SHORT REPORT

Freeze-thawing of breast milk does not prevent cytomegalovirus transmission to a preterm infant

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Freezing human milk is recommended to inactivate cytomegalovirus (CMV). A case of a preterm infant exclusively receiving frozen breast milk from his CMV seropositive mother showed that storage of breast milk for two months at -20°C did not prevent symptomatic postnatal CMV infection.

Breast milk is the main source of postnatal transmission of cytomegalovirus (CMV) to preterm infants, which in turn can cause severe illness.^{1–7} Various methods of virus inactivation in human milk have been described.^{8–11} Freezing human milk at -20°C for several days has been found to reduce or destroy viral infectivity *in vitro*. However, a certain level of infectivity remained, and there have been conflicting reports about the effectiveness of freeze-thawing. A range from 99% reduction of virus load after three days at -20°C to still detectable infectivity after 10 days at -15°C ¹¹ and after 10 days at -20°C in CMV-spiked milk has been reported, whereas in naturally infectious milk specimens, CMV was completely inactivated after storage at -20°C for seven days.⁸ On the other hand, no other treatment of human milk does less harm to its nutritional and immunological components than freeze-thawing.^{12–13} Because of the widespread and recommended^{14–15} use of freeze-thawing as a method to minimise infectious agents in human milk,¹⁶ we wanted to protect a preterm infant with high risk of postnatal CMV infection from breast milk by exclusive administration of freeze-thawed human milk.

PATIENT AND METHODS

Case report

A male preterm infant of 28⁺¹ weeks gestational age was born by caesarean section because of intrauterine growth retardation with increasingly pathological Doppler results of umbilical cord and fetal vessels. The Apgar score was 8/9/9 after one, five, and 10 minutes respectively, and arterial cord blood pH was 7.32. The 36 year old mother of Brazilian origin was CMV IgG seropositive. Therefore her own sequentially frozen breast milk samples were used exclusively from the start of feeding at the second day of life.

Methods

CMV analysis of breast milk, blood, urine, and bronchoalveolar lavage fluid (BAL) of the infant was performed as described.¹ After being thawed, the milk whey fraction was prepared.¹⁷ Aliquots of milk whey were used immediately for detection of virus infectivity and viral DNA load.¹⁸ To assess the viral load, micro cultures consisting of freshly prepared monolayers (four hours after seeding) of human foreskin fibroblasts (2.5×10^4 in 100 μl minimum essential medium/10% fetal calf serum per well) were used as previously described.¹⁸ After DNA isolation from milk whey, viral DNA was amplified by nested polymerase chain reaction (PCR)

using primer sequences of the CMV immediate early exon 4 region.¹⁷ Quantitative PCR of DNA from milk whey was performed using the Cobas Amplicor CMV Monitor test (Roche Molecular Biochemicals, Mannheim, Germany).¹⁸

Statistical analysis

For the description of DNA and virus decrease, logarithms of the measurements were fitted by linear regressions that correspond to an exponential decline after back transformation. For the quantitative PCR analysis, the geometric mean was estimated.

The study was approved by the ethics committee of the Medical Faculty of the University of Tübingen. Informed consent was obtained from the mother to test her milk.

RESULTS

The premature infant showed distinct growth retardation at birth (birth weight 490 g, length 28 cm, head circumference 21 cm, all values below the 3rd centile) with insufficient weight gain during the following weeks (1000 g after 10 weeks) despite full enteral feeding. Hepatopathy with an enlarged liver, rising liver enzyme activity, and increasing bilirubin concentrations gradually developed, strongly suggesting congenital liver disease. An extensive search for an infectious, metabolic, or genetic cause was inconclusive. Furthermore, a persisting thrombocytopenia and anaemia necessitated recurrent transfusions. All blood products transfused were CMV seronegative and inline filtered. CMV monitoring showed no infection during the first 16 weeks of life. At day 108, the infant's clinical condition deteriorated rapidly to a sepsis-like status with severe episodes of apnoea requiring reintubation (table 1).

Monitoring for CMV revealed positive qualitative PCR results for leucocytes, plasma, urine, and BAL (table 2), yet the bacterial blood cultures remained negative. In addition, viral isolates were obtained subsequently on day 111 from urine and BAL (table 2).

Antiviral therapy was not given. After six days, extubation was possible, and the condition was stable. However, the underlying pathology worsened, and the infant finally died on day 158 from respiratory failure due to the extreme hepatosplenomegaly. Post-mortem examination showed an idiopathic intrahepatic biliary duct hypoplasia.

Retrospective analysis of frozen breast milk samples from the mother of this preterm infant, given on the day of clinical deterioration and later, revealed the presence of infectious virus with a background of very high CMV DNA load. In the further course of lactation, both the viral DNA load and the amount of infectious virus decreased rapidly (fig 1). The predicted DNA decrease provides a very close fit to the observed results. The exponential decline had a daily reduction rate of the DNA load of 14%—that is, a half life

Abbreviations: CMV, cytomegalovirus; BAL, bronchoalveolar lavage fluid; PCR, polymerase chain reaction

Table 1 Laboratory test results and clinical symptomatology of the preterm infant (29 weeks gestational age, 490 g birth weight)

Day of life	Leucocytes (No/ μ l)	Granulocytes (No/ μ l)	Lymphocytes (No/ μ l)	Thrombocytes (No/ μ l)	CRP (mg/dl)	ALT (U/l)	AST (U/l)	γ -GT (U/l)	Direct bilirubin (μ mol/l)	Total bilirubin (μ mol/l)	Condition/symptoms
98	10950	2957	4599	85000	<0.4	70	28	64	83.8	102.6	Hepatosplenomegaly, CPAP. FiO ₂ 0.3, stable
103	21500	6665	11395	88000	1.3						1 apnoea (SaO ₂ 0.5), bagging
104	12950			83000	2.1			71	92.3	107.7	FiO ₂ 0.3, CPAP, stable
105	14920	7758	3282	86000		43	13	85	94.1	112.9	FiO ₂ 0.3, CPAP, stable
107	22680	8392	6350	78000	1.3						increasing FiO ₂
108	21290	9155	7664	72000	1.9						Sepsis-like symptoms, reintubation, anaemia, transfusion
111	12260	4904	5394	63000	0.8						Ventilation, FiO ₂ 0.3
112	12240	6732	2693	68000							Hepatosplenomegaly
114	13740	6320	4534	75000	0.9	44	12	23	66.7	148.8	$\uparrow \uparrow$, petechiae
117	16000	4480	6240	75000	0.9						Amelioration, extubation, CPAP
120	20300	6496	10353	94000	1.2	63	17	22	95.8	155.6	Hepatosplenomegaly, CPAP. FiO ₂ 0.3, stable
130	12850	5140	5269	120000	1.7	91	24	23	103	171	Hepatosplenomegaly, CPAP. FiO ₂ 0.3, stable

Sepsis-like symptoms without evidence of a bacterial origin occurred at day 108 requiring reintubation.

CRP, C Reactive protein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ -GT, γ glutamyl transferase; CPAP, continuous positive airway pressure; FiO₂, fraction of inspired oxygen; SaO₂, arterial oxygen saturation.

of 4.6 days. Owing to the vast abundance of breast milk in this case, the infant received freeze-thawed milk from day 43 when he was 108 days old, resulting in a freezing time of 65 days. CMV transmission probably occurred between day 72 (last CMV negative urine sample) and day 108 (beginning of the symptomatology). As genotyping using human CMV UL10–UL13 glycoprotein gene sequences¹ revealed, the viral strains isolated from breast milk and urine of the infant were identical (data not shown).

DISCUSSION

This case report provides strong evidence that freezing does not inactivate CMV in breast milk under clinical conditions and does not prevent CMV transmission. The fact that CMV-containing breast milk could still lead to a clinically relevant CMV infection of a preterm infant with idiopathic intrahepatic biliary duct hypoplasia after more than two months storage at -20°C has not been previously reported. Even after storage for one year at -20°C and three freeze-thawing cycles, we were able to isolate infectious virus from the whey fraction of these breast milk samples (data not shown). Genotyping of viral strains isolated from breast milk and urine¹ showed that breast feeding was the only source of

virus transmission to the infant. Recent publications^{4–5, 15} report that, despite the exclusive or predominant use of frozen breast milk, there can still be CMV transmission with apparent clinical symptoms.³

We can only speculate about the possible effect of the underlying disease in our index patient on the facilitation of the CMV infection and disease. However, he had a high risk of acquiring a postnatal CMV infection from untreated breast milk.⁷ The exponential decline in DNA load led to the speculation that a preceding increase was also exponential with a sharp peak load, which has been confirmed.^{18–19} Owing to the wide gap between the postnatal age of the preterm infant and the true age of the breast milk he received, and the reported unimodal dynamics of DNAlactia and virolactia during lactation,¹⁸ we must assume that, for many days before day 108 post partum, breast milk with a very high CMV load was fed to this infant. Unfortunately, we did not monitor the breast milk prospectively. Using both native milk from seropositive mothers and artificially infected milk from seronegative mothers, we recently showed that, during maximal virus excretion, CMV viral load can only be reduced but not eliminated by freeze-thawing.²⁰ When aiming to protect a population at risk from the hazards of postnatal

Table 2 Results of sequential cytomegalovirus (CMV) monitoring of a preterm infant (29 weeks gestational age, 490 g birth weight)

Material	Assay	Results				
		Day 5	Day 36	Day 72	Day 108	Day 111
Blood	PCR plasma	Negative			Positive	
	PCR leuco	Negative			Positive	
Urine	PCR		Negative	Negative		Positive
	IEA		Negative	Negative		Positive
	Culture		Negative	Negative		Positive
BAL	PCR					Positive
	IEA					Positive
	Culture					Positive

Sepsis-like symptoms without evidence of a bacterial origin occurred at day 108 requiring reintubation. BAL, Bronchoalveolar lavage fluid; PCR, polymerase chain reaction; IEA, short term culture with CMV immediate early antigen staining; leuco, leucocytes; culture, virus isolation.

Figure 1 Time course of quantitative cytomegalovirus (CMV) DNA and viral load in naturally infected breast milk samples (case report). The high speed centrifugation based microculture assay results are number of immediate early antigen positive (IEA+) fibroblast nuclei/ml whey; lower detection limit, 1 nucleus/ml whey. For the quantitative polymerase chain reaction (PCR) analysis, the lower detection limit was 400 copies/ml. The black bars represent test results below the corresponding detection limits. The black curve represents the statistical fit of the predicted quantitative PCR results with a half life of 4.6 days—that is, a daily reduction of the DNA load of 14%. First examination was at postnatal day 108 because of sepsis-like symptoms (black arrow). On postnatal day 108, milk was given from postnatal day 43.

CMV transmission through breast milk, freezing cannot be recommended.

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REFERENCES

- 1 **Hamprecht K**, Maschmann J, Vochem M, *et al*. Epidemiology of cytomegalovirus transmission of mothers to preterm infants by breastfeeding. *Lancet* 2001;**357**:513–18.
- 2 **Maschmann J**, Hamprecht K, Dietz K, *et al*. Cytomegalovirus-infection of extremely low birthweight infants via breast-milk. *Clin Infect Diseases* 2001;**33**:1998–2003.
- 3 **Cheong JL**, Cowan FM, Modi N. Gastrointestinal manifestations of postnatal cytomegalovirus infection in infants admitted to a neonatal intensive care unit over a five year period. *Arch Dis Child Fetal Neonatal Ed* 2004;**89**:F367–9.
- 4 **Jim WT**, Shu CH, Chiu NC, *et al*. Transmission of cytomegalovirus from mothers to preterm infants by breast milk. *Pediatr Infect Dis J* 2004;**23**:848–51.
- 5 **Doctor S**, Friedman S, Dunn M, *et al*. Cytomegalovirus transmission to extremely low-birthweight infants through breast milk. *Acta Paediatr* 2005;**94**:53–8.
- 6 **Miron D**, Brosilow S, Felszer K, *et al*. Incidence and clinical manifestations of breast milk-acquired cytomegalovirus infection in low birth weight infants. *J Perinatol* 2005;**25**:299–303.
- 7 **Meier J**, Lienicke U, Tschirch E, *et al*. Human cytomegalovirus reactivation during lactation and mother-to-child transmission in preterm infants. *J Clin Microbiol* 2005;**43**:1318–24.
- 8 **Dworsky M**, Stagno S, Pass RT, *et al*. Persistence of cytomegalovirus in human milk after storage. *J Pediatr* 1982;**101**:440–3.
- 9 **Friis H**, Andersen HK. Rate of inactivation of cytomegalovirus in raw banked milk during storage at –20 degrees C and pasteurisation. *BMJ* 1982;**285**:1604–5.
- 10 **Goldblum RM**, Dill CW, Albrecht TB, *et al*. Rapid high-temperature treatment of human milk. *J Pediatr* 1984;**104**:380–5.
- 11 **Welsh JK**, Arsenakis M, Coelen RJ, *et al*. Effect of antiviral lipids, heat, and freezing on the activity of viruses in human milk. *J Infect Dis* 1979;**140**:322.
- 12 **Evans TJ**, Ryley HC, Neale LM, *et al*. Effect of storage and heat on antimicrobial proteins in human milk. *Arch Dis Child* 1978;**53**:239–41.
- 13 **Liebhaber M**, Lewiston NJ, Asquith MT, *et al*. Alterations of lymphocytes and of antibody content of human milk after processing. *J Pediatr* 1977;**91**:897–900.
- 14 **Lawrence RA**. Storage of human milk and the influence of procedures on immunological components of human milk. *Acta Paediatr Suppl* 1999;**88**:14–18.
- 15 **Sharland M**, Khare M, Bedford-Russell A. Prevention of postnatal cytomegalovirus infection in preterm infants. *Arch Dis Child Fetal Neonatal Ed* 2002;**86**:F140.
- 16 **Bryant P**, Morley C, Garland S, *et al*. Cytomegalovirus transmission from breast milk in premature babies: does it matter? *Arch Dis Child Fetal Neonatal Ed* 2002;**87**:F75–7.
- 17 **Hamprecht K**, Vochem M, Baumeister A, *et al*. Detection of cytomegaloviral DNA in human milk cells and cell free milk whey by nested PCR. *J Virol Methods* 1998;**70**:167–76.
- 18 **Hamprecht K**, Witzel S, Maschmann J, *et al*. Rapid detection and quantification of cell free cytomegalovirus by a high-speed centrifugation-based microculture assay: comparison to longitudinally analyzed viral DNA load and pp67 late transcript during lactation. *J Clin Virol* 2003;**28**:303–16.
- 19 **Yasuda A**, Kimura H, Hayakawa M, *et al*. Evaluation of cytomegalovirus infections transmitted via breast milk in preterm infants with a real-time polymerase chain reaction assay. *Pediatrics* 2003;**111**:1333–6.
- 20 **Hamprecht K**, Maschmann J, Müller D, *et al*. Cytomegalovirus (CMV) inactivation in breast milk: reassessment of pasteurization and freeze-thawing. *Pediatr Res* 2004;**56**:529–35.