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BONE-MARROW DERIVED MACROPHAGES AS TARGETS FOR THE REPLICATION OF MOUSE HEPATITIS VIRUS TYPE 3

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1. Summary

Bone-marrow (BM) derived macrophages are sensitive target cells for replication of mouse hepatitis virus type 3 (MHV3). These cells can be grown in large numbers and the percentage of defined macrophages increased until day 10 when 100% of the cells represented macrophages. MHV3 replicated within these cells to high titers and caused the formation of multi-nucleated giant cells. This effect was seen with very low virus inocula in BM macrophages of C57BL/6 mice that are highly susceptible to *in vivo* infection with MHV3 whereas macrophages from resistant A/J mice did not show a cytopathic effect at these virus doses. 1000-fold higher virus doses, however, caused the cytopathic effect in macrophages of both C57BL/6 and A/J mice.

2. Introduction

Mouse hepatitis virus type 3 (MHV3), a member of the genus coronavirus [1] produces a characteristic cytopathic effect (CPE) in mouse peritoneal exudate cells (PEC) by fusing the macrophages into multi-nucleated giant cells [2]. The appearance of this CPE closely parallels viral replication and thus provides a useful tool for *in vitro* studies of virus macrophage interactions [3,4]. However, mouse PEC represent a heterogenous cell population [5]

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and divergent data have been reported on genetic differences between the capacity of PEC from different inbred mouse strains [6-8]. We have therefore used *in vitro* cultivated bone marrow (BM) macrophages for replication and titration of MHV3. These can be readily grown in large numbers and with a high degree of purity. MHV3 replicated to high titers in these cultures. The purpose of this report is two-fold: (a) we wish to report the usefulness of this tissue culture system for the replication of MHV3, and (b) we have reexamined the differences of *in vitro* MHV3 replication in macrophages derived from different inbred mouse strains.

3. Material and Methods

3.1. Virus

MHV3 originally a gift of R. J. Huebner (Baltimore, MD, USA) to Dr. G. Streissle (Wuppertal, F.R.G.) was passaged in 3-wk-old CF-1 mice (Winkelmann, Bochum, F.R.G.) by intraperitoneal (*i.p.*) infection. Livers from dead mice were pooled, and a homogenate was prepared in buffer and frozen at -70°C . The LD_{50} of this material for 8-wk-old C57BL/6 mice was 10^2 MID, and the LD_{50} for 8-wk-old A/J mice was 10^5 MID (see section 3.5 for explanation of the term MID).

3.2. Mice

Male A/J mice were obtained from Jackson Laboratories (Bar Harbor, ME, U.S.A.) and male C57BL/6 mice from the Gesellschaft für Versuch-

stierkunde (Hannover, F.R.G.). All mice were obtained at the age of 8 wk and used in the experiments within 3 to 6 wk of purchase. Upon arrival, they were tested for serum antibodies against MHV3 by indirect immunofluorescence (courtesy of Dr. W. Nicklas, German Cancer Research Center, Heidelberg, F.R.G.). The mice used in this study were consistently negative.

3.3. Cultures of BM macrophages

In vitro cultures of BM macrophages were set up according to a protocol described previously [9]. In brief, BM cells were obtained by flushing femurs and tibias with Balanced Salt Solution (BSS). A single cell suspension was prepared by passage through a 25 gauge needle. The cells were plated at densities of 200,000/ml in 24-well plates (Code 76-003-05, Linbro, Flow Labs, Bonn, F.R.G.) and cultured in Dulbecco's Minimal Essential Medium (D-MEM, Seromed, München, F.R.G.) supplemented with 15% heat inactivated fetal bovine serum (FBS), 5% heat inactivated horse serum (Seromed, München, F.R.G.), 1% essential and non-essential amino acids, 200 mM L-glutamine, 50 µg/ml gentamycin (Gibco, NY, U.S.A.) 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 8–10 days.

3.4. Cultures of PEC

PEC were obtained by rinsing the peritoneal cavity and cultured at a concentration of 3×10^6 /ml in 0.2 ml of medium RPMI 1640 supplemented with 5% FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine (Gibco, NY, U.S.A.) in the wells of flat-bottom 96 well plates (Code 3049, Falcon Plastics, Oxnard, CA, U.S.A.). These were incubated for 45 min at 37°C and rinsed three times with medium. The adherent cell population was further cultured for 2 days.

3.5. Titrations of MHV3

For titration of the virus, BM cells were grown in 24-well plates for 8–10 days. After this time the cells were infected with MHV3 and incubated for an additional 48 h. MHV3 caused 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).

4. Results

4.1. Characterization of the BM tissue culture system

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 8–10 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 previous data of one of us [10] and Falk et al. [11].

4.2. Comparison between PEC and BM macrophages of C57BL/6 mice

PEC or BM cultures of C57BL/6 mice were infected with 10 MID of the original virus pool. Culture supernatants were collected 4, 24, 48, and 72 h after infection and titrated in susceptible C57BL/6 BM cultures. The titration yielded titers of up to 10^9 MID in BM cultures of C57BL/6 mice after 48 h of culture and up to 10^7 MID in PEC cultures (Table 1).

4.3. Comparison of the CPE in BM macrophages of A/J and C57BL/6 mice

BM macrophages from mice of different strains at a comparable cell density were investigated for the appearance of multi-nucleated giant cells at different times after infection with graded dilutions of MHV3. Cultures derived from resistant A/J mice failed to produce giant cells except in cultures infected with high inocula of 10^4 MID or greater. In cultures in C57BL/6 macrophages, however, a CPE was observed after 48 h even when doses of 10 MID were inoculated (Fig. 1).

4.4. Virus replication in BM cultures of different strains of mice

In further experiments BM macrophages of A/J,

Table 1
MHV3 replication in bone-marrow (BM) macrophages and peritoneal macrophages (PEC) from C57BL/6 mice

Time after in vitro addition of MHV3 ^a	Virus titer ^b
I BM system	
4 h	∅
24 h	10 ⁷
48 h	10 ⁹
72 h ^c	10 ⁹
II PEC system	
4 h	∅
24 h	10 ⁵
48 h	10 ⁷
72 h ^c	10 ⁷

^a Virus dose: 10 MID.

^b Virus titers were tested in bone marrow cultures of C57BL/6 mice and are expressed as MID (see the definition in Material and methods).

^c Later than 72 h all cells were clumped and destroyed.

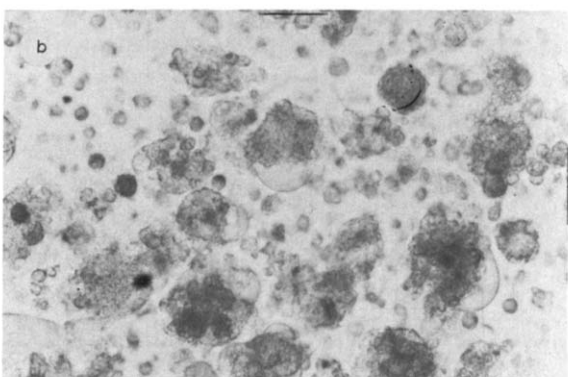
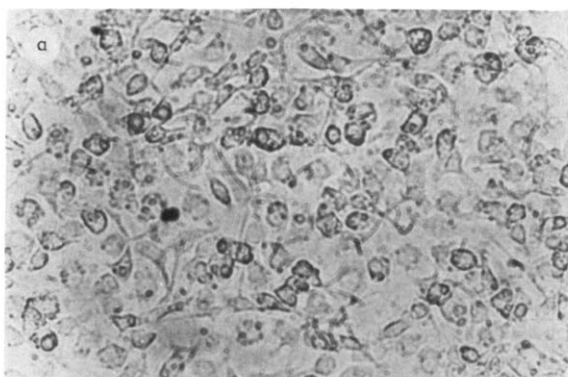


Fig. 1. Cytopathic effect (CPE) in MHV3 infected BM macrophages from C57BL/6 mice 48 h after infection. Magnification 150-fold; (a) uninfected cultures; (b) MHV3 infected cultures (CPE).

Table 2
MHV3 titers in BM macrophages of different strains of mice^a

Infecting dose (MID)	Mouse strain		
	A/J	C57BL/6	DBA/2
10 ¹	–	10 ⁵	10 ⁴
10 ²	–	10 ⁶	10 ⁵
10 ³	10 ²	10 ⁶	10 ⁶

^a The resulting virus titers expressed in MID were determined as described in Materials and methods.

C57BL/6 or DBA/2 mice were infected with graded doses of MHV3, and at 48 h the cell free supernatant fluids were recovered and tested for their viral titers as described in Materials and methods. As shown in Table 2, C57BL/6 and DBA/2 macrophages contained high virus titers in the supernatant fluid, whereas macrophages from A/J mice did not produce measurable titers of MHV3.

4.5. Testing of viral titers in the peritoneal wash-out fluid

In order to further document the applicability of the BM tissue culture system for viral titration, we have tested the virus titers in the peritoneal wash-out fluid of mice injected with MHV3. A/J or C57BL/6 mice were injected i.p. with 10³ MID and virus titers were determined at various times thereafter. Table 3 shows that there were marked differences in the virus titers between the two mouse strains. These were

Table 3
Virus titer in the peritoneal wash-out fluid of A/J and C57BL/6 mice after injection of 10³ MID^a of MHV3

Time after injection (h)	Virus titer ^{2b}	
	A/J	C57BL/6
5	–	–
24	–	10 ³
52	5 × 10 ⁴	5 × 10 ⁷
72	5 × 10 ⁵	5 × 10 ⁸
96	1 × 10 ³	^c

^a The virus dose injected was equivalent to 10 LD₅₀ for C57BL/6 mice.

^b Virus titers are expressed in MID = macrophage infecting dose.

^c Most C57BL/6 mice are dead at this time.

in the magnitude of $3 \log_{10}$ both 52 h and 72 h after infection. In further experiments (data not documented) we have also shown that virus titers in the blood and in homogenates of liver or spleen could be titrated readily in BM macrophages of C57BL/6 mice.

5. Discussion

In the murine model of infection with MHV3 resistance is genetically controlled. As established by Bang and Warwick [6] and confirmed by Virelizier and Allison [3] C57BL/6 mice are highly susceptible to MHV3 and die of hepatitis 3–4 d after infection. A/J mice, in contrast are resistant. Resistance is expressed on the level of the macrophages in that macrophages of resistant mice are unable to replicate MHV3. More recently, Macnaughton and Matterson [7] and Taguchi et al. [8] have studied the replication of MHV3 in mouse peritoneal macrophages and have observed virus replication both in macrophages of resistant and susceptible mouse strains. The reasons for the discrepancy between the data of Bang and Warwick and those of the other laboratories are not understood.

The PEC population is a mixture of lymphocytes, macrophages and small numbers of additional cells [5]. Since the lymphocytes by a variety of mechanisms, e.g. by interferon production, may massively interfere with virus replication, we feel that PEC are inferior for certain studies, and that for studies of virus macrophage interactions in the MHV3 system a culture of pure target cells is preferable. BM macrophages prepared as described in this study are pure macrophage cultures. Our data have shown that in A/J BM macrophage no CPE is formed at virus doses that readily cause a massive CPE in cultures of C57BL/6 mice.

Thus our data using BM macrophages have reiterated the results of Virelizier and Allison [3] that there are genetic differences in the *in vitro* replication of MHV3 in macrophages and that these differences reflect the *in vivo* situation. However, the block of replication in macrophages of A/J mice is

not absolute in that a CPE is observed when infecting with very high doses of MHV3.

Furthermore, our data have documented the usefulness of the BM macrophage system of C57BL/6 mice for titrations of MHV3. Although we have not explicitly compared our system to other titration systems [12–14] it appears obvious that the BM system represents a “physiological” system for studies of the interactions between MHV3 and macrophages. It is particularly useful since it consists of 100% macrophages and thus negative interference with other cell types (such as interferon production by lymphocytes) can be excluded.

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