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Interaction of mouse hepatitis virus 3 with Kupffer cells explanted from susceptible and resistant mouse strains. Antiviral activity, interleukin-1 synthesis

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1. SUMMARY

The genetic sensitivity of mouse strains to mouse hepatitis virus 3 (MHV 3) has been related in vitro to a delay of virus replication in liver sinusoidal cells. In vivo immuno-histochemical studies of the liver from infected mice have demonstrated that mechanisms other than direct viral injury are in operation. To examine potential mechanisms, the interaction of lipopolysaccharide (LPS)-stimulated Kupffer cells with MHV 3 was studied. We first observed a dramatic inhibition in viral replication in LPS-treated Kupffer cells explanted from A/J resistant mice. Second, we demonstrated that MHV 3 induced a dose-dependent interleukin 1 (IL-1) activity in the supernatants of infected Kupffer cells of both strains. These results led us finally to examine the antigen-processing function of the Kupffer cells of both strains of mice. No striking differences were observed in the ability of Kupffer cells from resistant or sensitive mice to collaborate with immuno-competent lymphocytes. Our data suggest that Kupffer cells play a double role which is crucial in the pathogenesis of MHV 3-induced hepatitis. First, they act directly as the genetically determined sensitivity of mice to MHV 3 infection is correlated with the efficiency of the antiviral activity induced in Kupffer cells by LPS. Second, they act indirectly through the synthesis of different amounts of IL-1 induced by MHV 3. This hypothesis is further borne out by the effects of indomethacin treatment on the course of MHV 3 infection in A/J resistant mice in vivo.

2. INTRODUCTION

Mouse hepatitis virus 3 (MHV 3), a member of the coronavirus family, induces a disease whose severity is host dependent [1]. Mice from the A/J strain are fully resistant to MHV 3; they develop a mild disease and survive. Mice from the C57Bl and Balb/c strains are highly susceptible to the virus and suffer from a fulminating hepatitis which leads to their death within 4–8 days after infection. Animals from the A2G and the C3H strains aged more than 3 months have an intermediate susceptibility to MHV 3; most of them survive the
acute stage of the infection but become chronic virus carriers with an evolving disease accompanied by signs of neurological involvement.

Genetic sensitivity displayed by mouse strains towards MHV 3 infection in vivo has been related in vitro to the degree of virus replication observed in hepatocytes [2], in peritoneal macrophages [3], in Kupffer cells and endothelial liver cells [4] from adult mice.

In vivo viral replication reaches a high level in Balb/c susceptible mice. However, the non-negligible amount of virus found in the livers of A/J resistant mice, suggests that the resistance of this strain is not directly linked to a restriction in viral multiplication. Recently, Levy et al. [5] demonstrated that the susceptibility or resistance to MHV 3 is genetically linked to the monocyte procoagulant activity (PCA) induced by the virus. Accordingly, severe abnormalities in liver microcirculation are observed early in the course of MHV 3 infection in Balb/c mice whereas, normal streamlined blood flow takes place in the livers of the resistant A/J animals [6].

On the basis of these data and of previous results concerning the role of sinusoidal liver cells in resistance to MHV 3 [7] we have carried out experiments in order to evaluate the effect of Kupffer cell infection on two of their functions which may be involved in the resistance displayed. Accordingly, we have compared the capacity of endotoxin to induce (i) restriction of MHV 3 replication, (ii) synthesis of interleukin-1 in infected Kupffer cells of both strains of mice. In addition, the accessory cell function of Ia (+) Kupffer cells in the presence of MHV 3 antigen has been studied. Our results have led us to suppose that the synthesis of IL-1 might play an important role in the pathogenesis of MHV 3 hepatitis. Experiments in which resistant mice were rendered susceptible to the infection by indomethacin treatment have borne out this hypothesis.

3. MATERIALS AND METHODS

3.1. Virus

MHV 3 was cultured and titrated on L 929 at 37°C, as described by Pereira et al. [8]. Where mentioned in the text, MHV 3 virus was inactivated either by a 5 h incubation at 56°C, or by a 2 h exposure to ultraviolet radiation.

3.2. Animals

Mice of the inbred A/J Orl and Balb/c strains from the Centre D’Elevage d’Animaux de Laboratoire, CNRS, Orléans, France were immunized by an I.P. injection of heat-inactivated MHV 3 at a dose of 2·10⁴ pfu/mouse in 200 µl.

For indomethacin treatment adult A/J mice were infected with 10³ pfu of MHV 3 intraperitoneally. They were treated with 100 µg indomethacin injected intraperitoneally at 24, 48 and 72 h post infection. All groups consisted of ten animals.

3.3. Preparation of Kupffer cells

Mouse Kupffer cells were isolated using the method already described for rats [9,10]. Briefly, perfusion fixation with a calcium-deprived medium was performed through the portal vein in the livers of mice killed by cervical dislocation. The livers cut into small pieces were then incubated for 45 min in a medium containing calcium and 0.05% collagenase in a New-Brunswick G7 gyrotory water-bath. The non-parenchymal cell suspension was filtered on nylon and separated from erythrocytes by centrifugation through a density cushion of metrizamide (Niegaard, final density 1.089 g/cm³).

The cell suspension was then introduced into the elutriation rotor (Beckman JE-6B). A fraction consisting essentially of Kupffer cells was collected at a flow rate of 42.4 ml/mn. The viability of this fraction was 90–95% as demonstrated by the Trypan blue exclusion test. The Kupffer cells were cultivated in Dulbecco-Hepes medium (pH 7.4) containing 20% filtered foetal calf serum (FCS). The cells (2.5·10⁵ cells/well) were used after a 24 h incubation at 37°C.

3.4. LPS

Escherichia coli 0127: B8 phenol-extracted lipopolysaccharide (DIFCO Laboratories, Detroit MI, U.S.A.) was used. The reaction of explanted Kupffer cells was studied using two parallel experimental groups of cultures, untreated or treated
for 24 h with LPS. After treatment, the medium was removed and the cells were used for the assays. Control Kupffer cells received complete medium without LPS.

All experiments, unless specified in the text, were carried out with 50 or 100 μg/ml of LPS.

3.5. Intrinsic antiviral assay

Kupffer cells were placed in 96-well plates at 3.0 × 10⁵ viable cells per well. They were pre-treated for 24 h with LPS; afterwards the cultures were infected with MHV 3 at a multiplicity of 0.1 pfu per cell. After 1 h adsorption, fresh medium was added to the wells. At different times of post-infection the plates were frozen until assayed for virus content. All experiments were repeated 3−5 times.

3.6. Collection of culture fluids for IL-1 assay

Normal or LPS-activated Kupffer cells were exposed or sham exposed for 1 h at 37°C to MHV 3 at multiplicities of 0.1 or 0.05 pfu/cell. Kupffer cells were then cultured in fresh medium with 5% FCS for an additional 24 h after which the supernatant fluids were collected, cleared of cells by centrifugation and stored at −80°C until assayed. In a subset of experiments, aliquots of Kupffer cells were exposed to polymyxin B (500 U/ml) or a combination of polymyxin B and virus to diminish any potential contribution of remaining LPS to the results observed.

In another subset of experiments, aliquots of Kupffer cells were exposed to indomethacin (10 μg/ml) or a combination of indomethacin and LPS or virus to prevent the co-production of prostaglandin E2 which adversely affects IL-1 assays [11].

3.7. IL-1 assay

IL-1 activity was measured using the standard mouse thymocyte proliferation assay [11]. In brief, 5−6-week-old A/J or Balb/c mice were ether anesthetized. The thymus glands were removed using aseptic techniques, teased into single cell suspensions and passed through a wire filter to remove particulates, washed twice in Dulbecco’s medium and resuspended in Dulbecco’s medium supplemented with 10% FCS, 1% glutamine, 2% sodium pyruvate, Heps buffer, antibiotics and 0.05% β-mercaptoethanol. Thymocytes were cultured for 72 h at 3 × 10⁵ cells/well in 96-well flat-bottomed plates in the presence of 1 μg/ml phytohaemagglutinin (PHA) and 50 μl of the test samples. Cultures were terminally pulsed with tritiated thymidine (20 μCi/ml) and the incorporation into lymphocytes was analyzed with a liquid scintillation counter (Beckman). These assays were carried out in triplicate. The assays of IL-1 incorporated several relevant controls, such as virus-containing culture medium, (without cell-derived factors) to ensure that the virus, when carried over into mouse thymocyte cultures, did not itself induce thymocyte proliferation. In order to eliminate the eventual role of contaminating endotoxin in the IL-1 assay, thymocytes were cultivated in the presence of PHA and different concentrations of LPS. A dose as high as 40 μg/ml did not induce any significant thymocyte proliferation, thus allowing us to rule out the role of contaminating LPS in our experiments.

3.8. Lymphoproliferative analyses

The technique has been adapted from Rogoff and Lipsky [12]

3.8.1. Preparation of spleen cell suspensions.

Spleens from immunized and control mice, aseptically removed, were gently teased apart to pass through a stainless-steel mesh. Cells were centrifuged, washed three times and incubated at 37°C for 6 h in a 25 cm² tissue culture flask (Falcon 3013). Non-adherent cells were collected and regarded as macrophage-depleted lymphocytes. Cell cultures were carried out in sterile microtiter plates (COSTAR 3596) in triplicate with 2 × 10⁵ responding splenic lymphocytes in each well under 100 μl medium.

3.8.2. Preparation of Ag-pulsed Kupffer cells.

Kupffer cells suspended in Dulbecco’s medium at approximately 2.10⁶ cells/ml were placed on a mechanical rotator and incubated for 45 min at 37°C with MHV 3 heat-inactivated virus as antigen or Dulbecco’s medium control. At the end of this period the cells were washed four times in large volumes of chilled Dulbecco’s medium and added to the splenic lymphocyte cultures.
3.8.3. Co-cultures. The co-cultures of splenic lymphocytes and Ag-pulsed Kupffer cells were incubated for 72 h at 37°C in a humidified 5% CO₂/95% air atmosphere. 18 h before harvesting, 1–2 μCi of tritiated thymidine was added to each well. Tritiated thymidine incorporation was then determined in a liquid scintillation counter. All data were expressed as the difference in counts per minute between the means of triplicate cultures containing MHV 3 antigen-pulsed or Dulbecco-pulsed Kupffer cells.

4. RESULTS

4.1. Multiplication of MHV 3 in Kupffer cells from different strains of mice activated or not with LPS

LPS-activated Kupffer cells from resistant and susceptible strains of mice were checked for their intrinsic antiviral activity in order to determine whether it paralleled the strain-related susceptibility or resistance to MHV 3.

Kupffer cells from susceptible animals (Fig. 1A) produced large amounts of virus. There was a 100-fold increase in virus yield 24 h after the infection of non-activated cells with a multiplicity of 0.1 pfu per cell (m.o.i). Treatment of Balb/c Kupffer cells with LPS failed to induce any noticeable restriction in the virus growth.

The multiplication of MHV 3 in Kupffer cells isolated from A/J resistant mice is shown in Fig. 1B. In nonactivated Kupffer cells from A/J mice, although the final yield of virus is the same as that found in Kupffer cells from Balb/c mice, there is a delay of 24–36 h in the onset of viral growth. In addition, activation of the A/J Kupffer cells with LPS lengthened the lag phase of viral growth and decreased the final virus yield.

All the experiments were repeated 3 to 5 times and the same effect was observed in all cases. The mean inhibition rate calculated at 48 h was 0% in the case of activated Balb/c Kupffer cells and 46% in the case of A/J-activated Kupffer cells (data not shown).

4.2. IL-1 production by activated Balb/c and A/J Kupffer cells

In order to insure that the apparent lack of antiviral activity in Balb/c Kupffer cells exposed to LPS was not due to a lower responsiveness of these cells to the LPS stimulus, we have evaluated the production of IL-1 in cultured Kupffer cells from A/J and Balb/c strains of mice after exposure to different concentrations of LPS. Super-
Fig. 2. LPS dose-dependent increase in IL-1 activity in the culture supernatants of A/J or Balb/c explanted Kupffer cells.

Fig. 2 shows that the dose-response curve of Balb/c Kupffer cells is similar to that of A/J Kupffer cells.

4.3. Induction of IL-1 synthesis by Kupffer cells from Balb/c and A/J mice after MHV 3 infection

Since LPS treatment of Kupffer cells from the Balb/c strain fails to counteract the multiplication of MHV 3, we wondered whether this altered property was accompanied by some other defects in the non-specific defense mechanisms of MHV 3-infected hepatic macrophages. An investigation was thus made concerning the induction of IL-1 synthesis in Kupffer cells infected with MHV 3.

Fig. 3A shows that MHV 3 induces the synthesis of IL-1 in Kupffer cells. Accordingly, supernatant fluids derived from normal non-activated Balb/c Kupffer cells, whether infected or not with MHV 3, did not contain any detectable amounts of IL-1 activity as measured by the standard mouse thymocyte proliferation assay. However, pretreatment of Kupffer cells with indomethacin shows them to display a virus dose-dependent increase in IL-1 synthesis. Finally, treatment of Kupffer cells with endotoxin in the presence of indomethacin still further increases the amount of IL-1 activity detected in the supernatants of infected Kupffer cells. Pretreatment of Kupffer cells with LPS in the absence of indomethacin led to variable results concerning the amount of detectable IL-1 activity but this effect was regularly virus dose dependent. Similar results were observed with Kupffer cells from A/J mice who also responded with a virus dose-dependent increase in IL-1 synthesis after treatment with indomethacin (Fig. 3B).

![Fig. 3. IL-1 activity produced in 24 h by cultured Kupffer cells exposed to MHV 3. (*) Thymocyte proliferation in the absence of Kupffer cell fluids.](image-url)
4.4. Effect of indomethacin treatment on the course of MHV 3 infection in A/J mice

MHV 3 induces an IL-1 activity detectable in the supernatant of Kupffer cells pretreated with indomethacin. Indomethacin is known to block the synthesis of prostaglandin E₂, which acts as an inhibitor of IL-1. Given the crucial role of the Kupffer cells in the resistance of the A/J mice to MHV 3, it was of interest to check the effect of indomethacin treatment on the course of MHV 3 infection in A/J mice (Table 1). Indomethacin alone had no deleterious effects on control animals when used at the dosages mentioned above. Animals infected with 2·10³ pfu of MHV 3 were fully resistant to the virus and showed no mortality. 92% of the animals that were treated with indomethacin and infected with MHV 3 died within 8 days of infection.

| Group                | Mortality rate (%) |
|----------------------|--------------------|
| Indomethacin         | 0                  |
| MHV 3                | 0                  |
| MHV 3 + Indomethacin | 92                 |

a Animals were treated with 100 µg indomethacin (I.P.) at 24, 48 and 72 h post infection.
b Animals were treated with 2·10³ pfu i.p.
c Mortality was determined by day 8 post infection.

4.5. Ability of A/J and Balb/c Kupffer cells to induce a lymphoproliferative response to MHV 3

Kupffer cells express the Ia-Ag [12] and may act as accessory cells in contributing to the local immune response in acute stages of liver diseases. It may be imagined that in Balb/c susceptible mice this function is diminished, thus leading to a decreased immune response. For this purpose we have examined the capacity of MHV 3 Ag-pulsed A/J and Balb/c Kupffer cells to induce proliferative responses in splenic lymphocytes from primed syngeneic mice. Kupffer cells pulsed with heat-inactivated MHV 3 at a multiplicity of 0.05 pfu/cell and washed to remove loosely bound Ag effectively triggered the splenic lymphocyte proliferative responses as shown in Table 2. The activity of the Kupffer cells from the A/J strain was similar to that of the Balb/c strain. A significant increase in ³H incorporation was found when Ag-pulsed Kupffer cells were incubated with primed syngeneic splenic lymphocytes.

| Kupffer cell strain | Spleen lymphocytes DNA synthesis b Expt. 1 Expt. 2 Expt. 3 |
|---------------------|-----------------------------------------------------------|
| Balb/c normal       | 740                                                       |
| immune              | 148                                                       |
| A/J normal          | 719                                                       |
| immune              | 6623                                                      |

a 2·10⁵ Kupffer cells/well cultured with 2·10⁵ splenic lymphocytes.
b Data are expressed as the difference in counts per minute between the means of triplicate cultures containing Ag-pulsed or control medium-pulsed Kupffer cells (± cpm). Results shown represent the mean of triplicate cultures. Standard deviations of the triplicate cultures were <15% and are not depicted.

5. DISCUSSION

Kupffer cell cultures provide a fruitful model for studying certain modalities of the inborn resistance of mice to MHV 3. Our results confirm that the genetically determined sensitivity of mice to MHV 3 infection is reflected in hepatic macrophages by an increased resistance to virus replication when these cells are activated by LPS. The efficiency of the antiviral state induced by LPS corresponds to the resistance and susceptibility shown by these strains in vivo.

Kupffer cells from the A/J and Balb/c strains of mice are both capable of responding to the LPS stimuli, as demonstrated by the synthesis of IL-1. Several experiments indicated that pretreatment with LPS induced an equivalent IL-1 production in Kupffer cells from A/J and Balb/c mice.

Our experiments were conducted with elevated concentrations of endotoxin, since the Kupffer cells are less sensitive to LPS in comparison to
peritoneal macrophages [10]. The inhibition of virus multiplication was dose dependent, the highest effect being obtained with 50–100 μg/ml. With higher doses of LPS this inhibition could no longer be observed, thereby suggesting that the relatively high concentration of LPS used in these experiments does not influence the viability of the cells as it allows the replication of the virus (data not shown). Although such high doses of LPS are never reached in vivo it must be kept in mind that, in the organism, other mediators such as interferon (released during the primary stages of viral infection) may decrease the threshold of sensitivity to LPS in the Kupffer cells. In vitro interferon does actually inhibit the multiplication of MHV 3 in Kupffer cells [13].

Our results corroborate recent data from our laboratory which demonstrated that A/J mice fed a hypercholesterolemic diet became susceptible to the virus and that, at the same time, the Kupffer cells lost their ability to be activated in vitro with LPS [14]. They correspond with the previous work of McNaughton and Patterson [15] who observed that MHV 3 replicated in A strain mouse macrophages, but they appear to be at variance with those of Virelizier and Allison [3] who suggested that macrophages from A/J mice were resistant to MHV 3 infection. This discrepancy could be due to the fact that the last authors used very low doses of virus. Likewise, Arnheiter et al. [2] demonstrated a restriction in viral multiplication for A/J mouse strain hepatocytes as compared with those of Balb/c.

Our results show that LPS-activated Kupffer cells from Balb/c mice infected with MHV 3 do not express their intrinsic antiviral activity as do the Kupffer cells explanted from the resistant strain. However, infection with MHV 3 does not impair the ability of the Kupffer cells to synthesize IL-1. More surprisingly, our data demonstrate that MHV 3 stimulates IL-1 production by Kupffer cells in vitro. Although viruses have long been considered to be effective inducers of IL-1, the data regarding virus-induced IL-1 production have been almost entirely derived from early animal studies [16]. To our knowledge, the only report of the ability of viruses to stimulate IL-1 production in vitro comes from Roberts et al. [17] who demonstrated the capacity of influenza virus to stimulate human IL-1 production in human macrophages. The finding that MHV 3 induces the synthesis of IL-1 in a dose-dependent manner is intriguing. IL-1 in the surrounding tissues is known to contribute to acute pathological changes such as cellular infiltration and hyperemia [16,18]. Recently, it has been shown to act directly and selectively on cultured vascular endothelial cells [18,19]: first, IL-1 induces endothelial biosynthesis and surface expression of a tissue factor-like procoagulant activity; second, IL-1 dramatically increases the adheriveness of the endothelial cell surface for peripheral blood polymorphonuclear leukocytes and monocytes.

The fact that (i) in nonactivated Kupffer cells from A/J mice viral multiplication is delayed, (ii) activation of Kupffer cells leads to a restriction in the multiplication of MHV 3 in A/J but not in Balb/c mice, and (iii) MHV 3 infection induces the synthesis of IL-1 by LPS-activated Kupffer cells in a dose-dependent manner, would suggest that a high replication of MHV 3 virus in susceptible mice may play a doubly crucial role, first, by a direct cytopathic effect on liver cells, and second, by an indirect induction of IL-1 synthesis. We report that indomethacin treatment alters the innate resistance of A/J mice. Results recently published by Abecassis et al. [20] have demonstrated that prostaglandin E 2 prevents fulminant hepatitis in susceptible Balb/c mice. Since indomethacin is known to block the synthesis of prostaglandin E 2, which is an inhibitor of IL-1 activity, these data further bear out the role of IL-1 in the pathogenesis of MHV 3 infection of mice. Thus, resistance to an acute infection appears to depend to a large extent on the replication potential of MHV 3 in cells of the reticuloendothelial system.

Le Prevost et al. [21–23] have shown that various manipulations affecting the host defence system may drastically enhance the susceptibility of genetically resistant mice. When the cellular or the humoral immune system is crippled by such non-selective methods as whole body irradiation or treatment with antilymphocyte serum, resistance can be abrogated. Under these conditions mice die with specific lesions of the liver and high virus titers. It is known that X-irradiation causes a
lymphoid depletion but also an impairment of the macrophage immunogenic function. It does not suppress the antigen uptake by macrophages but interferes with the processing stage. However, our results do in fact demonstrate that in genetically susceptible mice the Kupffer cells are perfectly able to process the MHV 3-Ag in a way which efficiently triggers off splenic lymphocyte proliferation. These data emphasize the role of the resistance of Kupffer cells to MHV 3 replication: a delayed viral replication in A/J resistant mice opens the way for the induction of the immune response to take place.

The data presented in this report show that the in vivo pattern of resistance to MHV 3 bears out the expression of the intrinsic antiviral activity of the hepatic macrophages. The in vitro induction of IL-1 by murine hepatitis virus type 3 is dose-dependent and could explain the acute changes in the microcirculation of the liver in inbred strains of mice following infection. The collaboration between the Kupffer cells and the immunocompetent lymphocytes is performed perfectly in A/J and Balb/c mice: (i) activated Kupffer cells synthesize IL-1, and (ii) they trigger lymphocyte proliferation when pulsed with heat-inactivated MHV 3-Ag. Our findings, together with the results published by other investigators [5,7,23] suggest that the hepatic macrophages play a doubly crucial role in the pathogenesis of MHV 3. First, in resistant strains of mice, they have the ability to delay viral growth, and second, in both strains of mice following infection. The collaboration between the Kupffer cells and the immunocompetent lymphocytes is performed perfectly in A/J and Balb/c mice: (i) activated Kupffer cells synthesize IL-1, and (ii) they trigger lymphocyte proliferation when pulsed with heat-inactivated MHV 3-Ag. Our findings, together with the results published by other investigators [5,7,23] suggest that the hepatic macrophages play a doubly crucial role in the pathogenesis of MHV 3. First, in resistant strains of mice, they have the ability to delay viral growth, and second, in both strains of mice, their infection by MHV 3 induces the synthesis of IL-1 in a dose-dependent manner. These data are corroborated by the dramatic effect observed after in vivo treatment of MHV 3-infected A/J resistant mice with indomethacin.

Histological and biochemical examinations of the liver, lung, spleen and kidney from indomethacin-treated mice will be undertaken to give further evidence of this hypothesis.

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