Evidence That both HIV and HIV-Induced Immunodeficiency Enhance HCV Replication among HCV Seroconverters

Marcel Beld,*1 Maarten Penning,* Vladimir Lukashov,* Martin McMorrow,2 Marijke Roos,3 Nadine Pakker,† Anneke van den Hoek,§ and Jaap Goudsmit‡

*Department of Human Retrovirology, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands; †Chiron Corporation, Amsterdam, The Netherlands; ‡Department of Clinical Viroimmunology, The Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands; and §Department of Public Health and Environment, Municipal Health Service, Amsterdam, The Netherlands

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The objective of this retrospective cohort study is to assess the mechanism by which human immunodeficiency virus type 1 (HIV) influences hepatitis C virus (HCV) replication in injecting drug users. Virological (HCV and HIV RNA levels) and immunological (CD4⁺, CD8⁺ cell counts, and anti-CD3 reactivity) parameters were determined in 19 HCV seroconverters in sequential samples over a period of 1 to 9 years. Among these subjects, 10 were HIV-seronegative (HIVneg), 4 were HIV-seropositive (HIVpos), and 5 seroconverted for HIV (HIVsc) during the observation period. HCV RNA levels were higher in HIVpos subjects than in HIVneg subjects. In subjects seroconverting for HIV, HCV RNA levels increased significantly immediately after HIV seroconversion (P < 0.0001), while they remained stable over time in HIVpos and HIVneg subjects. HCV RNA correlated inversely with CD4⁺ cell counts in both the HIVpos population (R = −0.22, P < 0.05) and the HIVneg population (R = −0.45, P < 0.0001). In addition, when subjects were stratified according to CD4⁺ cell counts a significant difference was found in HCV RNA levels between HIVpos and HIVneg subjects with CD4⁺ cell counts >500 cells/µl (P = 0.001), but not in the population with CD4⁺ cell counts <500 cells/µl. In no population was a correlation found between HCV RNA levels and CD8⁺ cell counts or anti-CD3 reactivity. Both HIV infection and CD4⁺ cell counts are apparently associated with HCV RNA levels. The direct association, independent of CD4⁺ cell counts, between HIV infection and HCV replication appears to be stronger than the association between HIV-induced CD4⁺ cell decline and HCV replication. We conclude that (i) HCV replication is in some way directly influenced by the presence of HIV; (ii) HCV-specific host immunity controls, in part, HCV replication; and (iii) HCV replication increases when the immune system is impaired by HIV.

INTRODUCTION

Hepatitis C virus (HCV) is a major cause of parenterally transmitted acute hepatitis (Choo et al., 1989) and is widespread among injecting drug users, with up to 90% being positive for antibodies to HCV (van den Hoek et al., 1990; Thomas et al., 1995). The clinical manifestations of HCV infection are diverse, and the clinical course is difficult to predict. Commonly, the course of HCV infection remains benign in the first few years (Eyster et al., 1993), although the majority (>80%) of HCV-infected individuals develop chronic disease and some may eventually progress to liver diseases, like liver fibrosis, cirrhosis, and hepatocellular carcinoma (Alter et al., 1992; Saito et al., 1990; De Mitri et al., 1995; Esteban, 1993). The natural history of HCV is poorly understood, especially the degree to which hepatocellular damage is mediated by the immune system or by the direct pathogenicity of HCV. HCV seems to show a higher replication rate in patients receiving immunosuppressive therapy and in patients co-infected with HIV (Sherman et al., 1993; Eyster et al., 1994; Cribier et al., 1995). Since HCV and HIV share the same parenteral route of transmission, co-infections with HCV and HIV are frequently found in injecting drug users (van den Hoek et al., 1990; Thomas et al., 1995) and in hemophiliacs who received multiple transfusions with clotting factor concentrates before 1985 (Eyster et al., 1993; Cribier et al., 1995; Watson et al., 1992).

HIV is known to cause immunosuppression characterized by a decline in CD4⁺ cell counts that is followed by clinical manifestations of acquired immunodeficiency syndrome (AIDS). Several reports have suggested that HCV replication is enhanced by HIV infection, either by HIV-induced immunosuppression or by HIV-HCV interaction (Thomas et al., 1996; Ghany et al., 1996; Chambost et al., 1995; Sherman et al., 1993; Eyster et al., 1994; Cribier et al., 1995); however, an association between HCV RNA levels and CD4⁺ cell counts in HIV-seronegative individuals remains to be demonstrated. In this study three immunological markers (CD4⁺, CD8⁺ cell counts, and anti-CD3 reactivity) were compared with

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HCV and HIV RNA levels in serial time points among 19 HCV seroconverters.

RESULTS
Impact of HIV infection on HCV RNA level

To determine the influence of HIV infection on HCV replication, we compared serial measurements of HCV RNA in subjects who remained HIV negative (HIVneg), were HIV positive (HIVpos), or seroconverted for HIV during the study period. None of the subjects were treated with any antiviral therapy.

HCV RNA levels were measured at 355 datapoints by the bDNA and at 130 datapoints with NASBA. The HCV NASBA was additionally used to study the concordance between the two assays and a significant association between the two quantitative HCV RNA assays was found when all values below the lower limit of both assays were omitted (R = 0.81, P < 0.0001; Fig. 1).

HCV RNA levels appeared to be higher among HIVpos subjects than among HIVneg subjects. This same phenomenon was observed among subjects who seroconverted for HIV: lower HCV RNA levels during the pre-HIV seroconversion period and higher levels after HIVsc (Fig. 2). To determine if the influence of HIV infection on HCV RNA replication was statistically significant, log-transformed HCV RNA copies were compared in HIVneg and HIVpos subjects. Over the whole observation period, mean HCV RNA levels within the HIVneg population were 10^5.62 ± 0.73 HCV RNA copies/ml, and within the HIVpos population 10^6.70 ± 0.84 HCV RNA copies/ml (P < 0.0001). Comparing the mean HCV RNA levels among the HIV seroconverters before (Fig. 3, left bar) and after (Fig. 3, right bar) HIVsc, HCV RNA levels increased from 10^5.47 ± 0.66 to 10^6.72 ± 0.81 HCV RNA copies/ml (P < 0.0001; Fig. 3). To determine whether this increase was due to HIV infection per se and not to the duration of HCV infection, we divided the mean study period of the HIVneg and HIVpos population into two equal parts, with a mean duration of approximately 2.5 years in both populations. Similarly, among the HIV seroconverters, we found that the mean period prior to HIV seroconversion was 2.4 years, whereas the mean period after HIV seroconversion was 1.9 years. No differences were found in HCV RNA levels between the first (Fig. 3, left bar) and second (Fig. 3, right bar) period of 2.5 years among the HIVneg and HIVpos subjects, indicating that the duration of HCV infection was not influencing HCV replication during the first 5 years of HCV infection. Mean HCV RNA levels were lower among HIVneg subjects than among HIVpos subjects, during both the first and the second period of 2.5 years (P < 0.0001). Moreover, comparing HCV RNA levels among HIV seroconverters before HIVsc with the levels among HIVneg subjects, levels of HCV RNA were indistinguishable (P > 0.1). On the other hand, HCV RNA levels were significantly higher after HIVsc (P < 0.0001), but indistinguishable from HIVpos (P > 0.1).

Using all datapoints above the lower limit of the quantification assay for both HIV RNA and HCV RNA concentrations, no significant association was found between parameters (R = 0.06, P = 0.60; Fig. 4). This lack of association between parameters might indicate that HIV
(or HIV proteins) enhances HCV replication when a certain threshold of HIV replication is reached.

Impact of immunological parameters on HCV RNA levels

To address the relationship between HCV RNA levels and CD4, CD8 cell counts and anti-CD3 reactivity, we used serial measurements of all parameters in 19 HCV seroconverters that remained HIVneg (n = 10), seroconverted for HIV (n = 5), or were HIVpos prior to HCVsc (n = 4). There were 292 samples that could be analyzed two-sided for the relationship between HCV RNA levels and CD4+ and CD8+ cell counts, as well as 233 samples with anti-CD3 reactivity. Only values above the detection limit of the bDNA assay were used in regression analyses of HCV RNA levels and immunological parameters.

The CD4 cell counts and HCV RNA levels were significantly associated across the entire study population ($R^2 = 0.52$, $P < 0.0001$; Fig. 5a). To address if this association was solely due to an HIV-induced decline in CD4 cell counts, we set aside a HIVneg population and a HIVpos population. The association of CD4 cell counts and HCV RNA levels was also significant in the HIVneg population ($R^2 = 0.45$, $P < 0.001$; Fig. 5b). A weaker correlation was found between CD4 cell counts and HCV RNA levels in the HIVpos population ($R^2 = 0.22$, $P < 0.05$; Fig. 5c).

No correlation was found, either across the whole population or in the two separated populations, between HCV RNA and CD8 cell counts or the anti-CD3 reactivity ($P > 0.1$; results not shown).

Influence of HIV infection on HCV RNA levels in populations stratified according to CD4 cell counts

To obtain evidence of HIV infection influencing HCV RNA levels, independently of CD4 cell counts, we separated the 19 subjects into three groups: those whose CD4 cell counts were low (400±500 cells/$\mu$L), interme-
diate (500±900 cells/μl), and relatively high (900±1300 cells/μl). These particular strata were made because none of the HIVneg individuals had a CD4⁺ cell count below 400 cells/μl, and in addition none of the HIVpos individuals had CD4⁺ cell counts greater than 1300 cells/μl. HCV RNA levels could thus be studied in groups of HIVneg and HIVpos subjects with comparable CD4⁺ cell counts. Despite the fact that no significant difference in CD4⁺ cell counts between the HIVneg and the HIVpos populations was found within strata, there was a significant difference in HCV RNA levels between the HIVneg and the HIVpos populations in both the intermediate and the high CD4⁺ cell counts group. In the low CD4⁺ cell counts group the same trend was observed, although the difference was not significant (Table 1).

DISCUSSION

Co-infections with HCV and HIV often occur in injecting drug users (van den Hoek et al., 1990; Thomas et al., 1995), and in multitransfused hemophiliacs treated with clotting factor concentrates before 1985, when HCV was not yet discovered or isolated and HIV testing was introduced (Eyster et al., 1993; Cribier et al., 1995; Watson et al., 1992). Interactions between HCV and HIV have been studied, but with some conflicting results. Most studies have found increased HCV RNA levels in subjects who seroconverted for HIV, without a correlation with CD4⁺ cell counts, suggesting that HIV infection affects the replication of HCV (Cribier et al., 1995; Eyster et al., 1994; Sherman et al., 1993; Telfer et al., 1994; Thomas et al., 1996). However, whether increased HCV replication is directly influenced by the infection with HIV in itself or indirectly mediated by the immune system remains unclear. Eyster et al. (1994) reported a 58-fold increase in HCV RNA levels in subjects who became HIVpos and a strong association between HCV RNA levels and CD4⁺ cell counts among HIVpos hemophiliacs. This latter association, however, was strongly influenced by values of 23 samples taken at least 2 years after HIV seroconversion. In a prospective study of HIVsc among injecting drug users, Thomas et al. (1996) found significantly lower HCV RNA levels before HIV seroconversion. They also found an association of CD4⁺ cell counts and HCV RNA levels, although this was related to the time from HIV seroconversion. Ghany et al. (1996) reported that levels of HCV RNA among HIVneg hemophiliacs were similar to levels among those who were HIVpos, suggesting that HIV infection is not the only factor that influences HCV replication. They found higher HCV RNA levels in subjects with severe immunodeficiency, i.e., those having CD4⁺ cell counts below 200 cells/μl. The lack of association between CD4⁺ cell counts and HCV RNA levels in some reports might reflect a deficit of serial measurements or sampling bias, of both HCV RNA levels and immunological parameters, and/or a lack of measurements taken before and after HIV seroconversion.

In our study, the impact of HIV infection and HIV-induced immunodeficiency was assessed by comparing
three immunological markers with HCV and HIV RNA levels as measured on serial samples of 19 HCVsc who were HIVneg, HIVsc, or HIVpos. HIV infection influenced the HCV replication rate significantly when HIVneg and HIVpos populations were compared in different strata of CD4+ cell counts above 500 cells/μL. This indicates that HIV infection per se, in an individual with a normal immune status, directly enhances the HCV replication level. The same was seen in HIV seroconverters, in whom HCV RNA levels were significantly higher after HIV seroconversion. Mean HCV RNA levels did not change significantly from the beginning of HCV infection to the

FIG. 5. Association between HCV RNA levels and CD4+ cell counts. The linear regression lines are shown. (a) Among the whole population ($R = -0.52$, $P < 0.0001$). (b) Among the HIVneg population ($R = -0.45$, $P < 0.0001$). (c) Among the HIVpos population ($R = -0.22$, $P < 0.05$). The dotted line represents the detection limit of the bDNA assay.
end of follow-up among individuals who remained HIVneg, or among individuals who were HIVpos at entry. One study found that, regardless of HIV status, HCV RNA levels were affected by the duration of HCV infection, although not very significant (Eyster et al., 1994). In our study no association was found between HCV RNA levels and duration of HCV infection, possibly because the mean follow-up in our study was only 5.4 years, while others found this correlation over the next 5 to 12 years or more.

No significant association was found between HCV RNA and HIV RNA levels when values below the lower limit of the bDNA assay were omitted in the regression analysis, which corroborates the results of Cribier et al. (1995). From our quantitative HCV and HIV data one might conclude that a minimal level of HIV replication is needed for the up-regulation of HCV replication. However, more HIV replication does not apparently lead to more HCV replication, suggesting that when a threshold of HIV replication is achieved, HCV replication is independent of HIV replication.

To assess the degree of immunological control for HCV replication, CD4+ and CD8+ cell counts and anti-CD3 reactivity were studied as surrogate markers for HCV-specific immunity in the whole population and in the separated groups according to HIV status. CD4+ cell counts and HCV RNA levels were strongly associated across the whole study population. This suggests that a decline of CD4+ cell counts, as a surrogate marker for HCV-specific immunity, increases HCV replication. To

### Table 1

<table>
<thead>
<tr>
<th>CD4+ 400±500 cells/μl</th>
<th>n</th>
<th>Mean HCV RNA ± SD</th>
<th>P</th>
<th>Mean CD4+ cells ± SD</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIVneg</td>
<td>9</td>
<td>6.328 ± .862</td>
<td>NS</td>
<td>467 ± 30</td>
<td>NS</td>
</tr>
<tr>
<td>HIVpos</td>
<td>10</td>
<td>6.758 ± .945</td>
<td></td>
<td>442 ± 29</td>
<td></td>
</tr>
<tr>
<td>CD4+ 500±900 cells/μl</td>
<td>79</td>
<td>5.953 ± .881</td>
<td>0.001</td>
<td>732 ± 124</td>
<td>NS</td>
</tr>
<tr>
<td>HIVneg</td>
<td>29</td>
<td>6.572 ± .782</td>
<td></td>
<td>690 ± 103</td>
<td></td>
</tr>
<tr>
<td>HIVpos</td>
<td>7</td>
<td>6.772 ± 1.110</td>
<td>0.0001</td>
<td>1098 ± 118</td>
<td>NS</td>
</tr>
</tbody>
</table>

Note. NS, not significant.
determine if this correlation was confounded by HIV infection, the association of CD4+ cell counts and HCV RNA levels in serial samples from the HIVneg population was studied. The inverse relationship between CD4+ cell counts and HCV RNA levels suggests even more that propagation of HCV is dependent on the quality of the immune system, implying that HCV replication is at least partially under the control of the immune system. In other words, when HCV-specific immune responses deteriorate, the level of HCV replication might increase. This will then be expressed in elevated HCV RNA levels, which are possibly accumulating over time among HIVneg individuals as has been previously suggested (Eyster et al., 1994). No significant association was found between HCV RNA and CD8+ cell counts. These data corroborate an earlier observation that HCV-specific CD8+ CTL responses do not exert much control over HCV (Rehermann et al., 1996), whereas HCV-specific CD4+ T-cell responses seem to be able to control HCV propagation by maintaining humoral responses and probably therefore play a more important role in the elimination of HCV infection (Missale et al., 1996; Botarelli et al., 1993; Diepolder et al., 1995). Conversely, the decline in the number of HCV-specific CD4+ T-cell responses may lead to increased HCV RNA levels. HCV RNA levels among HIVpos subjects were also significantly associated with CD4+ cell counts but less powerfully, most likely because they all had relatively low CD4+ cell counts due to HIV infection, with a relatively wide range in HCV RNA levels.

The effect of HIV infection on HCV RNA levels, independent of CD4+ cell counts, was shown across the whole population, divided in three groups according to their CD4+ cell counts. Significantly increased HCV RNA levels related to HIV seropositivity were found in the two groups with intermediate and high CD4+ cell counts, suggesting the direct role of HIV infection on the replication rate of HCV. In the group with CD4+ cell counts between 400 and 500 cells/µl, more or less the same phenomenon was observed: higher HCV RNA levels among the HIVpos population with comparable CD4+ cell counts. However, these higher HCV RNA levels were not significantly different, probably because of the small number of individuals present in this group compared with the other two groups. The mechanism for this direct regulation is still unclear, but it has been described that the liver, the primary HCV reservoir, contains the largest population of macrophages, the so-called Kupffer cells, and the presence of HIV was demonstrated in Kupffer cells and hepatocytes in AIDS patients by several groups (Hufert et al., 1993; Housset et al., 1993; Cao et al., 1992).

The HIV tat gene is a potent transactivator, capable of up-regulating viral gene expression in cultured cells by both transcriptional and posttranscriptional mechanisms, and has been shown to induce dermal lesions which resemble Kaposi’s sarcoma in transgenic mice (Vogel et al., 1988). Recently, a putative tat-binding motif was found in the gene coding for the NS4 protein of HCV, and it was assumed that the HIV tat protein may modulate HCV infections by directly up-regulating HCV replication (Ferbeyre et al., 1997). Although this assumption was not addressed by us and further studies are needed, it might be a possible explanation for the direct effect of HIV on the replication rate of HCV, provided that most HCV-infected cells are co-infected with HIV.

In summary, our data indicate that: (i) HIV infection leads to enhanced HCV replication; (ii) an inverse relationship between CD4+ cell counts and HCV RNA levels is seen in HIV infected individuals; and (iii) there is an inverse relationship between CD4+ cell counts and HCV RNA levels among HIVneg individuals, suggesting that HCV replication is under the control of the immune system. Therefore we conclude that HIV influences HCV replication in two ways (Fig. 6). HCV replication is in some way directly influenced by HIV and HIV may reduce the HCV-specific immunity by impairment of the overall immune response.

**PATIENTS AND METHODS**

**Participants**

The injecting drug users were recruited from a cohort started in December 1985 (van den Hoek et al., 1988), consisting of drug users living in Amsterdam and participating in the Amsterdam Cohort Studies on HIV and AIDS. In March 1996, we selected individuals who were followed for at least 3 years and seen at least seven times (n = 358). Their serum and plasma samples were stored initially at +4°C, then frozen at −20°C within 24 h of collection and handling, and ultimately stored at −70°C. Among 358 subjects, 19 HCV seroconverters were identified (Beld et al., 1998) and divided into three groups on the basis of their HIV status: (i) HIVneg throughout the study period (n = 10); (ii) HIVsc during the study (n = 5); (iii) HIVpos before HCVsc (n = 4). HCV RNA load was determined on every single sample for which the immunological data were available (n = 294). The average sampling interval between samples containing both data was 5 months (range 0.5±48 months).
with an average of 15 time-points per patient (range 2±22 time-points). The mean follow-up of HCV infection was 5.4 years (range 1±9 years), whereas the mean follow-up of HIV infection was 3.5 years (range 1±7 years). None of the 19 subjects received antiretroviral therapy during follow-up.

Serological data

Sera were tested for the presence of antibodies to HCV by the third-generation Enzyme Immunoassay (EIA 3.0; Abbott Laboratories, Chicago, IL). All positive EIA 3.0 assays were confirmed by the third-generation Strip Immunoblot Assay (SIA, RIBA, Chiron Corp., Emeryville, CA). Antibodies to HIV-1 were determined with commercial Enzyme Immunoassay (EIA; Abbott Laboratories) and confirmed by Western blot (Diagnostic Biotechnology, Herent, Belgium). All serological assays were performed according to the manufacturer's manual.

Viral RNA quantification

The HCV RNA load was determined longitudinally in the 19 HCV seroconverters, on 355 available samples by the branched DNA (bDNA) signal amplification assay 2.0 (Quantiplex HCV RNA, Chiron Corp.). All samples were tested in duplicate, and the mean value of the duplicate tests was used for data analysis. Viral load, expressed as HCV RNA copies/ml, was determined by comparison with an external standard curve with a quantitation limit of 3.0 HCV RNA copies/ml. HIV RNA load was also determined by the NASBA technology, which has a quantification limit of 10² HIV RNA copies/ml. All quantification assays were performed according to the manufacturer's manual.

T-cell subsets and T-cell reactivity

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood by density-gradient centrifugation on Ficoll–Hypaque (Pharmacia, Uppsala, Sweden). T lymphocyte immunophenotyping for CD4 and CD8 membrane markers was performed on a Coulter Epics-C cytofluorometer (Coulter Electronics, Hialeah, FL). PBMC were stained with CD4-mAb (Leu-3a-PE; Becton Dickinson, Mountain View, CA) or CD8-mAb (Leu-2a-PE; Becton Dickinson) according to the manufacturer's protocols. T-cell responsiveness was measured in triplicate as previously described (Schellekens et al., 1990), by measuring CD3-mAb (CLB, Amsterdam, The Netherlands)-induced proliferative capacity of PBMC in whole-blood cultures. The anti-CD3 response is given as counts per minute.

Statistical methods

Scatterplots were used to review the distributions of HCV RNA and HIV RNA, respectively, with all immunological markers. For statistical analysis, we used log-transformed viral RNA concentrations. The regression analyses were performed only with data above the detection limit of the quantification assays and values below the detection limit were omitted. The Mann-Whitney test was used for comparison of groups. A value of P < 0.05 was considered significant.

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