Inactivation of Hepatitis C Virus Infectivity by Human Breast Milk

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(See the editorial commentary by Jhaveri on pages 1932–3.)

Background. Hepatitis C virus (HCV) is spread through direct contact with blood, although alternative routes of transmission may contribute to the global burden. Perinatal infection occurs in up to 5% of HCV-infected mothers, and presence of HCV RNA in breast milk has been reported. We investigated the influence of breast milk on HCV infectiousness.

Methods/Results. Human breast milk reduced HCV infectivity in a dose-dependent manner. This effect was species-specific because milk from various animals did not inhibit HCV infection. Treatment of HCV with human breast milk did not compromise integrity of viral RNA or capsids but destroyed the lipid envelope. Fractionation of breast milk revealed that the antiviral activity is present in the cream fraction containing the fat. Proteolytic digestion of milk proteins had no influence on its antiviral activity, whereas prolonged storage at 4°C increased antiviral activity. Notably, pretreatment with a lipase inhibitor ablated the antiviral activity and specific free fatty acids of breast milk were antiviral.

Conclusions. The antiviral activity of breast milk is linked to endogenous lipase-dependent generation of free fatty acids, which destroy the viral lipid envelope. Therefore, nursing by HCV-positive mothers is unlikely to play a major role in vertical transmission.

Keywords. hepatitis C virus (HCV); transmission; breast milk; antiviral; free fatty acids.

Globally, an estimated 130 million people are chronically infected with hepatitis C virus (HCV) [1] and are therefore at a high risk for developing severe liver damages including hepatic steatosis, fibrosis, cirrhosis, and hepatocellular carcinoma [2, 3].

Being a blood-borne virus, the transmission of HCV occurs by exposure to infectious blood. Routes of transmission mainly include receipt of contaminated blood products, as well as needle stick injuries or injections with contaminated syringes. The latter mode of transmission is an important risk factor for intravenous drug users and represents the main route of transmission in developed countries [4–6]. Other modes of transmission involve vertical and sexual infections, with the latter occurring very infrequently [7]. In contrast, mother-to-infant transmission is regarded as the leading route of acquisition of HCV for children worldwide, even though transmission occurs only in about 5%–10% of deliveries [8, 9]. Risk factors in relation to perinatal transmission include HCV viremia, human immunodeficiency virus (HIV) coinfection, prolonged membrane rupture, and intrapartum exposure to maternal blood infected by HCV [10–12]. The role of breastfeeding for perinatal transmission is regarded to

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be neglectable, even though some studies have reported HCV RNA in breast milk and colostrum samples from infected women [13, 14]. Nevertheless, most studies agree that breastfeeding, as long as it does not involve bleeding or cracked nipples, does not increase the rate of perinatal transmission of HCV [10, 13, 15]. Therefore, there is no recommendation against breastfeeding from HCV-infected women neither from the American College of Obstetricians and Gynecologists (ACOG) nor the European Association for the Study of the Liver (EASL) [16, 17].

So far none of the previous studies have addressed the question about the infectivity and stability of HCV directly in human breast milk. Therefore, with the help of a productive HCV cell culture system based on the Japanese fulminant hepatitis (JFH1) HCV isolate, which reproduces the complete viral replication cycle in vitro, we present for the first time to our knowledge data analyzing the effect of human breast milk on HCV.

MATERIALS AND METHODS

Milk Samples and Infant Formula

Human milk samples of HCV-negative women were collected from healthy donors between 1 month and 12 month postpartum and kept frozen at −80°C until used in experiments. No fresh milk was included in this study because there were logistical issues with testing fresh milk directly. All mothers provided written informed consent for the collection of samples and subsequent analysis. Samples were tested against different viruses after storage for up to 1 year at −80°C or at various intervals at 4°C. Animal milk samples were obtained from a local dairy and frozen at −80°C until needed. Infant formulas of the following brands were used in experiments: Aptamil Pre, Beba Pro, Bebivita 1, Hipp Bio Combiotik Pre, Humana Pre, and Milumil Pre. The infant formulas were prepared according to the manufacturer's instructions.

Milk Preincubation Experiments and Virus Titration

To determine the effect of breast milk on viral infectivity, preincubation experiments were performed. Virus was incubated with breast milk at a ratio of 1:10, for 1 hour at room temperature. As control, virus was incubated with medium instead of milk. After the incubation period, target cells were infected in a limiting dilution assay on Huh7.5 cells, with slight modifications. The tissue culture infectious dose 50 ($TCID_{50}$) was determined 72 hours postinfection as described elsewhere [18]. Reduction factors (RFs) were calculated as the difference between the logarithmic virus titer of control and milk or fatty acid treated virus, using the following formula:

$$RF = a - b$$

RF: reduction factor; a: $\log_{10}TCID_{50}$/mL of control titration; b: $\log_{10}$ $TCID_{50}$/mL of milk/fatty acid titration.

RESULTS

Human Breast Milk Reduces HCV Infectivity

In order to analyze the effect of human breast milk on HCV infectiousness, preincubation experiments were performed. The chimeric genotype 2a Jc1 virus [19] was incubated with breast milk obtained from healthy donors in a ratio of 1:10 for 1 hour at room temperature before permissive Huh7.5 cells were infected. As a control, Jc1 was preincubated with cell culture medium. Testing milk samples from 13 different HCV-negative donors which were routinely stored at 4°C prior the experiment, we consistently observed a reduction of HCV infectivity by 2–3 orders of magnitude (Figure 1*A*). Even shorter preincubation periods of 1 minute were already sufficient to reduce viral titers (Supplementary Figure 1). Due to a cytotoxic effect of the milk on the target cells, viral titers below 10^2 $TCID_{50}$/mL could not be detected (n.d.). As HCV isolates are grouped into different subtypes that have variable biological properties influencing natural course and treatment response, we next assessed the effect of breast milk on all 6 major genotypes. We also included a novel HCV strain tentatively assigned to genotype 7 [20]. These analyses revealed that human breast milk inactivated HCV infectivity independent of the viral genotype (Supplementary Table 1) with reduction factors (RF's) of two or even higher. Dilution of the milk showed that the antiviral activity was concentration dependent with concentrations between 4% and 6% milk (depending on the donor) being sufficient to reduce HCV infectivity, whereas higher dilutions led to an abolishment of the antiviral effect (Figure 1*B*). It is of note that the HCV RNA copy numbers used in this study were approximately 1000-fold higher than detected in breast milk. Heat treatment of the milk prior to virus preincubation had no influence on the antiviral activity (Figure 1*C*). Furthermore, the antiviral activity of breast milk was specific to human milk and was not found in milk from horses, cows (colostral milk), or commercially available infant formula (Figure 1*D*). Taken together, these data demonstrate antiviral properties of human breast milk against HCV.

Human Breast Milk Has no Effect on HCV RNA nor Capsid, But Impairs the Integrity of the Viral Envelope

To further characterize the influence of human breast milk on HCV, we analyzed its effect directly on the viral particle. The experimental setup is shown in Figure 2*A*. First, we tested if the viral capsid was targeted by milk treatment. Because the HCV capsid is formed by core protein, we performed a core specific enzyme-linked immunosorbent assay (ELISA) after the preincubation period to detect whether the amount of core protein was changed. No difference between the milk and control treated virus could be detected (Figure 2*B*). Determination of viral RNA by quantitative real-time polymerase chain reaction (qRT-PCR) revealed that HCV RNA copies did not differ between the milk treated and the control-treated samples

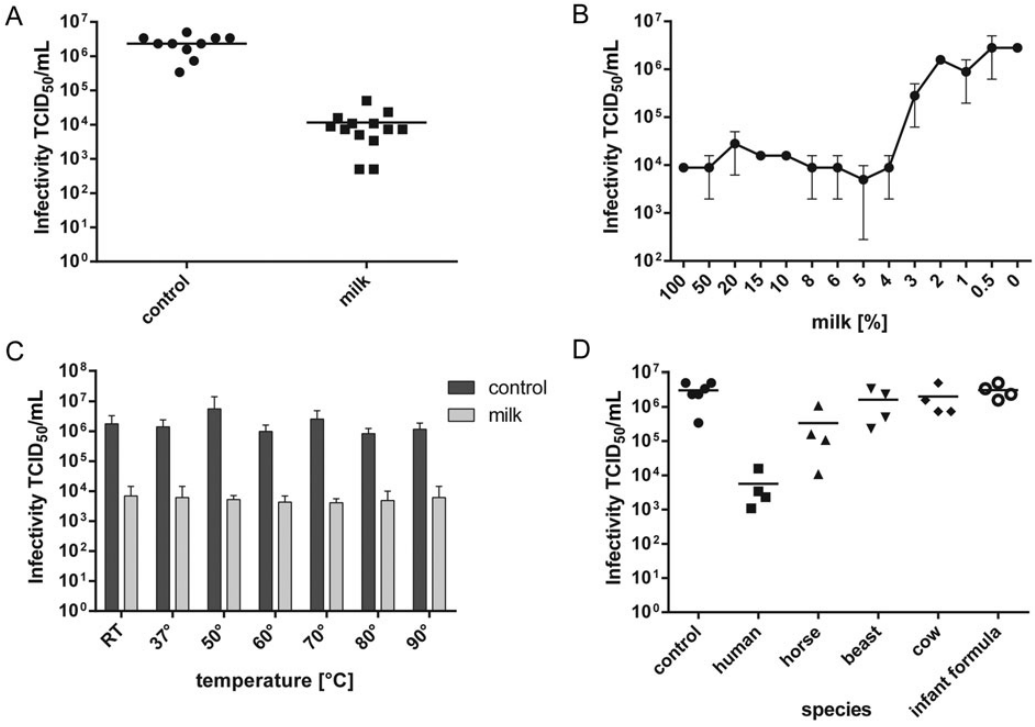

Figure 1. Human breast milk reduces HCV infectivity. *A*, Milk of 13 healthy donors was collected, routinely stored at 4°C for 4 days and incubated with HCV (Jc1) at a ratio of 10:1. As control, Jc1 was incubated with the same amount of culture medium. The mixture was incubated at room temperature for 1 hour followed by inoculation of Huh7.5 cells. After 72 hours, HCV infectivity was detected by an immuno-histochemistry staining against the viral protein NS5A to determine the tissue culture infectious dose 50 per mL ($TCID_{50}$/mL). Each symbol represents individual milk or control samples tested. The solid line represents the mean of the viral infectivity for each group. *B*, Human breast milk was diluted with PBS before incubation with Jc1 and inoculation of Huh7.5 cells. Infectivity was determined as in (*A*). A representative experiment from 1 donor out of 3 with standard deviation (SD) is shown. *C*, Milk or medium samples were heated at indicated temperatures for 1 hour before incubation with HCV and inoculation of Huh7.5 cells. Infectivity was determined as in (*A*). Shown is the mean + SD of 3 independent repetitions. *D*, Milk from different species (human, horse, cow, with beast milk as the colostral cow's milk) was collected and infant formula prepared according to the manufacturer's instructions. The milk was preincubated with Jc1 for 1 hour at RT before inoculation of Huh7.5 cells. Infectivity was determined as in (*A*). Each symbol represents individual milk or control samples tested. The solid line represents the mean of the viral infectivity for each group. Abbreviations: HCV, hepatitis C virus; RT, room temperature; $TCID_{50}$, tissue culture infectious dose 50.

(Figure 2*C*). To investigate if the viral genome itself was still infectious, we purified the viral RNA from milk or control-treated samples and transfected this virus particle associated RNA into highly permissive Huh7.5 cells. Viral replication was assessed via immunoflourescent (IF) staining against the viral protein NS3 as well as by qRT-PCR. Viral replication could be detected independent of the treatment, both by IF staining as well as genomic amplification via qRT-PCR (Figure 2*D*). To further analyze if infectious particles were released from these cells, the supernatant was harvested and used to infect naive Huh7.5 cells in a limiting dilution assay to determine the viral titer. Productive infection of target cells could be detected irrespective of the treatment (Figure 2*E*). These results indicate that neither the viral capsid nor the viral RNA was affected upon milk treatment, which left the viral envelope as possible target.

To test this assumption, a proteolytic protection assay was performed to determine the amount of protease-resistant, enveloped core protein after milk treatment. In the presence of an intact envelope, externally added proteinase K (PK) cannot cleave the viral capsid, because the protease has no access to the membrane enveloped core protein. In contrast, once the viral envelope is disrupted, digestion of core protein would occur, which can be measured via core-specific ELISA. As a positive control the detergent triton X-100 was added, which resolved all membranes and proved that the concentration of PK used was sufficient to cleave core protein. All of the tested milk samples showed a reduction in the amount of core after PK treatment with only minor differences observed between the different donors, whereas in the control sample no obvious reduction in the amount of core protein after PK treatment could be seen. In contrast, in each positive control PK led to a clear digestion of core protein (Figure 2*F*). These results indicate that human breast milk disrupts the viral envelope. Further analysis revealed that milk treatment also impaired the general biophysical properties of the viral particle as determined by iodixanol step gradient centrifugation. Fractions were collected and assayed for HCV core protein. Viruses treated as

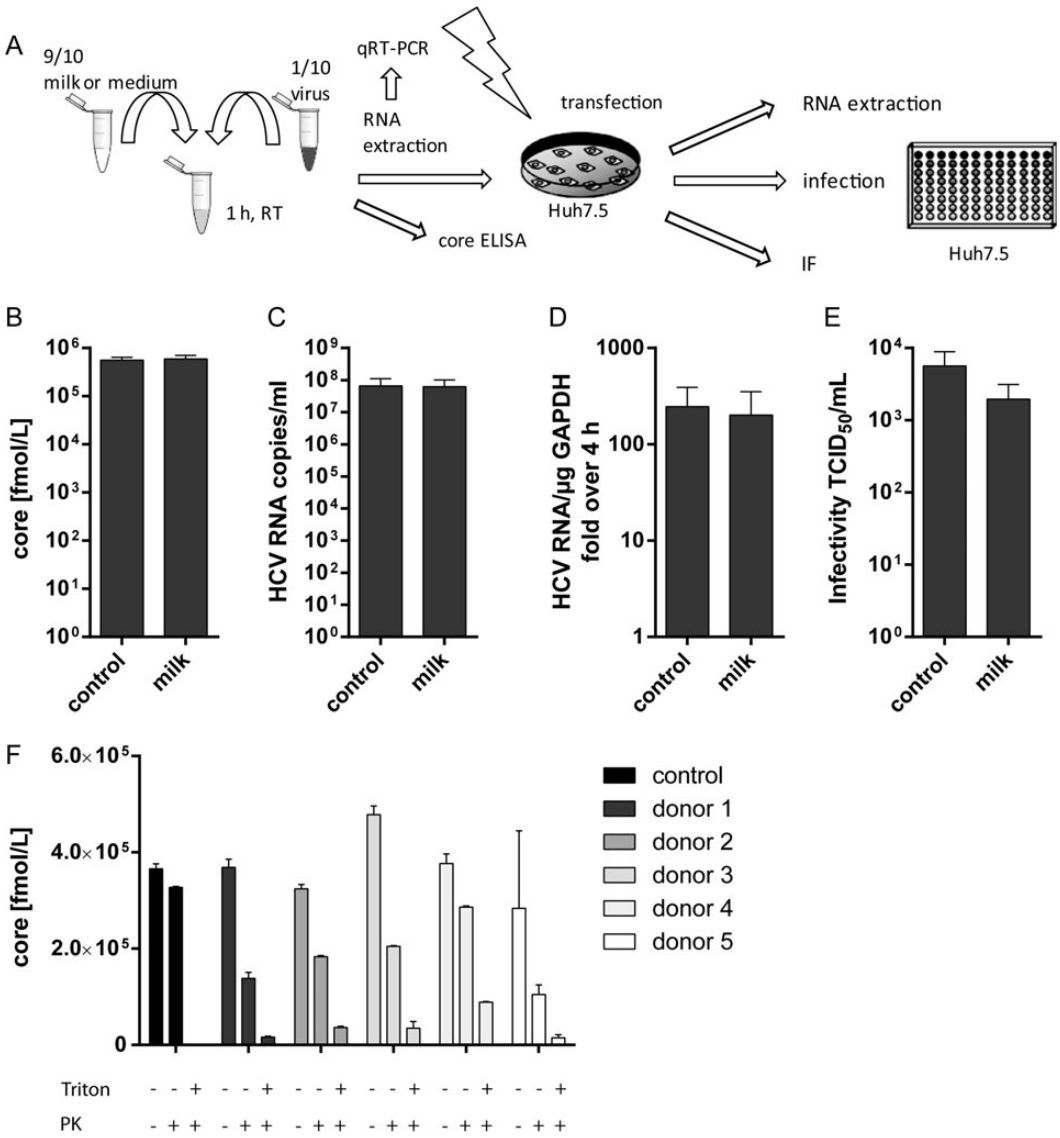

Figure 2. Human breast milk disrupts the HCV envelope. *A*, Experimental set up for studying the effect of breast milk on HCV stability. Milk and virus were mixed at a ratio of 10:1. The mixture was incubated at room temperature for 1 hour before the amount of core protein was determined via core-specific ELISA (*B*) as well as RNA extracted and measured by qRT-PCR (*C*). Extracted RNA was used to transfect naïve Huh7.5 cells by electroporation. After 72 hours, Huh7.5 cells were lysed and HCV RNA was analyzed by qRT-PCR (*D*). The supernatant of the cells was harvested and used to infect naive Huh7.5 cells to determine viral titer as $TCID_{50}$/mL (*E*). Shown is the mean + SD of 3 independent repetitions. *F*, Milk samples were incubated with Jc1 for 1 hour at RT before a proteolytic digestion protection assay was performed. Therefore, one part was left untreated, one part was treated with 50 µg/mL proteinase K (PK) for 1 hour at 4°C, and another part was lysed in 2% triton X-100 prior to PK treatment. The amount of protease-resistant core protein was quantified with a core-specific ELISA. Depicted are representative results for 5 different milk donors showing the mean values with standard deviations. Abbreviations: ELISA, enzyme-linked immunosorbent assay; HCV, hepatitis C virus; qRT-PCR, quantitative real-time polymerase chain reaction; RT, room temperature; $TCID_{50}$, tissue culture infectious dose 50.

control either with medium or cow's milk showed a distinct peak at a density of about 1.13 g/mL, whereas with the milk-treated virus a shift of the peak to a lower density of 1.10 g/mL was observed (Supplementary Figure 2), indicating human milk treatment alters particle density and lipoprotein association by disruption of the envelope.

Inactivation of Enveloped Viruses by Human Breast Milk

Besides providing optimal nutrition for the infant, human breast milk has been described to contain several components with antimicrobial activity including the potential to act antiviral against several viruses. Therefore, we investigated the effect of human breast milk on different enveloped and nonenveloped viruses in

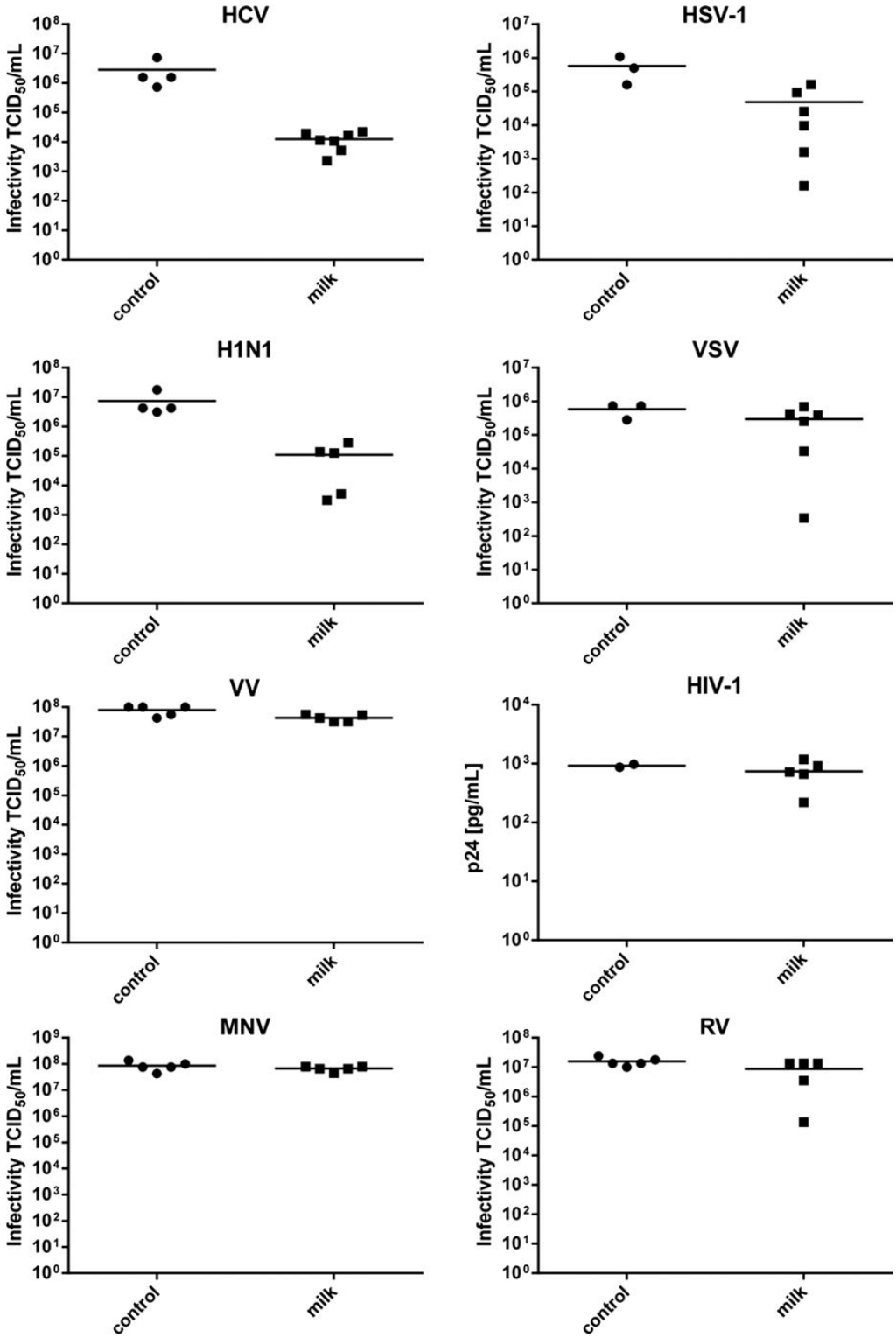

Figure 3. Human breast milk inactivates enveloped viruses from diverse virus families. Milk from 5 different donors was mixed with either HCV, HSV-1, H1N1, VSV, VV, HIV-1, MNV, or RV. The mixture was incubated for 1 hour at RT before infection of permissive cell lines. After 72 hours, the viral titers were determined as $TCID_{50}$/mL. For HIV, cells were infected with the milk/virus mixture and after 7 days the concentration of the antigen p24 was determined by ELISA. Each symbol represents individual milk or control samples tested. The solid line represents the mean of viral infectivity, or in case of HIV the mean p24 concentration, for each group. Abbreviations: ELISA, enzyme-linked immunosorbent assay; H1N1, influenza virus A; HCV, hepatitis C virus; HIV-1, human immunodeficiency virus type 1; HSV-1, herpes simplex virus type 1; MNV, murine norovirus; qRT-PCR, quantitative real-time polymerase chain reaction; RT, room temperature; RV, rotavirus; $TCID_{50}$, tissue culture infectious dose 50; VSV, vesicular stomatitis virus; VV, vaccinia virus.

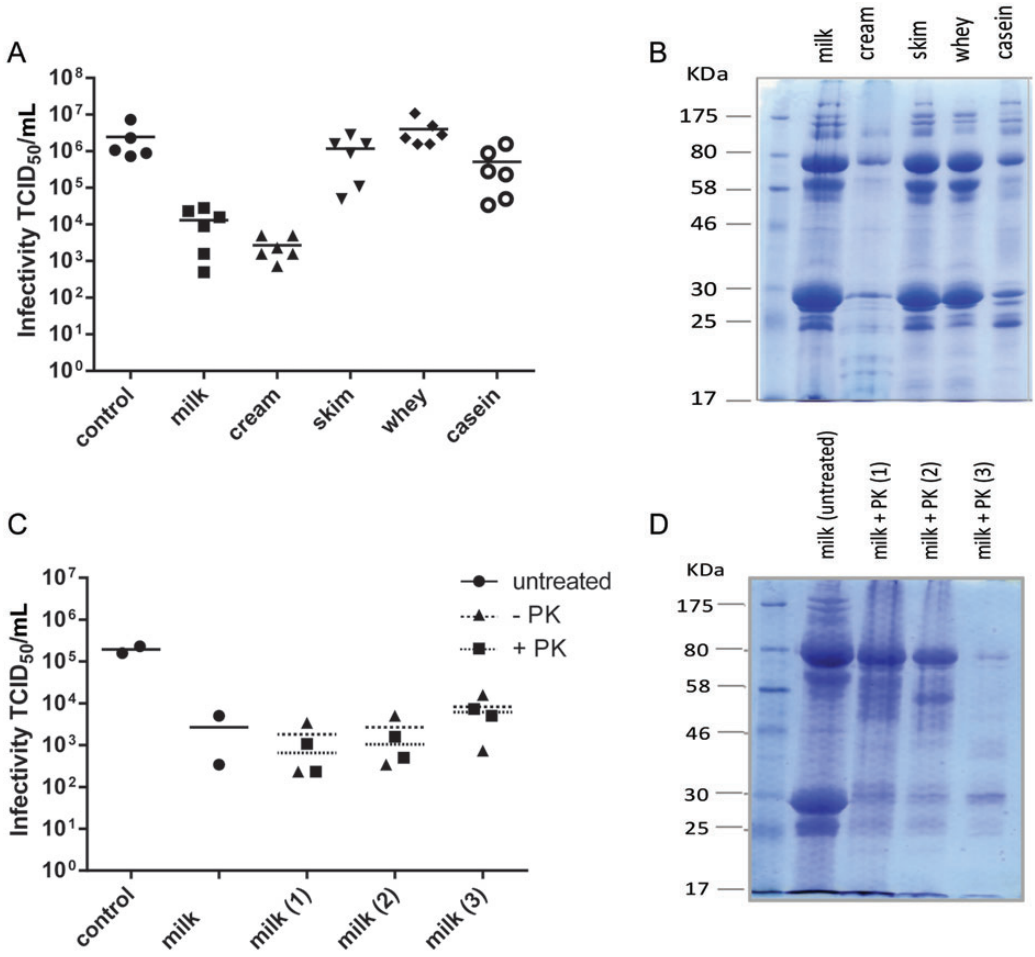

Figure 4. The antiviral activity of breast milk resides in the cream fraction and is not affected by proteolytic digestion. *A*, Milk fractions were separated upon ultracentrifugation and tested on their antiviral activity against HCV. Depicted is the residual viral infectivity as $TCID_{50}$/mL after incubation with each fraction. Symbols represent individual samples tested with the solid line representing the mean of the viral infectivity for each group. *B*, The fractionated samples were loaded onto a SDS 11% polyacrylamide gel, and proteins were visualized by coomassie blue staining (representative gel shown). *C*, Milk samples were treated either with 50 µg/mL active (+PK) or with inactivated (−PK) proteinase K and incubated for 1 hour at 37°C (1), 1 hour at 55°C (2) or 5 hours at 55°C (3). As a control some milk or medium samples were left untreated at 4°C. The samples were tested on their antiviral activity against HCV. Each symbol represents individual samples tested. The solid and dashed lines represent the mean of the viral infectivity for each group. *D*, Coomassie gel to detect proteins after digestion of milk, separated via a SDS 11% polyacrylamide gel and visualized by coomassie blue staining (representative gel shown). Abbreviations: HCV, hepatitis C virus; $TCID_{50}$, tissue culture infectious dose 50.

comparison to HCV. From the viruses tested, we observed a relatively strong antiviral effect of human milk on the enveloped viruses HCV (average between 2 and 3 orders of magnitude reduction in viral titer) and influenza virus (average about 2 orders of magnitude reduction), and a weaker effect against herpes simplex virus (average about 1 order of magnitude reduction) and vesicular stomatitis virus (VSV). No pronounced reduction in viral titers could be observed for the nonenveloped viruses murine norovirus and rotavirus. Similarly, we also did not observe a strong antiviral effect for vaccinia virus and HIV (Figure 3). Together these data indicate that human breast milk acts primarily against envelope viruses, although there were differences in the degree of inactivation for some viruses.

Identification of Breast Milk-derived Free Fatty Acids as Antiviral Agents Against HCV

To further resolve which component of human breast milk is responsible for the reduction in HCV infectivity, we performed biophysical analyses of breast milk components. Upon low speed centrifugation milk can be separated into cream and skim milk. The skim milk can further be subdivided by high speed centrifugation into the pellet containing caseins and into the supernatant called whey [21], a fraction very rich in milk proteins. The cream on the other hand, contains the milk fat consisting of a triglyceride core surrounded by a membrane called milk fat globular membrane (MFGM), in which proteins are incorporated [22]. First, we separated milk components by

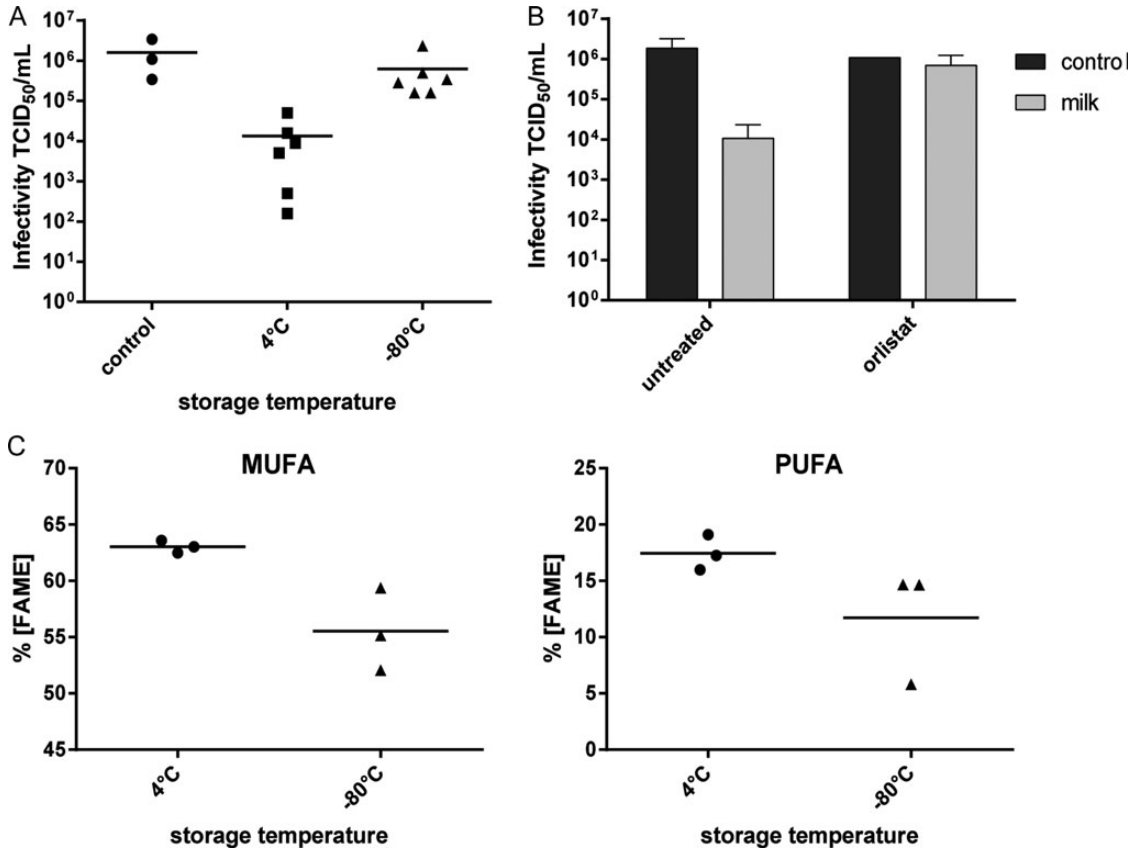

Figure 5. The antiviral effect of breast milk is storage and lipase dependent. *A*, Milk samples were collected and stored at either 4°C or −80°C for 4 days before they were tested on their antiviral activity against HCV. Each symbol represents individual samples tested. The solid line represents the mean of the viral infectivity for each group. *B*, Milk or as control medium samples were treated with 25 µg/mL of the lipase inhibitor orlistat before they were stored at 4°C for 4 days. After storage, the antiviral activity against HCV was determined as $TCID_{50}$/mL. Depicted is the mean + SD of 3 independent repetitions. *C*, Concentrations of MUFA and PUFA from 3 different donors were determined via thin layer chromatography after storage of milk at 4°C or −80°C for 4 days and are depicted as percent fatty acid methyl esthers (% FAME). Each symbol represents individual samples tested. The solid line represents the mean of the fatty acid concentration for each group. Abbreviations: HCV, hepatitis C virus; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SD, standard deviation; $TCID_{50}$, tissue culture infectious dose 50.

centrifugation and tested each fraction for their antiviral activity. Only the cream fraction led to a reduction in HCV infectivity comparable to the whole milk (Figure 4*A*). The successful separation of milk components was verified using SDS-PAGE and coomassie staining. These analyses confirmed that the bulk of the proteins was contained in the skim and subsequently in the whey fraction, whereas considerably fewer proteins were detected in the cream fraction (Figure 4*B*). It is known that some of the proteins of the cream fraction have a high antiviral property like lactadherin and lactoferrin [23]. To test if indeed a protein mediates the observed reduction in viral infectivity exerted by breast milk, we digested proteins by using proteinase K treatment. To increase the enzymatic activity, different incubation temperatures and incubation times were employed. Proteinase K treatment had no influence on the antiviral activity, irrespective of the reaction time or temperature (Figure 4*C*). The digestion of milk proteins was visualized via SDS-PAGE and coomassie staining (Figure 4*D*). Taken together, these data indicate that the reduction in viral infectivity exerted by treatment of HCV particles with breast milk was unlikely due to a protein. This finding left the major component of the cream fraction, the fat, as likeliest candidate for causing the observed antiviral effect. Interestingly, human breast milk only became antiviral after it was stored at 4°C but not −80°C, for 4 days (Figure 5*A*) and fresh human milk did not act antiviral (data not shown). Furthermore, the appearance of the antiviral activity could be prevented by treatment of the milk with the lipase inhibitor orlistat, which inhibits pancreatic and gastric lipases [24], as well as lipoprotein lipases [25], during the storage at 4°C (Figure 5*B*). By performing thin layer chromatography, we could show that the storage process of the milk led to an increase in both monounsaturated (MUFA) as well as polyunsaturated (PUFA) fatty acids at the 4°C stored sample compared to the −80°C stored sample (Figure 5*C*). Therefore, we

Table 1. Free Fatty Acids Act Antiviral Against HCV

Fatty acid		Conc. [mg/mL]	RF	SD
Butyric acid	4:0	10	−0.17	±0.53
Caproic acid	6:0	10	≥3.71	. . .
Caprylic acid	8:0	10	≥3.71	. . .
Capric acid	10:0	5	≥3.71	. . .
Lauric acid	12:0	5	≥3.71	. . .
Myristic acid	14:0	20	0.96	±0.71
Palmitic acid	16:0	20	0.58	±0.18
Stearic acid	18:0	20	1.08	±0.18
Palmitoleic acid	16:1	2	≥2.46	±1.7678
Oleic acid	$18:1^{cis}$	10	≥3.71	. . .
Elaidic acid	$18:1^{trans}$	20	0.21	±0.35
Linoleic acid	18:2	5	3.46	±0.3536
Linolenic acid	18:3	5	≥3.71	. . .
Arachidonic acid	20:4	1	≥3.71	. . .

Abbreviations: HCV, hepatitis C virus; RT, room temperature; SD, standard deviation; $TCID_{50}$, tissue culture infectious dose 50. Different concentrations of free fatty acids were incubated with Jc1 for 1 hour at RT before viral titers were determined as $TCID_{50}$/mL. Shown is the lowest concentration which reduced HCV titers by a reduction factor (RF) of at least 2 or the highest concentration tested. Reduction factors of viral titers below the detection range were declared as greater or equal (≥). The mean values ±SD of 2 independent experiments are shown.

hypothesized that the gain of antiviral activity of breast milk during storage at 4°C was linked to a lipase-dependent increase of free fatty acids. To test this hypothesis, we assessed the antiviral activity of different free fatty acids contained in breast milk and enriched during storage at 4°C. Among the fatty acids tested, some saturated as well as most of the unsaturated fatty acids reduced viral titers by several orders of magnitude (Table 1), thus confirming the potential of free fatty acids contained in human breast milk to act as antiviral factors against HCV.

DISCUSSION

In the present study, we used a productive cell culture system to show that HCV infectivity is markedly decreased after incubation with human breast milk. The observed antiviral effect was independent of the HCV genotype, not abolished on temperature treatment and not shared by milk of different species. Furthermore, we could show that the integrity of the viral envelope was impaired and that free fatty acids, likely produced in a milk lipase and temperature-dependent fashion, are responsible for the reduction in viral infectivity. Therefore, we propose the following working model (Figure 6): Due to milk-handling procedures, including freezing and thawing as well as mechanical disruption, the MFGM, which normally protects the triglyceride core, is disintegrated. Lipases present in human milk (lipoprotein lipase and bile salt stimulated lipase) then get access to the triglycerides, which results in the digestion of triglycerides and the release of free fatty acids (FA), diglycerides (DG), and monoglycerides (MG). These products of lipolysis are more polar than triglycerides, leading to the formation of micelles. These interact or even incorporate into the viral envelope destroying viral integrity, which results in a decreased infectivity.

Most studies agree that breast-feeding does not increase the risk of HCV transmission [26]. There are some studies claiming

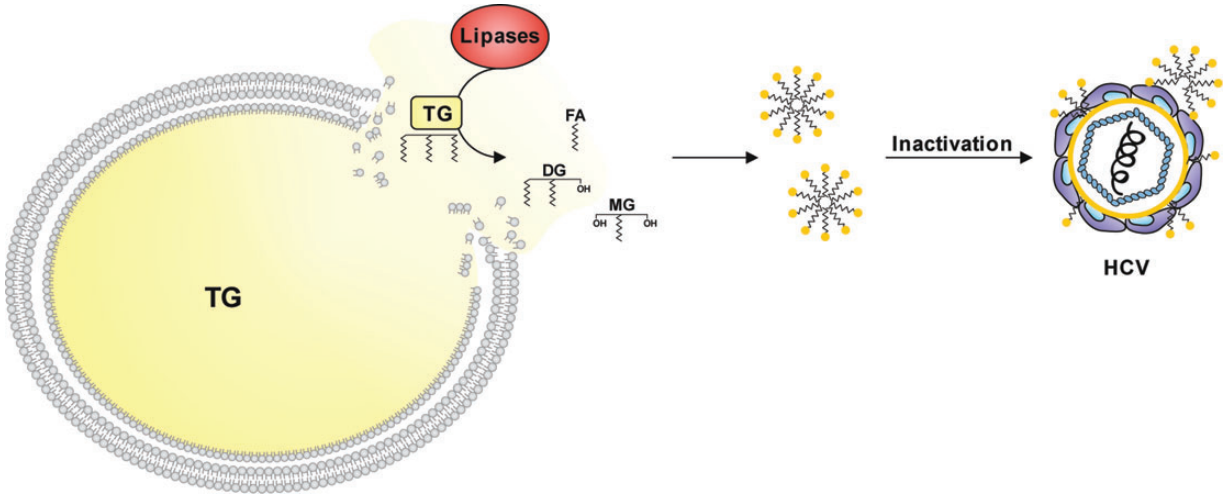

Figure 6. Hypothetical model for inactivation of HCV by human breast milk. Due to a disruption of the milk fat globular membrane (*gray*), milk lipases (*red*) get access to the triglyceride (TG) core (*yellow*). Following milk digestion free fatty acids (FA), monoglycerides (MG), and diacylglycerides (DG) are released. These are able to disrupt the viral envelope of HCV (schematic depiction, showing the glycoproteins E1 and E2 (*blue*), the viral envelope (*yellow*) and the capsid formed by the core protein (*light blue*), which protects the viral RNA). Abbreviation: HCV, hepatitis C virus.

that the amount of HCV RNA in the breast milk is too low to infect the newborn and that potentially low amounts of viruses are easily inactivated by gastric juices [12, 27]. We could not confirm this assumption in vitro as we observed that HCV is stable for 24 hours upon preincubation with gastric juices from healthy children as well as adults, confirming the ability of HCV to tolerate low pH's [28]. Therefore, different mechanisms are likely to exist that limit transmission of HCV via breast milk.

We could show that components of human breast milk exclusively interfere with the integrity of the viral envelope rendering core protein susceptible to proteolytic digestion by proteinase K, whereas the RNA and the capsid were unaffected. The fact that only the viral envelope was disrupted is in accordance with studies demonstrating the potential of free fatty acids to interact with enveloped viruses [29–32]. Furthermore, the interference with the viral envelope could also explain the observed cytotoxic effect on cells. It is known that fatty acids have an impact on membrane fluidity, therefore disintegrating the bilayer [31]. Further support comes from the observation that only enveloped viruses were affected by milk treatment, which we could demonstrate for HCV, influenza A virus (H1N1), and to a lesser extent for herpes simplex virus type 1 (HSV-1) and VSV. The assay used to determine HIV-1 infectivity differed from the assays used for the other viruses as it is based on the detection of p24 via ELISA, rather than the direct immune-histochemistry staining against a viral protein. Nevertheless, a reduction in the concentration of p24 antigen was observed for some milk donors. This is supported by a study of Wahl et al [33] who recently demonstrated the reduced oral transmission of HIV by breast milk in humanized mice. Our data hint that HCV is more efficiently inactivated by human breast milk compared to the other viruses tested. It is possible that HCV being a "lipovirosparticle" with a strong interaction between the virus and lipoproteins [34] is more susceptible to the interference of fatty acids than other viruses. The nonenveloped viruses tested in this study, murine norovirus and rotavirus, were not affected by milk treatment, supporting the hypothesis that the viral envelope acts as antiviral target. Interestingly, we tested also the effect of cow's milk on bovine viral diarrhea virus (BVDV) and in contrast to HCV an antiviral effect was observed (data not shown). Separation of milk components via centrifugation confirmed the importance of the fat, which is present in the cream fraction of the milk, for its antiviral activity. Fat is the second largest component of human milk and is composed mainly of triglycerides present in globules, coated with a cellular membrane called the milk fat globular membrane, in which proteins are incorporated (MFGM) [35, 36]. A protein involvement, for example, by lactoferrin, which has been described to have antiviral properties against several viruses, including HCV [37], could be excluded using a proteolytic digestion assay. Proteinase K treatment did not abolish the antiviral activity of the milk. Importantly, human breast milk becomes antiviral after storage of milk at 4°C for 4 days as described elsewhere [31], whereas the storage at −80°C did not result in the appearance of an antiviral activity. Furthermore, upon treatment of the milk with the lipase inhibitor orlistat, no reduction in viral titers could be observed, indicating that milk lipases play an important role in the appearance of the antiviral activity. In accordance with this, we could show that both monounsaturated as well as polyunsaturated fatty acids were increased after storage at 4°C compared to −80°C, which is the result of ongoing lipolysis in the milk samples by the activity of milk lipases at 4°C in contrast to −80°C [38]. There have been several studies highlighting the potential of free fatty acids to act as antimicrobial agents [29, 30, 39–42]. From the fatty acids tested, most unsaturated as well as some saturated fatty acids were able to reduce viral titers. It has been described before that there are structural determinants such as chain length, degree of unsaturation, and reactive groups that affect activity of the fatty acids [41, 43], with lauric (C12:0) and linoleic (C18:2) acid as the most active fatty acids, the latter amounting to 15% of total milk fatty acids [44]. Indeed, we did observe differences in the ability of the fatty acids to reduce HCV titers, with some being able to inhibit HCV at lower concentrations than others.

Based on this study we propose a so far undescribed mechanism of how HCV transmission upon breast-feeding from HCV infected women might be limited or prevented. Similar processes concerning the release of free fatty acids take place upon digestion of human breast milk by the infant. Milk fat digestion is a multistep process that already begins in the stomach of the infant with a partial hydrolysis of the milk fat globule core by lingual and gastric lipases and continuous in the duodenum, where the action of bile salt-stimulated lipase of human milk and pancreatic lipase completes the digestive process [45]. Therefore, milk digestion products, like free fatty acids, released in the stomach might be able to inactivate residual viral particles, which otherwise could be transmitted upon breastfeeding.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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