Inactivation of Cell-Associated and Cell-Free HIV-1 by Flash-Heat Treatment of Breast Milk

To the Editors:

Mother-to-child transmission (MTCT) is responsible annually for approximately 90% of the 370,000 newly acquired HIV infections in children younger than 15 years, of which 90% are in sub-Saharan Africa. Current World Health Organization recommendations state that HIV-positive mothers should breast-feed exclusively for 6 months unless replacement feeding is acceptable, feasible, affordable, sustainable, and safe, in which case avoidance of all breast-feeding is recommended. Cessation of breast-feeding should occur at 6 months only if a nutritionally adequate and safe diet is maintained, yet practical feeding alternatives that meet these criteria are frequently not available. In contrast to the estimated 200,000–350,000 infants who contract HIV through breast-feeding each year, WHO estimates that 1.5 million infants died because they were not breast-fed. Safe and nutritious feeding options are needed for these vulnerable infants. Heat treatment of breast milk is one of the methods recommended by WHO. We previously reported that flash-heat (FH), a simple in-home pasteurization method for use in developing countries (see below), is capable of inactivating cell-free HIV in “HIV-spiked” breast milk samples, as detected by reverse transcriptase assays, while retaining the milk’s nutritional, immunological, and antimicrobial properties. However, Becquart et al has found that cell-associated HIV, which includes latentely infected resting CD4 T lymphocytes, is a significant source of transmissible virus. This is further supported by the occurrence of transmission to the infant that is seen even after controlling for low levels of cell-free virus.

Our previous studies have shown that FH reduces reverse transcriptase activity to undetectable levels. Reverse transcriptase is a surrogate for infectivity because it is necessary but not sufficient for viral infectivity. Due to the limitations of this assay for detecting provirus or cell-associated virus, further investigation to determine true infectivity was needed. We performed multiple assays to comprehensively assess FH inactivation of cell-free and cell-associated HIV. Breast milk samples were spiked with “cell-associated virus” (infected cells), which included integrated latent viral genome and virus that has shed from the surface of cells and new progeny in the process of assembly. The virus used was HIV-1 clade C strain ZA12, grown in the continuous human T-Cell line “PM-1.”

Five breast milk samples were collected from healthy volunteer mothers in California, United States, and stored frozen at −70°C until thawed for use in experiments. The expressed breast milk was aliquoted into FH or unheated (UH) controls. Field conditions were simulated by using a 1-ounce peanut butter jar from South Africa for a water bath, a 16-ounce glass peanut butter jar to hold breast milk, and single burner butane stove to imitate intense heat of a fire. Briefly, a 50-mL sample of breast milk in an uncovered glass peanut butter jar was placed in 450 mL of water in the aluminum pan. The water and milk were heated together over a flame until the water reached 100°C and was at a rolling boil. The breast milk was immediately removed from the water and allowed to cool to 37°C.

Viral infectivity was quantitatively measured before and after FH by peripheral blood mononuclear cell (PBMC) neutralization assay. HIV-1 neutralization assays employ PBMCs from 4 normal blood donors prepared from buffy coats, pooled and frozen. The cells are stimulated for 72 hours with phytohemagglutinin P (PHA-P). FH and UH milk were diluted 10-fold and transferred to the 96-well assay plate in quadruplicate. 2.5 × 10^5 PBMC per well was added, and the plates were then incubated for 72 hours. Residual cell-free virus was removed by centrifuging and washing the plates and resuspending the cells in 200 µL medium. After an additional incubation of 24 hours, 1% Triton-X detergent was added to each well, and viral p24 quantified by enzyme immunoassay (Zeptometrix). The end point titer for infectivity was determined by the interpolated titer at which there is a 50% inhibition of p24 expression relative to controls.

Viral infectivity was also determined by using the TZM-bl assay. This assay employs a cell line (TZM-bl) that is engineered to express CD4 and CCR5 and integrated reporter genes for firefly luciferase and Escherichia coli β-galactosidase under control of an HIV long terminal repeat sequence. TZM-bl cells are permissive to a wide variety of HIV strains and allow the viruses to undergo a single round of replication.

Because a latent viral genome cannot be reactivated in a dead cell, we also examined the potential for cell-associated transmission by spiking uninfected PBMCs into breast milk and determining cell viability pre and post FH by flow cytometry.

The PBMC assay and detection by p24 antigen is a good way of measuring true infectivity and was able to show a decrease of up to 4.75 logs per mL of virus after FH. However, residual p24 of approximately 2.5 logs remained after FH regardless of the UH titer. This is due to an inherent artifact of the conventional PBMC assay. The conventional PBMC assay involves a rinse step on day 3, which consists of centrifuging and washing the cells in phosphate-buffered saline to remove residual p24 from cell-free inoculum. Because the inoculum used in this experiment was whole cells infected with HIV, this wash step is not able to remove inactivated virus in pelleted cells. The subsequent modified PBMC assay demonstrated this effect, in that new infectious virus did not replicate in the course of that 4-day assay, thus indicating that the residual viral particles are noninfectious. The TZM-bl assay was performed to further support that residual inoculum was noninfectious. This assay has a greater dynamic range than the PBMC/p24 assay due to not having the limitation of detecting defective virions and viral antigens in residual inoculum. This assay showed a complete loss of infectivity after FH. Flow cytometry measurements after FH demonstrated that 99.7% of the cells were dead.
In summary, FH inactivates a high titer of cell-free and cell-associated HIV-1 as confirmed by reverse transcriptase, PBMC, and TZM-bl assays. FH causes 99.7% cell death, thus predicting that latently infected lymphocytes will not transmit HIV after heating. FH treatment of expressed breast milk may be a safe infant feeding method for HIV-positive mothers in developing countries who do not have alternatives that fulfill the WHO acceptable, feasible, affordable, sustainable, and safe criteria, especially during times of high risk, such as during mastitis or during and after the addition of complementary foods.

ACKNOWLEDGMENTS
Special thanks to the mothers and infants who donated their breast milk for this study and to Jason Mooney, Mary Kate Morris, PhD, Vince Nguyen, and Chris Baker for their contributions.

Mylene L. Volk, MS*
Carl V. Hanson, PhD*
Kiersten Israel-Ballard, PhD†
Caroline J. Chantry, MD‡

*California Department of Public Health, Richmond, CA
†Division of Epidemiology, Berkeley School of Public Health, University of California, Berkeley, CA
‡Department of Pediatrics, University of California Davis Medical Center, Sacramento, CA

REFERENCES

A Single-Center Cohort Experience of Raltegravir in Salvage Patients Failing Therapy

To the Editors:

In the BENCHMRK-1 and BENCHMRK-2 studies (the blocking integrase in treatment-experienced patients with a novel compound against HIV, Merck studies), the entry criteria included patients with documented resistance to 3 classes of antiretrovirals.1,2 In those randomized studies, patients were assigned to receive an optimized antiretroviral regimen either alone or in conjunction with raltegravir (RGV), also known as MK-0518 or Isentress, and the antiretroviral regimens were individually designed on the basis of previous antiretroviral history and results of drug-resistance testing. Individuals who received RGV had higher rates of virologic suppression than those who received placebo, and the overall rates of viral suppression are among the highest reported for patients infected with HIV with triple-class resistance. Increases in the CD4-cell count were more pronounced in the RGV group than in the placebo group; the overall adverse-event profile did not differ between the 2 groups. Rates of cancer were higher in the RGV group; however, the rates of adverse events were low, and differences in the rates from the combined BENCHMRK studies and from a larger data set including other studies of RGV3 were not significant. One could speculate that the earlier occurrence of clinical events in the RGV group reflects a more robust immunologic response and unmasking of underlying conditions. Continued monitoring for these and other adverse events in patients receiving RGV are therefore important during its expanded use.

To study this further, 57 triple-class experienced individuals (47 males and 10 females) received RGV 400 mg twice a day in addition to optimized background therapy (OBT) at the Chelsea and Westminster Hospital, London, United Kingdom, as part of an expanded access study. A total of 31 individuals received RGV in combination with OBТ because of virologic failure on their prior regimen. A total of 26 individuals switched to RGV with an undetectable viral load from T-20 or a poorly tolerated boosted protease inhibitor.

In those individuals starting on RGV with virologic failure, the median number of prior antiretroviral regimens was 10 (range 3–20). As part of OBТ, 21 patients received darunavir; 23, etravirine; and 3, T20 of which 2 were naive. The median number of active drugs per patient being 2, the mean baseline CD4 count measured 206 cells per microliter, and the baseline viral load was 55,000 copies per milliliter. By 4 weeks, all patients achieved >1 log decrease in viral load, and by week 48, the mean rise in CD4 count was 80 cells per microliter (Fig. 1). By week 48, 2 individuals died of preexisting complications of cardiovascular disease, 1 patient stopped all antiretrovirals due to adherence issues, and 1 patient was lost to follow-up. No patients stopped because of RGV toxicity; of those who remained on RGV, all apart from 1 achieved an HIV-1 viral load <50 copies per milliliter and the patient who failed to suppress had additional adherence support and subsequently became undetectable.