorescence study of the problem provided evidence that the difference in susceptibility of different age groups to extracerebral inoculation of HSV was associated with a maturation of macrophages which rendered these cells more restrictive in the replication and dissemination of the virus (5). The importance of macrophage maturation in age-related resistance to HSV encephalitis was later confirmed by Allison's group employing an infectious center assay in vitro (4) and selective blockade of macrophage function in vivo by silica and antimacrophage serum (20). A thorough investigation of the nature of the macrophage restriction was provided in 1971 by Stevens and Cook (19), who showed that HSV undergoes an abortive infection in mature macrophages from adult mice.

The above-mentioned studies all dealt with experimental infections with HSV-1 and were almost exclusively concerned with the age-dependent barrier toward the final outcome of the infection in young mice, namely, death from encephalitis. In previous studies from this laboratory (10–14), an experimental model in mice of the extracerebral manifestations of disseminated HSV infections has been established, focusing on the focal necrotic hepatitis seen in most cases of fatal neonatal HSV-2 infections (15). In this model, an age-dependent resistance to liver infection was also noticed (11). Since macrophage restriction of virus growth was found to correlate with the difference in severity of HSV-1- and HSV-2-induced hepatitis (10) and with the genetically determined difference in resistance to HSV-2 hepatitis among inbred mouse strains (12), it seemed reasonable to test the possibility that macrophage maturation is also an important factor in age-dependent resistance to HSV-2 hepatitis, as it is to HSV-1 encephalitis.

The development of a gradual increase in the resistance of mice to induction of hepatitis by HSV-2 as the animals matured was found. In the studies of age-related resistance to HSV-1

encephalitis (4, 5), the attention was focused on the increase in resistance from the neonatal period to the age of 3 to 4 weeks, when the mice are weaned. The present study reveals that mice do not reach final maturity as regards resistance to HSV-2 hepatitis until they reach the fertile age, i.e., about 8 weeks of age. Even at this age, some of the animals showed a few necrotic lesions in the liver, but the mice seemed able to control and terminate the infection in visceral organs, since no virus was found on days 5 and 6 in the liver and spleen. However, 60% of the adult mice died from encephalitis, even though at a later time than the young ones. This shows that the barrier to virus penetration into the central nervous system can be overcome even in adult mice, provided the virus dose is high enough. When the virus enters this organ, it seems to be beyond the reach of the host defense mechanism terminating the infection in peripheral sites.

The nature of this age-dependent primary host defense mechanism in the liver seems to be macrophage maturation. Macrophages from adult mice were shown by the infectious center assay to restrict HSV-2 replication to a much higher extent than macrophages from young mice, since fewer macrophages from the adult mice supported virus replication satisfactorily to yield a plaque in the mouse embryonic cell overlay. Furthermore, the age-dependent resistance to HSV-2 hepatitis could be overcome by pretreatment with silica, an agent reported to be selectively toxic to macrophages (2, 6). The silica used in these experiments has been found active for macrophage blockade by A. C. Allison (20), from whom it originates, and it has been found to be able to abolish the macrophage-dependent difference in liver pathogenicity between HSV-1 and HSV-2 (13). Finally, the intravenous transfer of 2×10^6 syngeneic adult macrophages from adult to young mice 2 h before infection conferred to the latter a primary defense capacity against HSV-2 hepatitis almost as effective as seen in adult mice, as judged by the number of liver lesions and virus titers 4 days after the infection. Johnson (5) was unable to transfer resistance against HSV-1 encephalitis from 4-week-old mice to newborn mice with peritoneal macrophages, whereas Hirsch (4) found a significant protection after transfer, but only when more than 6×10^6 stimulated macrophages were inoculated. It thus seems that death from encephalitis is less sensitive than the degree of hepatitis as a parameter for macrophage protection, probably because macrophages are less important in the central nervous system than in the organs of the reticulo-endothelial system (1).

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