Failure to infect embryos after virus injection in mouse zygotes

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BACKGROUND: The intracytoplasmic injection of sperm raises the problem that viral elements may be transported into the oocyte by the spermatozoon or the surrounding medium. It also raises questions about how the developing zygote will behave. METHODS: We used the murine model to microinject murine cytomegalovirus (MCMV) into the zygote ooplasm and followed the changes in these microinjected zygotes in vivo and in vitro over time. RESULTS: 80% of zygotes microinjected with viral suspension, and 80% injected with medium alone, survived. Although MCMV DNA was detected in 56% of injected embryos, up until the blastocyst stage, the mice born from these injected zygotes developed normally and did not contain MCMV DNA. When embryonic stem cells were co-incubated with MCMV and then transferred into healthy blastocysts, the offspring were normal and did not contain any MCMV DNA. CONCLUSIONS: Our observations suggest that even if MCMV DNA persists from the zygote to the blastocyst stage, its presence has no detrimental effect on pre-implantation or post-implantation development.

Key words: ES cells/ICSI/MCMV DNA/mouse/zygote

Introduction

The intracytoplasmic injection of sperm has been particularly well developed recently as a means of medically assisted procreation (MAP). This technique raises the question of whether viral elements can be transported via the spermatozoon or the surrounding medium to the oocyte at fertilization and what effects this would have during development.

It is known that ejaculated semen can contain viral particles, such as human immunodeficiency virus (HIV: Baccetti et al., 1994, 1999), cytomegalovirus (HCMV: Mansat et al., 1997) papillomavirus (HPV: Lai et al., 1996) herpes simplex virus type 2 (HSV2: Moore et al., 1989) and hepatitis C virus (HCV: Leruez-Ville et al., 2000). Percoll gradients are generally used to select motile sperm from the ejaculate and this reduces the number of viral elements in the pool of selected sperm (Levy et al., 1997). Accordingly, the probability of selecting a viral particle with the motile spermatozoon when using ICSI pipettes is low. However, sperm may carry viral particles. This is particularly true of morphologically abnormal sperm (Huang et al., 1986; Dussaix et al., 1993; Baccetti et al., 1994), which are frequent in the case of male infertility. It is not always possible to select only motile sperm in oligoasthenospermic semen. In this case, the problem of transporting viral particles to the oocyte is more crucial. Conversely, ICSI is often recommended in azoospermia, and sperm are collected from the epididymis or testis biopsies. These biopsies consist of germinal, interstitial and blood tissues, which means that sperm may come into contact artificially with any viral particles that are present in interstitial or blood tissues. Furthermore, in certain ICSI cases, sperm are directly injected into the oocyte without being washed (De Croo et al., 2000).

The outcome of viral elements carried by the injected sperm or the surrounding medium is more of a problem with ICSI than with conventional IVF. The main studies on viruses and fertilization in mammals have concentrated on the co-incubation of viral particles with oocytes or embryos that are surrounded by a zona pellucida, which is known to be an efficient barrier against viruses (Neighbour and Fraser, 1978; Heggie and Gaddis, 1979; Tsutsui, 1995; Tsuboi and Imada, 1997; Baccetti et al., 1999; Vanroose et al., 1997, 2000). However, non-infectious viruses have been observed in the perivitelline space and are thought to have been carried by the sperm that were co-incubated with murine cytomegalovirus (MCMV) (Neighbour and Fraser, 1978). Furthermore the exposure of two-cell mouse embryos to the Coxsackie virus B4 and MCMV inhibited blastocyst formation, although only the Coxsackie virus B4 was recovered from the embryonic cells (Heggie and Gaddis, 1979). Some studies have been carried out on zona-free oocytes or embryos: Neighbour showed that exposing zona-free pre-implantation mouse embryos to MCMV does not affect development up to the blastocyst stage (Neighbour, 1978). Conversely, Vanroose et al. found bovine blastomeres expressing viral antigens in
morulae and blastocysts after the co-incubation of zona-free embryos with bovine herpes simplex virus-1 (Vanroose et al., 1997).

Purified viral RNA (Gamarnik et al., 2000) and DNA (Baskar et al., 1993) have been injected into the oocytes. When mengovirus RNA is microinjected into Xenopus oocytes it leads to the complete cycle of viral replication, yielding a high viral titre (10^7 pfu/oocyte, 30 h after microinjection; Gamarnik et al., 2000). Baskar et al. injected MCMV DNA into the male pronucleus of the zygote by use of the same technique as that used for transgenesis (Baskar et al., 1993). This resulted in smaller litter sizes, developmental retardation and fetal abnormalities in mice. MCMV DNA was demonstrated in fetuses by PCR and DNA--DNA cytohybridization.

DNA viruses introduced into the ooplasm during ICSI are predicted to have similar effects to viral DNA microinjected into the male zygote pronuclei. However, studies concerning the introduction of viral elements into the oocyte cytoplasm are scarce. Ivanova et al. discussed experiments with adenoviruses and concluded that the injection of adenoviruses into the ooplasm of mice and rabbits has no effect on development (Ivanova et al., 1999).

We introduced MCMV into the cytoplasm of mice zygotes at the pronucleus stage to evaluate the outcome of introducing viral elements into the ooplasm during fertilization. We chose MCMV as it is very similar to human CMV. This virus can occur in complete or incomplete forms or even as DNA and is known to induce severe fetal abnormalities. By injecting the virus directly into the zygote, we were certain of its presence in the zygote, which is not the case when it is simply co-incubated with sperm. We also evaluated the consequence of the virus in later stages of development by coincubating embryonic stem (ES) cells with MCMV before transferring them into healthy blastocysts.

Materials and methods

Virus preparation
The MCMV used in this study was the Smith strain obtained from American Type Culture Collection (ATCC, Maryland, Rockville, USA; VR-1399). It was maintained in the laboratory by serial passage in FVB/N mouse embryo fibroblast (MEF) cultures. The infected MEF cells were incubated for 3–5 days at 37°C to obtain characteristic MCMV cytopathic effects (CPE). The infected MEF were then collected and purified by use of a sucrose gradient [15% in phosphate-buffered saline (PBS)] and centrifuged as previously described (Teboobri et al., 2001). The concentration of the purified virus stock (MCMVp) was 10^8 pfu/ml.

Collection of eggs and blastocysts from mice

Collection of ova
Four to six week old female FVB/N mice from our pathogen-free breeding colony [Service d’Expérimentation Animale et de Transgénèse (SEAT), Institut André Lwoff, CNRS, Villejuif, France] were induced to ovulate by i.p. injection with 5 IU of pregnant mare’s serum gonadotrophin (PMSG) between 1300 and 1400 h, followed by 5 IU of HCG 48 h later. They were immediately placed with FVB/N males.

The following morning, the females with a vaginal plug were killed, the oviducts were removed and placed in M2 medium (Quinn et al., 1982) containing 3% hyaluronidase. The cumuli were discarded from the ampulla, the cumulus cells were removed by the enzymatic treatment and washed in M2 medium. After removing the defective oocytes, the intact oocytes were stored in M2 medium until microinjection.

Collection of blastocysts
Female FVB/N mice which had been induced to ovulate were mated with male FVB/N. The following morning, females with a vaginal plug were separated from the males. Four days later, they were killed, the uterine horns were recovered, and flushed with M2 medium. The recovered blastocysts were transferred into a fresh microdrop of M2 medium in a Petri dish and stored at 37°C until injection of ES cells.

MCMV infection

Microinjection of MCMVp into zygote ooplasm
Micromanipulations were carried out in microdrops of M2 medium covered with light paraffin oil in Petri dishes, to create a microinjection chamber.

The oocytes were maintained with a holding pipette. The injection pipette was filled by capillary action with MCMVp that had been centrifuged at 20 800 g for 5 min. The microinjection was performed into the ooplasm of the zygote, leading to a transient swelling of the oocyte. After ten washes with M2 (200 µl/drop), the zygotes were transferred into a fresh M2 microdrop before further manipulations.

The controls consisted of injecting the zygote ooplasm with medium alone. To ensure that the virus was present in each injected volume, an equal volume was placed in the bottom of tubes containing lysis buffer for PCR analysis.

MCMVp infection of ES cells

Cloned E14 was derived from murine ES cells from the ‘OLA 129’ strain of agouti mice (a gift from Doctor Boucheix, Laboratoire de Différenciation Hématopoïèse normale et leucémique, INSERM; U268). The ES cells were cultured on gelatine-coated tissue culture dishes containing a confluent monolayer of mitomycin C-treated MEF with 1000 IU/ml leukaemia inhibitory factor (LIF). ES cells and MEF were incubated at 37°C with high-glucose Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 20% fetal calf serum (FCS), 2 mmol/l glutamine, 1 mmol/l 2-mercaptoethanol and 1 mmol/l sodium pyruvate. Every 2 days, the medium was removed and the cells were trypsinized. After centrifugation, the cell suspension was reseeded in a gelatine-coated Petri dish for 1 h and non-adherent cells, enriched with ES cells, were recovered. They were incubated for 1 h in the presence of MCMVp (1×10^7 pfu/ml for 1×10^7 cells). After washing in M2 medium, the infected ES cells were centrifuged (2 ml at 500 g for 5 min). The pellet was resuspended in 100 µl fresh M2, and 20 µl of the suspension was placed into the microinjection chamber containing the blastocysts. Twelve to fourteen infected ES cells were injected into each blastocyst. The control group consisted of injecting non-infected ES cells into the blastocysts. These blastocysts were then transferred into pseudopregnant females.

Embryo transfer

C57BL/6J recipient mice were placed with vasectomized males to induce pseudopregnancy. One- or two-cell embryos that had been microinjected with MCMVp were then transferred into the recipient females the following day. The blastocysts which received ES cells were transferred into recipient females 72 h later.

Detection of MCMV DNA by PCR

The presence of MCMV DNA was tested in injected medium, two-cell embryos, blastocysts, fetuses, newborn mice and in the placentas and target organs of the recipient females.
A pair of 20 bp oligonucleotides based on the BamHI-B fragment of the Smith strain MCMV genome (Klotman et al., 1990) was used to amplify the sequence. The primer sequences were as follows: (bp 35708 to 35728), 5’ CGTCGCAAGGAACACCTCA 3’; and (bp 35946 to 35966, antisense), 5’ GTCGCCATGCGGAGGTTTC 3’ (Genset, Paris, France). This primer pair amplified a 258 bp fragment.

Samples were incubated in lysis buffer (Tris/EDTA 10/1; pH 7.8; proteinase K) for 2 h at 55°C or overnight at 55°C depending on the sample.

One microlitre of each lysate was amplified by use of Ready-to-Go® beads (Amersham, Pharmacia Biotech, Orsay, France). The PCR conditions were as follows: 94°C 5 min, 65°C 2 min and 70°C 4 min for the first cycle, followed by 30 cycles of 94°C 1 min, 65°C 1 min and 70°C 1 min. PCR products were separated on a 1.4% agarose gel and stained with ethidium bromide.

Results

Microinjection of zygotes with MCMVp

Effect of microinjection on zygotes

We studied the development in vivo and in vitro of microinjected zygotes. One hundred zygotes were microinjected with the viral suspension and 80 survived (80%). Twenty five zygotes were microinjected with the medium alone and 20 survived (80%).

Development of microinjected oocytes

Twenty zygotes were microinjected with the viral suspension and cultured for 4 days giving rise to 17 blastocysts (85%). The control zygotes resulted in a similar proportion of blastocysts (80%).

Of the 60 infected zygotes (30 of which were transferred at the two pronuclei stage and 30 at the two-cell stage), 40 developed normally and gave rise to apparently normal young (67%). This result was similar to that obtained in the control group—7/15, i.e. 47%, (Table 1).

Table I. In-vitro and in-vivo development of MCMVp microinjected zygotes

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<th>In vitro blastocyst stage</th>
<th>In vivo newborn mice</th>
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<td>MCMVp microinjected Controls</td>
<td>17/20a</td>
<td>40/60b</td>
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<td>4/5</td>
<td>7/15</td>
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aNumber of blastocysts obtained/number of microinjected zygotes.
bNumber of newborn mice/number of zygotes (two pronuclei) or embryos (two-cell stage) transferred.

ES cells co-incubated with MCMVp

Nine blastocysts that had been injected with 12–14 infected ES cells were transferred into two pseudopregnant mice. They gave birth to seven normal newborn, four of which where chimeras, as shown by their hair colour which was a mixture between FVB/N (white) and OLA 129 (agouti). In the control group, five of the seven offspring were chimeras, a similar result to that obtained with infected ES cells.

The organs (salivary glands, lung, liver and testis) of the seven mice born after the injection of infected ES cells were analysed on day 14 following birth and no specific MCMV DNA was recovered.

Discussion

The aim of this study was to evaluate the risks of viruses being transported by oocytes during ICSI fertilization in humans.

We found that injecting MCMV into fertilizing mice ooplasm had no effect on the pre-implantation development in vitro. The embryos obtained from these injected zygotes also developed normally in vivo and did not present anomalies like those described in cases of congenital CMV infection of human (Hansaw, 1966) or murine (Baskar et al., 1983) fetuses. We followed the development of the injected virus by testing for the presence of viral DNA at different stages of development (two-cell stage, blastocyst, fetus, newborn). The viral DNA, which was present in all the tested microinjection droplets, persisted in half of the two-
cell embryos and blastocysts, but disappeared in fetuses and newborn. As we were unable to eliminate viral DNA by repeatedly washing MCMV-injected embryos, we assume that PCR analysis detected DNA adherent to the outer layer of zona pellucidae as a result of contamination during microinjection procedures. However, viral DNA might correspond also to the microinjected DNA inside the cytoplasm of the zygote. This DNA might be stored either inside the embryo, or outside the embryo, in the perivitelline space or attached to the inner side of the zona pellucidae. Moreover it was not possible to determine whether the virus persists in its complete form or as DNA.

We also transferred ES cells previously co-incubated with MCMV into healthy blastocysts. No anomalies or viral DNA were observed in the embryos or newborn resulting from these blastocysts. In BDF1 mice, Tsutsui did not observe any anomalies or detect any virus in 11 day embryos after the injection of MCMV into blastocysts and transfer into pseudopregnant mice (Tsutsui, 1995).

It was reported that sperm can be viral DNA vectors (Bracket et al., 1971; Habrova et al., 1996), delivering imported replicating DNA to the oocyte and the embryo. The discrepancy between these reports and our results could be due to the fact that the above authors used purified forms of the viral DNA or modified DNA. Purified DNA (or plasmids) are known to fix and even to be internalized in the sperm, and are then expressed in the embryo as shown after IVF (Bracket et al., 1971: rabbit; Lavitrano et al., 1989: mouse) or ICSI (Chan et al., 2000: monkey).

Concerning MCMV, it was shown that microinjecting MCMV DNA into mouse male pronuclei causes malformations and that viral protein is subsequently found in the embryos (Baskar et al., 1993). However, to obtain such results, the authors injected purified MCMV DNA and used transgenic procedures as they injected DNA into the male pronucleus through the pronucleus membrane. The DNA integrated randomly, and in some cases viral proteins were expressed. In our experiments, which were similar to the ICSI procedure, purified DNA was not used. We injected either the complete, or incomplete form of the virus or unpurified DNA present in the viral suspension as shown at the ultrastructural level (Tebourbi et al., 2001).

These results only concern MCMV and cannot be extended to other viruses, even other DNA viruses. Ivanova et al. (1999) reported that the injection of adenoviruses into the cytoplasm of rabbit and mouse zygotes had no effect on subsequent development; however, these results have not been substantiated. Heggie and Gaddis recovered the Coxsackie virus B4 from murine blastomere extracts after the co-inoculation of the virus with two-cell embryos (Heggie and Gaddis, 1979). However, the Coxsackie virus B 4 is a very small RNA virus that is able to cross the zona pellucida.

Our result clearly show that even if complete and infectable MCMV persists in the mouse embryo until the blastocyst stage, it has no impact on the further development of the fetus or the newborn.

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References


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