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Original Communication

GB virus C in HIV co-infected individuals: Could there ever be a consensus on outcome?

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ABSTRACT

Beneficial effects of GB virus-C (GBV-C) on HIV disease reported by some investigators could not be observed by others. We therefore asked if GBV-C altered CD4 cell count in HIV co-infected individuals in two Kenyan cohorts: A commercial sex workers (CSW) cohort and an antenatal mother-child health (MCH) cohort. Anti-HIV antibodies were detected, in archived plasma samples from the Kenyan cohorts, using enzyme-linked immunosorbent assay (ELISA). CD4 and CD8 counts in whole blood were obtained using Tritest flow cytometry assay. Active GB virus-C infections were determined by polymerase chain reaction (PCR). Data were analyzed using Microsoft Excel, SPSS, and Epi Info statistical packages. Positive HIV serology with CD4 cell count of 200 cells/μl or less, at two consecutive and continuing determinations was regarded as suggestive of progressing HIV disease. More than half of the women in the sample were HIV-positive and about one-fifth were GBV-C positive. The risk of being HIV positive was significantly greater among the CSW cohort compared to the MCH cohort. HIV positive women co-infected with GBV-C had similar CD4 levels, compared to HIV positive women negative for GBV-C. These data provide no evidence that GBV-C co-infection protects against decline in CD4 cell counts in these cohorts.

KEYWORDS: CD4 cells, co-infection, commercial sex workers, HIV, GB virus C

INTRODUCTION

The suggestion that infection with GB virus C (GBV-C) slows the progression of HIV disease is controversial [1-3]. Many investigators have documented that in persons co-infected with GBV-C and HIV, the GBV-C produces beneficial effects, delaying progression to AIDS, and decreasing mortality in AIDS patients [4-13] and boosting the innate antiviral response to HIV infection [14-16]. It has also been demonstrated that GBV-C viremia in pregnant women curtails mother-to-child transmission (MTCT) of HIV [17, 18]. Intriguingly, the work of Supapol *et al*. [18] revealed that MTCT was decreased due to the GBV-C co-infection in the infant, whereas maternal GBV-C co-infection with HIV had no effect on MTCT. There have, however, been other reports registering marginal effects of GBV-C on

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protection against HIV infection [19] and others in which there were no discernible effects of GBV-C on HIV disease [20-24].

GB virus C and hepatitis C virus (HCV) are flaviviruses, sharing some degree of homology in terms of genome structure and amino acid sequence. However, unlike hepatitis C virus, GBV-C attacks lymphocytes [8, 25-27] it is not hepatotropic, and has not been shown to cause liver disease [8, 28-30]. Considering that it does not attack liver cells, the name GB virus C (GBV-C) is preferred over the alternative name hepatitis G virus (HGV) [8, 26].

In hepatitis C virus (HCV)/HIV individuals co-infected with GBV-C, and undergoing HCV antiviral therapy, clearance of GBV-C by the anti-HCV did not modulate short-term outcome in viremia and immune status [31]. On the longer term, Berzsenyi and associates [32] showed that in HIV/AIDS patients co-infected with HCV and/or GBV-C, liver cirrhosis-related deaths were much lower, indicating that GBV-C ameliorated the effects of HCV. On the contrary, GBV-C did not have an effect on deaths due to HIV infection alone, in which there was no liver cirrhosis.

By implication, the different results obtained from various studies investigating HIV/GBV-C co-infection underscore the fact that the tripartite interaction (HIV, GBV-C and the host) is complex, and that the effect of GBV-C infection on HIV may differ between cohorts.

To address whether GBV-C infection protects from HIV disease in cohorts of Kenyan women, we have assessed a collection of samples with known HIV status from two female cohorts. The main questions we have asked are, whether; 1) GB virus C infection slows HIV disease progression (as determined by CD4 counts); 2) GBV-C can be transmitted sexually; and 3) HIV infection is a risk factor for GBV-C infection.

METHODS

Samples

This work was a retrospective study using previously archived samples collected between the years 1989 and 2006 from the Pumwani Commercial Sex Worker Cohort (CSW cohort), in addition to the non-sex worker mother-to-child health (MCH) cohort, both located in Nairobi, Kenya. Plasma samples were obtained from these women and maintained at -20ºC. Because the samples were taken prior to the wide-spread availability of anti-retroviral therapy, HIV positive samples included in these studies were from therapy-naïve women. As of 2003 all women with CD4 counts less than 200 were placed on therapy. All study participants provided informed consent, and the study was approved by the ethics review committees of the University of Manitoba, Canada and the Kenyatta National Hospital, Kenya.

Of the women enrolled in the Kenyan Commercial Sex Workers (CSW) cohort, complete HIV and GBV-C data are available on 361 women. The mother-to-child health (MCH) cohort comprised 275 enrolled women for each of whom there are complete HIV and GBV-C data. CD4 counts following the date of GBV-C sampling were utilized as a marker suggestive of progression to AIDS. Women with a previous positive HIV serology who have at least two consecutive and continuing CD4 counts of 200 or less per microlitre were viewed as suggestive of the AIDS status. In this study, neither the GBV-C genotype, nor the viral burden due to HIV or GBV-C was determined.

Clinical laboratory tests

At the time of sample collection, serology was performed using an enzyme linked immuno-sorbant assay (ELISA) to detect anti-HIV antibodies (Recombingen, Trinity Biotech, Dublin, Ireland). Individuals whose plasma gave negative results in this assay are considered to be HIV negative. Positive tests were confirmed by a second ELISA. CD4 T cell counts were obtained on all samples using an aliquot of whole blood collected in sodium heparin vacutainers. Cell counts (CD4 and CD8) were assessed from whole blood using the Tritest flow cytometry assay (BD Pharmingen) [33].

Determination of active GB virus C infections: GB virus C PCR

Plasma samples were thawed overnight at 4ºC followed by centrifugation to remove any precipitated lipids and cellular debris, at the

National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, Manitoba, Canada. RNA was isolated from plasma using Qiagen RNeasy mini kit column isolations according to the manufacturer's instructions.

To detect active GB virus C infection, the positive sense strand of the genome was detected by RT-PCR using a Qiagen OneStep RT-PCR kit [34]. Briefly, a master mix containing 10 µl of 5X PCR Buffer (Qiagen), 1 µl of 10 mM dNTPs, 1.0 µl of 20 mM primer F (HGV108F-5' AGGTGGTGG ATGGGTGAT-3'), 1.0 µl of 20 µM primer R (HGV531R-5' TGCCACCCGCCCTCACCCGAA-3'), 2.0 µl enzyme mix, containing Omniscript RT, Sensiscript RT and HotStart Taq DNA Polymerase (Qiagen), 10.0 µl RNA template, 0.3 µl RNAse inhibitor, and water up to a final volume of 50.0 µl. Samples were then placed in a thermocycler and subjected to the following conditions: RT step (cDNA synthesis) 60ºC for 30min followed by 95ºC for 15 min. First stage PCR: 40 cycles of 95ºC for 20 sec, 55ºC for 30 sec, and 72ºC for 1 min. Using product obtained from the first stage, second stage PCR proceeded using Qiagen HotStar Taq DNA polymerase kit. Second stage PCR was carried out using a master mix containing 5 µl of 10X PCR Buffer (Qiagen), 1 µl of 10mM dNTP's, 0.5 µl of 20 µM primer F (HGV134F-5' TGGTAGGTCGTAAATCCCGGT-3'), 0.5 µl of 20 µM primer R (HGV476R-5' GG(A/G)GCTGGGTGGCC(C/T)CATGCA(A/T) T-3"), 0.2 µl of 5U/µl Taq DNA polymerase, 1.0-2.0 µl DNA template, and water up to a final volume of 50.0 µl. Samples were again placed in a thermalcycler and subjected to the following conditions: 95ºC for 15 min followed by 40 cycles of 95ºC for 20 sec, 55ºC for 30 sec, 72ºC for 30 sec. Samples were then qualitatively analyzed by gel electrophoresis using a 1.5% agarose gel stained with ethidium bromide and visualized using a BIORAD Gel Doc apparatus.

STATISTICAL ANALYSIS

Data were analyzed using Microsoft Excel, SPSS, and Epi Info statistical packages for Chi Square analysis, Mann Whitney *U*-Test, Spearman Rank Correlations (Bivariate), Relative Risk ratios, and Multivariate analysis.

RESULTS

As shown in Figure 1, more than half of the women in the sample were HIV-positive and about one-fifth were positive for GBV-C. As expected, the risk of being HIV positive was significantly greater among women in the sex trade worker cohort compared to the women in the mother-child group (*RR*=1.56, CI_{95}=1.34, 1.81; $X^2_{(1,635)}$=40.6, p<.0001); there was no difference in the proportion of GBV-C positive women between the two cohorts (*RR*=1.38, CI_{95}=0.91, 1.94). Of the total sample, 98 women (15.4%) were co-infected with HIV and GBV-C.

Most of the co-infected women in the sample (93.9%, n=92/98) had an HIV first-positive test result that preceded their first-positive GBV-C result (83.7%), or tested positive for both infections at the same time point (10.2%). To properly ascertain whether GBV-C is protective against or a risk factor for HIV infection, one would need to assess the HIV infection rate of GBV-C negative women compared to those who were infected with GBV-C prior to HIV infection. As only six women in the combined cohorts were infected with GBV-C prior to testing HIV-positive, the lack of an appropriate comparison group makes it difficult to assess the possible protective or risk effect imposed by GBV-C infection on subsequent HIV infection.

To determine whether HIV is a risk factor for GBV-C infection (research question #3), the GBV-C infection rate of the HIV negative women was compared to those who were infected with HIV prior to GBV-C infection. As demonstrated in Figure 2, one-fifth of the women in the combined cohorts were positive for GBV-C, regardless of HIV status; prior infection with HIV does not appear to pose a risk for GBV-C infection (*RR*=1.27, CI_{95}=0.92, 1.75). When the cohorts were analyzed separately (Table 1), HIV infection appeared to be a risk for subsequent GBV-C infection among the women in the mother-child cohort only (*RR*=1.73, CI_{95}=1.05, 2.83; $X^2_{(1,271)}$=4.80, p<.05).

Among the co-infected women, given that HIV was acquired before GBV-C for the majority of the women (83.7%), it is possible that GBV-C is sexually transmitted in this population. However,

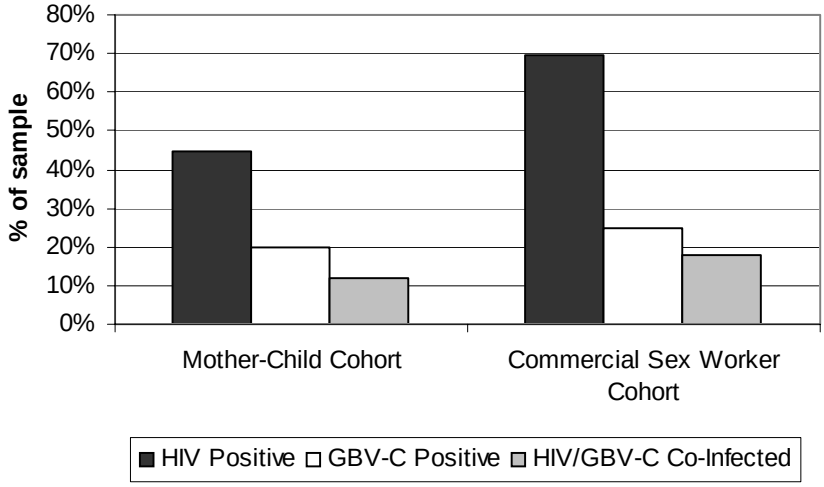

Figure 1. HIV and GBV-C status of Kenyan commercial sex worker study cohort and the non-sex worker mother-child women cohort.
The percentage of the commercial sex workers (CSW) cohort mono-infected with either HIV or GB virus C, or co-infected with both HIV and GB virus C was determined and compared with the corresponding percentages from the mother-child health cohort.

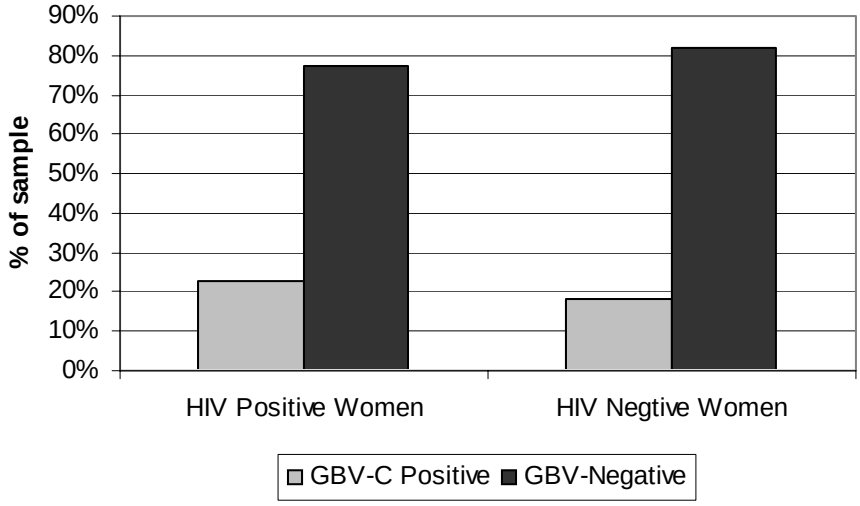

Figure 2. GBV-C infection rates among study participants (both cohorts) according to HIV status*.
*Participants with an HIV positive test date after or at the same time as a GBV-C positive test date were excluded. The percentage of the entire study participants that acquired GBV-C after HIV infection was determined and compared with the HIV - negative women who acquired GBV-C infection. Participants with an HIV positive test date after or at the same time as a GBV-C positive test date were excluded.

this would hold only if intravenous drug use, which is anecdotally becoming an issue currently in Kenya [35] did not have a substantial impact on the two study populations. In fact there is very little self-reported intravenous drug use in either cohort.

Also under investigation in these analyses is the role of GBV-C infection on the decrease in CD4 cells among GBV-C/HIV co-infected individuals. Of the 375 women infected with HIV, 34.4% met the consistently low ($\leq$200 cells/μl) CD4 cell count status (N=129; 20.3% of total sample).

Table 1. Differences in GBV-C infection rates between study cohorts according to HIV status.

		GBV-C Infection Status		
HIV Status		Positive	Negative	Total
Mother - Child Cohort	Positive* row %	30 25.0%	90 75.0%	120
	Negative row %	22 14.5%	130 85.5%	152
	Total row %	52 19.1%	220 80.9%	272
Sex Trade Worker Cohort	Positive* row %	52 21.8%	187 78.2%	239
	Negative row %	25 22.9%	84 77.1%	109
	Total row %	77 22.1%	271 77.9%	348

*Participants with an HIV positive test date after or at the same time as a GBV-C positive test date were excluded.

The risk of attaining this low CD4 cell count criterion was significantly greater among women in the sex trade worker cohort (41.3% achieved this low CD4 count) than women in the mother-child cohort (20.3% achieved this low CD4 count; RR=2.03, CI_{95}=1.39, 2.97; $X^2_{(1,374)}$ =16.07, p<.0001). HIV positive women co-infected with GBV-C, regardless of when they tested GBV-C positive had similar CD4 levels, compared to HIV positive women not infected with GBV-C (RR=1.09, CI_{95}=0.80, 1.49). It appears that infection with GBV-C is neither a risk nor a protective factor in progression to the low CD4 counts (Figure 3). This holds true for women in the mother-child and sex trade worker cohorts. Thus, these data do not support the hypothesis that GBV-C is protective against the development of low CD4 counts, and possibly AIDS.

It has also been hypothesized that GBV-C may delay the progression to AIDS (research question #1). To properly assess the impact of GBV-C co-infection on the time between HIV infection and development of low (≤200 cells/μl) CD4 cell counts, only women who seroconverted (from HIV-negative to positive status) during the study were analysed. Of 375 HIV-positive participants for whom we have matched GBV-C status, 72 (19.2%) were identified as seroconverters. Similar to the data presented in Figure 3, the proportion of HIV-positive participants who seroconverted during the study and progressed to low CD4 counts did not differ by GBV-C status (see Figure 4).

Next, the time for progression to low (≤200 cells/μl) CD4 cell counts for participants according to GBV-C infection status was calculated. Among the study participants the mean length of time of progression to CD4 counts ≤200 cells/microL following the first HIV-positive serology was 4.36 years (s.d.=3.02) and this did not differ according to GBV-C infection status ($F_{(1,26)}$ = 0.25, n.s.).

DISCUSSION

The data from the two Kenyan cohorts (Pumwani Commercial Sex Worker cohort and Mother-Child Health cohort) have shown that among co-infected women there is no association between GBV-C infection and amelioration of HIV disease, as measured by CD4 counts. Among those co-infected women, HIV positive test result either preceded or occurred at the same time as the GBV-C positive result. With our dataset, it

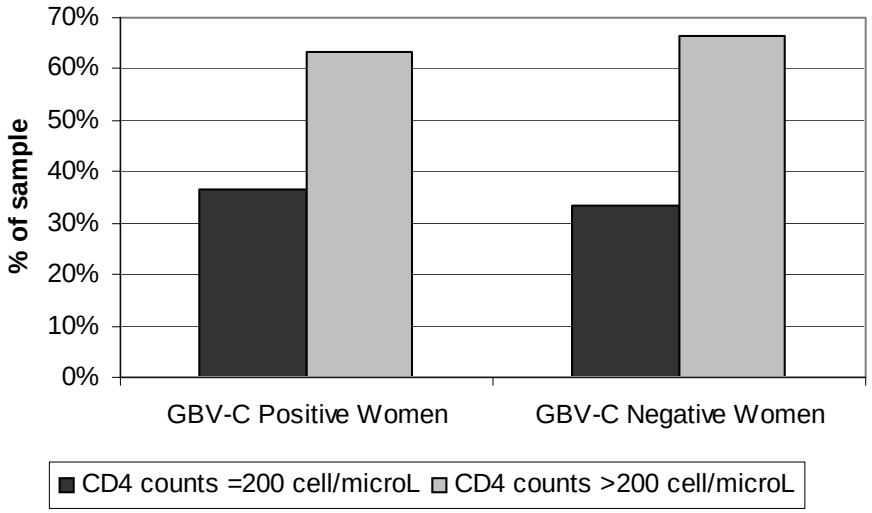

Figure 3. HIV disease status as represented by CD4 Counts ≤200 cell/microL.
HIV disease status was determined by CD4 counts (≤200 cells/microlitre) and compared between the GBV-C negative and GBV-C positive women in the population.

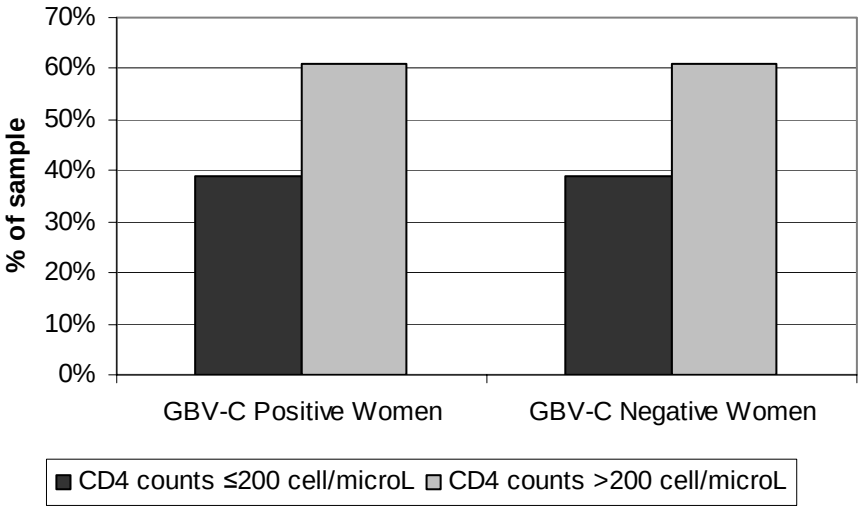

Figure 4. Progression from HIV seroconversion* to CD4 counts ≤200 cell/microL.
*Only participants who seroconverted from HIV-negative to positive status during the study were included in this analysis. Disease progression was determined as proportion of population which recorded a decline in CD4 counts from HIV infection (time of seroconversion) to very low CD4 counts (≤200 CD4 cells/microlitre).

was not possible to determine whether or not GBV-C infection protects against, or is a risk factor for, HIV infection.

It was previously recognized that GBV-C is transmitted through the parenteral route [9, 28]. However, evidence is now gradually accumulating from studies in diverse populations to the effect that this virus is sexually transmitted as well. Such evidence include: Observations among Taiwanese female licensed commercial sex workers [36]; observations among female commercial sex workers, and homosexual men in the Genitourinary Medicine (GUM) clinic in the Royal Infirmary of Edinburgh [37]. Observations among a group of Lebanese patients [38]; a study reported from the

University of Hawaii in Honolulu [39]; and among volunteers being treated for sexually transmitted infections (STIs) [40]. Indeed, recently, Supapol *et al*. [41] reported that in a cohort of pregnant women in Bangkok, Thailand, GBV-C infection may be linked with HIV infection, and that both parenteral and sexual routes are involved.

Ignoring any possible contributions from intravenous drug use which is rare in these Kenyan cohorts, the fact that among those co-infected individuals, the HIV infection was either concurrent with, or prior to GBV-C infection, may suggest that GBV-C can be sexually transmitted as well. However, in these data, the risk of GBV-C infection was greater among HIV-positive women compared to their HIV-negative counterparts only among participants in the mother-child cohort. While the elevated risk is suggestive that HIV infection may predispose one to GBV-C infection, it is curious that this relationship is not apparent in the sex trade worker cohort, despite their greater HIV risk via sexual transmission. The reason for this discrepancy thus remains unresolved. However, it is possible that there are differences in the sexual behaviours between the two cohorts that may elevate risk in one group but not in the other. Considering that GBV-C is sexually transmitted, as revealed by this work, and others [36-40], prior exposure of the CSW individuals to GBV-C could conceivably have led to the development of protective immunity; for instance, development of antibodies against GBV-C envelope 2 protein as published by Tillmann *et al*. [42]. However, anti-GBV-C E2 antibodies were not measured in this study. Further investigation in this area is, therefore, desired.

More than one-third of the participants with HIV showed evidence of continued CD4 counts $\leq$200 cells/μl. This proportion differed little according to GBV-C infection status. Because of the longitudinal nature of this dataset, it was possible to analyze separately those who seroconverted during the study. Of those who seroconverted during the study, 25.0% were co-infected with GBV-C, and the length of time for disease progression to this level was similar to that among the mono-infected HIV positive patients. While this finding differs from previous publications [4-6, 10-12, 14] it provides epidemiologic support for the alternative hypothesis that GBV-C infection offers little, if any, beneficial value on HIV disease progression [20-24].

It has been suggested that the beneficial effects of GBV-C infection in HIV disease (smaller decrease in CD4 count; slower progression to AIDS; lower mortality; improved quality of life) are due to GBV-C viremia, i.e., active GBV-C infection [7, 9, 43] monitored over a prolonged period [13]. Jung and associates [44] have demonstrated *in vitro* that early HIV replication is inhibited by the envelope E2 glycoprotein of GBV-C. Indeed, according to Herrera and associates, the E2 (269-296) peptide sequence interacts with, and modifies the conformation of the HIV-1 fusion peptide [45], presumably rendering it defective. Also, Mohr *et al.* (2010) [16] have demonstrated that the envelope protein E2 of GBV-C causes the production of antibodies that neutralize various isolates of HIV-1 by binding to host-derived antigen(s) on the HIV particle. It is note-worthy that viremia is only partial evidence in the diagnosis of infection since anti-E2 and detectable GBV-C RNA are mutually exclusive. Thus, viremia, may not absolutely be synonymous with infection in all cases.

Other multiple plausible reasons suggested include (i) Increased production of chemokines that naturally inhibit HIV infection [e.g., RANTES (regulated on activation, normal T-cell expressed and secreted), macrophage inflammatory protein (MIP) 1α and 1β, which are ligands for the CCR5; and stromal cell-derived factor (SDF-1), ligand for CXCR4)], as well as down regulation of the receptors (e.g., CCR5) for the chemokines [46]. (ii) Effective maintenance of an intact T-Helper 1 cytokine profile seemingly brought about by the GBV-C presence [47]. (iii) Activation of the interferon system, thereby protecting against HIV in the mixed infection [5]. (iv) GBV-C viremia [6], which may justify the observation that GBV-C nonstructural protein NS5A, or its component 30-amino acid segment when included in culture, inhibited replication of HIV-1 in Jurkat (a CD4 T-lymphocyte) cell line [48]. (v) GBV-C genotypic differences; and gender effects, considering that more men than women were found to be GBV-C positive [31]. However, a report on studies in Uganda [49]

suggests that the beneficial effects of GBV-C co-infection on survival among HIV infected adults were observed in both men and women. The same report also observed that the effects were obtainable with HIV subtypes A and D, in addition to B. (vi) Ethnogeographic differences in infections/co-infections. For instance, whereas in the USA, and in western Europe an estimated 30% of HIV-1-infected individuals are co-infected with Hepatitis C virus, the corresponding proportion in Spain is >50% [50].

These multiple mechanisms may be involved (to varying extents) in interactions of different components of innate immunity that may lead to the decrease in HIV-related morbidity and mortality in individuals co-infected with HIV and GBV-C [5]. It is conceivable that some of the complex interactions of the effectors of innate and adaptive immunity could compromise (rather than synergize with) others; thus, may diminish or enhance outcomes. This could be akin to the observation that complex allergen-induced airway inflammation protects against asthma, whereas it inhibits antimicrobial defense in animal models [51].

In the cohort studied by Williams, *et al*. (2004) [11], these authors did not observe any difference at 12 to 18 months after HIV seroconversion attributable to GBV-C viremia between individuals mono-infected with HIV, and those co-infected with HIV and GBV-C. However, five to six years after seroconversion, GBC-viremia was associated with greater survival from HIV disease. This tends to lend credence to the suggestion that there is an interaction of factors contributed by the GBV-C and the host reactions against the virus.

Coupled with the fact that persistent GBV-C replication in HIV individuals delays progression to AIDS and death (Williams *et al*. 2004 [11]; and as reviewed by Stoll, 2006 [9]), it may be that our observations could have been influenced by the limited time points of GBV-C measured. In addition, our operational view of progression of HIV disease purely on the basis of CD4 counts, and without clinical data, could have obscured any protective effect against HIV-related opportunistic infections or death.

In conclusion, the study of two female Kenyan cohorts presented here suggests that it is not possible to determine if GBV-C infection influences HIV infection in either direction because the number of the women who were GBV-C positive before acquiring HIV was too few. Further, it (1) provides no evidence that, in the presence of HIV co-infection with GBV-C, there is amelioration of the HIV disease. (2) suggests that GBV-C may be sexually transmitted. (3) suggests that prior infection with HIV may increase the risk of GBV-C infection only among women in the mother-to-child cohort, for some reason that is not immediately apparent from our data, but could involve better GBV-C immunity due to the increased exposure.

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