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Transmission of Mouse Minute Virus (MMV) but Not Mouse Hepatitis Virus (MHV) Following Embryo Transfer with Experimentally Exposed In Vivo-Derived Embryos[1]

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ABSTRACT

The present study investigated the presence and location of fluorescent microspheres having the size of mouse hepatitis virus (MHV) and of mouse minute virus (MMV) in the zona pellucida (ZP) of in vivo-produced murine embryos, the transmission of these viruses by embryos during embryo transfer, and the time of seroconversion of recipients and pups. To this end, fertilized oocytes and morulae were exposed to different concentrations of MMVp for 16 h, while 2-cell embryos and blastocysts were coincubated for 1 h. In addition, morulae were exposed to MHV-A59 for 16 h. One group of embryos was washed, and the remaining embryos remained unwashed before embryo transfer. Serological analyses were performed by means of ELISA to detect antibodies to MHV or MMV in recipients and in progeny on Days 14, 21, 28, 42, and 63 and on Days 42, 63, 84, 112, 133, and 154, respectively, after embryo transfer. Coincubation with a minimum of 10^5/ml of fluorescent microspheres showed that particles with a diameter of 20 nm but not 100 nm crossed the ZP of murine blastocysts. Washing generally led to a 10-fold to 100-fold reduction of MMVp. Washed MMV-exposed but not MHV-exposed embryos led to the production of antibodies independent of embryonic stage and time of virus exposure. Recipients receiving embryos exposed to a minimum of 10^7 mean tissue culture infective dose ($TCID_{50}$)/ml of MHV-A59 and 10^2 $TCID_{50}$/ml of MMVp seroconverted by Day 42 after embryo transfer. The results indicate that MMV but not MHV can be transmitted to recipients even after washing embryos 10 times before embryo transfer.

assisted reproductive technology, embryo transfer, health monitoring, mouse, mouse hepatitis virus, mouse minute virus

INTRODUCTION

The oocyte and the preimplantation embryo are surrounded by a zona pellucida (ZP) that protects the embryo from its environment [1, 2]. An intact ZP has been reported to act as an effective natural barrier against viruses for various species and prevents transmission of infectious agents during embryo transfer [3–10]. However, very small viruses belonging to the *Picornaviridae* family (e.g., the Mengo virus, which is 27–28 nm in size [11–13], and the Coxsackie B-4 virus, which is 30 nm [14]), have been shown to traverse the ZP of murine embryos, suggesting that this may also hold true for other viruses of similar size. The micropores in the ZP may allow entrapment of small mouse viruses even after extensive washing.

Mouse hepatitis virus (MHV), which is 80–160 nm in size, belongs to the coronavirus family of enveloped positive-strand RNA viruses. It is highly contagious in laboratory mice, being at present one of the most prevalent viruses in mouse colonies worldwide [15]. The symptoms of MHV infection in experimental studies range from subclinical manifestations in adult mice to high morbidity and mortality in neonatal or young mice, depending on virus strain, route of infection and genotype, age, and immune status of the host [16]. In a natural infection, enterotropic MHV is restricted largely to the intestine with excretion primarily in feces, while respiratory MHV is disseminated from the nasal mucosa to various target organs [17]. Transmission of MHV occurs via direct contact with infected mice and via exposure to contaminated bedding [16–18].

Mouse minute virus (MMV) is a nonenveloped linear positive-strand DNA virus of the *Parvoviridae* family having a diameter of approximately 20 nm [19]. The virus is highly contagious, but the outcome of natural infection in immunocompetent mice is essentially asymptomatic. Breeding mice become infected during the second and third months of life [20]. During acute infection, virus replication occurs in the small intestine, lymphatic organs, and liver. The virus can persist in the mesenteric lymph nodes for a long period and is transmitted primarily by urinary or fecal excretion. Contaminated food and bedding [20] also play a role because the virus is highly resistant to environmental factors and to a number of disinfectants [21, 22].

Previous reports showed that pups obtain colostral antibodies from their MHV- and MMV-seropositive mothers [16, 20, 23, 24]. These maternal antibodies protect them against MHV and MMV infections for approximately 2–4 wk. After this period, the maternal antibodies decline [16, 24]. The infected mothers may excrete virus even in the presence of antibodies, posing an immediate risk for other mice in the colony. This is of high relevance in health monitoring of embryo transfer recipients and in mouse colonies in general.

MHV and MMV were reported to considerably affect biomedical research [25–28]. They are undesirable agents and

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should be eliminated from infected colonies. Today, the technique of embryo transfer is the most widely used method to rederive mice that are microbiologically contaminated or to recover valuable mouse lines archived by assisted reproduction techniques (ARTs) such as cryoconservation of sperm or embryos. The risk of embryo transmission of viruses is prominent due to contamination of the reproductive tract in the abdominal cavity or due to the presence of blood from viremic mice in collecting and washing drops. Viruses can be transferred from the reproductive tracts to the holding media and can contaminate the embryos. Even mice with an acute infection can be used for ARTs, and, as such, the persistence of the virus is not an issue. The International Embryo Transfer Society recommends that embryos should be washed 10 times before transfer to suitable recipients [29]. The question remains as to whether small viruses can be removed by washing when embryos become contaminated.

The objectives of the study reported herein were to determine 1) the presence and location of fluorescent microspheres having the size of MHV-A59 and MMVp in the ZP after exposure of in vivo-produced murine embryos, 2) the immune response of recipients receiving unwashed and washed embryos that were previously exposed to various amounts of MHV-A59 and MMVp, and 3) the time at which seroconversion to MHV-A59 and MMVp occurs in mice infected during embryo transfer. Also discussed is the relevance of embryo transfer as a safe means to eliminate MHV and MMV from infected colonies, as well as the relevance MHV and MMV in health monitoring of recipients after embryo transfer.

The MHV-A59 and MMVp strains were used in the present study because of their ability to grow well in in vitro culture, permitting the production of viruses at high titers. In addition, the present work complements previous work done with MHV-A59 [5, 30]. Furthermore, the size of the viruses and the immune response they elicit mimic strains that are found in infected mice. The present study deals with the collection of in vivo embryos that are processed in vitro, a condition occurring when ARTs are used in a mouse facility.

MATERIALS AND METHODS

Cells and Viruses

Virus stocks of MHV-A59 (VR-764) and MMVp (VR-1346) and their permissive producer cell lines NCTC-1469 (CCL-9.1), A9 (CCL-1.4), and L929 (CCL-1) cells were obtained from American Type Culture Collection (Manassas, VA). NCTC-1469 and A9 cells were used for propagation of MHV-A59 and MMVp, respectively, while L929 cells were used for titration of MHV-A59 and MMVp. NCTC-1469, A9, and L929 cell cultures were maintained in Dulbecco minimum essential medium supplemented with 4.5 g/L of D-glucose/L-glutamine and 10% heat-inactivated fetal calf serum (L929 in 5% fetal calf serum). Propagation of virus stocks was performed in 75-cm^2 cell culture flasks (NCTC-1469 in cell culture flasks from Corning Costar, Cambridge, MA; and A9 and L929 in cell culture flasks obtained from Nunc, Roskilde, Denmark) at 37°C using 5% CO_2 in a humidified atmosphere. Cultures of permissive cells were infected with the appropriate virus for 1 h followed by removal of the virus suspension and replacement with 10 ml of cell culture medium. MHV-A59- and MMVp-infected permissive cells were frozen in their culture flasks after 20 h and 5 days, respectively. They were subjected to three freeze-thaw cycles to allow release of virus. The contents of the flasks were centrifuged at $3000 \times g$ for 5 min to separate virus from cell debris. The supernatant was passed through a Minisart filter having a pore size of 0.20 μm (Sartorius, Göttingen, Germany). For titration, L929 cells were seeded in 96-well plates at a concentration of 20×10^3/well for MHV-A59 and 3×10^3/well for MMVp and were cultured overnight. After removal of the culture medium, 12-fold wells were infected with 100 μl of each dilution. The cytopathic effect (CPE), observed as syncytia for MHV-A59 and as detachment of cells for the MMVp infection, was determined on the second day and the sixth day of culture, respectively. The mean tissue culture infective dose ($TCID_{50}$) for each viral stock was calculated according to the Spearman-Kaerber method [31, 32]. The MHV-A59 and MMVp stocks used in this study had titers of 10^9 $TCID_{50}$/ml and 10^6 $TCID_{50}$/ml, respectively, and were stored at −80°C until used.

Mice and Husbandry

Outbred Crl:CD1(Icr) mice were bred in a full barrier unit at the GSF–National Research Center for Environment animal facilities. Breeding colonies were kept in filter-topped type II Makrolon cages at 20°C–24°C, a humidity of 50%–60%, 20 air exchanges per hour, and a 12L:12D cycle. Wood shavings (Altromin, Lage, Germany) were provided as bedding. Mice were fed a standardized mouse diet (1314; Altromin) and were provided drinking water ad libitum.

Before entering a mouse room, staff were clothed in a clean suit and gown and wore disposable gloves, bonnets, and face masks. During routine weekly changes of cages that included lids, wire bars, and water bottles in class II laminar flow changing stations, mice were transferred to new cages using forceps padded with silicone tubing. Forceps were disinfected after each cage change with 70% ethanol. All materials, including individually ventilated cages (IVCs) (VentiRacks; BioZone, Margate, UK), Makrolon cages, lids, feeders, bottles, bedding, and water were autoclaved before use.

Mice were tested for microorganisms every 6 wk using 6-to 8-wk-old male Crl:CD1(Icr) sentinels from the colony as described [33]. Briefly, aliquots of approximately 5 cm^3 of soiled bedding were taken from each used cage on a rack. These aliquots were mixed in a sterile box with an equivalent amount of new sterile bedding, and the resultant mixture was distributed to the sentinel cage of the same rack. The serological examinations were performed according to the annual standard recommended by the Federation of European Laboratory Animal Science Associations [34], with the addition of *Leptospira* serogroups, ballum, canicola, hebdomadis and icterohaemorrhagiae, K virus, lactate dehydrogenase virus, polyoma virus, mouse thymic virus, and hantaviruses [35]. The mice were consistently negative for all of these infectious agents, including the ones examined in this study.

Experimental and control mice were kept in IVCs under positive pressure and the conditions already stated. All animal manipulations were performed in a class II laminar flow biological safety cabinet (Heraeus Instruments GmbH, Munich, Germany). All animal studies were approved by the Animal Care and Use Committee of the GSF–National Research Center for Environment and Health and by the Government of Upper Bavaria, Germany (211-2531–8/02).

Embryo Production

Six- to eight-wk-old females were superovulated by i.p. injections of 5 IU of eCG (Intergonan 1000; Intervet, Unterschleißheim, Germany) followed by 5 IU of hCG (Ovogest 1500; Intervet) 48 h later. Females were mated immediately after the hCG injection with males of proven fertility. The presence of vaginal plugs was determined the following morning (Day 0.5). Mice were killed on Day 0.5 for collection of fertilized ova or on Day 1.5 for collection of 2-cell embryos from the oviduct; mice were killed on Day 3 for collection of morulae or on Day 3.5 for collection of blastocysts from the uterus. Embryos were collected in M2 culture medium [36] and were stored for a short time in potassium simplex optimized medium (KSOM) [37] before experimental procedures.

Experimental Design

In a first in vitro step, the hypothesis was tested as to whether particles having the size of MHV and MMV are able to penetrate the ZP. In a subsequent in vivo approach, morulae were exposed to different concentrations of MHV-A59 for 16 h, fertilized embryos and morulae were exposed to different concentrations of MMVp for 16 h, and 2-cell embryos and blastocysts were exposed to different concentrations of MMVp for 1 h. These times were chosen to simulate overnight in vitro culture (16 h), after which 2-cell embryos and blastocysts develop from fertilized embryos and morulae, respectively, or short-term culture (1 h) on the day of embryo transfer. The four different embryonic stages were chosen because these are typical for various ARTs, and as observed for cattle embryos [38], the number of micropores in the murine ZP may differ according to the developmental stage, affecting its interaction with the viruses. Embryos were cultured in undiluted virus stocks or in 10-fold dilutions of the virus stocks in KSOM under silicone oil (Sigma, Deisenhofen, Germany) at 37°C in a moisture-saturated atmosphere of 5% CO_2/95% air in an incubator. Control embryos were cultured in virus-free KSOM.

Before embryo transfer to pseudopregnant recipients, a maximum of 80 2-cell embryos or 40 blastocysts were washed 10 times in KSOM by transferring them with a micropipette through 100-μl drops, and another group remained unwashed. The ratio of volume of medium containing embryos in the pipette

to volume of medium in each wash was approximately 1:100 [29]. A new micropipette was used for each washing step and transfer of embryos to a recipient. For each virus concentration, washed or unwashed embryos were transferred to three or four recipients. For control embryos, two or three recipients were used for washed or unwashed embryos. Because two different preimplantation embryonic stages were transferred, oviduct transfer for 2-cell embryos or uterus transfer for blastocysts was performed to simulate in vivo conditions. As described, in 0.1 μl of KSOM [39], 10 2-cell embryos were transferred to each oviduct of Day 0.5 pseudopregnant recipients, or five blastocysts were transferred to each uterus horn of Day 2.5 pseudopregnant recipients. Recipients were kept singly in IVCs. Pups born were kept with their mothers until weaning at 21 days postpartum. Pups were then kept singly in IVCs.

To determine if the recipients seroconverted, blood was collected from the tail vein on Days 14, 21, 28, 42, and 63 after embryo transfer, and sera were analyzed. Sera from progeny were prepared on Days 42, 63, 84, 112, 133, and 154 after embryo transfer and were analyzed for the presence of antibodies to MHV and MMV.

Manipulations of embryos and embryo transfers were performed by an experienced investigator. Control and experimental embryos were cultured in separate dishes. Control embryos were transferred before experimental embryos. From the same virus concentration, washed embryos were transferred before unwashed embryos. Aseptic precautions were observed throughout the study.

Permeability of Murine ZP to Fluorescent Microspheres

Fluorescent microspheres (Fluorospheres; Molecular Probes, Leiden, the Netherlands) were used to investigate the permeability of the ZP of murine blastocysts for particles with physical dimensions comparable to MHV (80–160 nm) and MMV (20 nm) as described [38, 40]. Crimson red fluorescent microspheres with a diameter of 100 nm (F-8763) and yellow-green fluorescent microspheres with a diameter of 20 nm (F-8803) were used. Five ZP-intact early blastocysts each were exposed for 6 h to 10^9, 10^5, 10^4, 10^2, and 10^1/ml of fluorescent microspheres of each of the two diameters in a culture dish. Two ZP-intact early blastocysts were used as negative controls and were cultured for 6 h in KSOM without microspheres. The localization of the microspheres was visualized using a Bio-Rad Radiance 2100 Blue Laser Diode BLDTM (Bio-Rad House, Hertfordshire, UK) linked to a Nikon Diaphot 300 inverted microscope (Nikon Corporation, Tokyo, Japan). Images were collected electronically using software provided by the manufacturer.

Exposure of Morulae (16 h) to MHV-A59 and MMVp Followed by Uterus Transfer

Morulae were exposed to the MHV-A59 stock (10^9 $TCID_{50}$/ml) or to KSOM containing 10^7, 10^6, and 10^5 $TCID_{50}$/ml of MHV-A59. Control embryos for the MHV-A59 group were cultured in KSOM. For the MMVp group, morulae were exposed to the MMVp stock (10^6 $TCID_{50}$/ml) or to KSOM containing 10^5, 10^4, 10^3, 10^2, and 10^1 $TCID_{50}$/ml of MMVp. Control embryos for the MHV group also served as controls for the MMV group. On the day of embryo transfer, 30–40 blastocysts were washed through each of 10 100-μl drops of KSOM. Another group of blastocysts was transferred to recipients without washing.

Exposure of Fertilized Embryos (16 h) to MMVp Followed by Oviduct Transfer

Fertilized embryos were exposed to the MMVp stock or to KSOM containing 10^5, 10^4, 10^3, 10^2, and 10^1 $TCID_{50}$/ml of MMVp. Control embryos were cultured in KSOM. Culture was performed as already described. On the day of transfer, 60–80 2-cell embryos were washed before transfer, and the remaining half were transferred to recipients without washing.

Exposure of Blastocysts (1 h) to MMVp Followed by Uterus Transfer

This experiment was performed in the same way as already described for morulae. However, blastocysts were exposed to the corresponding media for 1 h and not to 10^1 $TCID_{50}$/ml of MMVp.

Exposure of 2-Cell Embryos (1 h) to MMVp Followed by Oviduct Transfer

This experiment was performed in the same way as already described for fertilized embryos. However, 2-cell embryos were exposed to the corresponding media for 1 h and not to 10^1 $TCID_{50}$/ml of MMVp.

Serological Analysis

Sera from inoculated and control mice were heat inactivated at 56°C for 30 min immediately before the test and were diluted 1:10 in PBS (Oxoid, Hants, UK) containing 0.05% Tween 20 (R & L Slaughter, Essex, UK). Sera were tested for specific antibodies (IgG whole molecule) to MHV and MMV by means of ELISA using control non-viral-coated and viral-coated plates and negative and positive sera. The MHV antigen was obtained from Churchill Applied Biotechnology Ltd. (Cambridgeshire, UK), while the MMV antigen originated from Charles River Laboratories (Wilmington, MA). The optical density (OD) was read at 492 nm using a Multiskan ELISA plate reader (Thermo Life Sciences, Hampshire, UK). Serological results for MHV were equivocal low positive and positive when the ODs were 0.600–0.799 and greater than 0.799, respectively. For detection of MMVp, sera were equivocal low positive and positive when the ODs were 0.270–0.399 and greater than 0.399, respectively.

Virological Examination of Washing Drops

Only the washing drops from the MMVp experiments were analyzed. They were stored in sterile tubes at −20°C until analysis. All 10 washing drops were diluted 1:10 with the corresponding medium and were tested for the presence of MMVp by means of PCR and for virus infectivity by means of the in vitro infectivity assay in cell culture using the protocols described [41]. Briefly, for the in vitro infectivity assay, L929 cells were seeded in 96-well plates at a concentration of 3×10^3/well and were cultured overnight. After removal of the culture medium, 2-fold wells were infected with 100 μl of each dilution. The CPE, observed as detachment of cells resulting from the MMVp infection, was determined on the sixth day of culture.

For PCR analysis, 200 μl of the diluted washing drops were analyzed in duplicate. Total DNA from each diluted washing drop was extracted using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The primers used were those designed by Bootz et al. [42] as follows: 5′-GAGCGCCATCTAGTGAGC-3′ (forward) and 5′-ATTTGCCTGTGCTGGCTG-3′ (reverse), yielding a 483-bp product. A double-distilled water sample served as a negative PCR control. PCR was performed in a total volume of 20 μl using Taq DNA polymerase (Qiagen) for 40 cycles in a thermocycler (Biometra; Biomedizinische Analytik GmbH, Göttingen, Germany). Denaturation was performed at 94°C for 4 min. Each cycle consisted of 94°C (30 sec), 55°C (30 sec), and 72°C (30 sec). The last cycle was followed by a 7-min extension period at 72°C. PCR products (10 μl) were mixed with loading buffer (2 μl; MBI Fermentas, St. Leon-Rot, Germany), electrophoresed on a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light.

RESULTS

Permeability of the Murine ZP to Fluorescent Microspheres

The possibility of virus transmission during embryo transfer by attachment to or physical penetration into or through the ZP was investigated by 6-h exposure of blastocysts to fluorescent microspheres having sizes similar to those of MHV and MMV. In all five ZP-intact blastocysts, each cultured in KSOM with 10^9 or 10^5/ml of microspheres, the yellow-green fluorescent microspheres having a diameter of 20 nm crossed the ZP, whereas in embryos cultured with 10^4, 10^2, or 10^1/ml of microspheres, microspheres were detected only in the ZP. Independent of the concentration, beads having a diameter of 100 nm were deposited only in the outer half of the ZP.

Reproductive Performance and Seroconversion of Recipients and Their Progeny after 16-h Coincubation of Morulae with MHV-A59

With respect to the unwashed embryos, pups were born to recipients in all groups except those receiving embryos that had been exposed to 10^9 and 10^5 $TCID_{50}$/ml of MHV for 16 h. All recipients were seronegative for MHV on Day 14. By Day 21, one of three recipients receiving unwashed embryos from media containing 10^9 and 10^7 $TCID_{50}$/ml of virus seroconverted. On Day 28, two of three recipients receiving 10^9 $TCID_{50}$/ml of exposed embryos and one of three recipients

TABLE 1. Results of embryo transfer with unwashed and washed blastocysts following exposure of morulae to MMVp for 16 h and seroconversion of recipients and progeny.

Virus concentration for incubation of embryos ($TCID_{50}$/ml)	Embryo transfer with unwashed embryos: Total no. of pups born (no. of litters)	Seropositive mice days post embryo transfer*: 14 R	21 R	28 R	42 R	42 P	63 R	63 P	Embryo transfer with washed embryos: Total no. of pups born (no. of litters)	Seropositive mice days post embryo transfer*: 14 R	21 R	28 R	42 R	42 P	63 R	63 P
10^6	2 (1)[†]	2	2	2	2	0	2	0	2 (1)[†]	1	2	2	2	0	2	0
10^5	17 (3)[†]	2	3	3	3	12	3	8[a]	10 (2)[†]	2	3	3	3	8	3	7[c]
10^4	16 (3)[‡]	2	2	4	4	4	4	1[b]	4 (1)[‡]	0	1	1	1	0	1	0
10^3	6 (1)[†]	1	1	1	2	0	2	0	9 (2)[†]	0	0	0	0	0	0	0
10^2	5 (1)[‡]	0	0	1	1	0	1	0	3 (1)[†]	0	0	0	0	0	0	0
10^1	0[†]	0	0	0	0	NA	0	NA	2 (1)[†]	0	0	0	0	0	0	0
Control (KSOM)	5 (1)[†]	0	0	0	0	0	0	0	28 (3)[†]	0	0	0	0	0	0	0

* R, Recipients; P, progeny; NA, not available.
[†] Three recipients were used.
[‡] Four recipients were used.
[a] On Day 84, one pup was positive; and on Day 112, this pup was negative.
[b] On Day 84, this pup was negative.
[c] On Day 84, six pups were positive; on Day 112, four pups were positive; and no detectable MMV antibodies on Day 133.

receiving 10^7 $TCID_{50}$/ml of exposed embryos were seropositive. By Day 42, three of three recipients receiving 10^9 $TCID_{50}$/ml of exposed embryos and one of three receiving 10^7 $TCID_{50}$/ml of exposed embryos were seropositive. On Day 42, all pups tested were seronegative for MHV-A59.

With respect to the washed embryos, pups were born to recipients in all groups. Recipients and pups were seronegative for MHV-A59 throughout the experimental period.

Recipients receiving control unwashed and washed embryos gave birth to pups. Throughout the study, recipients and pups in the unwashed and washed control groups were seronegative for MHV-A59.

Reproductive Performance and Seroconversion of Recipients and Their Progeny after 16-h Coincubation of Morulae with MMVp

With respect to unwashed embryos, pups were born to recipients from all groups except when recipients received embryos that were exposed to 10^1 $TCID_{50}$/ml of MMVp for 16 h (Table 1). Recipients receiving embryos that had been exposed to 10^6–10^3 $TCID_{50}$/ml of MMVp were seropositive for MMVp by Day 14. One recipient receiving embryos that were exposed to 10^2 $TCID_{50}$/ml of MMVp seroconverted by Day 28. Recipients that were seronegative for MMVp at Day 42 did not develop antibodies by Day 63. Two of three recipients that received embryos exposed to 10^6 $TCID_{50}$/ml of MMVp seroconverted, and one of three had two pups; they remained seronegative until Day 63. From recipients receiving embryos that were exposed to 10^5 and 10^4 $TCID_{50}$/ml of MMVp, 12 of 17 pups and four of 16 pups, respectively, were seropositive on Day 42; the number of seropositive pups decreased thereafter by Day 63 to eight of 17 pups and one of 16 pups, respectively. By Day 84, one pup from the 10^5 $TCID_{50}$/ml of MMVp group was seropositive; by Day 112 after embryo transfer, this pup was seronegative. In the 10^4 $TCID_{50}$/ml of MMVp group, all mice were seronegative for MMVp by Day 84.

With respect to washed embryos, pups were born to recipients from all groups. Recipients receiving embryos exposed to 10^6 and 10^5 $TCID_{50}$/ml of MMVp seroconverted by Day 14. One recipient receiving embryos exposed to 10^4 $TCID_{50}$/ml of MMVp seroconverted by Day 21. Recipients receiving embryos exposed to 10^3 $TCID_{50}$/ml of MMVp or less did not develop antibodies to MMVp. Two pups were born in the 10^6 $TCID_{50}$/ml of MMVp group; these pups were seronegative on Day 42 and Day 63. In the 10^5 $TCID_{50}$/ml of MMVp group, eight of 10 pups showed antibodies on Day 42; seven of these were still seropositive on Day 63, and four of these were seropositive on Day 112. On Day 133, antibodies to MMVp were no longer detected.

Recipients receiving control unwashed and washed embryos gave birth to five pups and 28 pups, respectively. Recipients and pups in the control group did not have antibodies to MMVp.

Reproductive Performance and Seroconversion of Recipients and Their Progeny after 16-h Coincubation of Fertilized Embryos with MMVp

After exposure to 10^6 $TCID_{50}$/ml of MMVp for 16 h and transfer of washed and unwashed 2-cell embryos, the recipients did not give birth to pups (Table 2). The groups that were coincubated with 10^5, 10^4, 10^3, 10^2, and 10^1 $TCID_{50}$/ml of MMVp gave birth to 8, 2, 18, 1, and 21 pups, respectively, when the embryos were unwashed and to 2, 7, 14, 24, and 25 pups, respectively, when they were washed extensively before transfer.

In the groups of unwashed embryos, three of three, two of three, and three of four recipients receiving embryos exposed to 10^6, 10^5, and 10^4 $TCID_{50}$/ml of MMVp, respectively, seroconverted by Day 14. One of three recipients receiving embryos exposed to 10^3 $TCID_{50}$/ml of MMVp seroconverted by Day 42 and showed elevated antibodies until Day 63. All eight pups from the 10^5 $TCID_{50}$/ml group were seropositive on Day 42 and were seronegative by Day 63. Pups from the 10^4 and 10^3 $TCID_{50}$/ml of MMVp groups were seronegative on Day 42 and Day 63. From the 10^2 and 10^1 $TCID_{50}$/ml of MMVp groups, recipients and pups did not have antibodies to MMVp.

With respect to washed embryos, pups were born to recipients in all groups receiving embryos that had been exposed to 10^5–10^1 $TCID_{50}$/ml of MMVp. Recipients receiving washed embryos that were exposed to 10^6–10^4 $TCID_{50}$/ml of virus seroconverted by Day 21. Both pups from the 10^5 $TCID_{50}$/ml of MMVp group were seropositive on

TABLE 2. Results of embryo transfer with unwashed and washed two-cell embryos following exposure of fertilized embryos to MMVp for 16 h and seroconversion of recipients and progeny.

Virus concentration for incubation of embryos ($TCID_{50}$/ml)	Embryo transfer with unwashed embryos: Total no. of pups born (no. of litters)	Seropositive mice days post embryo transfer* 14 R	21 R	28 R	42 R	42 P	63 R	63 P	Embryo transfer with washed embryos: Total no. of pups born (no. of litters)	Seropositive mice days post embryo transfer* 14 R	21 R	28 R	42 R	42 P	63 R	63 P
10^6	0 (0)†	3	3	3	3	NA	3	NA	0 (0)†	3	3	3	3	NA	3	NA
10^5	8 (2)†	2	3	3	3	8	3	0	2 (1)†	2	3	3	3	2	3	0
10^4	2 (1)‡	3	3	3	3	0	3	0	7 (1)‡	1	1	1	1	0	1	0
10^3	18 (2)†	0	0	0	1	0	1	0	14 (2)†	0	0	0	0	0	0	0
10^2	1 (1)†	0	0	0	0	0	0	0	24 (3)†	0	0	0	0	0	0	0
10^1	21 (2)†	0	0	0	0	0	0	0	25 (3)†	0	0	0	0	0	0	0
Control (KSOM)	33 (3)†	0	0	0	0	0	0	0	16 (2)†	0	0	0	0	0	0	0

* R, Recipients; P, progeny; NA, not available.
† Three recipients were used.
‡ Four recipients were used.

Day 42 and seronegative by Day 63. All pups from the 10^4–10^1 $TCID_{50}$/ml of MMVp groups were seronegative on Day 42 and Day 63.

Recipients receiving control unwashed and washed embryos gave birth to 33 pups and 16 pups, respectively. Throughout the experimental period, seroconversion to MMVp occurred neither in recipients nor in their pups among the unwashed and washed control groups.

Reproductive Performance and Seroconversion of Recipients and Their Progeny after 1-h Exposure of Blastocysts to MMVp

After exposure of blastocysts to MMVp for 1 h and transfer to recipients without washing, pups were born in all groups (Table 3). Seroconversion to MMVp was observed in recipients receiving embryos that had been exposed to 10^6–10^3 $TCID_{50}$/ml of MMVp. All three recipients in the 10^6 and 10^5 $TCID_{50}$/ml groups seroconverted by Day 14. Two of three recipients in the 10^4 $TCID_{50}$/ml of MMVp group were seropositive by Day 28, and one of three recipients in the 10^3 $TCID_{50}$/ml of MMVp group seroconverted by Day 21. On Day 42, all three pups from the 10^6 $TCID_{50}$/ml of MMVp group and all 16 pups from the 10^5 $TCID_{50}$/ml of MMVp group were seropositive. By Day 63, three of three pups from the 10^6 $TCID_{50}$/ml of MMVp group and 12 of 16 pups from the 10^5 $TCID_{50}$/ml of MMVp group were seropositive. By Day 133 and Day 175, all pups from the 10^5 $TCID_{50}$/ml group and the 10^6 $TCID_{50}$/ml group, respectively, were seronegative. Pups from recipients of the 10^4 and 10^3 $TCID_{50}$/ml groups were seronegative at each time point. Recipients and pups from the 10^2 $TCID_{50}$/ml of MMVp group did not have antibodies to MMVp.

After transfer of washed embryos, pups were born in all groups. Two of three recipients from the 10^5 $TCID_{50}$/ml of MMVp group seroconverted by Day 14. By Day 21, seroconversion to MMVp was observed in recipients receiving embryos that were exposed to 10^6–10^4 $TCID_{50}$/ml of MMVp. On Day 42 and Day 63, four of 10, six of eight, and one of 13 pups from the 10^6, 10^5, and 10^4 $TCID_{50}$/ml of MMVp groups, respectively, had antibodies to MMVp; these pups were seronegative to MMVp by Day 84, Day 133, and Day 84, respectively. Recipients and pups from the 10^3 and 10^2 $TCID_{50}$/ml of MMVp groups did not have antibodies to MMVp.

TABLE 3. Results of embryo transfer with unwashed and washed blastocysts exposed to MMVp for 1 h and seroconversion of recipients and progeny.

Virus concentration for incubation of embryos ($TCID_{50}$/ml)	Embryo transfer with unwashed embryos: Total no. of pups born (no. of litters)	Seropositive mice days post embryo transfer* 14 R	21 R	28 R	42 R	42 P	63 R	63 P	Embryo transfer with washed embryos: Total no. of pups born (no. of litters)	Seropositive mice days post embryo transfer* 14 R	21 R	28 R	42 R	42 P	63 R	63 P
10^6	3 (1)†	3	3	3	3	3	3	3[a]	10 (2)†	0	1	1	1	4	1	4[c]
10^5	16 (3)†	3	3	3	3	16	3	12[b]	8 (2)†	2	2	2	2	6	2	6[d]
10^4	14 (3)†	0	1	2	2	0	2	0	13 (3)†	0	1	1	1	1	1	1[e]
10^3	25 (3)†	0	1	1	1	0	1	0	15 (3)†	0	0	0	0	0	0	0
10^2	20 (2)†	0	0	0	0	0	0	0	11 (3)†	0	0	0	0	0	0	0
Control (KSOM)	18 (2)‡	0	0	0	0	0	0	0	13 (2)‡	0	0	0	0	0	0	0

* R, Recipients; P, progeny.
† Three recipients were used.
‡ Two recipients were used.
[a] Pups were seronegative by Day 175.
[b] Pups were seronegative by Day 133.
[c] Pups were seronegative by Day 84.
[d] Pups were seronegative by Day 133.
[e] Pups were seronegative by Day 84.

TABLE 4. Results of embryo transfer with unwashed and washed two-cell embryos exposed to MMVp for 1 h and seroconversion of recipients and progeny.

Virus concentration for incubation of embryos ($TCID_{50}$/ml)	Embryo transfer with unwashed embryos: Total no. of pups born (no. of litters)	Seropositive mice days post embryo transfer* 14 R	21 R	28 R	42 R	42 P	63 R	63 P	Embryo transfer with washed embryos: Total no. of pups born (no. of litters)	Seropositive mice days post embryo transfer* 14 R	21 R	28 R	42 R	42 P	63 R	63 P
10^6	7 (1) †	3	3	3	3	7	3	7[a]	5 (1) †	3	3	3	3	5	3	2[c]
10^5	1 (1) †	3	3	3	3	1	3	1[b]	19 (3) †	2	3	3	3	18	3	4[d]
10^4	6 (2) †	1	1	1	1	0	1	0	13 (1) †	1	1	1	1	0	1	0
10^3	16 (2) †	0	0	0	1	0	1	0	27 (3) †	0	0	0	0	0	0	0
10^2	7 (1) †	0	0	0	0	0	0	0	19 (2) †	0	0	0	0	0	0	0
Control (KSOM)	9 (1) ‡	0	0	0	0	0	0	0	15 (2) ‡	0	0	0	0	0	0	0

* R, Recipients; P, progeny.
† Three recipients were used.
‡ Two recipients were used.
[a] Pups were seronegative by Day 133.
[b] Pups were seronegative by Day 112.
[c] Pups were seronegative by Day 84.
[d] Pups were seronegative by Day 84.

Recipients receiving control unwashed and washed embryos gave birth to 18 pups and 13 pups, respectively. Throughout the experimental period, seroconversion to MMVp occurred neither in recipients nor in their pups from the unwashed and washed control groups.

Reproductive Performance and Seroconversion of Recipients and Their Progeny after 1-h Exposure of 2-Cell Embryos to MMVp

After exposure of 2-cell embryos to MMVp for 1 h, pups were born to recipients of unwashed embryos from all groups (Table 4). Recipients receiving embryos exposed to 10^6–10^4 $TCID_{50}$/ml of virus seroconverted by Day 14, and one recipient receiving embryos exposed to 10^3 $TCID_{50}$/ml of virus seroconverted by Day 42. All pups from the 10^6 (n = 7) and 10^5 (n = 1) $TCID_{50}$/ml of MMVp groups were seropositive on Day 42 and Day 63. By Day 112 and Day 133, all of the pups from the 10^5 $TCID_{50}$/ml group and the 10^6 $TCID_{50}$/ml group, respectively, were seronegative. Pups from the 10^4 (n = 6) and 10^3 (n = 16) $TCID_{50}$/ml of MMVp groups were seronegative on Day 42 and Day 63. Among the 10^2 $TCID_{50}$/ml of MMVp group, recipients and pups had no antibodies to MMVp.

Pups were born to recipients of washed embryos in all groups. Three of three, two of three, and one of three recipients receiving embryos exposed to 10^6, 10^5, and 10^4 $TCID_{50}$/ml of MMVp, respectively, seroconverted by Day 14. By Day 21, three of three, three of three, and one of three recipients receiving embryos exposed to 10^6, 10^5, and 10^4 $TCID_{50}$/ml of MMVp, respectively, were seropositive. Five of five pups and 18 of 19 pups from the 10^6 $TCID_{50}$/ml of MMVp group and the 10^5 $TCID_{50}$/ml of MMVp group, respectively, were seropositive on Day 42; on Day 63, two of five pups and four of 19 pups from these two groups, respectively, were seropositive, and by Day 84 all were seronegative. Pups from the 10^4 $TCID_{50}$/ml of MMVp group (n = 13) were seronegative on Day 42 and Day 63. In the 10^3 and 10^2 $TCID_{50}$/ml of MMVp groups, recipients and pups had no antibodies to MMVp.

Recipients receiving control unwashed and washed embryos gave birth to nine pups and 15 pups, respectively. Throughout the experimental period, seroconversion to MMVp occurred neither in recipients nor in their pups among the unwashed and washed control groups.

Virological Examination of Washing Drops

PCR examination showed the presence of MMVp in some of the washing drops from the 10^6–10^4 $TCID_{50}$/ml of MMVp concentrations, independent of embryonic stage and time of exposure to the virus. A similar situation was observed in the in vitro infectivity assay except for the washing drops from the exposure of blastocysts for 1 h in 10^4 $TCID_{50}$/ml of MMVp, in which no infectious virus was detected. At higher viral concentrations, MMVp was found in more washing drops and vice versa. PCR also detected the presence of MMVp in the first washing drop from the 10^2 $TCID_{50}$/ml of MMVp groups for blastocysts and 2-cell embryos coincubated for 16 h and 1 h, respectively, as well as from the 10^1 $TCID_{50}$/ml of MMVp group for blastocysts coincubated for 16 h. The in vitro infectivity assay did not detect infectious virus in the groups. Control washing drops were negative for MMVp.

DISCUSSION

With the increasing demand for embryo transfers in the production of transgenic mice, revitalization of cryopreserved spermatozoa and embryos, and rederivation of mouse strains, inadvertent transmission of microorganisms into barrier areas is of major concern. Their main means of transmission to recipients during embryo transfers include contaminated personnel, instruments or equipment, carriage in transport or wash medium, and embryos themselves. In the present study, we investigated the risk of transmission of MHV-A59 and MMVp to Swiss mice by in vivo-produced embryos under aseptic routine working conditions for the production of rederived mice that precluded natural exposure to these agents.

Studies in species other than the mouse showed that fluorescent microspheres with a diameter of 20 nm but not 200 nm crossed the porcine ZP [40]; those with diameters of 40 nm and 200 nm did not traverse the bovine ZP [38]. In the present study, we showed that at concentrations of at least 10^5/ml of fluorescent microspheres with a diameter of 20 nm but not 100 nm crossed the ZP of murine blastocysts, indicating that binding of particles to early embryos plays a crucial role in viral transmission to recipients. Micropores are found in the ZP after loss of the cumulus cells during embryonic development and decrease centripetally in different species [38, 43–45]. The micropores in the outer surface of the ZP measure 182 nm in

cattle embryos [38], 50–100 nm in porcine embryos [29], and 140-1000 nm in murine embryos [45], indicating that the size of the micropores in the ZP is species specific.

Previous reports on murine species show that in vitro-derived 2-cell embryos [30] and in vivo-derived 1-cell embryos [10] and 2-cell embryos [4, 5, 7, 46] do not pose a risk of transmitting MHV during embryo transfer. As such, in the present study, only blastocysts were exposed to MHV. Washing blastocysts through 10 drops of media reduced the viral load to noninfectious levels or removed virus altogether, and recipients did not develop MHV antibodies even when doses as high as 10^9 $TCID_{50}$/ml were present in the culture media. This observation confirms results obtained when in vitro-fertilized embryos produced in media containing MHV were washed 10 times before transfer to suitable recipients [30] and when seronegative mice were rederived [4, 5, 7, 46]. These reports indicate that MHV does not become tenaciously attached to the ZP, enabling removal by washing to a level that does not constitute an infectious dose for the recipient and precluding the risk of infection. The results also show that recipients seroconvert to MHV when doses of 10^7 $TCID_{50}$/ml or higher are present in the culture media.

MHV is excreted only during the first 10–14 days after infection and is not persistent in seropositive mice [47–49]. Seroconversion of MHV-infected mice generally occurs between Day 7 and Day 20 after infection [17, 27, 50–52]. In this study, MHV antibodies were detected in some mice in the positive groups as early as Day 21 and as late as Day 42 after embryo transfer (i.e., at the time of weaning of the pups). As such, microbiological examination of the killed mother after weaning of the pups would detect the presence of MHV in recipients. However, the absence of antibodies in some recipients receiving embryos from the same culture dish implies that recipients may not necessarily become infected, despite the presence of virus in the transferred embryos. Therefore, pertinent to microbiological monitoring, each recipient rather than representative samples or cohorts should be examined. The difference in immune response by mice to the same viral dose may be due to individual differences in the outbred mice used in the present study [41].

With respect to MMVp, the serological data obtained after unwashed MMVp coincubated embryos were transferred to suitable recipients showed that media containing at least 200 $TCID_{50}$/ml led to antibody production. MMV antibodies were found in recipients by Day 42 after embryo transfer, enabling microbiological examination immediately after weaning of the pups. MMV persists in infected mice for a longer period than MHV [53]. In our experimental colony, mice were shown to excrete MMVp in the feces for at least 1 yr after infection (unpublished results), posing a high risk of transmission to other mice, a condition that warrants appropriate measures to prevent infection of neighboring mice. Because the ODs dwindled with time and the pups became seronegative, we conclude that the MMV antibodies found in the pups were of maternal origin.

Washed embryos that were previously exposed to media containing at least 10^4 $TCID_{50}$/ml of MMVp led to antibody production. Independent of embryonic stage and time of exposure, PCR detection of MMV in some washing drops and seroconversion in recipients indicate that washing of embryos through 10 drops sometimes only reduced the viral load 10-fold to 100-fold. This is further supported by the fact that 60 embryos that were previously exposed to 10^6 $TCID_{50}$/ml of MMVp and were washed 10 times had a titer of 10^5 $TCID_{50}$/ml after titration on L929 cells (data not shown).

The reported absence of parvoviral antibodies in recipients of embryos from mouse parvovirus (MPV)-infected donors [10] indicates that such high MMVp doses as those used in the present study may not normally be found in media during collection and preparation of embryos. Nevertheless, the data show that MMVp at high concentrations was not removed by 10 washes. This may also hold true for other murine viruses that are of similar size to MMV such as MPV (20–26 nm), Theiler murine encephalomyelitis virus (28–30 nm), and lactic dehydrogenase virus (30–55 nm). A lack of MMV detection by immunofluorescent assay using rat embryo fibroblast cells [23] after 2-h exposure of intact 2-cell embryos to MMV is in contradiction to the present findings. This discrepancy may be due to the coincubation of MMV-inoculated embryos with MMV antibody for 30 min [23], which may have neutralized viruses present. In our study, washing was performed with medium lacking MMV antibody. Further research is needed to optimize removal of viruses such as MMV from mouse embryos before embryo transfer.

The confocal laser scanning microscope data showed that particles having the size of MMV can penetrate the ZP at concentrations of at least 10^5/ml, while particles at a lower concentration were found in the ZP. At a concentration of 10^9/ml of plaque forming units, the Mengo virus penetrated the ZP within 10 min, but about 60 min were required to infect all of the vitelli [13]. Little is known about the mechanism of attachment of viruses to the ZP. In vitro studies showed that lectin-binding sites are found more densely in the exterior regions of the mouse ZP than in the interior region [54–56], suggesting that the viruses may bind to the glycoprotein moiety of the ZP surface.

Viruses that were found in the murine ZP include Sendai virus (having a size of 100–200 nm) [57–59], while Mengo virus (27–28 nm) was found to penetrate the ZP [11–13]. Viruses such as bovine herpesvirus (BHV-1) (180–200 nm) and pseudorabies virus (150–250 nm) adhered to the ZP even after 10 washings [29]. BVDV, having a size of 35–60 nm [60–62], and BHV-1 [63] remained attached to bovine embryos, despite washing. In contrast to a previous report of transzonal infection of the porcine ZP-intact embryo by porcine parvovirus [64], most porcine viruses adhere to but do not penetrate the ZP [40, 65] and can lead to seroconversion in recipients [66]. Sendai virus adhered to the murine ZP even after 12 washings with trypsin [58]. In the present study, whether MMV was in or within the ZP was not investigated in detail. However, serological and PCR data showed that embryos served as vectors for MMVp, suggesting that penetration of the ZP by MMVp should not be excluded. This could be the reason why, in some cases, MMVp was no longer detected in the washing drops but led to seroconversion of recipients. Another plausible reason is that MMV may get deeply lodged in the micropores of the ZP and cannot be removed even by frequent washings. Further studies are underway to determine if MMVp was in the ZP or if this virus had penetrated the ZP.

From the present results, we conclude that embryo transfer is an adequate tool for the elimination of viruses, provided that they do not adhere to or penetrate the ZP, enabling removal by washing to a noninfectious dose. However, our findings also show that embryo transfer does not exclude the risk of transmitting small viruses such as MMVp.

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