Dual Infection with Human Immunodeficiency Virus Type 1 and Type 2: Impact on HIV Type 1 Viral Load and Immune Activation Markers in HIV-Seropositive Female Sex Workers in Abidjan, Ivory Coast

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ABSTRACT

To determine the impact of dual infection with HIV-1 and HIV-2 on HIV-1 viral load and markers of immune activation among HIV-seropositive FSWs in Abidjan, we analyzed blood samples obtained from consenting HIV-seropositive FSWs attending a confidential clinic between September 1996 and June 1997 in Abidjan. Among HIV-1 and HIV-2 dually seropositive FSWs, polymerase chain reaction (PCR) testing with HIV-1 and HIV-2 primers was used to differentiate between FSWs who were PCR positive only for HIV-1 and those positive for both HIV-1 and HIV-2 (dually infected). Of the 203 FSWs, 151 (74%) were HIV-1 seropositive only (median age, 26 years), 4 (2%) were HIV-2 seropositive, and 48 (24%) were dually seropositive (median age, 30 years). Of the 48 dually seropositive FSWs, 33 (69%) were dually infected and 15 (31%) were dually seropositive. Median CD4+ T cell counts per microliter were not significantly different among the three groups (525 for HIV-1 positive only, 502 for dually infected, and 416 for dually seropositive) (p = 0.14). Median viral load (log_{10} copies/ml) was not significantly different among the HIV-1-only FSWs (4.8 log_{10} copies/ml) compared with the 32 dually infected FSWs (4.6 log_{10} copies/ml) and 14 dually seropositive FSWs (4.7 log_{10} copies/ml; p = 0.95). Median levels of HLA-DR immune activation were increased in both CD4+ and CD8+ T cells for the dually infected (n = 27) FSWs compared with those infected with HIV-1 only (n = 123) (p = 0.019 and p = 0.01, respectively). Dual infection does not appear to influence levels of HIV-1 viral load in vivo. However, levels of HLA-DR are higher among FSWs dually infected with HIV-1 and HIV-2 than among those infected with HIV-1 only.

INTRODUCTION

IN WEST AFRICA, the epidemic of the human immunodeficiency virus (HIV) is characterized by the presence of both HIV-1 and HIV-2.1 For instance, in Abidjan, Ivory Coast, 13% of pregnant women are HIV-1-seropositive, 1.1% are HIV-2 seropositive, and 0.8% are dually seropositive.2 Polymerase chain reaction (PCR) testing of samples from dually seropositive persons shows that about 70% are indeed infected with both viruses.3,4 In vivo studies of the virologic and immunologic consequences of dual infection are lacking. Such studies could provide important clues to the pathogenesis of dual infection and may be useful in treating and counseling persons who are dually infected. In addition, they may provide valuable information for vaccine design in the context of interaction of two closely related retroviruses. Attempts to gain insight into the pathogenesis of dual infection by using in vitro studies have yielded mixed results. In one in vitro study, superinfection with HIV-1 and HIV-2 resulted in phenotypically mixed virus particles with an expanded cellular host range, raising the possi-
bility of faster disease progression in vivo. However, other in vitro studies have shown that HIV-2 inhibits or downregulates HIV-1 replication, and peripheral blood mononuclear cell (PBMC) cultures of persons infected with HIV-2 have been shown to protect against subsequent infection with HIV-1. Epidemiologic studies have also yielded contrasting results: some but not others have reported that HIV-2 protects against HIV-1. We hypothesized that, if HIV-2 prevents a subsequent HIV-1 infection, it is conceivable that it might also moderate the course of HIV-1 disease in vivo in persons dually infected with HIV-1 and HIV-2.

To verify this hypothesis, we compared HIV-1 viral load, lymphocyte subsets, and immune activation markers among female sex workers (FSWs) in Abidjan, Ivory Coast, who were infected with HIV-1 and HIV-2 and FSWs who were infected with HIV-1 only. FSWs in Abidjan are a unique population in which to address these questions, because the proportion who are dually seropositive is about 30%.

MATERIALS AND METHODS

Study population

Since 1992, we have provided HIV counseling and testing and diagnosis and treatment of sexually transmitted diseases (STDs) for FSWs attending a confidential clinic in Abidjan. From September 1996 through June 1997, consenting FSWs were consecutively enrolled in this study. None of the participants were receiving HIV antiretroviral therapy at the time of specimen collection.

Laboratory analysis

Whole blood was drawn from the FSWs into EDTA (K$_3$) tubes (Becton Dickinson, San Jose, CA). Within 4 hr of blood draw, plasma was separated from cells by centrifugation at 2000 g and then aliquoted and stored at −70°C.

HIV serology

We determined HIV antibody status by an enzyme-linked immunosorbent assay (ELISA)-based testing parallel algorithm. For HIV type-specific serodiagnosis, we used a synthetic peptide line immunoassay (Peptilav 1-2; Sanofi Diagnostics, Marnes La Coquette, France). DNA PCR testing

We performed DNA PCR testing on uncultured PBMCs as described elsewhere. In brief, we prepared total cellular DNA by lysing 4 × 10$^6$ PBMCs/ml in lysis buffer (10 mM Tris-HCl [pH 8.3]–0.05% Triton X-100), adding 10 mg of proteinase K per milliliter, incubating at 56°C for 1 hr, followed by 95°C for 10 min to inactivate the proteinase K. For HIV-1 and HIV-2 PCR testing we used specific PCR primers from the protease gene as reported elsewhere.

HIV-1 RNA viral load assay. We quantified HIV-1 RNA viral load in plasma by the reverse transcriptase-PCR (RT-PCR) Amplicor HIV-1 Monitor assay, version 1.5 (Roche Diagnostics Systems, Branchburg, NJ). This assay accurately quantifies HIV-1 subtype A/G recombinant viruses, which are the predominant subtypes in Ivory Coast.

HIV-1 RNA viral load assay. HIV-2 RNA plasma viral load was quantified by an RT-PCR prototype assay (Roche Diagnostic Systems). Briefly, RNA from 300 µl of plasma along with a spiked quantitation standard (50 copies) was extracted. A single-tube RT-PCR was performed with Tth DNA polymerase (Roche Molecular Systems, Alameda, CA), using 50-µl specimen equivalents per reaction. Primer sequences and genome positions (based on the HIV-2Rod strain) used for the targeted HIV-2 long terminal repeat (LTR) region were forward primer LTR-3 (5'-GCTGGCAGATTGAGCCCTGGGAGGT-3' ; nucleotides 574–601), reverse primer LTR-2 (5'-GAATGACCAGGGCGAGCTAGGAGAGAT-3' ; nucleotides 749–776), and LTR probe 5'-TGGCTGTTCCCTGCTAGA-CTCTACCACTGACT-3' (nucleotides 625–656). Cycler conditions were as follows: initial heating step at 50°C for 2 min followed by 60°C for 30 min to complete the RT reaction; PCR initiated by 4 cycles of 95°C for 10 sec to denature, 55°C for 10 sec to anneal, 72°C for 10 sec to extend; PCR continued with 30 cycles of 90°C for 10 sec to denature, 60°C for 10 sec to anneal, 72°C for 10 sec to extend, and finishing with a final extension step at 72°C for 15 min. Amplicon detection and quantitation were achieved using a microwell plate-based colorimetric assay format. Stocks of SIVmac251 and HIV-2NIH-Z with associated virus particle counts and core antigen values were used as assay standards to minimize variability. This assay has a limit of detection of 100 copies/ml.

Immunophenotyping. We carried out three-color flow cytometric measurements by applying to a FACScan flow cytometer (Becton Dickinson) fresh peripheral whole blood within 4 hr of collection into an EDTA tube. Aliquots of cells were stained with commercially available monoclonal antibodies (Becton Dickinson). The markers analyzed were CD4, CD8, CD25, CD28, CD38, CD45RO, CD45RA, and HLA-DR. CD25, CD38, and HLA-DR were used to assess activation of T cell subsets; CD45RO and CD45RA were used to quantify memory and naive cells, respectively.

Assays for serum activation markers

We measured levels of tumor necrosis factor type II receptors (sTNFαRII) and β$_2$-microglobulin concentrations in the plasma specimens by ELISA (Quantikine; R&D Systems, Abingdon, UK).

Analysis of data

All samples that were below the detection limit of the Amplicor Monitor assay were assigned a value of 1.0 log$_{10}$ copies/ml, which was below the assay cutoff value of 2.3 log$_{10}$ copies/ml. The immunologic data were analyzed as the percentage of lymphocytes for the major cell subsets (CD4$^+$ and CD8$^+$ T cells) or as the percentage of a major lymphocyte subset (activation and memory markers) stained with each or a combination of monoclonal antibodies. We considered FSWs as dually infected if both HIV-1 and HIV-2 PCR testing were positive and as HIV dually seropositive if only HIV-1 PCR test-
ing was positive and HIV-2 PCR testing was negative. The Kruskal–Wallis and the Mann–Whitney U tests were use to assess statistical difference between more than two groups (α was set at 0.05). If significant differences were found, the Mann–Whitney U test was performed to analyze difference between two groups. Correlation analysis was done by Spearman correlation coefficients. Because not all of the assays were done for every subject, the number of samples analyzed per assay is indicated where necessary in Results.

RESULTS

Characteristics of the study populations

Of the 343 FSWs enrolled, 203 were HIV seropositive, and 140 were HIV seronegative. Of the 203 HIV-seropositive FSWs, 151 (74%) were HIV-1 seropositive, 4 (2%) were HIV-2 seropositive, and 48 (24%) were dually seropositive. The median age for the HIV-1-seropositive women was 26 years, 30 years for those who were dually seropositive, and 24 years for those who were HIV seronegative. The median duration of sex work was 24 months (8–48 months) for the HIV-1-positive FSWs, 44 months (24–84 months) for the HIV-dually seropositive, and 12 months (6–36 months) for the HIV seronegative ($p < 0.001$). CD4+ T cell counts were available for 161 HIV-seropositive FSWs: 16 (10%) had CD4+ T cell counts of fewer than 200/μl, 64 (40%) had CD4+ T counts of 200–499/μl, and 81 (50%) had counts of >500 CD4+ T cells per microliter.

PCR results of dually seroreactive FSWs

To determine the proportion of the 48 HIV dually seropositive FSWs who were infected with both HIV-1 and HIV-2, we performed PCR testing, using HIV-1- and HIV-2-specific primers. All the samples were positive by HIV-1 DNA PCR testing, and HIV-1 RNA viral load was quantified in all the samples by Amplicor HIV-1 Monitor assay. Of the 48 HIV dually seropositive FSWs, 33 (69%) were positive by HIV-2 PCR testing (HIV dually infected), and 15 (31%) were negative by HIV-2 PCR testing (HIV dually seropositive).

CD4+ and CD8+ T lymphocyte cell counts

Median CD4+ T cell counts among the HIV-1-infected FSWs (525 cells/μl) were similar compared with those who were dually infected (502 cells/μl) and slightly lower in the dually seropositive FSWs (416 cell/μl; $p = 0.14$) (Table 1).

HIV-1 RNA viral load in HIV-1 and HIV-2 dually infected FSWs

To determine whether dual infection influences HIV-1 replication in vivo, we compared HIV-1 RNA viral load among the three groups of FSWs (HIV-1 infected, dually infected, and dually seropositive). Median HIV-1 RNA viral load did not differ significantly in the three groups: 4.8 log_{10} copies/ml among the HIV-1-infected FSWs; 4.6 log_{10} copies/ml among the dually infected FSWs; and 4.7 log_{10} copies/ml among the dually seropositive ($p = 0.95$) (Table 1). Of the 23 FSWs with quantifiable HIV-2 RNA viral load, we observed a positive correlation between HIV-1 and HIV-2 viral load (Spearman correlation, $r = 0.50; p = 0.01$) (Fig. 1). Median HIV-2 plasma viral load was generally low among the dually infected persons (median, 2.5 log_{10} copies/ml; range, 2.2–4.2).

Serum levels of immune activation markers

Increased levels of CD8+ T cells expressing the CD38 molecule and the HLA-DR antigen lymphocyte subsets are strong prognostic markers of HIV disease progression. The median percentage of CD8+ T cells expressing CD38 for the 123 HIV-1 infected FSWs (95%; interquartile range, 91–97%) was similar compared with those for the 27 dually infected FSWs (93%; interquartile range, 89–95%), and the 14 dually seropositive FSWs (95%; 93–98%) (Fig. 2). Median levels of expression of HLA-DR in CD8+ T cells were significantly higher among the 27 FSWs who were dually infected (68%; interquartile range, 63–73%) ($p = 0.01$), and the 14 who were dually seropositive (67%; interquartile range, 63–75%; $p = 0.037$) (Fig. 2) than the 123 HIV-1-infected FSWs (62%; interquartile range, 51–69%).

We also determined whether CD8+ T memory cells (CD8+ CD45RA−) differed in HIV-1-infected and dually infected FSWs. The median percentage of CD45RA-negative CD8+ T cells was higher, but not significantly so, for the 27 FSWs who were dually infected (64%; interquartile range, 49–77%) and the 14 FSWs who were dually seropositive (64%; 50–74%) compared with the 123 HIV-1-infected FSWs (54%; interquartile range, 42–66%; $p = 0.086$) (Fig. 2). CD8+ T cells expressing CD28 antigens have antiviral suppressive activity that is lost during HIV-1 disease progression. The median percentage of CD8+ T cells expressing CD28 did not differ among the 123 HIV-1-infected FSWs (47%; interquartile range, 38–57%) and the 27 FSWs who were dually infected (43%; 37–49%), and 14 dually seropositive FSWs (46%; 32–55%) ($p = 0.28$) (Fig. 2).

We found significantly higher median levels of expression of HLA-DR in CD4+ T cells for the 27 dually infected FSWs (38%; interquartile range, 32–58%) ($p = 0.019$) and the 14 dually seropositive FSWs (42%; interquartile range, 31–52%) ($p = 0.056$) compared with the 123 HIV-1-infected FSWs (34%; interquartile range, 24–46%). The expression of CD25 on CD4+ T cells was higher, but not significantly so, for the 27 FSWs who were dually infected (50%; interquartile range, 49–57%) compared with the 14 dually seropositive (50%; 45–55%) ($p = 0.037$) (Fig. 2). Median levels of expression of HLA-DR in CD8+ T cells for the 27 dually infected FSWs (38%; interquartile range, 32–58%) ($p = 0.019$) and the 14 dually seropositive FSWs (42%; interquartile range, 31–52%) ($p = 0.056$) compared with the 123 HIV-1-infected FSWs (34%; interquartile range, 24–46%). The expression of CD25 on CD4+ T cells was similar for HIV-1-infected and dually infected FSWs and CD45RO expression on CD4+ T cells was higher for dually infected than HIV-1-infected FSWs ($p = 0.099$) (Fig. 2).

Correlation between HIV-1 RNA viral load and phenotypic T cell markers

When we considered all the HIV-seropositive FSWs together, plasma RNA HIV-1 viral load was correlated signifi-
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<td>HIV-1 RNA viral load (log₁₀ copies/ml)</td>
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<td>4.8 (4.1–5.4)</td>
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<td>CD8⁺ T cells/µl</td>
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<td>β₂-Microglobulin (pg/ml)</td>
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<td>sTNF-α RII (pg/ml)</td>
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<td>3.9 (2.5–5.5)</td>
<td>33</td>
<td>4.3 (2.5–6.0)</td>
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**Abbreviation**: ND, Not done.

Differences between the three HIV-seropositive groups were tested for significance by the nonparametric Kruskal–Wallis test. Because no significant differences were found, no further analysis was performed. Differences between each of the HIV-positive groups and the HIV-negative group were tested by the Mann–Whitney U test, and for each variable the difference was highly significant (p < 0.0001).
cantly with CD38 on CD8⁺ T cells ($r = 0.55; p < 0.01$), and with HLA-DR on both CD4⁺ ($r = 0.38; p < 0.0005$) and CD8⁺ T cells ($r = 0.3; p < 0.005$). We then considered these markers (CD38 and HLA-DR) separately for HIV-1-infected and HIV dually infected FSWs: for HIV-1-infected FSWs, viral load correlated with CD38 on CD8⁺ T cells ($r = 0.61, p = 0.001$), with HLA-DR on CD8⁺ cells ($r = 0.38, p < 0.001$), and with HLA-DR on CD4⁺ cells ($r = 0.44, p < 0.001$) (Fig. 3). Among HIV dually infected FSWs, viral load correlated only with CD38 on CD8⁺ T cells ($r = 0.61, p = 0.001$), and not with HLA-DR on CD8⁺ cells ($r = 0.25, p = 0.23$), and with HLA-DR on CD4⁺ cells ($r = 0.22, p = 0.62$) (Fig. 3).

DISCUSSION

In this comprehensive cross-sectional study, we found a striking similarity in viral load and immunologic parameters among HIV-1-infected and dually infected FSWs. Our findings suggest that in persons dually infected with HIV-1 and HIV-2, HIV-2 does not seem to have any influence on in vivo HIV-1 viral load. This assertion is further supported by the observation in this study of a positive correlation between HIV-1 and HIV-2 RNA viral load. Indeed, we had reasoned that if HIV-2 moderates HIV-1 replication in vivo, in dually infected persons an inverse correlation should exist between HIV-1 and HIV-2 RNA viral load. Indeed, we had reasoned that if HIV-2 moderates HIV-1 replication in vivo, in dually infected persons an inverse correlation should exist between HIV-1 and HIV-2 RNA viral load. Our results are at variance with one other study that reported a significantly lower HIV-1 RNA viral load among 14 HIV dually infected tuberculosis patients compared with tuberculosis patients infected only with HIV-1.19 One limitation of our study is that we do not know the order of infection of the viruses; this may be important in determining any disease-protective effect that HIV-2 may have. Thus, longitudinal studies are needed to gain insights into the pathogenesis of dual infection. Despite this limitation, our results indicating similar viral load in HIV-1 and HIV dually infected persons are consistent with the comparable HIV clinical manifestations and disease course that have been reported for persons infected with HIV-1 and those who were dually seropositive.20 Moreover, the high prevalence of HIV dually infected FSWs (33 [16%] of 203) compared with the 2% who were infected with HIV-2 suggests indirectly the lack of possible protection from HIV-1 infection conferred by HIV-2.

The significantly increased levels of HLA-DR immune activation expressed both in CD4⁺ and CD8⁺ T cells, and of CD45RO expression on CD4⁺ T cells and CD45RA expression on CD8⁺ T cells for the dually infected FSWs, compared with those infected with HIV-1 only, may suggest an increased immune activation status of the host that does not appear to result in an upregulation of virus replication as assessed by plasma RNA levels. However, viral load correlated positively with HLA-DR on both CD4⁺ and CD8⁺ cells for all the HIV-positives FSWs, which is consistent with what has been reported by others previously.21 We believe that the lack of correlation between viral load and HLA-DR in CD4⁺ and CD8⁺ cells among HIV dually infected FSWs is likely due to the small sample size, since the $r$ values were similar.

We observed remarkable similarities in virologic and immunologic parameters in dually infected and dually seropositive FSWs. A possible explanation is that the FSWs we classified as dually seropositive were indeed dually infected but that HIV-2 had not been confirmed by PCR testing because of the low provirus load of HIV-2.22 However, our finding that 69% of FSWs who were dually seropositive were dually infected is consistent with what we and others have reported previously.3,4 Alternatively, dually seropositive FSWs may represent persons that have been infected with HIV-1 who have been exposed to, but who are not infected with, HIV-2.

In summary, dual infection with HIV-1 and HIV-2 does not appear to influence levels of HIV-1 replication in vivo; however, longitudinal studies of persons infected with HIV-2 and HIV-2 DUAL INFECTION

FIG. 1. Correlation between HIV-1 and HIV-2 RNA plasma viral load. The diagonal lines indicate the calculated linear regression curve (solid line) and the 95% confidence limits (dashed lines). The 23 dots represent the 23 HIV-2 plasma samples that were quantified.
subsequently infected with HIV-1 will provide more insights into the interaction of these two viruses in vivo. Levels of HLA-DR on both CD4+ and CD8+ T cells were higher among FSWs dually infected with HIV-1 and HIV-2 than they were among those infected with HIV-1 only.

**ACKNOWLEDGMENTS**

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FIG. 3. Correlation between HIV-1 plasma RNA viral load and markers of immune activation among female sex workers who were infected with HIV-1 only (solid lines and solid rectangles) and those who were dually infected (dashed lines and open rectangles). Correlations of viral load with the markers were as follows: for FSWs infected only with HIV-1: CD38 on CD8\(^+\) T cells \((r = 0.61, p = 0.001)\), HLA-DR on CD8\(^+\) cells \((r = 0.38, p < 0.001)\), and HLA-DR in CD4\(^+\) cells \((r = 0.44, p < 0.001)\); for HIV dually infected FSWs, the correlations of viral load were as follows: CD38 on CD8\(^+\) T cells \((r = 0.61, p < 0.001)\), HLA-DR on CD8\(^+\) cells \((r = 0.25, p = 0.23)\), and HLA-DR on CD4\(^+\) cells \((r = 0.22, p = 0.62)\).
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