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Protection of Mice against Infection with Mouse Hepatitis Virus Type 3 by Injection of Silica

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Abstract

Injection of silica did not brake the resistance against MHV3 conferred to C57BL/6 mice by injection of C. parvum. However, silica itself had a marked protective effect against MHV3 infection that was maximal when injecting 1 mg 2 hrs before virus infection. The protective effect of silica was observed in a number of inbred mouse strains that differ in their relative resistance to MHV3 infection. No viral titers were observed in the spleen and liver of mice which had received MHV3 plus silica, whereas high titers were observed in the virus-infected controls. Injection of silica caused a marked decrease in the number of esterase-positive macrophages in the peritoneal wash-out population, that may be compatible with the possibility that the cause of the protection is the depletion of target cells for the viral infection. This latter effect, however, was short-lived and 24–48 hrs after injection of silica, high numbers of esterase-positive cells were again observed. This may explain why only little protection was observed when silica was administered 2 days before virus infection.

Introduction

Mouse hepatitis virus type 3 (MHV3) is a coronavirus naturally occurring in mice that, upon systemic administration, causes lethal hepatitis within a few days (see references 14, 21 and 22 for reviews). The strong tropism of this virus for macrophages is well established (2). Thus, high virus titers after intraperitoneal (i.p.) infection are observed in the peritoneal wash-out fluid, presumably derived from viral replication in macrophages. We have, in addition, shown that MHV3 replicated to high titers in cultures of pure macrophages which are grown from mouse bone marrow using conditioned media (SCHINDLER, L. and KIRCHNER, H., manuscript in preparation).

However, in addition to serving as a target cell of viral replication, macrophages may have a role in antiviral defense by a variety of mechanisms, some of which are yet ill-defined (15). One of these may be the production of interferon (9). One way of blocking macrophage function in vivo is the injection of silica (1) and with certain viruses it has been shown that the injection of silica brakes antiviral resistance (24). We have recently observed that injection of *C. parvum* caused a protection of mice against

MHV3 infection (18). Since *C. parvum* is known to activate macrophages (19), we have speculated that macrophage activation may be related to antiviral protection. Therefore, we have attempted to brake the antiviral effect of *C. parvum* by injection of silica. These experiments were not successful, but, unexpectedly, we observed that silica had a protective effect against MHV3 infection by itself.

Materials and Methods

Virus

Our starting virus material has been described (18). This virus was replicated in macrophage cultures derived from peritoneal exudate cells (PEC) of C57BL/6 mice resulting in a virus pool the titer of which was 10^6 MID (macrophage infecting doses; see below). One pool of virus was used in almost all experiments. In a single series of experiments (Table 2) an additional preparation was used which was kindly provided by Dr. H. Wege (Würzburg, FRG). It was grown in Sac (−) cells, (a Moloney sarcoma virus transformed cell line) and contained 4.7×10^5 $TCID_{50}$ (tissue culture infective doses) per ml.

Virus titrations

In vitro cultures of BM macrophages were set up according to a protocol described previously (11). In brief, BM cells were obtained by flushing femurs and tibias with Balanced Salt Solution (BSS). A single suspension was prepared by passage through a 25-gauge needle. The cells were plated at densities of 200.000/ml in 24-well plates (Cole 76-003-05, Linbro, Flow Labs, Bonn, FRG) and cultured in Dulbecco's Minimal Essential Medium (D-MEM, Seromed, München, FRG) supplemented with 15 % heat inactivated fetal bovine serum (FBS), 5 % heat inactivated horse serum (Seromed, München, FRG), 1 % essential and non-essential amino acids 200 mM L-glutamine, 50 µl/ml gentamycin (Gibco, N. Y., USA) and 20 % L-cell conditioned medium (the conditioned L-cell medium was prepared in our own laboratory). BM cells were refed daily starting on day 5 and were cultured for a total of 8 to 10 days.

BM macrophages can be grown easily in large numbers and their yield is considerably higher than the yield of peritoneal macrophages from the same number of mice. In BM cultures grown in L cell-conditioned medium, the number of morphologically defined macrophages increased linearly until days 8 to 10 and then remained constant. From day 10 on 100 % of the cells were macrophages, according to the criteria of phagocytosis, adherence to plastic surfaces and to non-specific esterase staining. These data (that are not extensively documented here) are in full accordance with the data of KLIMETZEK and REMOLD (11).

For titration of the virus, BM cells were grown in 24-well plates for 8 to 10 days. After this time, the cells were infected with MHV3 and incubated for an additional 3 days. MHV3 causes the formation of giant cells in these cultures, subsequently referred to as CPE. Virus titers are expressed as the reciprocal value of the virus dilution that still caused this effect (also designated MID = macrophage infecting dose).

Mice

Male mice of several inbred strains were obtained from Bomholtgard (Ry, Denmark) at the age of 8 weeks and used in the experiments within the subsequent 4 weeks.

Reagents

Silica (DQ 12 < 5 µm) was obtained from Dr. M. Reisner (Steinkohlebergbauverein, Postfach 13 01 40, 4300 Essen 13, FRG). It was suspended in sterile Hanks' Balanced Salt Solution.

C. parvum (strain CN 6134, Burroughs Wellcome, Beckenham, England) was a killed suspension of bacteria containing 7 mg of protein per ml and thiomersal. In the experiments described here, 700 µg of C. parvum were injected 2 hrs before virus infection.

Experimental protocol

Mice were infected intraperitoneally (i.p.) with MHV3 (in most experiments, with a dose equivalent to 10 LD_{50} for the given mouse strain). The tested compounds were injected i.p. at various times before or after virus infections. Dead mice were recorded twice daily of several days. Since all mortality occurred between days 3 and 5, the experiments were terminated after 7 days.

Esterase staining

In order to identify the percentage of macrophages in the peritoneal wash-out population, the method of esterase staining was used as described by KOSKI et al. (10). Peritoneal macrophages show a dark-red cytoplasma when stained by this method, whereas other cell types (lymphocytes) either remain unstained or show a slight green colour after counterstain with methyl green.

Results

Lack of effect of silica on C. parvum-mediated protection against MHV3 infection

C57BL/6 mice that are highly susceptible to infection with MHV3 were infected i.p. with 10 LD_{50}. As previously reported (18), injection of 700 µg of *C. parvum* simultaneously with the virus caused a protection of 90 % of the mice (Table 1). This protection was not affected when 1 mg silica was injected 2 hrs before *C. parvum* (plus MHV3). Unexpectedly, however, the dosage of silica in the control group caused a protection against MHV3. The experiments described in the following paragraphs were undertaken to analyze this protective effect of silica against the lethal outcome of the i.p. infection with MHV3.

Protection of C57BL/6 mice against MHV3 by injection of silica

A range of doses of silica were injected i.p. 2 hrs before i.p. infection with MHV3, and tested for their protective effect. As can be seen in Figure 1,

Table 1. Effect of Silica and of C. parvum on MHV3- induced Lethality in C57BL/6 Mice (intraperitoneal route of infection, virus dose 10 LD_{50})

Treatment Schedule	Dead Mice/Group
MHV3 alone	19/20
C. parvum plus MHV3 (simultaneously)	2/20
Silica (2 hrs before) + C. parvum + MHV3	2/20
Silica 2 hrs before MHV3	4/20

Dose of silica 1 mg/mouse, dose of C. parvum 700 µg/mouse; in controls it was shown that neither compound alone caused any lethality.

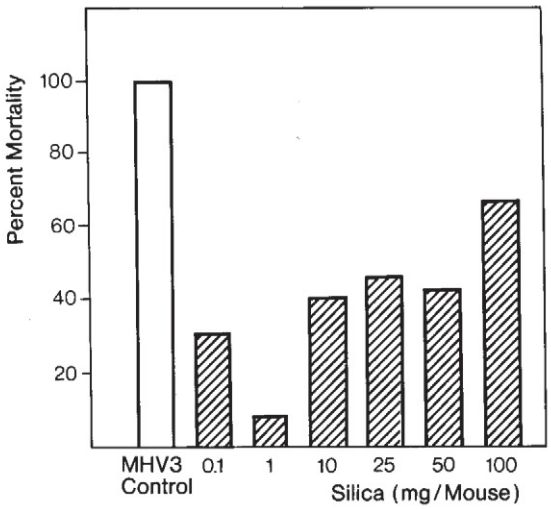

Fig. 1. Protection of C57BL/6 Mice Against MHV3 Infection by Simultaneous Injection of Different Doses of Silica. Mice were infected with 10 LD_{50}.

protection was optimal when a dose of 1 mg was injected. This dose subsequently was used in all experiments.

Figure 2 shows that equal protection was observed when 1 mg of silica was injected at different times before virus infection or simultaneously with the virus. However, injecting silica more than 72 hrs before virus infection had only marginal protective effects, if any.

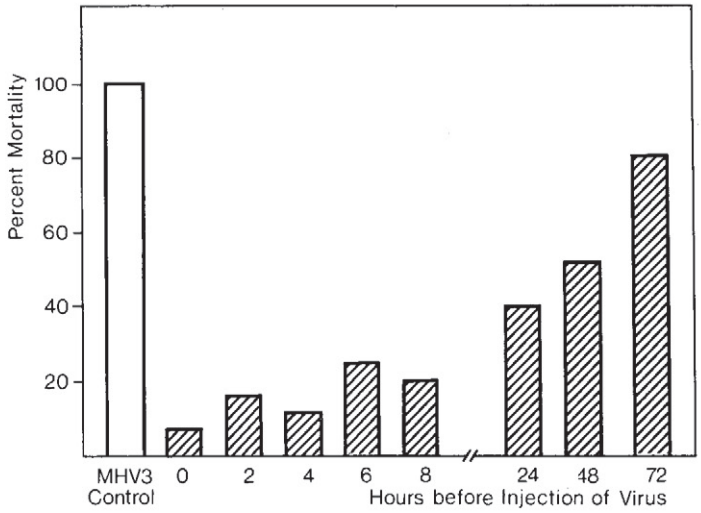

Fig. 2. Protection of C57BL/6 Mice Against 10 LD_{50} of MHV3 by i.p. Injection of 1 mg of Silica at Different Times before i.p. Infection.

Table 2. Effect of Silica[a] against Different Doses of MHV3 in C57BL/6 Mice

Virus Dose (LD_{50})	Silica Injected	Dead Mice/Group
10	no	18/20
(regular virus pool)	yes	2/20
10^2	no	20/20
	yes	12/20
10^3	no	20/20
	yes	19/20
10[b]	no	18/20
	yes	2/20

[a] 1 mg silica per mouse was injected 2 hrs before virus infection, both silica and the virus were given i.p.

[b] This virus preparation was obtained from Dr. Wege (see Materials and Methods). This was the only experiment in which our regular virus pool was not used.

In still additional experiments, we injected 1 mg of silica 2 hrs before virus infection and varied the dose of MHV3 (Table 2). As above, a marked effect was observed when 10 LD_{50} were injected. Protection could also be observed against 100 LD_{50}, but there was no protection against higher virus doses. In this set of experiments, we have also tested another isolate of MHV3 and have obtained the same results as with the isolate which we used routinely.

Finally, we have shown that intravenous injection of silica is not protective against i.p. infection with MHV3, indicating that protection is not due to systemic effects but rather represents a local phenomenon (data not documented).

Determination of viral titers

In C57BL/6 mice after i.p. infection with 10 LD_{50} MHV3, high virus titers are observed in the peritoneal wash-out fluid, in the spleen, and in the

Table 3. Virus Titers in Different Organs of C57BL/6 Mice after i. p. Infection with 10 LD_{50} MHV3

Material Tested	Silica Injected[a]	Virus Titer[b]
Peritoneal wash-out fluid	no	10^5
	yes	< 10
Liver tissue homogenate	no	10^7
	yes	< 10
Splenic tissue homogenate	no	10^7
	yes	< 10

[a] 1 mg silica per mouse was injected simultaneously with the virus.

[b] MID per ml of test material, testing was performed in cultures of bone marrow-derived macrophages of C57BL/6 mice.

Table 4. Estimation of the Percentage of Macrophages by Esterase Staining in the Peritoneal Wash-out Fluid of C57BL/6 Mice after Injection of Silica

Material Injected	Time After Injection (hrs)	Percentage of Positive Cells
Silica (1 mg)	4	8[a]
	24	30
	48	40
Silica plus MHV3 (10 LD_{50})	4	1
	24	20
	48	60
PEC of untreated mice		52

[a] Note that this number may represent an overestimation since esterase-positive cells by morphology did not represent typical macrophages, and their staining pattern was weak.

liver (Table 3). However, no virus could be detected at these sites in mice injected with 1 mg silica 2 hrs before infection.

Estimation of the number of peritoneal macrophages after injection of silica

C57BL/6 mice were injected with 1 mg of silica and the number of macrophages in the washed-out PEC was determined by esterase staining (Table 4). It can be seen that there was a drastic drop in esterase-positive cells already at 4 hrs after injection of silica. The number of positive cells that represented about 50 % in untreated control mice dropped to about 8 %. Even these, by morphology, were not typical large macrophages but

Table 5. Effect of Silica (1 mg/Mouse) on MHV3 Infection of Different Strains of Inbred Mice

Mouse Strain	Virus Dose[a]	Silica Injected	Dead Mice/Group
DAB/2	10^2	no	20/20
		yes	1/20
C57BL/6	10^2	no	19/20
		yes	2/20
A/J	10^5	no	18/20
		yes	12/20
	10^4	no	19/20
		yes	6/20
(C57BL/6 × A/J)F_1	10^2	no	18/20
		yes	0/20
	10^3	no	19/20
		yes	7/20

[a] The virus dose is expressed in MID (which were based on titration of the virus pool in C57BL/6 bone marrow macrophages).

rather small atypical cells, perhaps macrophage precursors and showed only a weak staining pattern (latter data not shown).

Testing of additional strains of mice

All experiments reported thus far have been performed with C57BL/6 mice which are highly susceptible to infection with MHV3. In additional experiments, mice of other strains or F1 hybrids were tested (Table 5). As can be seen, all mice could be protected against lethal doses of the virus by injection of 1 mg of silica 2 hrs before virus infection.

Discussion

In mouse models of viral infection, and probably in virus infections of man, macrophages may have a pivotal role. In certain systems, it has been shown that they are involved in antiviral defense, for example, in a mouse model of infection with Herpes Simplex Virus (HSV) – (8). These findings have been reiterated by our recent data, that endogenously produced interferon may play a role in resistance (6), and that macrophages are the producer cells of this interferon (9).

On the other hand, macrophages are important target cells of a number of different viruses, including HSV (13), Lactatdehydrogenase Virus (LDV) – (5) and also MHV3 (2). Thus, viral replication in macrophages represents a key element in viral pathogenesis. This is certainly true for MHV3 for which it was shown that the genetic control of viral resistance is expressed on the level of the macrophage (2). It is, however, not firmly established if macrophages play a role in antiviral resistance in the MHV3 system.

In recent studies, we (10) and others (7, 17, 20) have tested »immunostimulatory« bacteria for their effects in experimental viral infections. In our studies, we have found *C. parvum* to be active against HSV infection when given several days before the virus. In contrast, protection against MHV3 was observed when *C. parvum* was injected on the day of virus infection (18). The reasons for protection in the latter system were not understood, but one of the most prominent effects of *C. parvum* is the activation of macrophages (19). Thus, we have made an attempt to reverse the protective effect of *C. parvum* by injection of silica, an agent known to be toxic for macrophages (1). This approach unexpectedly has yielded the result that silica itself had a protective effect against MHV3. Again, the time relationship was such that silica protected when given close to the time point of virus infection.

Another virus that replicates in mouse macrophages is LDV, and DUBUY (3) has done experiments similar to ours. His experiments were initiated under the assumption that silica, by killing the macrophages, will deplete the peritoneal cavity of macrophages and thus, be protective. However, in the LDV system, there was no protection by silica. In addition, it was

shown that, although silica was toxic for macrophages, it additionally caused the rapid influx of new macrophage precursors into the peritoneal cavity. BUTZKO et al., however, found that infection of silica was capable of protecting mice against infection with Junin Virus (3). However, the reasons for this protection were not elaborated on.

Yet, it has to be added that in the system of HSV infection of mice, also where virus replication occurs in peritoneal macrophages, resistance is broken by injection of silica (24). These data were reproduced in our laboratory using C57BL/6 mice and the same batch of silica used as in the present study (unpublished data). Thus, as far as the in vivo effects of silica are concerned, a complex picture emerges from the data in three different virus systems.

The protective effect of silica is not caused by a direct effect on MHV3 since incubation of MHV3 with silica in vitro, followed by low-speed centrifugation, did not impair infectivity of the virus which remained in the supernatant (unpublished data). Also, the protective effect does not appear to involve host genetics, since highly susceptible C57BL/6 mice were equally protected as resistant A/J mice that, in order to evoke lethality, had to be injected with much higher virus doses. Finally, protection appears to represent a local phenomenon and not a systemic effect of silica since intravenous injection of silica was not protective against i.p. virus infection.

Investigating the number of esterase-positive cells in the peritoneal wash-out fluid, we found that there was a marked decrease (8 % as compared to 45 % positive cells in control mice). Perhaps this decrease was even more pronounced since the remaining positive cells by morphology were not typical macrophages and were only slightly stained. Our data may be – in contrast to the data of DUBUY (3) – still compatible with the conclusion that the protective effect of silica is caused by killing of the target cells for replication of MHV3. Depletion of esterase-positive cells after injection of silica was only short lived and after 24–48 hrs, high numbers of positively staining cells were found again. This result may explain why only little protection is observed when silica is injected 48–72 hrs before infection.

However, the protective effect of silica in its time relationship was similar to the one of *C. parvum* (18) and glucan (23). These two compounds certainly do not kill the macrophages in the peritoneal cavity (unpublished data), but both compounds cause, among other effects, enhanced macrophage phagocytosis. It will have to be determined whether there is a common mechanism underlying the protective effect of these two compounds and of silica on MHV3 infection.

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