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GB Virus C (GBV-C) coinfection in advanced HIV disease is associated with low CCR5 and CXCR4 surface expression on CD4⁺ T cells

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Abstract

Background—Co-infection with the flavivirus GB virus C is frequent in patients suffering from human immunodeficiency virus (HIV) due to shared routes of transmission. GBV-C co-infection has been proposed to exert a beneficial influence on HIV infection. *In vitro* studies demonstrated down-regulation of CCR5 as a potential mechanism of GBV-C to modulate HIV disease progression. We therefore studied surface expression of the two major HIV co-receptors, CCR5 and CXCR4, on CD4⁺ and CD8⁺ T-cells in 128 HIV patients stratified with respect to their GBV-C status, immune function and HAART *in vivo*.

Methods—GBV-C infection was studied in 128 HIV patients by nested RT-PCR. FACS analysis was used to measure CCR5 and CXCR4 surface expression on CD4⁺ and CD8⁺ T cells.

Results—GBV-C RNA replication was detected in 30% (38/128) of patients. In HIV patients with advanced immunodeficiency, we found up-regulation of CCR5 surface expression on CD4⁺ T cells. However, in patients with GBV-C co-infection, no up-regulation of CCR5 CD4⁺ T cells was detected. Furthermore, CXCR4 surface expression was reduced in GBV-C co-infected patients. These findings were independent of HAART and HIV viral load. HIV co-receptor expression on CD8⁺ T cells was not altered in patients with GBV-C co-infection.

Conclusion—Thus, GBV-C co-infection in HIV disease leads to reduced expression of the two major HIV co-receptors, CCR5 and CXCR4, on CD4⁺ T cells in patients with advanced stage of immunodeficiency providing a possible molecular explanation for the clinical benefit of GBV-C co-infection in late stage HIV disease.

Keywords

CD4; CD8; viral infections

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Introduction

Co-infection with the flavivirus GB virus C (GBV-C; previously known as hepatitis G virus) is frequent in patients with human immunodeficiency virus (HIV) infection due to shared routes of transmission. Parenteral, sexual, and vertical transmission of GBV-C have been reported [1]. GBV-C is not known to cause any human disease [2]. However, several studies have reported a beneficial influence of GBV-C on the course of HIV disease progression, although this has not been confirmed in all studies (reviewed in [3]). In persons with HIV, GBV-C co-infection is associated with high CD4⁺ T cell counts, low plasma HIV RNA levels, improved response to antiretroviral therapy, and prolonged AIDS-free survival.

It is well known that in addition to CD4, HIV-1 entry into target cells depends on its interaction with a secondary receptor, most often the chemokine receptors CCR5 or CXCR4. In general, HIV-1 isolates that are transmitted *in vivo* use CCR5 (R5), while CXCR4-tropic (X4) variants often appear late in HIV infection and are associated with a more rapid decrease in CD4⁺ T cell counts. Furthermore, it has previously been shown that CCR5 expression on CD4⁺ T lymphocytes is dependent on CD4⁺ T cell decline and is upregulated during advanced HIV disease [4].

The molecular mechanisms of HIV/GBV-C interaction remain unclear; however, downregulation of CCR5 and CXCR4 on CD4⁺ T cells has been associated with GBV-C infection *in vitro* [5–7]. Furthermore, downregulation of CCR5 on CD4⁺ T cells was previously reported in a small group of HIV/GBV-C coinfecting patients [5]. Recently published data by Maidana-Giret *et al.* [8] underline the impact of GBV-C co-infection on cellular activation markers CD25, CD38 and CD69, as well as CCR5.

Based on a large HIV-1 cohort, here we report that GBV-C replication downregulates not only CCR5, but also CXCR4 surface expression on CD4⁺ T-lymphocytes *in vivo* when patients reach an advanced stage of immune deficiency. Importantly, this effect of GBV-C co-infection is independent of HAART (highly active antiretroviral therapy) and HIV viral load.

Patients and Methods

We included 128 consecutive patients with known chronic HIV-1 infection from the Bonn HIV clinic. Patients with hepatitis B or hepatitis C co-infection were excluded to eliminate potential confounding effects due to other viral co-infections on HIV co-receptor expression. The study was approved by the local ethics committee.

GBV-C RNA detection and CCR5-Δ32 analysis

Detection of GBV-C RNA was performed using nested RT-PCR as previously described [9]. GBV-C positivity was confirmed with a semi-quantitative real time PCR on a LightCycler (Roche), with a lower limit of detection of 1000 geq/ml. The CCR5-Δ32 gene polymorphism was analyzed by real-time PCR on a LightCycler (Roche) using primers flanking the Δ32 deletion site: 5'-AAGGTCTTCATTACACCTGC-3' and 5'-GATGACCATGACAAGCAGCG-3'; thermal profile: 1s at 60°C, 4s at 72°C, and 1s at 90°C.

Immunofluorescent staining and flow cytometry

CCR5 and CXCR4 surface expression on CD4⁺ and CD8⁺ T cells were determined by flow cytometry. Briefly, 100μl of fresh blood was subjected to erythrocyte lysis (BD, San Jose CA, USA). The following antibodies were used: CD3 FITC, CD4 PE, CD8 PerCP (BD, San

Jose, CA, USA), CCR5 APC (BD San Jose, CA, USA), and CXCR4 APC (BD, San Jose, CA, USA). Four-color flow cytometric analysis was performed on a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) and analyzed by FlowJo (Figure 1).

Statistics—All statistics were performed using SPSS 17.0 (Chicago, IL, USA). Differences were compared by Fisher's exact tests and ANOVA as appropriate. P values <0.05 were considered statistically significant.

Results

In the present study, 78% (98/128) were male, and MSM was the risk factor for HIV transmission in 39% (50/128) of patients. 73% (93/128) of patients were on HAART, and the plasma HIV viral load was below the detection limit of 50 copies/ml in 60% (77/128).

GBV-C infection status

Of the 128 studied HIV patients, 38 (30%) had detectable GBV-C RNA. Demographic and biochemical profiles are shown in Table 1 according to GBV-C status. GBV-C RNA was more frequent in men than women ($p=0.025$), and GBV-C RNA was detected more often in HIV patients less than 40 years of age ($p=0.04$). There were no significant differences in baseline CD4⁺ T cell counts or HIV viral loads with respect to GBV-C replication status.

Expression of surface CCR5 and CXCR4 according to CCR5/Δ32 mutation status

We first analyzed the CCR5/Δ32 mutation status in all 128 HIV patients. The heterozygous CCR5/Δ32 mutation was found in 14/128 (11%). No patient was homozygous for CCR5/Δ32. There was no association between GBV-C replication and the presence of CCR5/Δ32 ($p=0.152$). However, in HIV patients with a heterozygous CCR5/Δ32 allele, we found a significantly lower expression of CCR5 on CD4⁺ and CD8⁺ T cells (GeoMean 8.4 versus 10.2, $p=0.038$, and GeoMean 8.9 versus 14.7, respectively, $p<0.01$). CXCR4 expression on CD4⁺ and CD8⁺ T cells was not altered in patients with a CCR5/Δ32 mutation (data not shown). To exclude any potential influence of CCR5/Δ32 on the relationship between GBV-C replication status and surface expression of CCR5, all patients with a heterozygous CCR5/Δ32 genotype ($n=14$) were excluded from further analysis.

CCR5 surface expression and GBV-C replication on CD4⁺ T cells

Previous studies have shown a correlation between the decline of CD4⁺ T cells and the density of HIV co-receptor expression on CD4⁺ T cells [4]. Thus, we stratified HIV patients according to their immune status (cut-off value of < 200 CD4⁺ T cells/μl defined as advanced immunodeficiency). As previously described by Maidana-Giret *et al.* [8], the overall percentage of CCR5 expressing CD4⁺ T cells was not significantly altered: 14.7% CCR5 positive CD4⁺ T cells (< 200 CD4⁺ T cells/μl) versus 9.4% (≥ 200 CD4⁺ T cells/μl) in GBV-C negative and 10.6% (< 200 CD4⁺ T cells/μl) vs. 11.0% (≥ 200 CD4⁺ T cells/μl) in GBV-C positive patients ($p=0.1$). However, the density of CCR5 surface expression on CD4⁺ T cells was significantly higher in HIV mono-infected patients with CD4⁺ T cell counts < 200/μl compared to those with counts ≥ 200 (GeoMean 13.4 versus 9.8; $p=0.036$) (Figure 2A). Importantly, in GBV-C co-infected patients, this up-regulation of CCR5 surface expression on CD4⁺ T cells was not detected (GeoMean 9.7 versus 10.5; $p=0.5$). Furthermore, adjusting for HIV treatment in HIV mono-infected patients on HAART, we also found a significantly higher density of CCR5 surface expression on CD4⁺ T cells during advanced immunodeficiency (GeoMean 12.0 versus 9.2, $p=0.031$). CCR5 upregulation was likewise not present in GBV-C co-infected patients. Instead, CCR5 surface expression in patients with GBV-C co-infection and advanced immunodeficiency showed a trend towards being reduced (GeoMean 8.6 versus 10.7, $p=0.053$; Figure 2B). In untreated

HIV mono-infected patients, CCR5 surface expression on CD4⁺ T cells was higher in patients with advanced immunodeficiency (GeoMean 19.2 vs. 11.0). However, due to small numbers of patients in this group, this effect did not reach statistical significance. In patients with GBV-C co-infection, we found no significant difference in CCR5 surface expression with respect to their immune status (GeoMean 12.9 versus 9.8; $p=0.35$) (Figure 2C).

CXCR4 surface expression and GBV-C replication on CD4⁺ T-cells—As seen earlier with CCR5 surface expression, the percentage of CXCR4 expressing CD4⁺ T cells was not altered by GBV-C co-infection: 35.5% of CXCR4 positive CD4⁺ T cells (< 200 CD4⁺ T cells/ μ l) versus 45.6% (≥ 200 CD4⁺ T cells/ μ l) in GBV-C negative and 27.5% (< 200 CD4⁺ T cells/ μ l) versus 44.8% (≥ 200 CD4⁺ T cells/ μ l) in GBV-C positive patients ($p=0.3$). Furthermore, in HIV mono-infected patients, there was no significant difference in CXCR4 surface expression with respect to their immune status (GeoMean 14.0 versus 12.7, $p=0.37$). However, in HIV/GBV-C co-infected patients, a significant reduction of CXCR4 surface expression was found in patients with advanced immunodeficiency (GeoMean 9.5 versus 13.0), $p<0.001$) (Figure 3A). Likewise, adjusting for HIV treatment HIV mono-infected patients on HAART showed no significant difference of CXCR4 surface expression on CD4⁺ T cells with respect to their immune status (GeoMean 12.4 versus 12.0, $p=0.71$), whereas in GBV-C co-infected patients down-regulation of CXCR4 surface expression was associated with advanced immunodeficiency (GeoMean 9.5 versus 12.7, $p=0.007$; Figure 3B). In contrast, in untreated HIV mono-infected patients CXCR4 surface expression on CD4⁺ T cells was even higher in patients with advanced immunodeficiency (GeoMean 21.1 versus 14.3, $p<0.001$), whereas GBV-C co-infected patients even showed down-regulation of CXCR4 surface expression (GeoMean 9.3 vs. 13.6, $p=0.12$; Figure 3C). Due to small numbers of patients in this group statistical significance was not reached.

In contrast to the effects related to CD4⁺ T-cell count and GBV-C co-infection, no effect of HIV viral load on CCR5 and CXCR4 co-receptor surface expression was observed ($p=0.73$ and 0.12, respectively). Furthermore, CCR5 and CXCR4 surface expression on CD8⁺ T cells were not altered by GBV-C co-infection ($p=0.211$ and 0.63, respectively) independent of stage of immunodeficiency.

Discussion

GBV-C replication has been associated with a clinical benefit in HIV infected patients in the majority of retrospective clinical studies. However, the mechanisms of interaction between GBV-C and HIV still are incompletely understood. It is known that the expression of the two major HIV co-receptors, CCR5 and CXCR4, are important in HIV transmission and progression. Thus, we studied the influence of GBV-C replication on the expression of these co-receptors in CD4⁺ and CD8⁺ T cells in a large cohort of HIV-1 infected patients. In our cohort of 128 HIV positive patients, the overall prevalence of GBV-C co-infection was 30%, which is similar to data from other HIV-1 positive populations. We found a higher prevalence of GBV-C RNA in younger patients [9]. This may be explained by the fact that clearance of GBV-C replication may be associated with development of GBV-C E2 antibodies that protect older patients from reinfection [10].

As the CCR5/ $\Delta 32$ mutation has been associated with slower HIV disease progression, we analyzed CCR5/ $\Delta 32$ in our cohort. We found 11% of all HIV patients to harbor a heterozygous CCR5/ $\Delta 32$ mutation. In agreement with Tillmann *et al.* [11], there was no association between GBV-C infection and CCR5/ $\Delta 32$, indicating that the effect of GBV-C viremia on HIV disease is independent of mutations in the CCR5 receptor.

Furthermore, our cross-sectional analysis demonstrates that the surface density of both major HIV co-receptors, CCR5 and CXCR4, was markedly reduced on CD4⁺ T lymphocytes among patients with an advanced stage of immunodeficiency.

Together with the fact that the percentage of CCR5 and CXCR4 expressing CD4⁺ T cells was not altered, which is in line with recent data [8], we conclude that the average number of CCR5 and CXCR4 receptors per single CD4⁺ T cell was reduced. This effect was seen in patients on HAART and untreated patients. However, due to current HIV treatment guidelines recommending initiation of HAART < 350 CD4⁺ T cells/ μ l, only small numbers of untreated patients with advanced immunodeficiency were included. Thus, statistical significance was not reached for untreated patients. Reynes *et al.* [4] have previously shown that increased CCR5 density on CD4⁺ T lymphocytes is logarithmically correlated with the loss of CD4⁺ T cells during advanced stages of HIV infection. We found that this up-regulation of CCR5 expression in HIV patients with advanced disease was blunted by GBV-C co-infection. This effect may represent an important mechanism contributing to delayed HIV progression in patients co-infected with GBV-C, since HIV-1 cell-to-cell transmission is related to the level of CCR5 and CXCR4 expression. Our data are in accordance with clinical epidemiological data presented by Williams *et al.* [12] showing an association of GBV-C co-infection with decreased mortality only in the late stage of HIV infection, whereas two other reports [13] [14] studying HIV patients without significant immunological compromise were unable to demonstrate a beneficial effect of GBV-C co-infection.

As found for CCR5 expression in HIV mono-infected patients with advanced immunodeficiency, CXCR4 expression in the subgroup of untreated HIV patients was up-regulated in HIV mono-infected patients with advanced immunodeficiency. We found this effect of CXCR4 up-regulation to be compensated by HAART treatment. This is in accordance with a recent study reporting down-regulation of CCR5 and CXCR4 in HIV patients receiving HAART [15]. Xiang *et al.* [7] previously reported that Jurkat cells stably expressing GBV-C NS5A inhibited HIV-1 replication by decreasing surface density of CXCR4 *in vitro*. Interestingly, this effect could only be partially explained by internalization of CXCR4 subsequent to the release of SDF-1 ligand.

GBV-C replication has been detected in CD4⁺ and CD8⁺ T lymphocytes and B lymphocytes [16]. GBV-C replication leads to decreased CCR5 and CXCR4 surface density on CD4⁺ T lymphocytes, whereas in our study CCR5 and CXCR4 surface density on CD8⁺ T lymphocytes was not influenced by GBV-C co-infection status. GBV-C replication leads to decreased CCR5 and CXCR4 surface density on CD4⁺ T lymphocytes, whereas in our study CCR5 and CXCR4 surface density on CD8⁺ T lymphocytes was not influenced by GBV-C co-infection status. In our cohort surface expression of CD4 was not altered by GBV-C co-infection, an effect that Xiang *et al.* [17] have observed *in vitro* (data shown). Currently, it remains unclear how GBV-C infection leads to altered expression of CCR5 and CXCR4. These data suggest that GBV-C and HIV-1 may interact directly in co-infected CD4⁺ T-lymphocytes. Thus, further studies are warranted to clarify the mechanisms of interaction between GBV-C and HIV-1 at the cellular level.

In summary, our data show that the HIV co-receptors, CCR5 and CXCR4, are downregulated on CD4⁺ T lymphocytes in HIV-1/GBV-C co-infected patients with advanced immunodeficiency. This effect is independent of HAART treatment and HIV viral load. Our data support *in vitro* data of down-regulation of CCR5 and CXCR4 on CD4⁺ T-lymphocytes and provide additional evidence of a possible mechanism by which GBV-C co-infection could delay HIV progression, particularly during advanced HIV disease.

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Figure 1.

Representative histograms of CCR5-specific and CXCR4-specific immunofluorescence determined on CD4⁺ T lymphocytes from GBV-C non-infected individuals (4 upper panels) and GBV-C co-infected subjects (4 lower panels). The dotted lines represent the isotype control.

Figure 2.

Surface density of CCR5 on CD4⁺ T cells in a large cohort of HIV-1 patients (A = all patients, n = 114; B = patients on HAART, n = 81; C = untreated patients, n = 33). All further comparisons of GBV-C negative and positive patients in this analysis demonstrate overlapping confidence intervals.

Figure 3. Surface density of CXCR4 on CD4⁺ T cells in a large cohort of HIV-1 patients (A = all patients, n = 114; B = patients on HAART, n = 81; C = untreated patients, n = 33). Comparisons of GBV-C negative and positive patients additionally revealed a significant difference in the group of patients with advanced immunodeficiency in panel A and C.

Table 1

Clinical characteristics of study cohort.

	GBV-C STATUS			P-value
	Total n=128	GBV-C RNA- n=90	GBV-C RNA+ n=38	
Gender				Fisher's exact test:0.025
Male	98	64 (65%)	34 (35%)	
Female	30	26 (87%)	4 (13%)	
Age				0.04
<40	63	39 (62%)	24 (38%)	
≥40	65	51 (78%)	14 (22%)	
HIV risk factor				0.06
MSM	50	40 (80%)	10 (20%)	
Non-MSM	78	50 (64%)	28 (36%)	
Ethnicity				0.191
Caucasian	102	69 (68%)	33 (32%)	
Non-Caucasian	26	21 (80%)	5 (20%)	
HAART				0.865
No HAART	35	25 (79%)	10 (21%)	
HAART	93	65 (68%)	28 (32%)	0.384
3 × Nuc	6	4 (67%)	2 (33%)	
NNRTI	19	15 (79%)	4 (21%)	
PI	64	42 (66%)	22 (34%)	
NNRTI/PI	4	4 (100%)	0 (0%)	
HIV RNA				0.419
50	77	56 (73%)	21 (27%)	
>50	50	33 (66%)	17 (34%)	
Mean CD4 (cells/μl)		476	385	ANOVA:0.06
Mean log ₁₀ HIV RNA (cp/ml)		2.47	2.5	0.854