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## EFFECT OF COINFECTION WITH GB VIRUS C ON SURVIVAL AMONG PATIENTS WITH HIV INFECTION

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### ABSTRACT

**Background** Previous studies have suggested that people with human immunodeficiency virus (HIV) infection who are coinfecting with GB virus C (GBV-C, or hepatitis G virus) have delayed progression of HIV disease. GBV-C is related to hepatitis C virus but does not appear to cause liver disease.

**Methods** We examined the effect of coinfection with GBV-C on the survival of patients with HIV infection. We also evaluated cultures of peripheral-blood mononuclear cells infected with both viruses to determine whether GBV-C infection alters replication in vitro.

**Results** Of 362 HIV-infected patients, 144 (39.8 percent) had GBV-C viremia in two tests. Forty-one of the patients with GBV-C viremia (28.5 percent) died during the follow-up period, as compared with 123 of the 218 patients who tested negative for GBV-C RNA (56.4 percent;  $P < 0.001$ ). The mean duration of follow-up for the entire cohort was 4.1 years. In a Cox regression analysis adjusted for HIV treatment, baseline CD4+ T-cell count, age, sex, race, and mode of transmission of HIV, the mortality rate among the 218 HIV-infected patients without GBV-C coinfection was significantly higher than that among the 144 patients with GBV-C coinfection (relative risk, 3.7; 95 percent confidence interval, 2.5 to 5.4). HIV replication, as measured by the detection of p24 antigen in culture supernatants, was reproducibly inhibited in cultures of peripheral-blood mononuclear cells by GBV-C coinfection. Coinfection did not alter the surface expression of HIV cellular receptors on peripheral-blood mononuclear cells, as determined by flow cytometry.

**Conclusions** GBV-C infection is common in people with HIV infection and is associated with significantly improved survival. (N Engl J Med 2001;345:707-14.)

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**T**HE course of human immunodeficiency virus (HIV) infection is extremely variable among infected persons, although the reasons are incompletely understood. Recent reports suggest that persons with HIV infection who are coinfecting with GB virus C (GBV-C, also called hepatitis G virus) have delayed progression of HIV disease.<sup>1-4</sup> GBV-C, a member of the Flaviviridae family, is the human virus most closely related to hepatitis C virus (HCV).<sup>5,6</sup>

GBV-C was first identified in persons with non-A, non-B, non-C hepatitis<sup>5,6</sup>; however, in subsequent studies it did not appear to replicate primarily in hepatocytes or to cause acute or chronic liver disease.<sup>7-9</sup> In fact, no specific disease has been convincingly associated with this virus. Infection with GBV-C can persist for decades with no apparent clinical illness or death.<sup>10</sup> Persistent GBV-C infection is common, with rates of infection of approximately 1.8 percent in healthy blood donors, 15 percent in HCV-positive persons, and up to 35 percent in HIV-positive persons.<sup>11-13</sup> Clearance of infection occurs in approximately 60 to 75 percent of immunocompetent GBV-C-infected persons, along with the development of antibodies to the envelope glycoprotein E2.<sup>14</sup> Plasma-derived GBV-C has been propagated in vitro with the use of cultures of peripheral-blood mononuclear cells.<sup>15</sup> In addition, we recently demonstrated that virus derived from an infectious molecular clone replicated in the CD4+ cells in cultures of peripheral-blood mononuclear cells.<sup>16</sup>

In 1998, Heringlake et al. described an association between GBV-C viremia and prolonged survival

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in a study involving 33 subjects who were coinfecting with HIV and GBV-C and 164 HIV-infected subjects without GBV-C infection.<sup>1</sup> Subsequently, three other studies also demonstrated better survival among 69 persons coinfecting with GBV-C and HIV (46 subjects with hemophilia and 23 subjects without hemophilia) than among 404 HIV-infected persons without GBV-C infection.<sup>2-4</sup> In one study, these results were found to be independent of age, HIV load, HCV load, CD4+ and CD8+ T-cell counts, and CC chemokine receptor 5 (CCR5) (HIV-coreceptor) genotype.<sup>4</sup>

We sought to determine whether GBV-C infection was associated with prolonged survival in a large population of HIV-infected persons who had acquired HIV through a variety of modes of transmission. In addition, we evaluated cultures of peripheral-blood mononuclear cells infected with both HIV and GBV-C to determine whether GBV-C infection altered HIV replication *in vitro*.

## METHODS

### Clinical Evaluation

We evaluated consecutive patients who were treated in our HIV clinic between April 1988 and June 1999 if they gave written informed consent and provided blood samples sufficient for the purposes of the study. All serum and plasma specimens were prepared within two hours after the blood was sampled and were stored at  $-80^{\circ}\text{C}$  until use. Demographic and clinical data for the patients were prospectively entered into a relational data base (Paradox, Borland International, Scotts Valley, Calif.), and data on deaths were obtained from medical and state health department records. Data on mortality were not available for subjects who left the state of Iowa.

Information about prescribed HIV treatment and prophylaxis against *Pneumocystis carinii* pneumonia was abstracted from clinic records. The standard of care was defined for three periods — before 1993, 1993 to 1996, and after 1996. Before 1993, treatment with a single nucleoside reverse-transcriptase inhibitor was considered to be the standard of care. Between 1993 and 1996, the standard was considered either cycling of more than one nucleoside reverse-transcriptase inhibitor, or treatment with two simultaneous nucleoside reverse-transcriptase inhibitors. After 1996, three-drug regimens that included two nucleoside reverse-transcriptase inhibitors and either a nonnucleoside reverse-transcriptase inhibitor or an HIV-protease inhibitor were considered standard.

If the CD4+ cell count was 500 or higher, the standard of care was to prescribe no therapy, and if the plasma HIV RNA level was less than 400 copies per milliliter, any therapy prescribed was considered standard. Before 1989, trimethoprim-sulfamethoxazole, dapsone, or aerosolized pentamidine was considered standard for patients with a history of *P. carinii* pneumonia. After 1988, the standard of care included treatment with trimethoprim-sulfamethoxazole, dapsone, aerosolized pentamidine, or atovaquone for all patients with fewer than 200 CD4+ cells per cubic millimeter.<sup>17</sup> Documentation of the prescribed anti-HIV therapy and prophylaxis against *P. carinii* pneumonia was available for 96.4 percent and 98 percent of the patients, respectively. The study was approved by the institutional review board of the University of Iowa, and all patients provided written informed consent.

RNA was extracted from serum or plasma (200  $\mu\text{l}$ ) with the use of a previously described guanidinium-isothiocyanate extraction method<sup>18</sup>; one quarter of the RNA preparation was used in a nested reverse-transcriptase polymerase chain reaction (RT-PCR) to amplify GBV-C RNA (with the use of primers from the 5' untranslated region).<sup>18,19</sup> PCR products were identified by agarose-gel electrophoresis and ethidium bromide staining.<sup>18</sup> Negative control samples and positive control samples were included with each

sample undergoing PCR testing. To be considered positive, the sample had to test positive on two separate occasions or two samples obtained on different dates had to test positive. Laboratory personnel were not aware of the clinical status of the patients whose samples they tested.

### Cells and Viruses

Peripheral-blood mononuclear cells were isolated from healthy blood donors and incubated in RPMI 1640 medium containing phytohemagglutinin and interleukin-2 for 48 hours before being infected with GBV-C, HIV, or both, as previously described.<sup>15</sup> Cell viability was measured by trypan-blue exclusion studies. We determined the extent of protein synthesis in mock-infected (control) and GBV-C-infected peripheral-blood mononuclear cells by metabolically labeling cellular proteins with [<sup>35</sup>S]methionine and determining the counts per minute of their incorporation by acid precipitation, as previously described.<sup>20</sup> The GBV-C isolate used in this study was derived from supernatant fluids from cell cultures that had previously been transfected with full-length RNA transcripts of GBV-C and passed three to six times in cultures of peripheral-blood mononuclear cells.<sup>16</sup> Similarly, mock-infected control preparations were derived from the supernatant fluids from uninfected cultures of peripheral-blood mononuclear cells.<sup>16</sup> The volume of mock-infected supernatant was normalized to equal that of the GBV-C-infected supernatant.

The HIV isolate used in these studies was a nonsyncytium-inducing strain (National Institutes of Health AIDS Research and Reference Reagent Program strain 92UG031; catalogue no. 1741). The preparations of HIV were propagated as previously described.<sup>21</sup> After activation in phytohemagglutinin and interleukin-2 for two days,  $1 \times 10^6$  peripheral-blood mononuclear cells were washed and then resuspended in 100  $\mu\text{l}$  of a preparation containing HIV,

**TABLE 1. BASE-LINE CHARACTERISTICS OF THE 362 PATIENTS WITH HIV INFECTION.\***

VARIABLE	GBV-C STATUS	
	POSITIVE (N=144)	NEGATIVE (N=218)
Mean age — yr	34.5	35.1
Female sex — no. (%)	17 (11.8)	26 (11.9)
White race — no. (%)	128 (88.9)	189 (86.7)
Received standard of care — no./no. with data (%)†		
Anti-HIV therapy	131/142 (92.3)	183/201 (91.0)
Prophylaxis against <i>P. carinii</i> pneumonia	144/144 (100.0)	210/211 (99.5)
Mean base-line CD4+ cell count — per mm <sup>3</sup>	313.5±252.2	263.5±258.5‡
Mode of HIV transmission — no. (%)		
Intravenous drug use	23 (16.0)	36 (16.5)
Sexual contact§	113 (78.5)	162 (74.3)
Blood or blood products	8 (5.6)	20 (9.2)

\*Plus-minus values are means  $\pm$ SD.  $P > 0.05$  for all comparisons between the GBV-C-positive group and the GBV-C-negative group; Pearson's chi-square test was used for the comparison of categorical variables, and a t-test was used for the comparison of continuous variables.

†The standards of care for anti-HIV therapy and prophylaxis against *P. carinii* pneumonia are defined in the Methods section.

‡ $P = 0.07$  for the comparison with the GBV-C-positive group.

§Men who had sex with men accounted for 80 percent of the patients who had acquired HIV by sexual transmission. There was no significant difference between the GBV-C-positive group and the GBV-C-negative group in the distribution of modes of transmission.

GBV-C, or both (multiplicity of infection, approximately 0.1). We also varied the timing of infection with HIV or GBV-C in some cultures. Viral isolates were added to cells for four hours at 37°C before 2 ml of fresh medium was added, after which cells were incubated overnight. The cells were washed, and samples of the culture supernatant were collected immediately and then twice weekly. HIV replication was determined by the measurement of HIV p24 antigen in culture supernatants,<sup>21,22</sup> and GBV-C replication was determined by the measurement of positive-sense RNA in culture supernatants and positive- and negative-sense RNA in cell lysates, as previously described.<sup>16</sup>

### Flow Cytometry

The level of expression of HIV receptors (CD4) and major coreceptors (CXCR4 [CXCR4] and CCR5) on peripheral-blood mononuclear cells after infection with GBV-C was determined by flow cytometry (FACScan, Becton Dickinson, San Jose, Calif.).<sup>16,21</sup> Cells were infected with GBV-C or were mock-infected. Cells were pelleted and then resuspended in 10 µg of murine anti-CD4 (IgG1 conjugated with fluorescein isothiocyanate [FITC]) per milliliter, biotinylated anti-CXCR4 (IgG2a), and anti-CCR5 antibodies (IgG2a conjugated with R-phycoerythrin [R-PE]; PharMingen, San Diego, Calif.) or murine isotype control antibodies (murine IgG1-FITC, IgG2a-biotin, and IgG2a-R-PE, respectively) for 30 minutes at 4°C. CXCR4 and the cells stained with the appropriate isotype control were incubated with streptavidin-conjugated CyChrome (PharMingen) for 30 minutes. After each step, the cells were washed twice with phosphate-buffered saline.

### Statistical Analysis

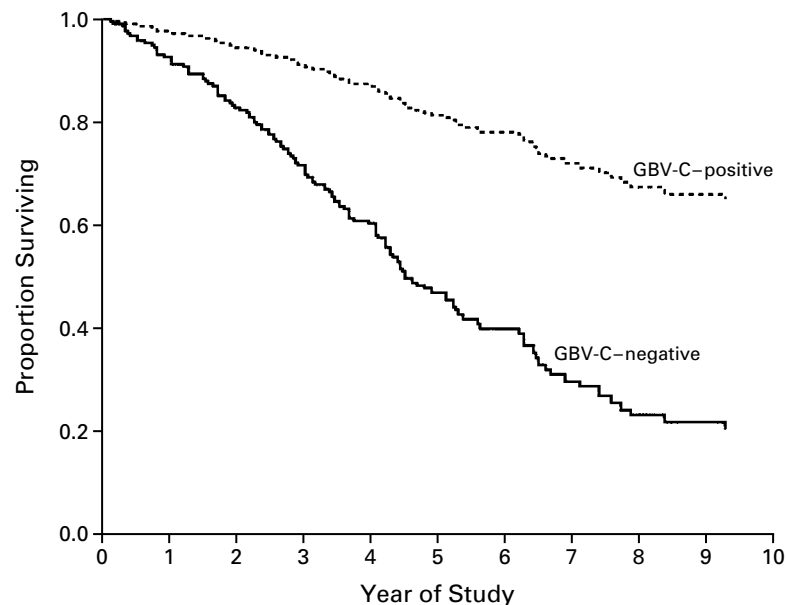
A chi-square or Fisher's exact test was used for the comparison of categorical variables, and a two-sample t-test was used for the

comparison of continuous variables, with correction for unequal variances when appropriate.<sup>23</sup> A Cox proportional-hazards model<sup>24</sup> was used to compare survival from the time of entry into our clinic between patients who were infected with GBV-C and those who were not, with adjustment for age at enrollment (base line), receipt of anti-HIV therapy and prophylaxis against *P. carinii* pneumonia, base-line CD4+ cell count, sex, race, HCV-antibody status, and mode of HIV transmission. The age at enrollment and the base-line CD4+ cell count were entered into the model as continuous variables; the other variables were categorical. The time-dependent variables (receipt of anti-HIV therapy and prophylaxis against *P. carinii* pneumonia) were defined for each period as described above and were coded as categorical variables according to whether or not the patient received treatment according to the standard of care for each period. The significance level was set at 0.05, and all P values were two-tailed. All statistical analyses were performed with the use of SPSS software (version 8.0, SPSS, Chicago).

## RESULTS

### Mortality among Patients with and Patients without GBV-C Coinfection

We tested 362 HIV-infected patients for GBV-C RNA in our HIV clinic between 1988 and 2000. As in previous studies,<sup>12,13</sup> GBV-C viremia was common; 144 of the patients (39.8 percent) had viremia. There were no significant differences in base-line clinical or demographic characteristics between the group of patients who tested positive for GBV-C RNA and the group that tested negative, although there was a trend toward a higher base-line CD4+ cell count among

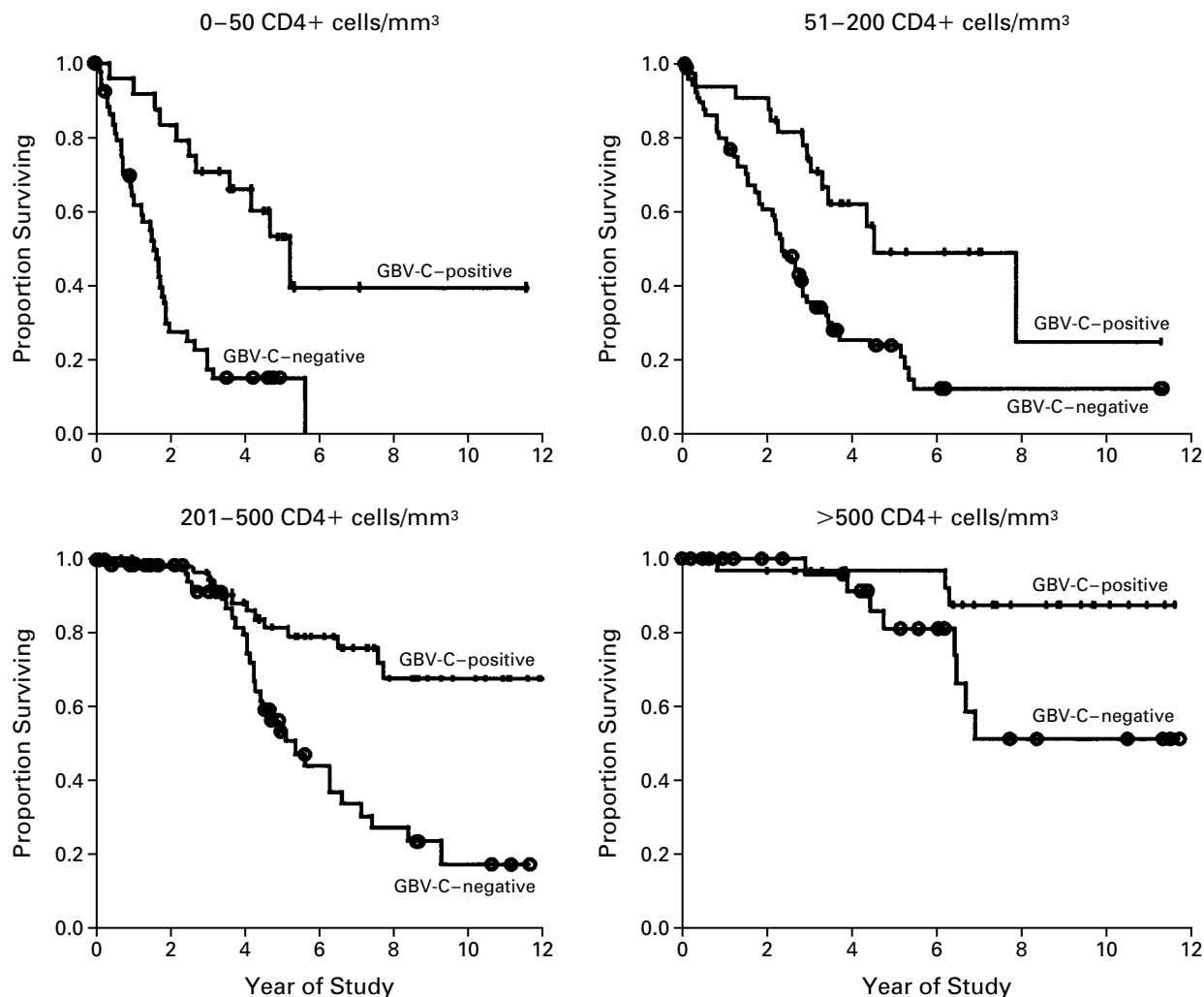


No. AT RISK

GBV-C-positive	144	135	111	68	43	20
GBV-C-negative	218	156	91	42	20	12

**Figure 1.** Survival Curves for HIV-Infected Patients with and Patients without GBV-C Viremia.

With the use of a Cox proportional-hazards model in which we adjusted for other important prognostic variables, we found that HIV-infected patients with GBV-C coinfection survived significantly longer than HIV-infected patients without GBV-C coinfection ( $P < 0.001$ ). The mean duration of follow-up for the entire cohort was 4.1 years.



**Figure 2.** Kaplan-Meier Survival Curves According to the Base-Line CD4+ Cell Count.

In each stratum of CD4+ cell counts, fewer patients with GBV-C viremia than without GBV-C viremia died — 44 percent versus 75 percent among patients with fewer than 50 CD4+ cells per cubic millimeter ( $n=73$ ;  $P=0.01$ ); 44 percent versus 76 percent among patients with 51 to 200 CD4+ cells per cubic millimeter ( $n=98$ ;  $P=0.003$ ); 23 percent versus 44 percent among patients with 201 to 500 CD4+ cells per cubic millimeter ( $n=122$ ;  $P=0.02$ ); and 7 percent versus 21 percent among patients with more than 500 CD4+ cells per cubic millimeter ( $n=69$ ;  $P=0.32$ ). The circles and tick marks on the curves represent times when data were censored for particular patients.

the patients with GBV-C viremia ( $P=0.07$ ) (Table 1). The rate of GBV-C infection among patients who had acquired HIV by sexual transmission (41.0 percent) was similar to the rate of GBV-C infection in those who acquired GBV-C through percutaneous modes of transmission (35.6 percent). Those data support the results of previous studies suggesting that sexual transmission of GBV-C is common.<sup>25</sup>

The mean duration of follow-up for the entire cohort was 4.1 years. Of the patients with GBV-C viremia, 41 (28.5 percent) died during the follow-up period, as compared with 123 patients in the GBV-

C-negative group (56.4 percent;  $P<0.001$ ). In a Cox proportional-hazards model, the adjusted relative risk of death in the GBV-C-negative group as compared with the GBV-C-positive group was 3.7 (95 percent confidence interval, 2.5 to 5.4;  $P<0.001$ ) (Fig. 1). There were no significant differences between the GBV-C-positive group and the GBV-C-negative group in the number of patients lost to follow-up (data not shown).

The Kaplan-Meier survival curves for the two groups of patients, stratified according to base-line CD4+ cell count, are shown in Figure 2.<sup>26</sup> In a sub-

group analysis of patients who entered our clinic before 1990 (six years before highly active antiretroviral therapy became available), we found that 9 of 27 GBV-C–positive patients died, as compared with 48 of 67 GBV-C–negative patients ( $P < 0.001$ ). Of the 47 HIV-infected patients who entered the study after 1995, only 1 died before July 1, 2000, when the collection of data ceased. Thus, our data do not allow us to assess the potential interaction between GBV-C infection and highly active antiretroviral therapy.

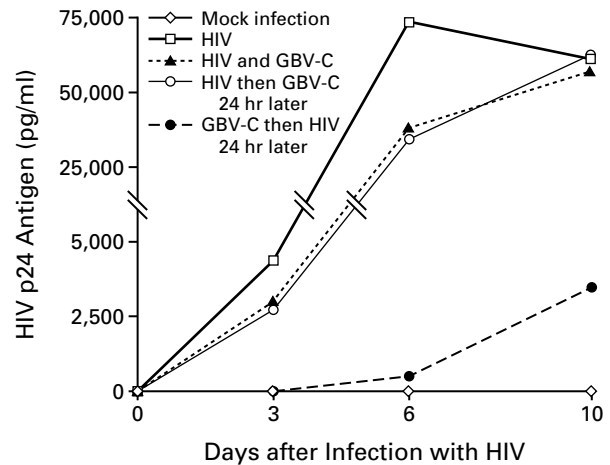
#### Effect of GBV-C Infection on HIV Replication in Vitro

To determine whether GBV-C infection altered HIV replication in vitro, duplicate cultures of peripheral-blood mononuclear cells were infected with HIV alone, GBV-C alone, or HIV and GBV-C. Mock-infected peripheral-blood mononuclear cells served as the negative controls. HIV replication, demonstrated by the production of p24 antigen in the supernatant fluid from the cell culture, was inhibited by 23.0 percent after three days in culture and by 49.4 percent after six days in culture when GBV-C and HIV were used to infect cells simultaneously (Fig. 3).

To determine whether inhibition occurred when GBV-C infection was initiated after HIV infection was already established, cells were infected with HIV and incubated for 24 hours before being infected with GBV-C. In these cells, HIV replication was inhibited by 31.6 percent three days after infection with HIV and by 58.1 percent six days after infection — values similar to the extent of the inhibition seen in the cells that were infected simultaneously with HIV and GBV-C. Cells infected with GBV-C 24 hours before being infected with HIV demonstrated more profound inhibition of HIV; in these cultures, replication was almost completely halted by three and six days after infection (reduced by 87.4 percent three days after infection with HIV and by 99.0 percent six days after infection) (Fig. 3). When fresh peripheral-blood mononuclear cells stimulated by phytohemagglutinin and interleukin-2 were added to the cultures six days after infection, HIV replication was detected four days later, illustrating that GBV-C did not prevent the entry of HIV (Fig. 3). These experiments with coinfection were performed in duplicate a total of seven times with similar results.

Three separate passages of the GBV-C isolate were tested against three separate preparations of HIV, and the inhibitory effect was reproducible. GBV-C replication was documented by the finding of positive-sense RNA in cell-culture supernatants by RT-PCR, and by the detection of negative- and positive-sense GBV-C RNA in cell lysates (data not shown).

To determine whether GBV-C infection altered the expression of HIV receptors on the surface of peripheral-blood mononuclear cells, resulting in decreased attachment and entry of HIV, we used flow cytometry to measure the expression of the HIV receptor



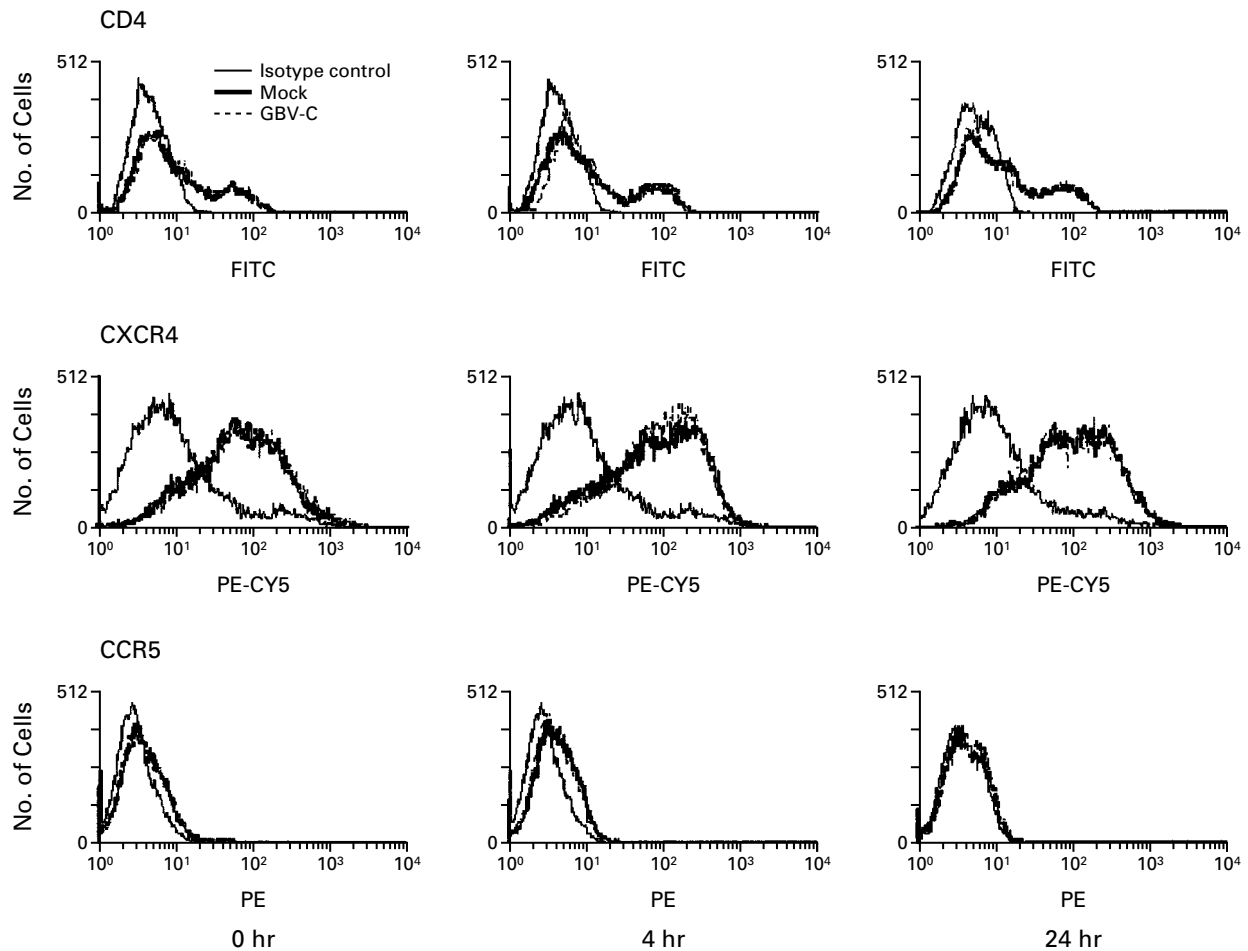
**Figure 3.** GBV-C Infection of Peripheral-Blood Mononuclear Cells and Inhibition of HIV Replication.

Cultures of peripheral-blood mononuclear cells stimulated with phytohemagglutinin and interleukin-2 were mock-infected or infected (as described in the Methods section) with HIV alone, HIV and GBV-C simultaneously, HIV followed by GBV-C 24 hours later, or GBV-C followed by HIV 24 hours later. HIV replication was highest in cells infected with HIV alone and lowest in cells that were infected with GBV-C 24 hours before being infected with HIV. We measured HIV replication by determining the concentration of HIV p24 antigen in culture supernatants immediately after infection and 3, 6, and 10 days later. Fresh peripheral-blood mononuclear cells were added after samples were obtained for a determination of HIV replication on day 6.

(CD4) and coreceptors (CXCR4 and CCR5) on GBV-C–infected and mock-infected cells.<sup>16,21</sup> There were no differences in the expression of CD4, CXCR4, or CCR5 between mock-infected and GBV-C–infected cells immediately after infection or 5 minutes, 20 minutes, 1 hour, 4 hours, or 24 hours after infection (Figure 4 shows the data for three of the time points). To ensure that GBV-C infection did not cause cell toxicity leading to diminished HIV replication, we used trypan-blue exclusion microscopy and measurements of the incorporation of [<sup>35</sup>S]methionine-labeled cellular proteins<sup>20</sup> to demonstrate that GBV-C infection did not result in increased cell death or diminished metabolic activity for eight days after infection (data not shown).

#### DISCUSSION

The variability of HIV disease progression and mortality among infected persons is incompletely understood. Although virologic and immunologic factors have been found to be associated with the delayed progression of HIV disease, these are often not found in long-term survivors.<sup>27</sup> Our study confirms that there is a high rate of GBV-C viremia among HIV-



**Figure 4.** GBV-C Infection and Surface Expression of HIV Receptors.

Stimulated cultures of peripheral-blood mononuclear cells from normal donors were either mock-infected or infected with GBV-C, and cells were evaluated by flow cytometry immediately (0 hr) and after 4 and 24 hours. The expression of CD4, CXCR4, and CCR5 are shown in the top, middle, and bottom panels, respectively. The comparison with the isotype control antibody demonstrates the specificity of the surface labeling. There were no significant differences between the mock-infected and the GBV-C-infected peripheral-blood mononuclear cells in the cell-surface expression of any of these HIV receptors. FITC denotes fluorescein isothiocyanate, PE phycoerythrin, and CY5 CyChrome.

infected persons<sup>12,13</sup> and extends the findings of previous studies regarding persons who are coinfecting with HIV and GBV-C.<sup>1-4</sup> We studied 40 percent more patients with HIV and GBV-C coinfection than were enrolled in these four studies combined, and our patients had acquired HIV through a variety of routes. The patients who were coinfecting with GBV-C and HIV were similar to those who were HIV-positive but GBV-C-negative in terms of both base-line characteristics and treatment received. Among the HIV-infected patients, the mortality rate was significantly lower among those with GBV-C viremia, independently of the prescribed anti-HIV therapy or prophylax-

is against *P. carinii* pneumonia, base-line CD4+ cell count, age, race, sex, or mode of HIV transmission. GBV-C infection did not prevent the depletion of CD4+ cells, since the base-line CD4+ cell counts were less than 50 cells per cubic millimeter in 25 coinfecting patients (17 percent) and were 51 to 200 cells per cubic millimeter in 32 coinfecting patients (22 percent). Nevertheless, in all subgroups defined by base-line CD4+ cell counts, the patients infected with GBV-C had a lower mortality rate than HIV-infected patients without GBV-C viremia (Fig. 2).

HIV replication was diminished *in vitro* by coinfection with GBV-C. GBV-C replication in periph-

eral-blood mononuclear cells appeared to be noncytopathic and did not inhibit the synthesis of cellular proteins; thus, the effect on HIV replication did not appear to be a result of cellular toxicity. The inhibitory effect of GBV-C replication on HIV growth in cell culture was evident when HIV infection preceded GBV-C infection, and GBV-C infection did not alter the expression of CD4, CXCR4, or CCR5. The mechanism of inhibition therefore appears to operate during a stage of HIV replication after attachment and entry.

In vitro models of other viruses that inhibit HIV replication have been described<sup>28,29</sup>; however, no epidemiologic data indicate that these infections have a role in delaying HIV disease progression or HIV-associated death. GBV-C and its close relative HCV are unusual among RNA viruses of humans in that they cause persistent infection without a DNA intermediate or a known latent stage in their replication cycle. The mechanism by which these persistent flaviviruses evade the natural antiviral systems of cells is not well understood. A flavivirus related to HCV and GBV-C, bovine viral diarrhea virus, induces interferon- $\alpha$  in cattle<sup>30</sup> and attenuates experimental infections with bovine respiratory syncytial virus in calves.<sup>31</sup> Interferon- $\alpha$  is known to inhibit HIV replication.<sup>32,33</sup>

GBV-C infection has not been associated with any known disease and does not appear to represent a substantial health hazard in humans.<sup>10,34</sup> For this reason, the Food and Drug Administration has not recommended screening blood donors for GBV-C RNA, although approximately 1.8 percent of donors have GBV-C viremia.<sup>11</sup>

In summary, we found a significantly lower mortality rate among patients coinfecting with HIV and GBV-C than among HIV-infected patients who were not infected with GBV-C. In addition, in a model of coinfection in cell cultures, we found that GBV-C infection of peripheral-blood mononuclear cells inhibited HIV replication. The possible role of GBV-C infection as a treatment for HIV infection remains to be evaluated.

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