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Role of Macrophages in Hepatitis Induced by Herpes Simplex Virus Types 1 and 2 in Mice

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A marked difference was found between herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) in the induction of hepatic necrotic lesions in mice inoculated intraperitoneally. Although HSV-2 produced many large, progressive liver lesions in 4-week-old BALB/c mice, HSV-1 only occasionally induced a few, self-limiting foci, which eventually healed. This was reflected in the isolation of HSV from the liver and spleen, two organs that are rich in macrophages. Although HSV-1 could be only temporarily isolated, HSV-2 was found in the two organs until the mice died. On the other hand, no such difference was found in the isolation of virus from the brain, which contains no macrophages, and the mice eventually died from encephalitis. This difference in hepatic involvement caused by the two virus types was found to parallel a marked difference in the restriction of HSV-1 and HSV-2 replication by macrophages as measured by an infectious center assay in vitro. HSV-2 produced 17 times as many infectious centers in infected peritoneal macrophage cultures as did HSV-1. Furthermore, the HSV-2 plaques in the cell overlay were large and increasing in size, whereas the HSV-1 plaques were small and showed regression on prolonged incubation. It was shown that this diversity was unique to the macrophage population and not caused by differences in the uptake of virus by macrophages. This model involving two closely related virus types shows the importance of tissue macrophages in the primary host defense against virus infections.

Herpes simplex virus (HSV) exists in two closely related antigenic types, 1 (HSV-1) and 2 (HSV-2). In spite of their close relationship, a number of differences in clinical and epidemiological as well as biochemical and biological characteristics have been disclosed (14).

In previous studies (10, 11), a striking difference in liver involvement in intraperitoneally (i.p.) infected mice was observed. On i.p. inoculation, HSV-2 strains induce macroscopic focal necrotic lesions in the liver, whereas HSV-1 strains only occasionally produce a few tiny lesions that are hardly visible to the naked eye.

This difference in pathogenicity between two closely related virus types seems to offer a convenient model for evaluating host defense mechanisms that are decisive for the outcome of an infection. Therefore, a closer examination of the course of infection caused by the two virus types was desired.

Since numerous studies have demonstrated the importance of macrophages in the primary defense against virus infections (2, 5-7, 9, 15, 16, 19-21), the question of whether differences existed in the relationship of this particular cell population of the two types of herpesvirus was investigated.

MATERIALS AND METHODS

Mice. Inbred, specific-pathogen-free mice of the BALB/c/A/BOM strain were obtained from the Gl. Bomholtgaard Laboratory Animal Breeding and Research Center, 8680 Ry, Denmark, and used at an age of 4 weeks.

Viruses. The MacIntyre (HSV-1) and MS (HSV-2) strains were used throughout this study. Their origin, site of isolation, and serological typing as well as their production and titration in human embryonic lung cell cultures were previously described (10, 11). The viruses were plaque purified before production of the virus pools used in these studies.

Media. Human embryonic lung (HEL) cells and primary mouse embryonic cells (MEC) were grown in Eagle minimal essential medium (MEM) containing 10% calf serum and 0.08% sodium bicarbonate.

Peritoneal exudate (PE) cells were harvested in RPMI 1640 medium containing 20% fetal calf serum (FCS), 2% HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, and 10 IU of heparin per ml. The cells were grown in the same medium without heparin. MEM containing 5% FCS was used in the washing procedures.

The semisolid overlay media were MEM containing 5% calf serum for virus titration and RPMI 1640 medium containing 20% FCS for the infectious center assay. Both media contained 1% methyl cellulose.

All virus dilutions for animal inoculation and various assays were done in MEM containing 5% calf serum. To all media were also added 200 IU of penicillin per ml, 200 μg of streptomycin per ml, and 2.5 μg of mycostatin per ml.

In vivo experiments. Four-week-old BALB/c mice were inoculated i.p. with 10^5 plaque-forming units (PFU) of virus in 0.1 ml of diluent. The mice were observed for morbidity and mortality. After infection, five mice were killed each day, and their livers were carefully examined for macroscopic lesions. The liver, spleen, and brain were removed aseptically from each mouse and kept at −70°C until assayed for virus.

Assay of organs for virus. Livers, spleens, and brains pooled from five exsanguinated mice were homogenized to a 10% suspension in a Hannover homogenizer (Ernst Schutt Jr.). After centrifugation at 4,000 × g for 30 min at 4°C, the supernatant was tested for virus. The virus titrations were performed in duplicate HEL cell cultures by a plaque method previously described (10).

PE cells. Four-week-old BALB/c mice were killed by cervical dislocation. The skin over the abdomen was removed, and 3 ml of medium was injected i.p. After gentle massage of the abdomen, the abdominal fluid was harvested into a 10-ml syringe with an 18-gauge needle. The number of macrophages and lymphocytes in the suspension was determined in a fluorescent microscope after cytoplasmic staining with acridine orange as the macrophage marker.

Infectious center assay. PE cells from 4-week-old BALB/c mice were plated on plastic petri dishes (35 mm, Falcon) in a concentration of 5 × 10^5 macrophages in 2 ml of RPMI medium containing 20% FCS. The next day, the petri dishes were vigorously washed three times with MEM containing 5% FCS, and the wash medium was collected for preparation of lymphocyte suspensions. After three washes, more than 95% of the cells on the petri dishes were adherent, spindle-shaped, phase-dark macrophages as judged by phase-contrast microscopy. The cultures were infected with either MacIntyre or MS strains that contained 5 × 10^5 PFU in 0.2 ml of diluent. After 1 h of adsorption at 37°C, the dishes were washed three times with 3 ml of MEM containing 5% FCS. This was followed by incubation for 1 h with 1 ml of MEM containing 10% heat-inactivated rabbit HSV-1 hyperimmune serum with 50% neutralizing titers of 256 and 128 against HSV-1 and HSV-2, respectively. The macrophages were then overlaid with 3 ml of an RPMI-methyl cellulose medium containing 3 × 10^5 MEC/ml, and after 2 days the plaques were counted after staining with methylene blue.

Nonadherent PE cells from the macrophage cultures were collected in plastic culture flasks (25 cm^2, Falcon) and treated with 100 mg of carbonyl iron per 10 ml of medium for 45 min at 37°C. The cell suspension consisted entirely of lymphocytes after two successive treatments with a magnet. A 10^6 concentration of peritoneal lymphocytes was infected with 10^6 PFU of HSV-1 or HSV-2. After 1 h of adsorption at 37°C, the lymphocyte suspensions were washed three times with 15 ml of MEM containing 5% FCS; the last time, they were washed with 10% HSV-1 hyperimmune serum. The cells were distributed on preformed MEC monolayers (10^5 per monolayer) and overlaid with 3 ml of RPMI-methyl cellulose medium. After 3 days, the dishes were stained with methylene blue for plaque visualization.

Virus growth in tissue culture. The plaque assays were performed as previously described (10). The plaques were counted in an enlarger set at a ×9 magnification. For an assessment of the areas after 2 days of incubation, all of the plaques except marginal and confluent plaques in four dishes were traced on paper at a magnification of ×17.5. The areas were calculated using a Hewlett-Packard calculator digitizer (model 9107A) and expressed in square millimeters. The virus growth in BALB/c embryonic fibroblast cultures was measured by harvesting the supernatant from two dishes of fibroblasts infected with 10^3 PFU of HSV-1 or HSV-2. The supernatants were stored at −70°C until titration in duplicate HEL cell cultures.

Virus adsorption in macrophage cultures. Macrophage cultures containing 5 × 10^5 macrophages were prepared as described for the infectious center assay. Duplicate cultures were infected with 2 × 10^4 PFU of HSV-1 or HSV-2 in 0.2 ml of diluent. After 60 min of adsorption at 37°C, the cultures were removed from the incubator and the adsorption was stopped by dilution of the inoculum in 20 ml of cold medium. Samples from two cultures were pooled and assayed for nonadsorbed virus by inoculating 0.1 ml of the sample in four HEL cell monolayers. The percentage of adsorption was calculated as the percentage of virus lost from the inoculum compared to the amount lost from control petri dishes that lacked macrophages.

RESULTS

Course of infection. To compare the course of infections with HSV-1 and HSV-2, groups of 4-week-old BALB/c mice were inoculated i.p. with 10^5 PFU of the two virus types. Signs of illness started on day 6 in both groups, with the symptoms being of central nervous system origin (posterior paralysis, encephalitis). The only specific symptom was severe gastric retention, which was seen in about 20% of the HSV-2-infected mice. In mortality control groups of 10 mice, the overall mortality was equal in the two groups (80%). The mean survival time was, however, shorter in the HSV-2-infected group (7.6 versus 9.0 days), but this was not statistically significant because of a great variation between survival times of individual mice.

As previously described (10, 11), the macroscopic examination of the livers of mice killed at daily intervals revealed lesions in most animals that were inoculated with HSV-2 from day 3 to day 7. On the other hand, the HSV-1-infected mice showed only a few tiny lesions in a few cases on days 3 and 4, whereas no lesions

were seen on days 5 to 7. No other visceral organs showed any lesions.

On testing the liver and spleen for herpesvirus, an obvious difference between animals infected with HSV-1 and HSV-2 appeared (Fig. 1). The initial steps in the virus growth curves were almost identical, and although no virus was grown from the liver and spleen of HSV-1-infected mice on days 5 to 7, the two organs from HSV-2-infected animals contained virus until their deaths. The two groups of mice showed virus in the brain from days 4 and 5 onward, respectively.

Replication of HSV-1 and HSV-2 in mouse peritoneal macrophages. As the previous experiment had shown a marked difference in the restriction of HSV-1 and HSV-2 infection in the liver and spleen, two organs "well armed" with cells of the reticuloendothelial system, the question of whether this diversity could be caused by differences in the ability of mouse macrophages to allow replication of the two virus types was investigated.

Macrophages from 4-week-old BALB/c mice were grown in petri dishes, infected with HSV-1 or HSV-2, and overlaid with MEC. After 2 days of incubation, a marked difference between HSV-1 and HSV-2, in terms of the number and size of the plaques appearing, was observed. The HSV-1-infected macrophage cultures showed only a few small plaques, which on prolonged incubation regressed further (Table 1). On the other hand, the HSV-2-infected cultures showed a large number of plaques, which after 2 days of incubation were larger than the HSV-1 plaques and continued to grow on further incubation (Fig. 2).

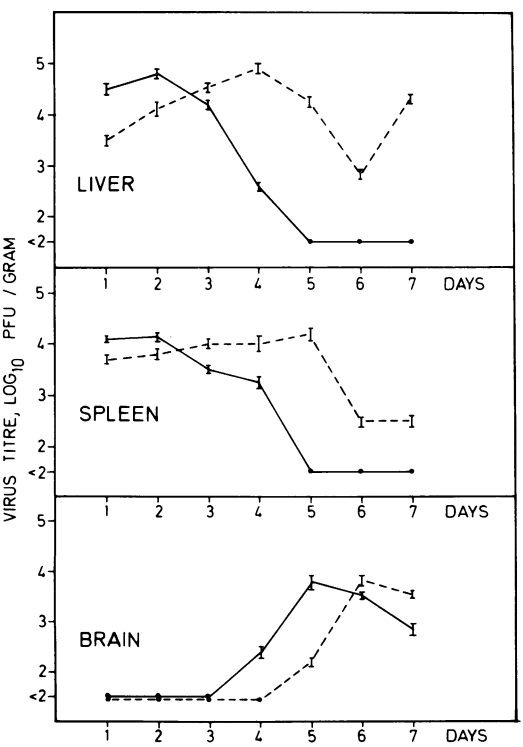

FIG. 1. *Titers of virus in pooled organs from five 4-week-old BALB/c mice inoculated i.p. with 10^5 PFU of HSV-1 (——) or HSV-2 (- - - - -). Bars represent two standard deviations.*

Growth of HSV-1 and HSV-2 in other mouse cells. To rule out the possibility that the difference observed in the replication of HSV-1 and HSV-2 in mouse peritoneal macrophages should be a more general feature of mouse cells, the replication of the two virus types in peritoneal lymphocytes and in BALB/c mouse embryonic fibroblasts was investigated.

The infectious center assay with peritoneal lymphocytes infected with HSV-1 or HSV-2 at a multiplicity of infection of 1 showed no replication of any of the virus types in these cells.

Simultaneous titration of different pools of HSV-1 and HSV-2 in BALB/c mouse embryonic fibroblast and HEL cell cultures showed a slightly higher plaque count of HSV-1 compared with HSV-2 in MEC as judged by the HEL/MEC ratio (Table 2). The mean size of HSV-2 plaques was, however, significantly larger than that of HSV-1 plaques (1.24 versus 2.24 mm^2). The virus growth curves in BALB/c MEC showed a 19- to 50-fold higher multiplication of HSV-1 as compared with HSV-2 (Fig. 3).

Adsorption of HSV-1 and HSV-2 to mouse macrophages. One reason for the difference in the yield of infectious virus from peritoneal macrophages infected with HSV-1 and HSV-2 could be a relative inability of HSV-1 to adsorb to mouse macrophages. Therefore, studies comparing the adsorption rates of HSV-1 and HSV-2 to macrophage cultures were performed. Macrophage cultures were infected with 2×10^4 PFU of HSV-1 or HSV-2, and after 60 min of

TABLE 1. *Number of infectious centers in peritoneal macrophage cultures from 4-week-old BALB/c mice infected with 5×10^5 PFU of either HSV-1 or HSV-2 and overlaid with MEC*

Virus type	No. of dishes	Mean no. of plaques[a]	Type of plaques
HSV-1	5	11 ± 7.4	Small, regressive
HSV-2	4	185 ± 43.2	Large, progressive

[a] Duplicate control dishes without macrophages showed no plaques. The mean number is given as a range ± two standard deviations.

adsorption the amount of virus that was lost from the supernatant medium and which could not be washed off the cells was assayed. In two such experiments, the percentages of adsorption of HSV-1 to macrophages were found to be 32 and 31%, respectively, compared to 36 and 34% adsorption of HSV-2, respectively.

DISCUSSION

Several studies have demonstrated the importance of macrophages in the primary defense against virus infections (2, 5–7, 9, 15, 16, 19–21). One of the most extensively studied viruses in this respect is HSV-1. In 1965, Johnson (7) used immunofluorescence studies to show that the age-dependent resistance in mice to HSV-1, first described by Andervont in 1929 (1), was associated with the lack of dissemination of virus from mature macrophages. Later Hirsch et al. (6) confirmed these studies using an infectious center assay, and Zisman et al. (20) were able to break down this age-dependent resistance by using antimacrophage serum and macrophage-toxic silica injections. In 1971, Stevens and Cook (18) showed that the restriction of HSV replication in adult macrophages was the result of an abortive infection that is specific to the macrophage population.

The purpose of the present study was to provide a more thorough examination of the difference in the pathogenic action of HSV-1 and HSV-2 in mice by using the background of the previously observed diversity between the two closely related virus types of liver pathogenicity in i.p. inoculated mice (10, 11). Furthermore, the role of macrophages in this difference was studied.

The results of the infectious center assay with mouse peritoneal macrophages showed a 17-fold difference in the restriction of HSV-1 and HSV-2 replication as judged by the number of infectious centers appearing. Hirsch et al. (6) found that a sevenfold higher replication of HSV-1 in unstimulated macrophages from suckling mice as compared with the replication in macrophages from adult mice was satisfac-

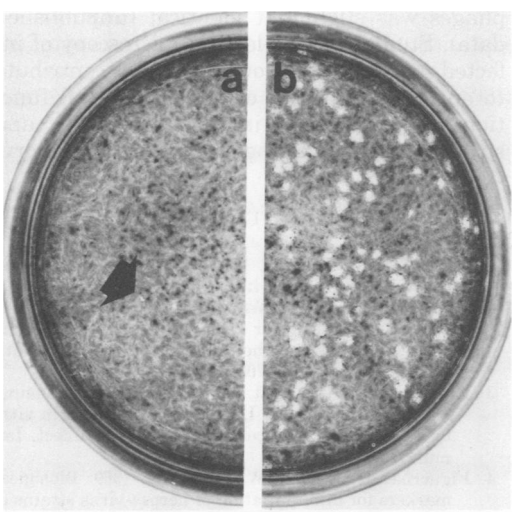

FIG. 2. *Infectious centers in macrophage cultures infected with 5 × 10⁵ PFU of either HSV-1 (a) or HSV-2 (b) after 2 days of incubation. The arrow indicates a small HSV-1 plaque.*

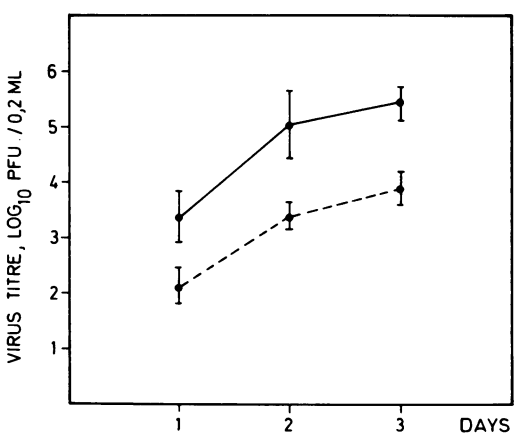

FIG. 3. *Virus titers in supernatant of BALB/c mouse embryonal fibroblast cultures infected with 10³ PFU of either HSV-1 (——) or HSV-2 (----). Bars represent two standard deviations.*

TABLE 2. *Simultaneous titration of pools of HSV-1 and HSV-2 in HEL and MEC (BALB/c) cell cultures*

Virus type	Virus titer (PFU/ml)[a]		HEL/MEC
	HEL	MEC	
HSV-1	$3.5 \times 10^8 \pm 0.5 \times 10^8$	$8.9 \times 10^7 \pm 1.6 \times 10^7$	3.9
	$3.2 \times 10^5 \pm 0.4 \times 10^5$	$9.0 \times 10^4 \pm 0.8 \times 10^4$	3.6
HSV-2	$1.4 \times 10^7 \pm 0.4 \times 10^7$	$2.8 \times 10^6 \pm 0.1 \times 10^6$	5.0
	$1.8 \times 10^6 \pm 0.6 \times 10^6$	$3.9 \times 10^5 \pm 0.3 \times 10^5$	4.6

[a] Mean of three dishes ± two standard deviations.

tory in explaining the age-dependent difference in resistance to this virus type. Compared with this, the 17-fold difference between HSV-1 and HSV-2 found in this study might easily explain the diversity in the number, degree, and duration of the hepatic necrotic lesions seen in 4-week-old BALB/c mice inoculated i.p. with the two virus types.

Furthermore, the HSV-2 infection as measured by the size and development of individual plaques in the MEC overlay was halted by the macrophages to a much lower degree than the HSV-1 infection, which was completely stopped, leading to regression of the plaque on prolonged incubation. The observation that not only the number of plaques but also their size can be affected by macrophages is in agreement with that of Lodmell et al. (8), who found that the size of fluorescent foci after incubation of HSV-1-infected primary rabbit kidney monolayers with rabbit PE cells depended on the number of peritoneal leukocytes in the culture. However, they used stimulated PE cells, which are known to be more restrictive to HSV-1 replication than unstimulated ones (6). Using spleen cells, Ennis (3) and Simmons et al. (17) found a similar plaque-reducing effect that was observed only when HSV-sensitized cells were employed.

The data found in the infectious center assay correlate well with the results of the in vivo experiments. Although the liver lesions in HSV-2-infected animals were large, numerous, and increasing in size, HSV-1-infected mice only occasionally developed a few small necrotic foci on days 3 and 4, which apparently healed. The self-limiting character of the HSV-1 liver infection was also reflected in the virus titrations from liver and spleen. Although the multiplication of HSV-1 in these two organs was completely suppressed after day 4, the replication of HSV-2 continued until the death of the animals. The overall outcome of the infection as measured by gross mortality and mean survival time was not, however, influenced by the arrest of the virus spread in visceral organs. Mice belonging to both groups and having symptoms of infection to the central nervous system died, and no obvious difference in the virus titers of the brain was seen.

This is in agreement with the hypothesis that the macrophages are decisive in the outcome of infection in visceral organs that are rich in macrophages, although the course of infection in the central nervous system is not influenced by macrophage restriction when the peripheral barrier has been overcome because of the lack of tissue macrophages in this system.

The difference observed in the restriction of HSV-1 and HSV-2 was found to be unique to the macrophages in the three cell populations studied. It was found that no replication of any of the two virus types in peritoneal lymphocytes from mice occurred, which is in agreement with the observation of Nahmias et al. (13) who found that HSV-1 cannot replicate in untransformed human peripheral blood lymphocytes. The replication of the two virus types in BALB/c fibroblasts does not lend support to the hypothesis that structural cells are involved in the pathogenic distinction between the two virus types: the relative plaquing efficiency in MEC as compared with HEL cells was almost identical; HSV-1 yielded more infectious virus than HSV-2, whereas the HSV-2 plaques were larger than the HSV-1 plaques, all general characteristics of the two virus types (4, 12).

The basic nature of the difference in the restriction of HSV-1 and HSV-2 replication in macrophages was not revealed by this study. No difference was found in the adsorption of the two virus types to peritoneal macrophages, and the amount of interferon produced by macrophages was slight but identical (unpublished data). Studies using electron microscopy of infected macrophages would probably contribute to our understanding of the differential function of macrophages in HSV infections, and such studies are in progress in this laboratory.

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