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Role of Activated Macrophages in Resistance of Congenitally Athymic Nude Mice to Hepatitis Induced by Herpes Simplex Virus Type 2

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Congenitally athymic nude (nu/nu) mice of a BALB/c genetic background were found considerably more resistant to the induction of focal necrotic hepatitis by herpes simplex virus type 2 (HSV-2) than were phenotypically normal littermates (nu/+) or BALB/c mice. The augmented resistance was age dependent, as it was only manifested in mice from 4 to 5 weeks of age. Studies of the course of infection showed that nude mice were able to restrain virus multiplication in the liver far better than normal mice in the early phase of infection. However, they seemed inferior to normal mice in eliminating the infectious process. In vitro investigation of periteonal macrophages revealed that macrophages from 6-week-old nude mice exhibited accelerated spreading and were three times as restrictive in the replication of HSV-2 as macrophages from normal mice. However, no difference was found in the efficiency of adsorption/phagocytosis between macrophages from nude and normal mice. The increased resistance of nude mice could be abolished by blockade of the macrophage function of the mice by silica. Nude mice reconstituted at birth with thymus cells were just as susceptible to infection as normal mice. These data suggest that the increased resistance of nude mice to HSV-2 hepatitis is due to the presence of nonspecifically activated macrophages before infection.

Extensive studies of the mouse mutant nude with congenital absence of thymic tissue have revealed fundamental defects in the thymus-dependent immune functions of this animal (28). It is widely accepted that nude mice, because of the immunodeficiency, are highly susceptible to bacterial and viral infections (25). It was therefore very surprising to find these mice more resistant than normal euthymic mice of the genetic background strain to the induction of focal necrotic hepatitis by herpes simplex virus type 2 (HSV-2) (17).

Since the resistance of mice to HSV hepatitis has been found to be correlated with the degree of restriction of virus replication in mouse macrophages in vitro (16, 18, 19), the question of whether macrophages represent the cellular expression of the augmented resistance of nude mice to this infection was investigated. Recent reports on enhanced activity of macrophages from congenitally and induced thymus-deficient mice toward *Listeria monocytogenes* (5, 26, 29), tumor cells (15), and vaccinia virus (23) lend further support to a hypothesis of a crucial role of macrophages in the resistance of nude mice to HSV-2 hepatitis.

MATERIALS AND METHODS

Mice. Congenitally athymic nude (nu/nu) mice and phenotypically normal littermates heterozygous for the nu gene (nu/+) as well as normal inbred BALB/c/A/BOM mice were obtained as specific-pathogen-free animals from the Gl. Bomholtgaard Laboratory Animal Breeding and Research Center, Ry, Denmark. A full description of the history of the nude mice used and the conditions of husbandry at Gl. Bomholtgaard has been given by Rygaard (25). Briefly, progeny of the "genuine Scotch stock" were obtained in 1969 from the Institute of Animal Genetics, Edinburgh, Scotland. At Gl. Bomholtgaard, a complete transfer of the nu gene to the BALB/c strain of inbred mice has been accomplished by 10 successive backcrosses of heterozygous male nu/+ mice to female BALB/c mice. The breeding unit, from which the mice used in this study were achieved, was established 8 months ago by transferring fetuses from hysterectomized animals to germfree isolators, where the mice were foster-nursed. Adaptation to a defined bacterial envirnoment was achieved by supplying the mice with an aerobic and anaerobic nonpathogenic flora obtained from Zentralinstitut für Versuchstierforschung, Hannover, Germany, and Central Animal Laboratory, Nijmegen, Holland, respectively. The production of nude mice was established by mating nu/nu male mice with nu/+ female mice. The breeding unit is main-

tained in a barrier-sustained animal house with appropriate ventilation, temperature, and relative humidity and use of autoclaved food and water. The mice were used within 3 days of arrival in a special room in our animal house. Only female mice were used.

Virus. HSV-2, strain MS, used in this study has been described previously (21).

In vivo experiments. Mice were inoculated intraperitoneally (i.p.) with 10^6 plaque-forming units (PFU) of HSV-2 in 0.1 ml of diluent. In most experiments, mice were killed after 4 days of infection by exsanguination under ether anesthesia. The course of infection was followed by killing mice at daily intervals after the infection. The number of liver lesions was graded semiquantitatively from 0 to 4: 0, no lesions; 1, <5 lesions; 2, 5 to <20 lesions; 3, 20 to <100 lesions; 4, 100 or more lesions.

Assay of organs for virus. Livers and brains collected aseptically were frozen at −70°C until homogenized individually to a 10% suspension in Eagle minimum essential medium supplemented with 5% calf serum and antibiotics, using a Hannover homogenizer (Ernst Schutt, Jr.). After centrifugation at 4,000 × *g* for 30 min at 4°C, the supernatants were titrated in human embryonic lung fibroblast cultures by a plaque method previously described (17).

Histology. Liver specimens from exsanguinated mice were fixed in 4% Formalin. The fixed livers were embedded in paraffin, and histological sections were prepared and stained with hematoxylin-eosin at the Institute of Anatomy, University of Aarhus.

Infectious-center assay. The infectious-center assay used for the determination of virus replication in macrophages was described in detail earlier (16). Briefly, unstimulated peritoneal resident cells were harvested by lavage of the peritoneal cavity of mice with RPMI 1640 medium containing 20% fetal calf serum, antibiotics, and 10 IU of heparin. The relative number of macrophages in the peritoneal lavage was determined in a fluorescent microscope by using cytoplasmic staining of lysosomes with acridine orange as the macrophage marker. The peritoneal cells were plated on plastic petri dishes (35 mm, Falcon) in a concentration of 5×10^5 macrophages per dish. The next day nonadherent cells were removed by washing, and the macrophages were infected with 5×10^5 PFU of HSV-2. After 60 min of adsorption, the nonadsorbed virus was removed by washing and HSV-hyperimmune serum treatment, and the macrophages were overlaid with mouse embryonic cells in methyl cellulose medium. Plaques appearing in this target cell monolayer were counted after 2 days.

Adsorption of HSV-2 to macrophages. The adsorption of HSV-2 to macrophages was assessed as previously described (16). Macrophage cultures were prepared as described above and infected with 2×10^4 PFU of HSV-2. After 60 min of adsorption, the original inoculum was diluted in 20 ml of cold medium and assayed for virus. The percentage of adsorption ($A\%$) was calculated from the formula $A\% = [(M - C)/I] \times 100\%$, where M and C are the amounts of virus lost from the inoculum (I) in macrophage cultures and in control petri dishes without macrophages, respectively.

Silica pretreatment. A silica suspension of 15 mg/ml was prepared as previously described (20). Mice were inoculated intravenously with 0.2 ml (3 mg/mouse) 2 h before the virus inoculation.

Immunological reconstitution of nude mice. Thymus tissue from 3-day-old nu/+ and 8-week-old BALB/c mice was disintegrated in a glass homogenizer in RPMI 1640 medium with 5% fetal calf serum, 10 IU of heparin, and antibiotics and passed through a few layers of gauze. The cells were washed twice in RPMI 1640 medium and adjusted to 10^8 viable cells per ml. The lymphocytes were inoculated i.p. in 1-day-old nu/nu mice in a volume of 0.2 ml.

The proportion of T lymphocytes in lymph nodes of reconstituted mice and control mice was assessed by a cytotoxicity test. Cervical lymph nodes were processed as described above for thymus tissue, and the cell suspension was adjusted to about 5×10^6 lymphocytes per ml. Fifty-microliter samples of cells, anti-Thy 1.2 serum (1:40), and agarose-absorbed (6) guinea pig serum as complement source were mixed and incubated for 1 h at 37°C. Finally, 50 μl of trypan blue was added, and the number of viable cells was determined by counting the dye-excluding cells. The anti-Thy 1.2 serum used was raised in AKR mice by nine weekly inoculations i.p. of 5×10^6 to 1×10^7 thymocytes from C3H mice, as described by Reif and Allen (24). It had a cytotoxicity titer against BALB/c thymocytes of 640, and the 1:40 dilution used killed more than 95% of BALB/c thymocytes.

RESULTS

In normal specific-pathogen-free-maintained mice no spontaneous liver lesions have ever been detected. However, during these experiments a total of four nude mice, all aged 6 weeks or more, showed liver lesions at autopsy that differed from the ordinary experimental HSV-2 lesions. This constitutes 2.9% of the total number of mice used and 6.7% of mice aged 6 weeks or more, a frequency of spontaneous liver lesions of an order found by others (25). Both macroscopically and microscopically the lesions were similar to those described for mouse hepatitis virus infection in nudes by Ward et al. (27). All four mice were excluded from the experiments.

Susceptibility of nu/nu, nu/+, and BALB/c mice to HSV-2 hepatitis. Groups of nu/nu, nu/+, and BALB/c mice of varying ages were inoculated i.p. with 10^6 PFU of HSV-2. Scoring of liver lesions 4 days after infection revealed that from the age of about 5 weeks the nude thymus-aplastic mice were considerably more resistant than normal littermates (nu/+) or BALB/c mice to the induction of hepatitis by HSV-2, as judged by the number of necrotic foci in the liver (Fig. 1). However, younger nude mice were just as susceptible as normal mice, which showed a gradual age-related increase in the resistance, as previously described (19). Corre-

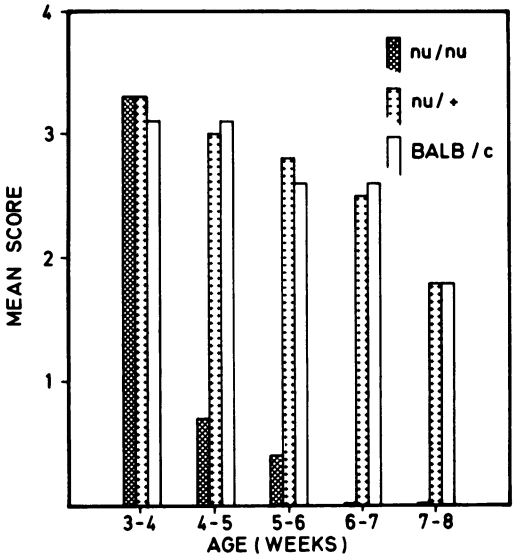

FIG. 1. *Mean score of liver lesions in nu/nu, nu/+, and BALB/c mice of increasing ages 4 days after i.p. inoculation of 10^6 PFU of HSV-2. The lesions were graded semiquantitatively from 0 to 4. Groups consisted of 4 to 17 mice. Summary of experiments.*

sponding to this, virus titer levels in nude mice beyond the age of 5 weeks were either below the detection limit (10 PFU/0.1 g) or low in most animals (Fig. 2).

Course of infection in nu/nu, nu/+, and BALB/c mice. Groups of 6-week-old nu/nu, nu/+, and BALB/c mice were killed at daily intervals after infection for further analysis of the course of infection. Liver lesions developed in nu/+ and BALB/c mice as previously described (16), becoming visible on day 3 and reaching a final size of about 1 mm in diameter on days 4 to 5. On the other hand, only 6 out of 21 nude mice showed a few tiny lesions on the liver margin. The virus titers obtained in the livers revealed fundamental differences between nude and normal mice (Fig. 3). In normal mice, increasing amounts of virus were recovered from the liver during the first 4 days of the infection, followed by an abrupt decrease from day 5 onward, so that most mice were free of detectable virus on days 6 and 7. Contrary to this, virus titers obtained in livers of nude mice were low during the first 5 days of infection, but in these mice virus titers increased during the last 2 days of the experiment. It was not possible to follow the course of infection beyond day 7 because both nude and normal mice of this age and with the virus dose used died from ascending myelitis or encephalitis between days 7 and 10 after infection. Virus growth curves from the brain of nude and normal mice were, in agreement with this, fundamentally alike, with virus first appearing in the brain of some mice on day 5 and showing increasing virus titers on the following days (Fig. 4).

Histological examinations. During the course of infection, the development of liver lesions was followed in all groups of mice by histological examination of the livers from each day of the experiment. In BALB/c and nu/+ mice the microscopic development of the lesions was quite comparable to earlier descriptions (20, 21), whereas regular lesions were absent in nude mice infected at 6 weeks of age. The livers of nude mice examined at any day of the experiment were further characterized by a general Kupffer cell proliferation. In some sections sparse infiltration with mononuclear cells was also present in the nude mice. The infiltrations, consisting of 5 to 10 cells per focus, were often located around a few degenerated liver cells. Histological examinations of nude mice infected at 3 weeks of age, at which time of life macroscopic lesions are induced, revealed, however, that at this age the histological picture of the lesions 4 days after infection was quite comparable to the findings in nu/+ and BALB/c mice.

Replication of HSV-2 in macrophages from nu/nu, nu/+, and BALB/c mice. Mac-

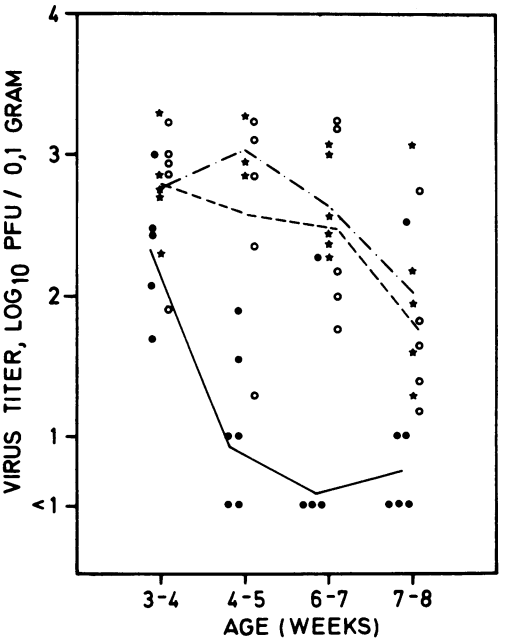

FIG. 2. *Virus titers in livers of nu/nu (●, ——), nu/+ (*, — · — ·), and BALB/c (○, -----) mice of increasing ages 4 days after i.p. inoculation of 10^6 PFU of HSV-2. Each point represents one mouse. Lines are drawn between the means of each group.*

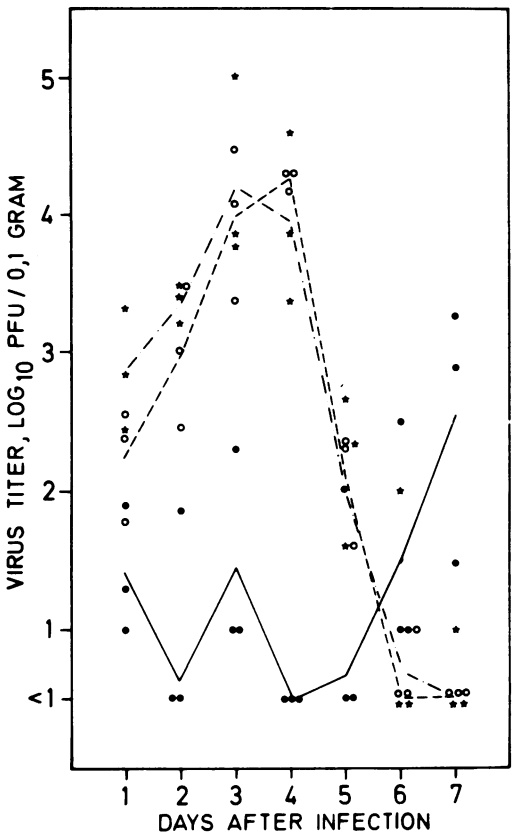

FIG. 3. *Course of infection in the liver of 6-week-old nu/nu (●, ——), nu/+ (*, — · — ·), and BALB/c (○, -----) mice inoculated i.p. with 10^6 PFU of HSV-2. Each point represents one mouse. Lines are drawn between the means of each group.*

rophages were harvested from the peritoneal cavity of 6-week-old nu/nu, nu/+, and BALB/c mice. No difference was found in the total number of peritoneal cells obtained from the three types of mice; neither was there any difference in the relative number of lymphocytes and macrophages recovered (Table 1). The cells were plated on plastic petri dishes in a concentration of 5×10^5 macrophages per dish. After 1 h of adsorption at 37°C, a marked difference between the three types of cultures was seen by phase-contrast microscopy. BALB/c and nu/+ macrophages were still rounded, although they were phase-dark with a very few spindle-shaped cells in between. Nude macrophages, on the other hand, showed a much higher degree of "spreading," with most of the cell being phase-dark and spindle-shaped. After 24 h of incubation, this difference had disappeared and the cultures were indistinguishable from each other, with the majority of cells being phase-dark and spindle-shaped.

The replication of HSV-2 in such macrophage cultures was assessed by an infectious-center assay, with the number of plaques appearing in a mouse embryonic cell overlay representing the number of macrophages that had replicated the virus efficiently. The number of infectious centers in five such cultures from nu/nu mice was from 40 to 72 (mean, 54 ± 13), whereas the number in nu/+ and BALB/c cultures ranged from 128 to 191 (mean, 166 ± 23) and 141 to 187 (mean, 161 ± 17), respectively. The restriction of HSV-2 replication in macrophages from nude mice was thus three times as high as that in macrophages from normal mice (Table 1).

The difference in the yield of infectious centers in macrophage cultures from normal and athymic mice was not due to differences in the adsorption of the virus to macrophages. After 60

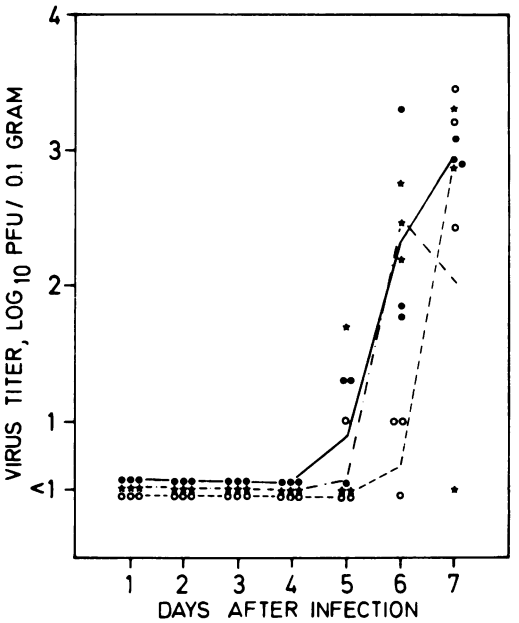

FIG. 4. *Course of infection in the brain of 6-week-old nu/nu (●, ——), nu/+ (*, — · — ·), and BALB/c (○, -----) mice inoculated i.p. with 10^6 PFU of HSV-2. Each point represents one mouse. Lines are drawn between the means of each group.*

TABLE 1. *Data on peritoneal cells and the interaction of HSV-2 and macrophages from 6-week-old nu/nu, nu/+, and BALB/c mice*

Mouse	No. of cells/mouse ($\times 10^6$)	Macrophage (%)	No. of infectious centers[a]	Adsorption (%)
nu/nu	3.5	56	54 ± 13	29.0
nu/+	3.1	53	166 ± 23	26.5
BALB/c	3.7	55	161 ± 17	31.5

[a] The mean numbers of five cultures are given ± standard deviation.

min of adsorption, the amounts of virus that was lost from the supernatant medium and could not be washed off the cells were 29.0% (nu/nu), 26.5% (nu/+), and 31.5% (BALB/c), respectively, of the inoculum (Table 1).

Effect of silica on HSV-2 hepatitis in nude mice. In previous studies, silica has been found effective in blocking the macrophage restriction of HSV replication in livers of mice (19, 20). Therefore, it was investigated whether the augmented resistance of nude mice could be abolished by silica treatment. Five mice received 3 mg of silica intravenously 2 h before receiving the virus i.p. After 4 days of infection it appeared that blockade of the macrophages had rendered nude mice almost as susceptible to HSV-2 liver infection as were normal mice (Fig. 5).

Susceptibility of T-cell-reconstituted nude mice to HSV-2 hepatitis. Newborn nude mice received a mixture of about 2×10^7 thymocytes from 3-day-old nu/+ and 8-week-old BALB/c mice. The reconstituted mice were reared behind specific-pathogen-free barriers and developed normally. At 6 weeks of age the reconstituted mice were found to be just as susceptible to the induction of hepatitis by HSV-2 as were normal mice, as judged by the mean score of liver necrosis and virus titers reached in the livers (Fig. 5).

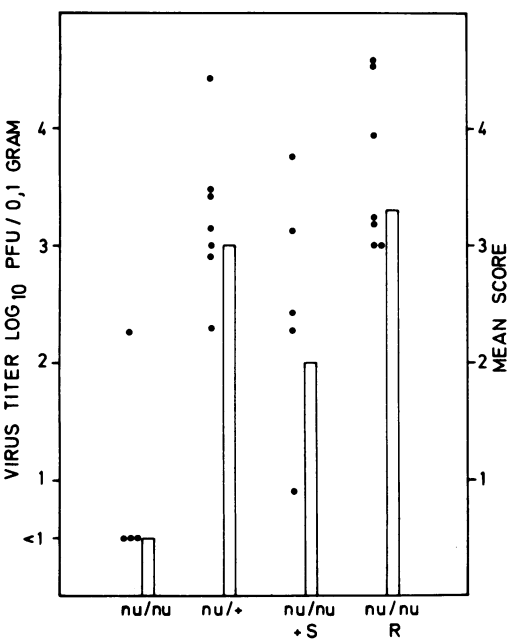

FIG. 5. *Effect of silica treatment and thymus cell reconstitution on virus titers (●) and mean score of liver lesions (□) in 6-week-old nude mice 4 days after i.p. infection with 10^6 PFU of HSV-2. Groups: nu/nu, control nude mice; nu/+, control heterozygous littermates; nu/nu + S, nude mice receiving 3 mg of silica intravenously 2 h before infection; nu/nu + R, nude mice immunologically reconstituted at birth with thymus cells. Each point represents one mouse. Liver lesions were graded semiquantitatively from 0 to 4.*

The functional ability of the T-cell graft to reconstitute nude mice was assessed by measuring the relative number of anti-Thy 1.2 serum-sensitive lymphocytes in peripheral lymph nodes at autopsy. The reconstituted nude mice showed from 29 to 50% (mean, 38 ± 7) Thy 1.2-positive lymphocytes as compared with from 45 to 61% (mean, 55 ± 6) in nu/+ mice. Two non-reconstituted nude mice showed 4 and 6% dead lymphocytes in this test.

DISCUSSION

This study was undertaken to examine a previously reported high resistance of nude mice to the induction of focal necrotic hepatitis by HSV-2 (17). This resistance was at first very surprising in view of the immunodeficiency of these mice. A genetic basis for the resistance, similar to that described for the GR mouse strain (18), could be rejected. The nude mice were more resistant than congenic mice of the background strain and heterozygous littermates, differing only in the genetic constitution from the nudes by the nu gene. Another possible explanation, namely, that the lesions in normal mice are mediated immunopathologically by T lymphocytes (8), was also rejected, since weanling nudes were just as susceptible to infection as were normal mice. Only mice aged 5 weeks or older were more resistant than normal mice.

Macrophage restriction of virus replication has been found important in other aspects of mouse resistance to HSV (14, 16, 18–20). Furthermore, reports on the role of activated macrophages in resistance of nude mice to other pathogens (5, 10, 29) stimulated an investigation of the handling of HSV-2 by peritoneal macrophages from nude mice. First of all, macrophages from 6-week-old nude mice showed accelerated spreading after 1 h in culture, as compared with macrophages from normal mice, a well-accepted characteristic of activation (1, 22). This was also found by Zinkernagel and Blanden (29). Another sign of macrophage activation in nude mice was the general Kupffer cell proliferation in the liver seen microscopically. Despite the obvious signs of activation of macrophages from nudes, no difference in the total cell number or macrophage percentage in peritoneal washings from the various groups of mice was noted. This is also in agreement with the findings of others (5, 23).

Assessment of the replication of HSV-2 in macrophage cultures revealed that restriction of

virus growth was higher in macrophages from mature nude mice as compared with cells from normal mice, since the number of macrophages from nude mice that supported virus replication was only one-third the number from normal mice. However, adsorption/phagocytosis was not more efficient in macrophage cultures from nude mice than in those from normal mice. Varying results concerning these two parameters, uptake and intracellular handling of the microorganism, in macrophages from nude and thymectomized mice have been reported with *L. monocytogenes*: augmented phagocytosis-deficient bactericidal activity (29); augmented phagocytosis and bactericidal activity (5); normal phagocytosis-suppressed bacterial multiplication (26). The reasons for these differences with the same microorganism are obscure, but they may be due to differences in the experimental conditions used. The only in vitro investigation of the fate of a virus in macrophages from nude mice showed failure of replication of vaccinia virus in these cells in contrast to a 30-fold increase in the virus titer in macrophages from control mice (23). Virus adsorption was not investigated. However, this study was carried out with paraffin-oil-induced macrophages, which do not necessarily reflect the properties of the native peritoneal cells. No in vivo correlate of the enhanced macrophage activity toward the vaccinia virus was reported.

As further evidence of the role of activated macrophages in the resistance of nude mice to HSV-2 hepatitis, silica blockade of nude macrophage function was found to abolish this resistance. Using another macrophage-toxic agent, Emmerling et al. (10) were likewise able to break down the resistance of nude mice to *L. monocytogenes*.

The causal relationship between the absence of the thymus and virus resistance was tested by reconstituting nude mice with nu/+ and BALB/c thymocytes. Six weeks after this procedure, nude mice were found to be just as susceptible to infection as were normal mice, suggesting a normal, nonelevated level of macrophage defense. This is compatible with the observations of Rao et al. (23) that macrophages obtained from thymus-transplanted nude mice behaved functionally as macrophages from untreated nu/+ mice in their vaccinia virus replication assay.

Studies of the virus growth in the liver of nu/nu, nu/+, and BALB/c mice revealed that the superiority of nude macrophages in sustaining virus multiplication in the liver is only temporary. From day 5 onward a dramatic decrease in the amount of virus recovered from livers of normal mice occurs, probably mediated by recruitment and activation of macrophages triggered by specifically sensitized T lymphocytes (2–4). On the other hand, in nudes, virus titers seemed to increase during the same period, suggesting that the immune, thymus-dependent response is necessary for the final elimination of the infectious process. These data are in agreement with the observations of Zinkernagel and Blanden (29), who found that the elimination of *L. monocytogenes* from the tissue of thymectomized mice was impaired from days 3 to 6 of infection. Similarily, Emmerling et al. (9, 10) found that nude mice showed increased resistance to *L. monocytogenes* during the initial phase of infection, but this was followed by continued infection with a chronic trend not seen in normal mice.

Explanations for the development of activated macrophages in nude mice have been dealt with by others (5, 15, 23, 29). Most authors point to the role of an increased bacterial gut flora, maybe because of the inability of these mice to produce immunoglobulin A (7). It has been shown that nude mice have poorly developed Peyer patches and a low level of gut immunoglobulin A plasma cells (12). Nonspecific macrophage activation by bacterial lipopolysaccharide (11, 13) absorbed from the gut might thus be responsible for a sustained, nonspecific state of macrophage activation. An alternative explanation of the phenomenon could, however, be suggested. This would imply the presence of suppressor T cells or lymphotoxins with macrophages as targets in normal mice and reconstituted nude mice. The absence of such suppressors in nude mice could thus account for the more active state of macrophages from these mice.

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