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Maternally-derived passive immunity to enterotropic mouse hepatitis virus

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Summary. Maternally-derived antibody to enterotropic mouse hepatitis virus (MHV) strain Y was transferred to pups by both intrauterine (IgG) and lactogenic (IgA and IgG) routes. Antibody present in the gastric whey of pups suckling immune dams dropped to undetectable levels by weaning age (21 days post partum). MHV-specific IgG was found in the serum of passively immune pups up to 10 weeks of age. Immune dams transferred equal levels of antibody to 3 consecutive litters of pups, without evidence of decline. Immunoblots showed that IgA and IgG in whey and serum were directed against nucleoprotein N and glycoprotein S. MHV-specific IgM was not detected in any sample.

Introduction

Coronaviruses are important pathogens in many species of birds and mammals, including man. They are usually associated with either respiratory or enteric disease. Mouse hepatitis virus (MHV), family *Coronaviridae*, genus *Coronavirus*, is a common, highly contagious virus of laboratory mice. The MHV group contains numerous, closely related and highly mutable viruses with either respiratory or enteric tropism [3]. MHV is important both as a natural pathogen of laboratory mice and as a model of coronaviral infection, particularly enteritis in the neonate. As with other enterotropic coronaviruses, outbreaks of enterotropic MHV in naive mouse populations cause enteritis with high mortality among neonates. Older pups and adult mice show minimal or no apparent disease [4, 10]. Observations in enzootically infected mouse populations give reason to suspect that maternally-derived passive immunity is very significant in protecting newborn mice against apparent disease [5, 13, 15].

The passive transfer of immunoglobulins from dam to young in mice differs from that of most other mammals. IgG is absorbed in utero by means of specific receptors in the yolk sac wall, and is selectively absorbed postnatally for up to

16 days via IgG receptors in the intestine [6]. Pilot studies with MHV-Y, an enterotropic strain of MHV [1], have shown that pups born to immune dams and transferred to naive dams at 1 week of age, then challenged with MHV-Y, all developed enteritis. In contrast pups born to naive dams and foster-nursed by immune dams were protected against disease. This finding suggested that ingestion of immune milk is essential for passive immunity to enterotropic coronavirus infection and that locally active, intraluminal antibody such as IgA, might play an important role in protecting pups from enteritis. The purpose of the present study was to evaluate the route of transfer and the role of the different immunoglobulins in passive immunity against enterotropic MHV and to determine which viral proteins are recognized by these immunoglobulins.

Materials and methods

Virus

MHV-Y was isolated in NCTC-1469 cells during a natural outbreak of enteritis in infant mice [1], passaged in infant CD 1 mice and used in the form of a clarified intestinal homogenate. MHV-S was obtained from the American Type Culture Collection, Bethesda, MD and passaged twice in NCTC-1469 cells. NCTC-L 929 cell-adapted MHV-3 was obtained from the Institut für Labortierkunde, Universität Zürich, Switzerland and passaged in NCTC-L 929 cells.

Mice, inoculations and sample collections

Outbread CD 1 (Crl-CD 1 Br) albino mice were obtained from Charles River Breeding Laboratories, Portage, MI in filtered containers. All mice were kept in autoclaved micro-isolator cages (Lab Products, Maywood, NJ) containing pine shavings. Cages were changed aseptically in a laminar flow hood as previously described [2]. All animals were seronegative to MHV. Dams were orally inoculated with a single dose of MHV-Y containing 10^3 median neonatal enteritis doses of virus or with sterile tissue culture fluid (controls) and bred 10 days later. Pilot studies have shown that oral inoculation results in higher MHV antibody titers than parenteral inoculation and mimics more closely immunity in naturally infected animals. The 10 day interval between immunization and breeding ensured that the dams were not shedding virus when the pups were born (approximately 30 days after inoculation). Adult mice were killed with carbon dioxide gas and neonates by decapitation. Serial blood samples were taken with capillary tubes by periorbital puncture, under methoxyflurane (Metofane, Pitman-Moore Inc., Washington Crossing, NJ) anesthesia. Terminal blood samples were obtained from pups by decapitation and from dams by cardiac puncture. Whey was collected by removing the stomach curds from pups, suspending them in phosphate buffered saline (50% w/v) and centrifuging the homogenate at 12,000 × **g** for 60 min. The supernate was stored at − 20 °C.

Serology

Sera and whey were tested for MHV-specific IgG by an enzyme immunoassay (EIA) using MHV-S-infected formalin-fixed NCTC-1469 cells as antigen [18]. MHV-S is the prototype strain to which MHV-Y, which grows poorly in cell culture, is most closely related [1]. MHV-specific IgA was best detected with an emzyme-linked immunosorbent assay (ELISA) using purified MHV-3 as antigen. MHV-3 grown in NCTC-L 929 cells was pelleted at 95,000 × **g** for 2 h. The resulting pellet was resuspended in STE buffer (100 mM NaCl,

10 mM Tris, 1 mM EDTA), layered on a continuous sucrose gradient (50%/30% w/v) and centrifuged for 2 h at 95,000 × **g**. The visible band was collected, stored at − 70 °C and diluted in bicarbonate buffer (pH 9.6) for coating wells of 96-well microtiter plates (Dynatech Laboratories, Alexandria, VA). A peroxidase-labeled goat-anti-mouse IgA conjugate and ABTS substrate (both Kirkegaard and Perry Laboratories, Gaithersburg, MD) were used in the ELISA [12]. Sera of sentinel mice and of dams of replacement litters were screened for MHV antibody by indirect immunofluorescence assay [17]. Attempts to detect MHV-specific IgM used all of the assays described above.

Immunoblots were performed on nitrocellulose paper using MHV-S as antigen. MHV-S was propagated in NCTC-1469 cells, and clarified culture fluid was treated overnight at 4 °C with polyethylene glycol-6000 (8 g/100 ml) in the presence of NaCl (2.2 g/100 ml). The resulting precipitate was centrifuged at 6,000 × **g** for 60 min. The pellet was resuspended in STE buffer, and layered on a discontinuous gradient (70%/20% sucrose in STE buffer) and centrifuged at 95,000 × **g** for 90 min. The visible band was collected and stored in aliquots at − 70 °C. Purified MHV-S was boiled for 3 min in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and electrophoretically separated on a 12.5% acrylamide gel at 12 mA for 13 h. The proteins from the gel were then electroblotted onto nitrocellulose paper at 100 V for 6 h [20]. The nitrocellulose paper was stained with Ponceau-S, cut into strips along the visible lanes and saturated in blocking buffer (10% fetal calf serum) overnight. Nitrocellulose strips were twice washed for 10 min in TRIS-buffered saline, covered with test serum or whey at different dilutions and incubated for 3 h at 20 °C. After two more wash cycles, phosphatase-labeled goat anti-mouse IgG, IgA, or IgM conjugate was added and incubated for another 2 h at 20 °C followed by two 10 min washes. The strips were then stained with BCIP/NBT (5-bromo-4-chloro-3-indolyl-phosphate/Nitroblue tetrazolium) phosphatase substrate system (Kirkegaard and Perry Laboratories) and air dried.

Results

The isotype and titer of MHV immunoglobulins transferred in utero and in milk, as well as the duration of secretion during lactation were first examined. Immune and naive adult females were bred and allowed to whelp. Serum was collected from half of each litter immediately after birth before suckling to assess intrauterine transfer of passive immunity. The rest of the litter was sacrificed 24 h later to collect serum and whey, then replaced by a litter from a naive dam to maintain the nursing stimulus. On days 5, 10, and 20 post partum, the pups were removed for 2 h and replaced by naive pups which had previously fasted for 4 h. After feeding, serum and whey were collected from these pups. Sera and whey were tested for MHV-specific IgA, IgG, and IgM by EIA or ELISA. MHV-specific IgA and IgG but not IgM could be found in the milk of immunized dams throughout the 20 days of lactation (Table 1). During this period, IgA titers remained relatively constant, whereas IgG titers peaked at 5 to 10 days and declined by 20 days post partum. Sera of pups sacrificed immediately after birth (day 0) contained MHV-specific IgG, indicating in utero transfer of antibody (Table 2). Virus-specific IgM could not be detected at any time. In 7 of the 14 replacement litters used to collect whey on days 5, 10, and 20, IgG antibody could be found in the serum (data not shown). This showed that IgG can appear in the serum of naive pups after only 2 h of suckling on an immune dam.

Table 1. MHV antibody titers in gastric contents of naive pups nursing MHV-Y-immunized or naive dams for 2 h at intervals after parturition

Days post partum	Immune dams			Naive dams		
	n	IgA	IgG	n	IgA	IgG
1	5	139 (14)[a]	229 (40)	5	–[b]	–[c]
5	5	91 (24)	918 (160)	5	–	–
10	4	226 (46)	1131 (230)	5	–	–
20	5	242 (220)	527 (98)	5	–	–

[a] Reciprocal of geometric mean titer (standard deviation)
[b] < 1 : 10
[c] < 1 : 50

Toward the end of lactation, suckling activity declined and pups started to eat solid food. To find out how this change affected passive immunity, adult females were MHV-Y- or sham-inoculated and bred 10 days later. At 1, 2, 3, and 4 weeks of age, two pups from each litter were sacrificed and serum and stomach contents were collected and tested. MHV-IgG, but not IgA or IgM, could be found in the serum of pups suckling immune dams. MHV serum IgG titers peaked at 14 days (Table 2), when they reached levels higher than the ones found in their dams' sera collected immediately post partum. Serum IgG titers dropped markedly after weaning at 21 days (Table 2). MHV-specific IgM could not be detected in sera from either pups or dams. Stomach contents of pups suckling immune dams yielded constant IgG titers and gradually declining IgA titers up to the age of 14 days, but neither isotype of MHV antibody was detected at or beyond 21 days (Table 3). This coincided with the macroscopically obvious change of the stomach contents from milk curd to solid food mass.

Table 2. MHV IgG titers in serum of immunized and naive dams and their pups at intervals after parturition

Days post partum	Pups			Dams		
	n	immune	naive	n	immune	naive
0	5	5571 (640)[a]	–[b]	6	11403 (1920)	–
1	5	3200 (0)	–			
7	6	11403 (3850)	–			
14	6	18101 (7706)	–			
21	6	10159 (4103)	–	11	7731 (1920)	–
28	6	1837 (1073)	–			

[a] Reciprocal of geometric mean titer (standard deviation)
[b] < 1 : 50

Table 3. MHV IgA and IgG titers in stomach curds of pups suckling dams previously immunized with MHV-Y, at intervals after parturition

Days post partum	Immune dams			Naive dams		
	n	IgA	IgG	n	IgA	IgG
1	5	139 (14)[a]	229 (40)	5	–[b]	–[c]
7	6	80 (0)	257 (136)	4	–	–
14	6	50 (27)	224 (109)	4	–	–
21	6	–	–	4	–	–
28	6	–	–	4	–	–

[a] Reciprocal of geometric mean titer (standard deviation)
[b] < 1 : 10
[c] < 1 : 50

In a final experiment, the titer and duration of passively acquired MHV IgG in serum were evaluated in first, second, and third litter pups weaned from immune vs. naive dams to determine the duration of detectable passive antibody and if the concentration of passively transferred antibody declined with sequential litters. Pups from three consecutive litters born to immune and naive dams were exsanguated at 2, 4, 6, 8, and 10 weeks of age and sera were tested for MHV-specific IgG. Naive males used for breeding also served as sentinels to verify that dams were not re-exposed to MHV, thereby causing booster infections. Passively acquired MHV antibodies persisted in the sera of pups born and nursed by immunized females for 4 to 10 weeks. The MHV serum IgG titers of two week old pups correlated closely with those of their corresponding dam, but dropped substantially during the next two weeks (Table 4). Antibodies persisted for 4 weeks in pups born to dams with low MHV IgG serum titers and then declined to undetectable levels. In other animals, serum antibodies could be found through 10 weeks. The duration of passive immunity did not decrease with the second and third litter of individual dams. To ensure that the persisting titers were not due to an inadvertant MHV infection, all sera were tested for IgA, which could be found in the immunized dams later than five months after inoculation but not in any of the offspring (data not shown).

Selected whey and serum samples were also examined by Western blot to determine the viral protein specificity of the immunoglobulin isotypes. Serum of mice experimentally infected with other prototype strains of MHV cross-reacted with MHV-S. MHV-JHM IgG reacted with all three structural proteins of MHV-S, including glycoprotein S (Fig. 1, lane 1). MHV-Y serum IgG reacted with the nucleoprotein N and glycoprotein S (Fig. 1, lane 2). Passively acquired MHV-Y serum IgG in pups (Fig. 1, lane 3), as well as whey IgG and IgA (Fig. 1, lanes 4 and 6) also reacted with N and S. Reactivity to the M protein of MHV-

Table 4. MHV IgG titers in serum of dams and their pups of the first three consecutive litters

Dam no.	Weeks after birth						
	dam		pups				
	0	3	2	4	6	8	10
			1st litter				
1	6400[a]	6400	12800[b]	800	100	50	–
2	12800	6400	9050	800	100	–	–
3	3200	3200	3200	100	–	–	–
4	25600	6400	25600	1600	200	100	–
5	25600	12800	25600	2262	400	50	–
6–10[c]	–[d]	–	–	–	–	–	–
			2nd litter				
1	12800		12800	1600	1600	800	100
2	6400		4525	800	100	–	–
3	800		4525	100	–	–	–
4	3200		4525	800	400	141	200
5	3200		4525	1131	282	50	50
6–10	–		–	–	–	–	–
			3rd litter				
1	12800	800	25600	800	400	282	200
2	6400	ND[e]	6400	200	70	–	–
3	100	200	400	50	–	–	–
4	3200	1600	4525	200	70	50	–
5	6400	3200	4525	800	–	100	–
6–10	–	–	–	–	–	–	–

[a] Reciprocal MHV IgG titer
[b] Reciprocal geometric mean of MHV IgG titer, 2 replicates
[c] Uninfected controls
[d] $< 1:50$
[e] Dam died at weaning

S was not detected in sera or whey of MHV-Y-immune mice (Fig. 1, lanes 2–4 and 6).

Since IgM against MHV could not be detected in any samples, sera from mice infected for 3, 5, 7, 10, or 14 days with MHV-Y were tested. All were IgM-negative using ELISA, EIA, and Western blot. The goat-anti-mouse IgM conjugates that were used yielded positive results when tested by ELISA and immunoblots with a different antigen (*Borrelia burgdorferi* [14]).

Discussion

The mouse selectively transfers IgG to fetuses in utero through yolk sac receptors and to pups for about two weeks via specific IgG receptors on intestinal epi-

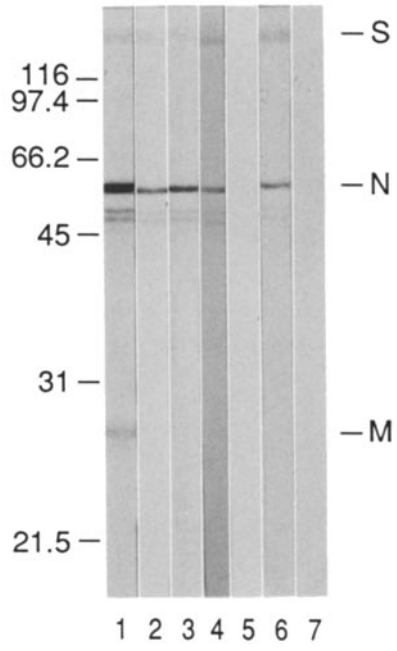

Fig. 1. Immunoblots of MHV IgA and IgG antibody from experimentally infected mice showing specificity to structural proteins of MHV-S. *1* Serum IgG of an adult mouse inoculated oronasally with MHV-JHM [1 : 8 in Tris-buffered saline (TBS)]. *2* Serum IgG of adult female immunized orally with MHV-Y (1 : 5 in TBS). *3* Passively acquired serum IgG of pups suckling dams immunized with MHV-Y (1 : 5 in TBS). *4* IgG in whey of pups nursed by MHV-Y immune dams (1 : 5 in TBS). *5* Serum IgG of naive control (1 : 5 in TBS). *6* IgA in whey of pups suckling dams immunized with MHV-Y (undiluted). *7* IgA in whey of pups suckling naive dams (undiluted). Molecular weight (kDa) is expressed on the left, location of S, N, and M on the right

thelium. Other immunoglobulins (IgA and IgM) are secreted in milk and may play a locally active role in the intestinal lumen. This study demonstrated that IgG to enterotropic MHV-Y was transferred in utero as well as through the milk. The same results were found previously with dams immunized intraperitoneally with killed MHV strain DVIM, another enterotropic strain of MHV [13]. Intrauterine antibody transfer could not be detected from dams inoculated oronasally with respiratory MHV-JHM [5]. The present study also demonstrated lactogenic transfer of IgA, but absorption from the intestine into the blood of pups did not occur. Intraluminal IgA and IgG in the gastro-intestinal tracts of immune pups could be detected until the suckling activity declined and pups started to eat solid food.

Serum antibody in pups nursed by MHV-Y-immune dams persisted much longer than expected. Earlier studies with mice inoculated either oronasally or intraperitoneally with non-enterotropic MHV-JHM had shown that IgG in the serum of passively immune pups persisted for about two weeks after the receptor-mediated absorption of IgG in the intestine ceased at approximately two weeks

of age [5, 15]. In contrast, passively acquired IgG to MHV-Y persisted at low titer for more than 10 weeks in some mice. This finding suggests that results of serologic monitoring should be interpreted cautiously among animals of this age group. The possibility must be considered that low positive results are due to maternally-derived passive immunity. The current study has also shown that an IgA assay could be used to differentiate between passive and active immunity, since serum IgA was only found in mice that were actively immune.

Compared with a similar study on respiratory strains of MHV [5], serum IgG titers of dams inoculated with enterotropic MHV-Y did not so obviously decline over the course of their first three litters. MHV IgG titers passed on to the second or third litter were not substantially reduced. Serum IgG titers of pups dropped to the same level in all three consecutive litters after weaning and duration of passive immunity did not decrease in later litters. This was not due to repeated infections of the dams, since sentinel mice remained seronegative.

MHV-specific IgM was not detected in these experiments using three independent assays. Literature reports of MHV IgM could not be found, implying that no specific IgM response is generated by MHV infection. In vitro studies showing that plasma cells in culture cease to produce IgM when infected with MHV may support this contention [7].

Among the three major structural proteins of MHV, nucleoprotein N is moderately and membrane glycoprotein M highly conserved among MHV strains, whereas spike glycoprotein S is very variable. A fourth protein, hemagglutinin esterase HE, appears in only a few MHV strains [8, 9, 16, 19]. MHV-S virions possessed N, M, and S bands on immunoblots, but not HE when stained with MHV-JHM antiserum. Additional bands at 50 kDa are virus-related proteins of cell origin antigenetically related to N protein and vary in expression dependent on culture conditions of the virus [11]. In this study, MHV-Y-specific IgG and IgA were directed against structural proteins N and S of MHV-S. That no specific reaction to the M protein was found is most likely due to insufficient antibody titer of the samples.

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