Protection of Mice Against Mouse Hepatitis Virus by Corynebacterium parvum

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C57BL/6 mice that are highly susceptible to infection with mouse hepatitis virus type 3 were protected against intraperitoneal viral infection by simultaneous intraperitoneal injection of Corynebacterium parvum. No protection was observed when C. parvum was given intravenously or when it was injected intraperitoneally 3 days before viral infection. Protective effects were, however, consistently found when C. parvum was given 2 h before or 2 h after viral infection. Activity was seen only against 10 50% lethal doses and not against 100 50% lethal doses. C. parvum also caused a significant decrease of virus yield in cultures of peritoneal exudate cells infected with mouse hepatitis virus type 3. These data suggest a direct effect of C. parvum on virus-susceptible cells. Injection of C. parvum in mice caused activation of natural killer (NK) cells and of interferon production. However, these two effects were equally demonstrable at high and low doses of C. parvum, whereas protection against mouse hepatitis virus type 3 was not demonstrable at low doses of C. parvum. Thus, antiviral protection may be dissociated from activation of NK cells and induction of interferon.

We have reported that a preparation of killed Corynebacterium parvum cells protected mice against lethal infection with herpes simplex virus (HSV) (10). Similar observations have been made independently by other groups which treated various experimental viral infections with C. parvum or other bacteria, e.g., Bacille-Calmette-Guerin (3, 6, 16, 22). In our model, C. parvum had to be given several days before viral infection to achieve protection (13). Recently, Budzko et al. (2) reported that C. parvum protected mice against Junin virus infection when the bacteria were given on the day of virus infection but that C. parvum injected before infection was not effective. In this paper, we present some data on the protective effect of C. parvum on mice infected with mouse hepatitis virus type 3 (MHV3). Similarly to the Junin Virus system, protection of NHV3-infected mice was achieved when C. parvum was administered simultaneously with the virus infection.

MATERIALS AND METHODS

Mice. C57BL/6J BOM mice were obtained from Gl. Bomholtgard Ltd. (Ry, Denmark). Only male mice of 10 to 12 weeks of age were used in the experiments described. Experimental groups consisted of 20 mice which were housed in groups of 10 in two separate cages.

Virus. MHV3 originally obtained from B. H. Hübner, Baltimore, Md., was passaged in 3-week-old CF-1 mice (Winkelmann, Bochum, Germany) by intraperitoneal (i.p.) infection. Livers from dead mice were pooled, and a homogenate was prepared in buffer and frozen at −70°C. The 50% lethal dose (LD50) of this material for 8-week-old C57BL/6 mice was 10−2.5. When assayed in cultures of peritoneal exudate cells (PEC) of C57BL/6 mice, the cytopathic effect occurred up to a dilution of 10−7. Thus, the macrophage infecting dose (MID) of the virus was considered to be 103.

Virus titration. PEC were obtained by rinsing the peritoneal cavities of untreated C57BL/6 mice with balanced salt solution. Cells were cultured for 2 days at a cell concentration of 3 × 104/ml in 0.2 ml of RPMI 1640 medium supplemented with 5% fetal bovine serum in the wells of a 96-flat-bottom-well plate (Falcon Plastics, Oxnard, Calif.). They were then washed with balanced salt solution and infected with serial 10-fold dilutions of the virus material. After 4 more days of incubation at 37°C, the plates were examined for the formation of giant cells which are readily detectable by microscopic screening of the tissue culture well. The viral titer is expressed as the reciprocal value of the virus dilution that still caused this effect.

C. parvum. C. parvum (strain CN 6134, Burroughs Wellcome, Beckenham, England) was a formalinkilled suspension of bacteria containing 7 mg of protein per ml and 0.01% thiomersal. The bacteria were washed three times by centrifugation and resuspended in sterile saline.

Experimental schedule. For in vivo experiments, C. parvum was injected i.p. at a dose of 700 µg simultaneously or at various times before or after i.p. infection of mice with 10 LD50s of MHV3. The number of dead mice was recorded daily for 10 days. Deaths that occurred were due to histopathologically proven hepatitis.

For in vitro experiments, PEC were obtained by
lavage from the peritoneal cavity of C57BL/6 mice. They were cultivated as described above for virus titration but C. parvum was added to the experimental cultures in addition to MHV3. Viral titers were read after 4 days.

Activation of natural killer (NK) cells and induction of interferon production by injection of C. parvum. Mice were injected with different doses of C. parvum, and after 24 h PEC were washed out and tested for both NK cell activity and interferon production with methods previously described (4). Briefly, NK cell activity was measured in a 4-h assay against 51Cr-labeled YAC-1 lymphoma cells. Results were expressed as percent specific release. Parallel sets of cultures were cultivated at 37°C for 24 h without addition of any further stimulant. The cell-free supernatant was then recovered and frozen at -70°C until testing for interferon was performed. The interferon assay was a standard assay with L cells and vesicular stomatitis virus and international standards (25). Results were expressed as international units per ml.

RESULTS

Protection of mice against MHV3 infection by injection of C. parvum. In initial experiments, we injected C. parvum at various times before experimental infection of the mice with MHV3. There was no protection when C. parvum was given 3 days before MHV3 infection (Table 1). However, mice were protected when C. parvum was administered simultaneously with MHV3. Protection was observed equally when C. parvum was given 2 h before or 2 h after viral infection and when C. parvum was injected i.p. but not after intravenous injection. Washed bacteria caused the same degree of protection as did the regular vaccine containing thiomersal (data not shown). Finally, protection was dependent on the dose of challenge virus. Protective effects were observed when the mice were injected with 10 LD50. However, no protection occurred at 100 LD50.

In vitro effect of C. parvum on the replication of MHV3 in mouse peritoneal cells. The preparation of MHV3 used in our studies caused a formation of giant cells in PEC of C57BL/6 mice up to a dilution of 10^-7. Thus, the MID was 10^7. When 140 μg/ml of C. parvum was added 1 h before viral infection, the viral titer was reduced to 10^6 MID (Table 2). The same effect was observed when C. parvum was injected in vivo, 1 h before recovery and infection of PEC.

Activation of NK cells and induction of interferon by different doses of C. parvum. Mice were injected with different doses of C. parvum, and protection against MHV3 was tested. PEC were washed out from a parallel group of mice, and NK cell activity and interferon production were assayed. Interestingly, the latter two phenomena were equal in mice in-
Therefore, evidence suggested that the targets of interferon inducers (5). In contrast, in recent studies from our laboratory it has been shown that the titers of serum interferon in mice injected 7 days previously with C. parvum and challenged with HSV do not differ from control mice that received HSV only (H. Kirchner, unpublished data).

C. parvum represents one of the most powerful stimulants of the lymphoreticular system of mice (see reference 21 for a review). A multitude of effects on different cells of the lymphocyte-macrophage system has been ascribed to the activity of C. parvum, e.g., activation of macrophages (7) and NK cells (17). On the other hand, certain lymphocyte functions, such as mitogen responses or lymphocytotoxicity, were depressed in spleen cell cultures of C. parvum-treated mice (9, 11, 20). Interestingly, 7 days after injection with C. parvum, NK cell activity was also depressed (18, 19). We and others have therefore stressed that compounds such as C. parvum should be called immunomodulators since they have both suppressing and stimulatory capacities. However, protection against viral infections may represent immunopotentiation in a true sense.

The reasons why C. parvum protects mice from infection with MHV3 are not known. Two lines of evidence suggested that they are different from those that cause the previously described protection against HSV. First, protection could be observed when C. parvum was given simultaneously with MHV3, whereas under these conditions no protection was observed against HSV. Second, i.v. injection of C. parvum did not protect mice against MHV3 infection. However, protection against HSV occurred regardless of the route of C. parvum infection. Therefore, protection against HSV may be caused by a systemic effect, whereas local effects may be predominant in the protection against MHV3.

Differences in genetic susceptibility and pathogenesis between HSV and MHV3 may be important in this context. C57BL/6 mice are resistant to infection with HSV (12, 14), but highly susceptible to infection with MHV3 (1). Adult mice of strains susceptible to HSV infection die from encephalitis 7 to 10 days after i.p. infection (14). In contrast, after i.p. infection with MHV3 adult mice die from fulminant hepatitis after 3 to 5 days (1).

Previously, it has been assumed that newborn, but not adult mice, are susceptible to HSV infection and that the resistance of adult mice was caused by the capacity of their macrophages to actively restrict HSV replication (23). However, the situation appears to be more complex since it is now well established that adult mice of certain inbred strains are fully susceptible to HSV infection (12, 14). Nevertheless, the percentage of macrophages that may be infected by HSV in vivo (in the peritoneal cavity) or in vitro appears to be very low (15, 22). In contrast, MHV3 readily replicates to high titers in macrophages of genetically susceptible mice, such as C57BL/6, both in vivo and in vitro, and the replication in PEC seems to play the key role in the in vivo pathogenesis of the virus (1).

Presently, there are many possibilities to explain the protective effect of C. parvum against infection with MHV3. C. parvum induces interferon (8) and activates NK cells (18). Its activating effect on macrophages has been established for some time (21). In our laboratory we are probing these various effects of C. parvum, but their respective contribution to the protective effect of C. parvum against MHV3 remains to be further investigated. However, we have shown in this paper a clear-cut dissociation between antiviral protection on the one side and activation of NK cells and induction of interferon production on the other side. Low doses of C. parvum did not protect against MHV3 infection but yet efficiently induced these two defense mechanisms. Therefore, by exclusion, we tend to believe that C. parvum does not protect via one of these two mechanisms. Perhaps, and this conclusion is supported by our in vitro data, C. parvum protects by a direct effect on macrophages known to represent the targets of MHV3 replication (1, 24).

**LITERATURE CITED**


