JOURNAL OF VIROLOGY, Mar. 2006, p. 2529–2538 Vol. 80, No. 5
0022-538X/06/$08.00+0 doi:10.1128/JVI.80.5.2529–2538.2006

Natural Killer Cell Function Is Well Preserved in Asymptomatic Human Immunodeficiency Virus Type 2 (HIV-2) Infection but Similar to That of HIV-1 Infection When CD4 T-Cell Counts Fall

Samuel Victor Nuvor, Marianne van der Sande,† Sarah Rowland-Jones, Hilton Whittle, and Assan Jaye*

MRC Laboratories, Fajara, Banjul, The Gambia

Received 20 October 2005/Accepted 7 December 2005

Natural killer (NK) cells are potent effectors of natural immunity and their activity prevents human immunodeficiency virus type 1 (HIV-1) viral entry and viral replication. We sought to determine whether NK immune responses are associated with different clinical course of HIV-1 and HIV-2 infections. A cross-sectional analysis of NK cell responses was undertaken in 30 HIV-1 and 30 HIV-2 subjects in each of three categories of $CD4^+$-T-cell counts (>500, 200 to 500, and <200 cells/μl) and in 50 HIV-uninfected control subjects. Lytic activity and gamma interferon (IFN-γ) secretion were measured by chromium release and enzyme-linked immunospot assays, respectively. Flow cytometry was used to assess intracellular cytokines and chemokines. Levels of NK cytotoxicity were significantly higher in HIV-2 than in HIV-1 infections in subjects with high $CD4^+$-T-cell counts and were similar to that of the healthy controls. In these HIV-2 subjects, cytolytic activity was positively correlated to NK cell count and inversely related to plasma viremia. Levels of intracellular MIP-1β, RANTES, tumor necrosis factor alpha, and IFN-γ produced by NK $CD56^{bright}$ cells were significantly higher in HIV-2- than HIV-1-infected subjects with high $CD4^+$-T-cell counts but fell to similar levels as CD4 counts dropped. The data suggest efficient cytolytic and chemokine-suppressive activity of NK cells early in HIV-2 infection, which is associated with high $CD4^+$ T-cell counts. Enhancement of these functions may be important in immune-based therapy to control HIV disease.

AIDS in West Africa is caused by two human immunodeficiency virus (HIV) strains of differing pathogenicities (23). HIV-2 infection progresses more slowly to AIDS and infected subjects survive longer than those with HIV-1 infection (20, 37). Plasma viral load is lower (3), the CD4 count declines more slowly (14), and transmission rates are lower in HIV-2 than in HIV-1 infection (24, 29). The immunopathogenic basis for the difference in clinical course between HIV-2 and HIV-1 remains unclear. However, some HIV-2-infected subjects have a high viral load and die as quickly as HIV-1-infected subjects (35), suggesting that the virus may be no less pathogenic per se. In tissue culture both HIV-1 and HIV-2 cause similar levels of cytopathicity (31). Studies in West Africa have shown that individuals with HIV-2 infection whose CD4 counts have fallen below 500 cells/μl have a mortality similar to that of those infected with HIV-1 or with dual infection (30). Thus, it appears that early events in the interaction between HIV and the immune system may be pivotal in determining the subsequent outcome for individuals with HIV-2 infection.

Natural killer (NK) cells are potent effectors of natural immunity and they constitute the initial immune defense reaction against variety of viruses, fungi, parasites, and bacteria (28). NK cells are able to lyse tumor and virus-infected cells without prior activation. In addition, they can produce higher levels of cytokines and chemokines (4) that may enhance the adaptive immune response to pathogenic infection and therefore play an important role in controlling infections. A few studies in HIV-1 infection have suggested that NK cell activity may indeed be important in protective immunity: Rapid progression to AIDS has been associated with reduced NK cell activity and a decrease in NK cell numbers (6, 32). NK cells from HIV-1-infected subjects were also found to secrete CC chemokines with the ability to suppress autologous HIV replication in vitro (10, 17, 25) and high gamma interferon (IFN-γ) release was found to be associated with NK cell number in interleukin (IL)-2 therapy during AIDS (16). Recently, exogenous cytokine stimulation with IL-15 in combination with IL-12 and IL-18 was reported to increase IFN-γ production from NK cells in HIV-infected subjects (22). Moreover, increased NK activity has been observed in HIV-exposed but uninfected individuals, indicating that NK cells may also contribute to the protection against primary HIV infection (33).

In general, apart from exerting their functions by secretion of antiviral soluble factors, NK cells also kill viral infected cells. Such cytotoxicity is non- major histocompatibility complex (MHC) restricted but occurs via a pattern of receptors specific for MHC class I molecules. The binding of these receptors transmits inhibitory signals to NK cells, thus making them nonfunctional. However, when there is downregulation of MHC or altered MHC expression as is observed during HIV infection (13), the inhibitory receptors are not engaged to their MHC ligands, allowing the activation of NK cells and lysis of their target cells. Such cytolysis is mediated through the per-

* Corresponding author. Mailing address: Medical Research Council Laboratories, Fajara, P. O. Box 273, Banjul, The Gambia. Phone: (220) 4495442. Fax: (220) 4496513. E-mail: ajaye@mrc.gm.
† Present address: RIVM, Bilthoven, The Netherlands.

TABLE 1. Characteristics of HIV-infected patients and healthy controls

Group	CD4 T-cell count/μl	No. of subjects	No. male	No. female	% CD4 (mean ± SE)[a]	% CD8 (mean ± SE)[a]
HIV-1	>500	30	10	20	28.58 ± 1.1	41.47 ± 2.1
	(200–500)	30	7	23	21.03 ± 1.7	49.93 ± 1.9
	<200	30	10	20	6.03 ± 0.7	63.73 ± 2.4
HIV-2	>500	30	7	23	33.80 ± 1.6	37.47 ± 2.1
	(200–500)	30	19	11	23.30 ± 1.4	45.87 ± 2.4
	<200	30	16	14	9.17 ± 1.0	59.13 ± 3.1
Controls		50	36	14	37.12 ± 1.0	26.80 ± 1.1

[a] Percentage cells of total lymphocytes measured from whole blood by flow cytometry using the MultiSET program.

forin/granzyme B or Fas lytic pathway (19); or by antibody-dependent cell cytotoxicity mediated by CD16 receptors expressed on NK cells (2). It has, however, been difficult to ascertain in vitro NK lysis of autologous HIV-infected targets and a recent study has indeed shown the inability of NK cells to lyse HIV-infected $CD4^+$ T cells in vitro (5). Assessment of in vitro functional NK lysis of targets has therefore relied on the use of NK-sensitive cell line such as erythroleukemia cells (17, 33).

The secretion of CC chemokines may be the most important mechanism of suppression of HIV replication in vitro (10) and this effect was found most pronounced in patients with low-level viremia as a result of antiviral therapy (17, 21). Thus, the activity of NK cells may be influenced by the level of viremia. However, the use of antiretroviral drugs in treating HIV-infected individuals enhances NK activity (36) and may not give a true reflection of the relationship between the state of HIV infection and NK cell activity. We therefore chose to analyze NK activity in HIV-2 infection, where plasma virus load can be 20-fold lower than in HIV-1-infected asymptomatic patients (15) and the majority of HIV-2-infected people have low or undetectable virus load. In this context, we hypothesized that the basis for a protracted stable $CD4^+$-T-cell counts and low plasma virus load in HIV-2 may relate to increase NK functional activity early in infection. This study compares NK cell frequencies and functional activities between HIV-1- and HIV-2-infected subjects in a cross-sectional analysis at various levels of $CD4^+$-T-cell counts.

MATERIALS AND METHODS

Subject population. Subjects were selected from a clinic-based cohort of HIV-infected subjects at the MRC Laboratories in Fajara, The Gambia (30). These subjects included blood donors, female sex workers, cases of tuberculosis, or suspected cases of sexually transmitted disease as well as those showing clinical symptoms suggestive of HIV infection. The patients attended the clinic every 3 months and a sample was requested for CD4 assessment every 6 months. They are supported through regular counseling and treatment for opportunistic infection and home visits are conducted by trained field workers to determine the vital status of those patients who do not regularly attend the hospital for their 3-month follow-up examination. Antiretroviral therapy is now being offered to HIV-infected patients in the Gambia according to national treatment guidelines but was not available to this cohort at the time of the study.

This study recruited 90 antiretroviral naïve HIV-1- and HIV-2-infected individuals each from this cohort after informed consent was obtained. Subjects were classified into three categories of 30 patients according to the latest $CD4^+$-T-cell counts: high (>500 cells/μl), medium (200 to 500 cells/μl) and low (<200 cells/μl) (Table 1). HIV-1 and HIV-2 plasma RNA loads were measured by reverse transcription-PCR using specific long terminal repeat primers, as described elsewhere (3). Cutoff values for the detection of viral RNA were estimated to be 100 copies/ml, and any sample with a value below the cutoff was assigned a value of <100 copies/ml. Subjects who had dual HIV-1 and HIV-2 infection were excluded from the study. When comparing NK cell frequencies and functional activities, we included healthy HIV-seronegative control subjects whose blood samples were obtained from the blood bank of the Royal Victoria Teaching Hospital in Banjul. The Gambia Government/MRC Ethics Committee approved the study.

Effector and target cells for NK assays. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation and were cultured in RPMI 1640 (Sigma) supplemented with 10% fetal calf serum (Gibco BRL), 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM glutamine, a medium we refer to as R-10. PBMCs were used in NK assays with NK-sensitive cell line K562, an erythroleukemia cell line derived from a chronic myeloid leukemia patient in blast crisis. The K562 cells were maintained in R-10 medium.

Surface staining for T-cell subsets, NK cell count, and NK phenotyping. Lymphocyte subsets ($CD4^+$ and $CD8^+$ T cells) and NK cell percentage were assessed from recruited subjects by the use of four-color MultiTEST CD3/CD8/CD45/CD4 and CD3/CD16+CD56/CD45/CD19 reagents (Becton Dickinson). Fifty microliters of whole blood were stained with 10 ul of MultiTEST reagent, mixed, and incubated in the dark for 15 min. The red cells were lysed with 1× FACSLysing solution (Becton Dickinson) for 15 min at room temperature and cells were centrifuged, fixed with 2% formalin, acquired in flow cytometry, and analyzed with MultiSET software.

NK phenotyping was done with PBMCs that were separated from whole blood following gradient density centrifugation. Staining was carried out with the use of the following fluorochrome-conjugated monoclonal antibodies: CD56-allophycocyanin (Coulter), CD3-peridinin chlorophyll protein, and CD16-phycoerythrin (Becton Dickinson). Analysis was performed on a FACScalibur cytometer using CellQuest-Pro software (Becton Dickinson). $CD56^{dim}$ and $CD56^{bright}$ NK subpopulations were distinguished by establishing two regions based on the mean fluorescence channel (cell surface density) that detected $CD3^-CD56^{dim}$ and $CD3^-CD56^{bright}$.

Intracellular Cytokine and CC chemokine staining by flow cytometry. The detection of intracellular secretion of CC chemokines (RANTES, MIP-1α, and MIP-1β) and cytokines (IFN-γ and tumor necrosis factor alpha [TNF-α]) by NK cells was carried out by stimulating PBMCs with K562 cells at a ratio of 50:1. We added 10^4 K562 cells in a 50-μl volume to 10^6 PBMC/100-μl volume in a 96-well U-bottomed plate. Brefeldin A (Becton Dickinson) was then added at a final concentration of 10 μg/ml to inhibit the secretion of cytokines and plates were incubated at 37°C in a 5% CO_2 humidified incubator for 17 h. Cells were washed and stained with anti-CD3-peridinin chlorophyll protein, anti-CD56-allophycocyanin, or anti-CD16 phycoerythrin for 20 min. The cells were then fixed and permeabilized with 1× permeabilization solution 2 (Becton Dickinson) after which, cells were stained with carboxyfluorescein-conjugated monoclonal antibodies to RANTES, MIP-1α, MIP-1β, TNF-α, and IFN-γ at room temperature for 30 min. Isotype-matched antibodies labeled with phycoerythrin or fluorescein isothiocyanate was used to optimize specific fluorochrome staining. Cells were acquired and analyzed by FACScalibur using Cell-Quest Pro software. For each sample, acquisition of PBMCs was continued until 300,000 events were gated for $CD56^+$ $CD3^-$ analysis.

ELISPOT assays for IFN-γ detection. IFN-γ release by NK cells was also measured by ELISPOT assay using PBMCs as effectors and K562 NK-sensitive cells as targets at an effector-to-target cell (E:T) ratio of 25:1. Polyvinylidene difluoride-backed plates (MAIP S45; Millipore) were coated with anti-IFN-γ antibody (MABTECH) in carbonate-bicarbonate buffer by incubation at 4°C overnight. PBMCs (1.25×10^5) in 100 μl R-10 were added to 50 μl K562 target

TABLE 2. Frequency and phenotypes of NK cells in HIV-1- and HIV-2-infected subjects and healthy controls

Group	CD4 count/μl	Median % NK cells,[a] median (interquartile)	Mean % $CD56^{dim}$ cells[b] ± SE	Mean % $CD56^{bright}$ cells[b] ± SE
HIV-1	>500	9.50 (7.0–12.0)	4.43 ± 0.6*	0.09 ± 0.0
	(200–500)	7.50 (6.0–12.0)	5.85 ± 0.7	0.06 ± 0.0
	<200	8.00 (4.5–18.50)	4.96 ± 0.7	0.09 ± 0.0
HIV-2	>500	9.0 (6.5–13.0)	5.13 ± 0.8	0.06 ± 0.0
	(200–500)	10.50 (7.5–15.0)	4.86 ± 0.6	0.07 ± 0.0
	<200	9.50 (5.50–12.5)	4.46 ± 0.6*	0.07 ± 0.0
Control		14.00 (11.0–20.0)	6.95 ± 1.0	0.07 ± 0.0

[a] Percent NK cells of total lymphocytes measured from whole blood using the MultiSET program. The percentage of NK cells in healthy control was significantly higher than in either the HIV-1 or HIV-2 group for all three categories of CD4.

[b] Percentage of $CD56^+$ $CD3^-$ cells measured from PBMCs using CellQuest. The NK $CD56^{dim}$ subpopulation was significantly higher in healthy controls than in the HIV-1 group at the high and HIV-2 group at the low $CD4^+$-T-cell level (*).

cells (5×10^3) in duplicate wells. Effector cells in R-10 medium alone served as negative controls and effector cells with phytohemagglutinin (2 μg/ml) were used as positive controls. Cells were incubated for 16 h at 37°C and 5% CO_2 and plates were washed and developed with chromogenic alkaline phosphatase substrate after the two-step incubation with biotinylated anti–IFN-γ and streptavidin antibodies.

IFN-γ responses were measured as the number of spot-forming cells per well, and a reaction was considered positive if there was a >95% probability that the number of spot-forming cells appearing in the test well was at least twice that in wells that contained the negative control medium under the assumption of a Poisson distribution. No individual showed spontaneous responses by PBMCs without K562 targets. The specific spot-forming cells were measured by subtracting the negative control value from that of the test spots and the results were expressed as number per 10^6 PBMCs.

NK cell cytotoxicity assay. Ex vivo NK cytolysis (NK-CTL) was carried out by ^{51}Cr release assay using K562 cells as targets. Target cells were labeled with 100 μCi/ml sodium [^{51}Cr]chromate (Amersham International) for 1 h at 37°C and washed twice with nonsupplemented RPMI 1640. Labeled targets were resuspended in R-10 medium and dispensed in 96-well U-bottomed plates at 5×10^3 cells/well. PBMCs were then added to the K562 cells at an effector-to-target ratio of 50:1. Cultures were incubated for 4 h before 25 μl culture supernatant was collected and mixed with 125 μl of scintillation fluid (Optiphase Supermix; Wallac). The amount of ^{51}Cr released in culture supernatants was measured in a gamma counter (1450 Microbeta Trilux; Wallac) and the percentage of cytotoxicity was determined by using the formula $(E - M/D - M) \times 100$, where E is the experimental ^{51}Cr release, M is the ^{51}Cr released by targets in the presence of culture medium (which was 15 to 25% of the total release), and D is the total release of ^{51}Cr in the presence of 5% Triton X-100 detergent.

Statistical analysis. Statistical analysis was done using Graphpad prism and $P < 0.05$ was used as the significance level. Viral load data were log transformed and data are presented as means with standard error or medians with interquartile. Differences between HIV-1 and HIV-2 at all $CD4^+$-T-cell categories were tested by Mann-Whitney U test or Student t test. Analysis of variance was carried out for multiple comparison and adjustment of P values was done by Bonferroni correction. Function of NK cells was related to NK cell frequency and plasma viral load using the Spearman rank correlation test.

RESULTS

Frequency of NK cells and NK cell phenotypes in HIV-1 and HIV-2 infections. The percentage of NK cells among total lymphocytes measured from whole blood in HIV-1 and HIV-2 subjects in different categories of $CD4^+$-T-cell counts and in uninfected subjects are shown in Table 2. The percentage of NK cells was similar in both HIV-1 and HIV-2 subjects in all categories of $CD4^+$-T-cell counts. Equally, no differences in the NK percentages were seen with changes in $CD4^+$-T-cell counts in either of the two infections. However, the NK percentages in both HIV subjects in all categories of $CD4^+$-T-cell counts were significantly lower than in uninfected controls ($P < 0.05$).

The percentages of NK cell subpopulations in PBMCs were also similar in both infections. There were no significant differences between HIV-1- and HIV-2-infected subjects in their percentage of NK $CD56^{dim}$ or NK $CD56^{bright}$ populations. However, there were significantly higher NK $CD56^{dim}$ populations in uninfected controls than in HIV-1 subjects at high $CD4^+$-T-cell counts and in HIV-2 subjects at low $CD4^+$-T-cell counts ($P < 0.05$).

Higher cytolytic activity of NK cells in HIV-2- than HIV-1-infected individuals with a normal $CD4^+$-T-cell count. Cytolytic activity (% mean maximal lysis ± standard error) was significantly higher in HIV-2 than in HIV-1 subjects with high $CD4^+$-T-cell counts (18.8 ± 2.5% versus 11.5 ± 1.7%; $P = 0.03$) (Fig. 1a). However, cytolytic activity was similar in those with intermediate $CD4^+$-T-cell counts but was lower in HIV-2 than in HIV-1 subjects (15.1 ± 2.0% and 9.0 ± 1.8%; $P = 0.01$) in the low range of $CD4^+$-T-cell counts.

NK cytolytic activity in the healthy controls was similar to that of HIV-2 subjects with high $CD4^+$-T-cell counts (20.1 ± 1.2% versus 18.8 ± 2.5%; $P = 0.25$), and significantly higher than in HIV-1 subjects (20.1 ± 1.2% versus 11.5 ± 1.7%, $P < 0.0001$). Levels of NK cytolysis in the healthy controls were also higher than in HIV-1 or HIV-2-infected patients with intermediate $CD4^+$-T-cell counts (13.0 ± 1.7% for HIV-1, $P = 0.001$, and 14.4 ± 2.5% for HIV-2, $P = 0.006$) and with low $CD4^+$-T-cell counts (15.1 ± 2.0% for HIV-1, $P = 0.01$ and 9.0 ± 1.8% for HIV-2, $P < 0.0001$). This suggests NK immune response during early HIV-2 infection is more efficient than in HIV-1 infection. NK cytolytic activity in HIV-2, however, became similar to that of HIV-1 as infection progressed to lower $CD4^+$-T-cell counts and reached a significantly lower level than in HIV-1 subjects at the low category of <200 cell/μl (15.1 ± 2.0% for HIV-1 versus 9.0 ± 1.8% for HIV-2, $P = 0.01$). There was a weak positive correlation between the frequency of percent NK cells and cytolytic activity in HIV-2 subjects ($r = 0.270$, $P = 0.01$; Fig. 1b) but an association was absent in HIV-1 subjects ($r = 0.126$, $P = 0.24$; Fig. 1c).

No differences in IFN-γ secretion by NK cells between HIV-1- and HIV-2-infected subjects. We also carried out an ELISPOT assay after stimulation of PBMCs with K562 cells to determine the secretion of interferon gamma by NK cells. Similar levels of interferon gamma secretion were found in both HIV-1 and HIV-2 infections (Fig. 2). The mean spot-forming cells (SFC ± standard error) per million PBMCs in HIV-1-infected subjects with a high $CD4^+$-T-cell count (>500 CD4 cells/μl) was 638.1 ± 105.6 compared to 467.9 ± 55.0 in HIV-2-infected subjects. These levels were also similar between the two infections at the lower $CD4^+$-T-cell categories: mean SFC/10^6 PBMCs at medium category was 655.5 ± 91.4 for HIV-1 and 640.8 ± 97.6 for HIV-2; and in the low category they were 640.3 ± 116 and 450.3 ± 71.9 respectively. The secretion of IFN-γ in HIV-uninfected controls (1,053.0 ± 69.0) was significantly higher than in both HIV-1 and HIV-2 infections at all the $CD4^+$-T-cell categories ($P < 0.05$). The values

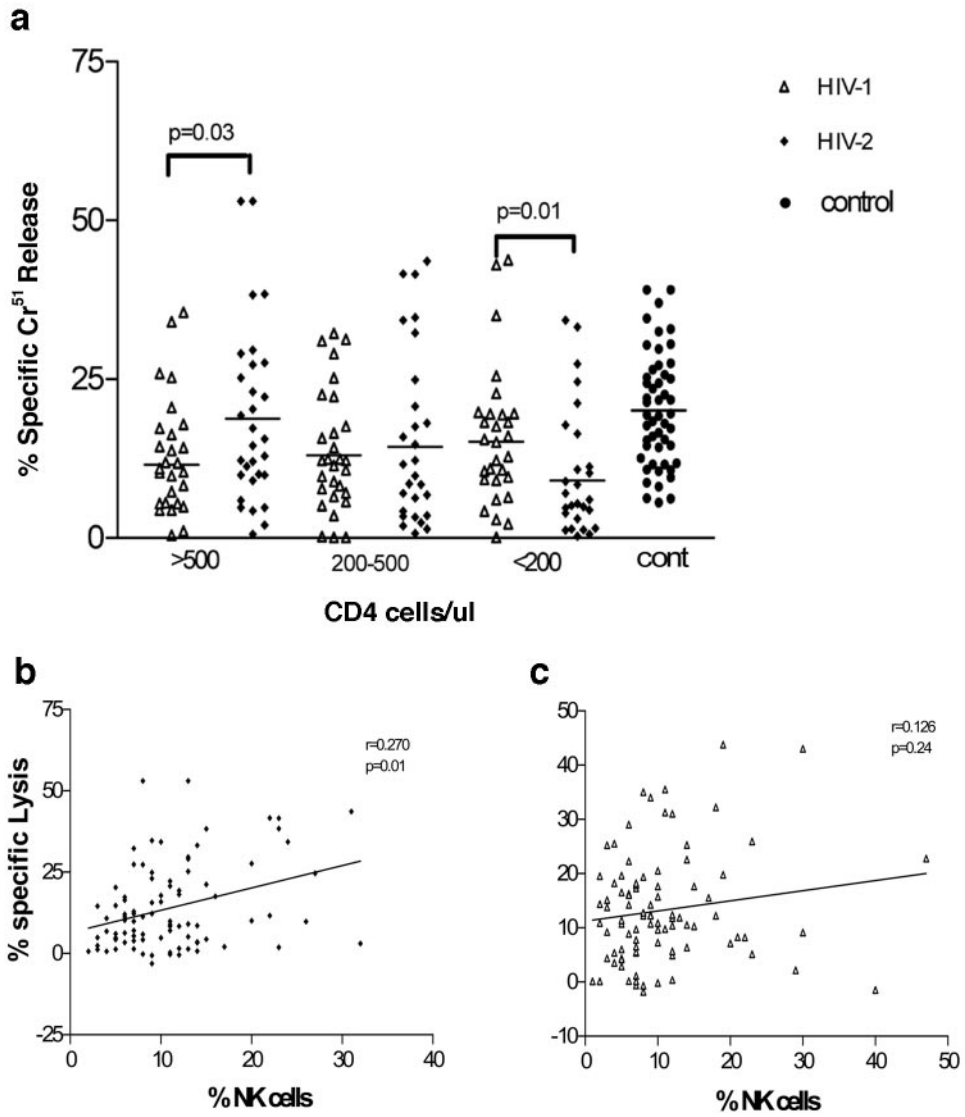

FIG. 1. Cytolytic activity of NK cells in HIV-1- and HIV-2-infected and HIV-uninfected subjects. Cytolytic activity of NK cells was determined with fresh PBMCs by chromium release assay at an E:T ratio of 50:1 using K562 cells as target cells. The specific lysis for the three categories of $CD4^+$ T cells is shown (a). The mean percent specific lysis is represented as a horizontal line. Correlation between NK cell count and specific lysis in all HIV-2 ($r = 0.270$; $P = 0.01$) (b) and in all HIV-1 ($r = 0.126$; $P = 0.24$) (c)-infected individuals.

of SFC/PBMC were not significantly different between the three categories of $CD4^+$ T-cell counts in either HIV-1 ($P > 0.05$) or HIV-2 ($P > 0.05$) subjects.

Higher number of NK $CD56^{bright}$ cells producing intracellular CC chemokines and cytokines in asymptomatic HIV-2 infection. The percentage of NK cells producing CC chemokines as well as proinflammatory cytokines was determined by intracellular cytokine staining. The percentage of NK $CD56^{dim}$ cells producing CC chemokines was similar in both infections and at each $CD4^+$ T-cell stratum but differences were found in the percentages of NK $CD56^{bright}$ cells producing MIP-1β, RANTES, TNF-α, and IFN-γ between HIV-1- and HIV-2-infected individuals.

Representative dot plots of NK subsets ($CD56^{dim}$ and $CD56^{bright}$) and the percentages of $CD56^{bright}$ cells secreting chemokines and cytokines are shown in Fig. 3a and b. NK $CD56^{bright}$ cells in HIV-2 subjects with a high $CD4^+$-T-cell counts had significant higher levels of MIP-1β and RANTES than HIV-1 subjects in the same category: 23.5 ± 8.3% versus 2.3 ± 0.8%, $P = 0.04$, for MIP-1β; 14.9 ± 6.6% versus 0.8 ± 0.5%, $P = 0.04$, for RANTES (Fig. 3c and d). There were no differences between the two infections in the levels of MIP-1β-secreting cells at intermediate $CD4^+$ T-cell counts (0.5 ± 0.3% for HIV-1 versus 5.39 ± 4.2% for HIV-2, $P = 0.79$) and at low CD4 count (8.7 ± 2.9% for HIV-1 versus 3.1 ± 1.2% for HIV-2, $P = 0.34$).

Equally, there were no significant differences in the levels of RANTES between HIV-1 and HIV-2 at medium (0.1 ± 0.0% for HIV-1 versus 2.7 ± 1.6% for HIV-2, $P = 0.24$) and low CD4 counts (5.7 ± 4.7% for HIV-1 versus 13.5 ± 6.0% for

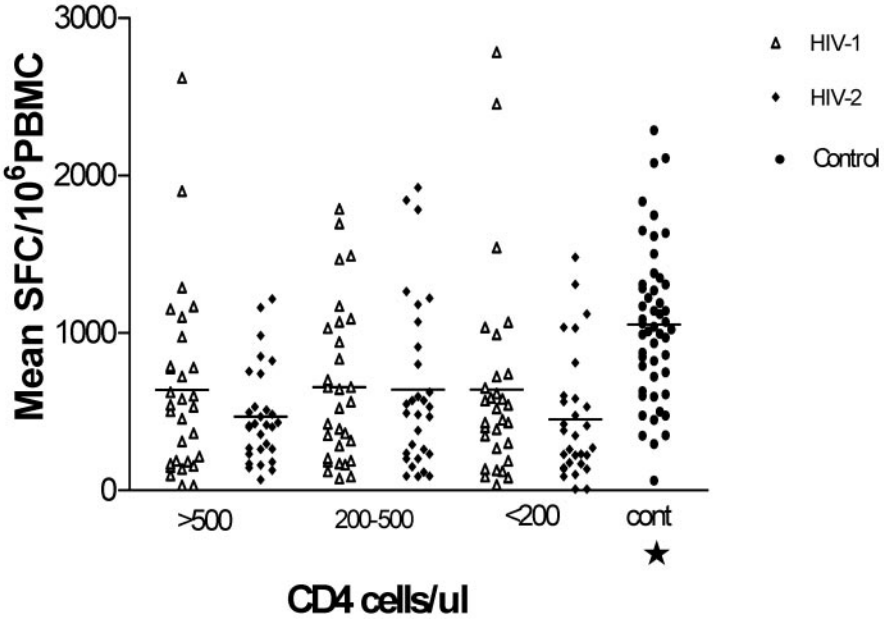

FIG. 2. IFN-γ production by NK cells in HIV-1- and HIV-2-infected and HIV-uninfected subjects as determined by ELISPOT assay. Cells were incubated together with NK-sensitive cells (K562) as target cells at an E:T ratio of 25:1 overnight. The specific IFN-γ spot-forming cells (SFC) are shown in the three categories of $CD4^+$ T cells. Differences between infections were not significant but healthy controls (*) had higher levels than all categories of patients ($P < 0.05$ in each case).

HIV-2, $P = 0.27$). The levels in HIV-2 subjects decreased significantly for MIP-1β at lower $CD4^+$-T-cell counts ($P = 0.02$ for intermediate and $P = 0.04$ for low $CD4^+$ T-cell counts), whereas there was a nonsignificant increase of MIP-1β in HIV-1 at low $CD4^+$-T-cell counts ($P = 0.48$). The levels of RANTES, however, dropped in those with medium $CD4^+$-T-cell counts in HIV-2 infections (14.9 ± 6.6% versus 2.7 ± 1.6%, $P = 0.18$) but surprisingly increased in the low $CD4^+$-T-cell group to a level similar to that in those with high $CD4^+$-T-cell counts (14.9 ± 6.6% versus13.5 ± 6.0%, $P = 0.65$) and a similar increase was noted for HIV-1 (0.8 ± 0.5% versus 5.7 ± 4.7%, $P = 0.14$) subjects.

The frequencies of $CD56^{bright}$ cells producing MIP-1α were low (Fig. 3e). There were no differences between the two infections in all groups of $CD4^+$-T-cell counts: 0.7 ± 1.8% versus 0.1 ± 0.5%, $P = 0.20$, for high; 1.9 ± 4.2% versus 1.2 ± 5.5%, $P = 0.17$, for medium; and 3.2 ± 5.0 versus 0.5 ± 1.2%, $P = 0.07$ for low $CD4^+$-T-cell counts in HIV-2 and HIV-1 subjects, respectively. In HIV-2 subjects, there was a nonsignificant increase in the level of MIP-1α-secreting cells with decreasing CD4 count.

NK $CD56^{bright}$ cells secreting the cytokines tumor necrosis factor alpha and Interferon gamma followed a similar pattern. There were significantly higher frequencies of the cytokine-secreting cells in HIV-2 than in HIV-1 subjects with high $CD4^+$-T-cell counts: 21.7 ± 7.6% versus 1.4 ± 0.9%; $P = 0.01$, for TNF-α and 13.5 ± 5.1% versus 2.5 ± 1.1%; $P = 0.04$, for IFN-γ, (Fig. 3f and g). The proportion of both TNF-α- and IFN-γ-secreting cells decreased to similar levels in both infections: for TNF-α, at the medium $CD4^+$-T-cell stratum, the proportions were 2.1 ± 0.9% in HIV-1 versus 5.9 ± 4.9% in HIV-2, $P = 0.81$; and for IFN-γ, they were 2.2 ± 0.7% in HIV-1 versus 7.9 ± 3.7% in HIV-2, $P = 0.60$. Whereas in the low-$CD4^+$-T-cell group, TNF-α levels were 1.6 ± 1.0% in HIV-1 versus 8.8 ± 4.9% in HIV-2, $P = 0.10$; IFN-γ levels were 5.4 ± 2.2% in HIV-1 versus 9.2 ± 4.2% in HIV-2, $P = 0.44$. The proportion of NK $CD56^{bright}$ cells producing IFN-γ and TNF-α in the uninfected controls was similar to that of HIV-2 subjects but higher than in HIV-1 individuals ($P < 0.05$, data not shown).

Association between plasma virus load and NK cytolytic activity. Plasma virus loads in HIV-1 and HIV-2 subjects at different $CD4^+$-T-cell categories are shown in Fig. 4a. The mean log plasma virus load was significantly higher in HIV-1 than HIV-2 subjects in both the high (4.3 ± 0.1 in HIV-1 versus 3.21 ± 0.1 in HIV-2, $P < 0.0001$) and medium $CD4^+$-T-cell categories (4.6 ± 0.1 in HIV-1 versus 3.42 ± 0.2 in HIV-2, $P < 0.0001$) but was similar to HIV-2 at low $CD4^+$-T-cell counts (5.2 ± 0.1 in HIV-1 versus 4.9 ± 0.2 in HIV-2, $P = 0.07$). In both infections the plasma viral load was similar between the high and medium $CD4^+$-T-cell groups ($P = 0.09$ for HIV-1 and $P = 0.54$ for HIV-2), but increased significantly at low $CD4^+$-T-cell counts ($P = 0.0003$ for HIV-1, $P < 0.0001$ for HIV-2).

Correlation between NK lysis activity and log plasma viral load, adjusting for CD4 as a partial variable, showed a weak but significant inverse relationship in HIV-2 infection ($r = -0.27$, $P = 0.009$; Fig. 4b), but this relationship was not evident in HIV-1 infections ($r = -0.03$, $P = 0.786$; Fig. 4c). The proportion of subjects with undetectable viral load in HIV-2 infection was seen in both the high and medium CD4 categories, which showed similar viral loads (Fig. 4a) and NK cytolysis was still higher in HIV-2 subjects in the combined group with CD4 counts of >200 cells/μl (data not shown). This suggests

that there could be an effect of viral load on NK cytolysis. However, correlation between viral load and NK specific lysis of each of the various groups of $CD4^+$-T-cell counts was not significant, most likely due to the reduced numbers of patients. Unlike cytolysis, no significant relationship was observed between plasma viral load and the cytokine- or chemokine-secreting activities of NK cells in all combined groups or in any category of CD4 count.

DISCUSSION

In this study we demonstrated higher levels of NK activity in HIV-2 than HIV-1 subjects when the $CD4^+$-T-cell counts were greater than 500 cells/μl. In both infections NK activity was found to decrease markedly as the $CD4^+$-T-cell counts fell, suggesting that NK cell functions are affected by HIV progression. Similar findings have been noted for HIV-specific $CD8^+$ T-cell suppressive activity, which declines with disease progression (18, 26). Though, unlike $CD8^+$ T cells, the assessment of NK function by use of in vitro autologous lysis of $CD4^+$ T cells has been found difficult (5). However, studies using NK-sensitive cell lines have allowed the role of NK cells in HIV infection to be documented. Our findings are therefore consistent with previous reports that used similar techniques and showed the loss of NK cells and function as disease progresses in HIV-1 infection (1, 6, 26). However, our data highlight that people with HIV-2 who have normal $CD4^+$ counts appear to constitute a distinct group, and that with disease progression, most of the differences between HIV-1 and HIV-2 infection disappear.

The consistently higher levels of ex vivo expression of NK effector functions in HIV-2 infection, as reflected both in cytolysis of an NK-sensitive cell line and secretion of chemokine and cytokine antiviral factors, indicate that sustained NK responses is efficient early in HIV-2 infection and may contribute to the control of viremia in many of HIV-2-infected subjects. Enhanced NK cell activity has been detected in high-risk exposed but uninfected individuals, suggesting that NK cells may protect against infection (33). This finding contrasts to studies of $CD8^+$ T-cell responses in HIV-1 and HIV-2 infection which failed to find more efficient $CD8^+$ T-cell responses in HIV-2 infection (15, 38).

Although both $CD8^+$ and NK cells have been found to suppress HIV replication in vitro, NK cell activity has been more clearly related to aviremia following antiretroviral therapy (17, 21). The strong and multifunctional NK cell activity in HIV-2 infection, which is associated with substantially lower levels of plasma viremia than with HIV-1 in the asymptomatic phase, strengthens the concept that early effective innate immunity influences the rate and level of viral replication. Taken together, the observations that the levels of cytolysis in asymptomatic HIV-2 subjects were similar to that of healthy controls and that this activity was positively correlated with percentage NK cells and inversely correlated with plasma viremia suggest that NK activity may suppress viral replication in HIV-2 infection and imply that NK function influences the clinical course of HIV-2 disease.

Interestingly, NK cytolysis decreased significantly in HIV-2 subjects with <200 CD4 cells/μl compared to HIV-1-infected individuals. Though this pattern appeared absent in the other functional analysis, a careful interpretation of this to reflect a difference in rate of progression between the two infections once this stage of clinical HIV infection is reached will warrant a follow-up study over time.

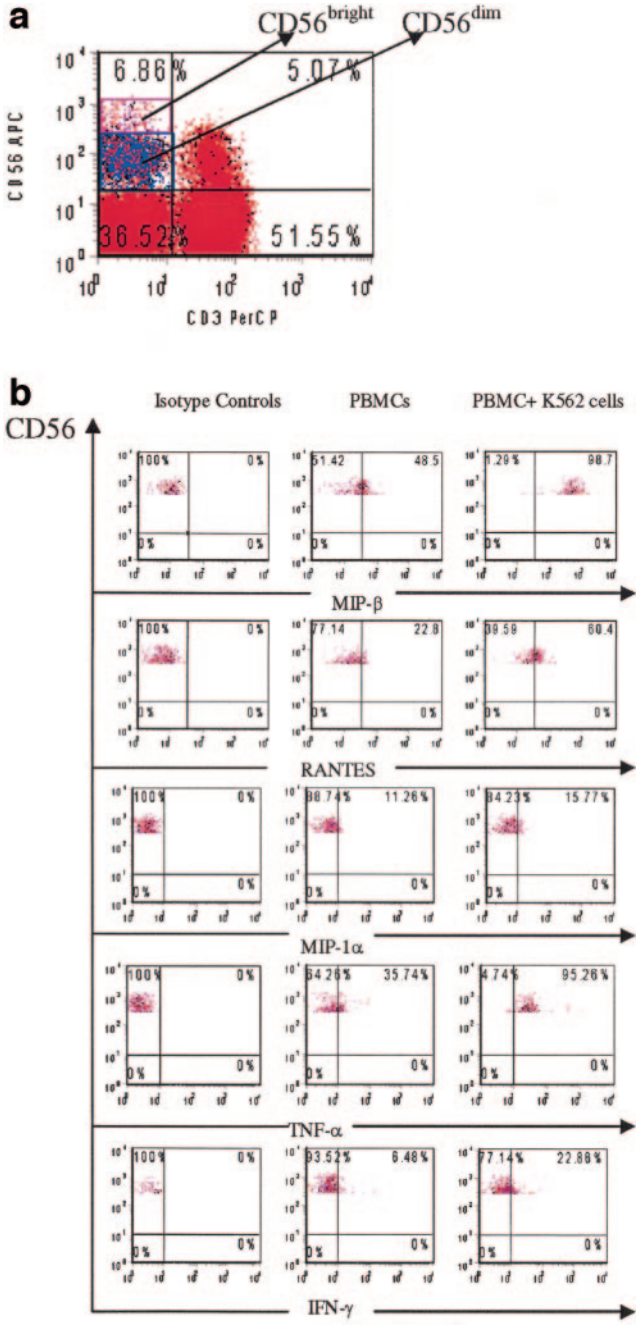

FIG. 3. Frequencies of NK $CD56^{bright}$ cells secreting CC chemokines and proinflammatory cytokines in HIV-1 and HIV-2 subjects. A combined surface marker staining for NK $CD56^{bright}$ and $CD56^{dim}$ subsets (a) and intracellular cytokine staining techniques were used to determine the percentage of NK $CD56^{bright}$ cells secreting MIP-1β, RANTES, MIP-1α, TNF-α, and IFN-γ (b). For each subject, PBMCs were incubated alone as a negative control and PBMCs with K562 cells as a test sample. The mean percent secreting cells for MIP-1β, RANTES, MIP-1α, TNF-α, and IFN-γ in different groups of CD4 T-cell counts in the HIV-1 and HIV-2 groups (c to g) is represented with the standard error. *P* values, when significant, are shown.

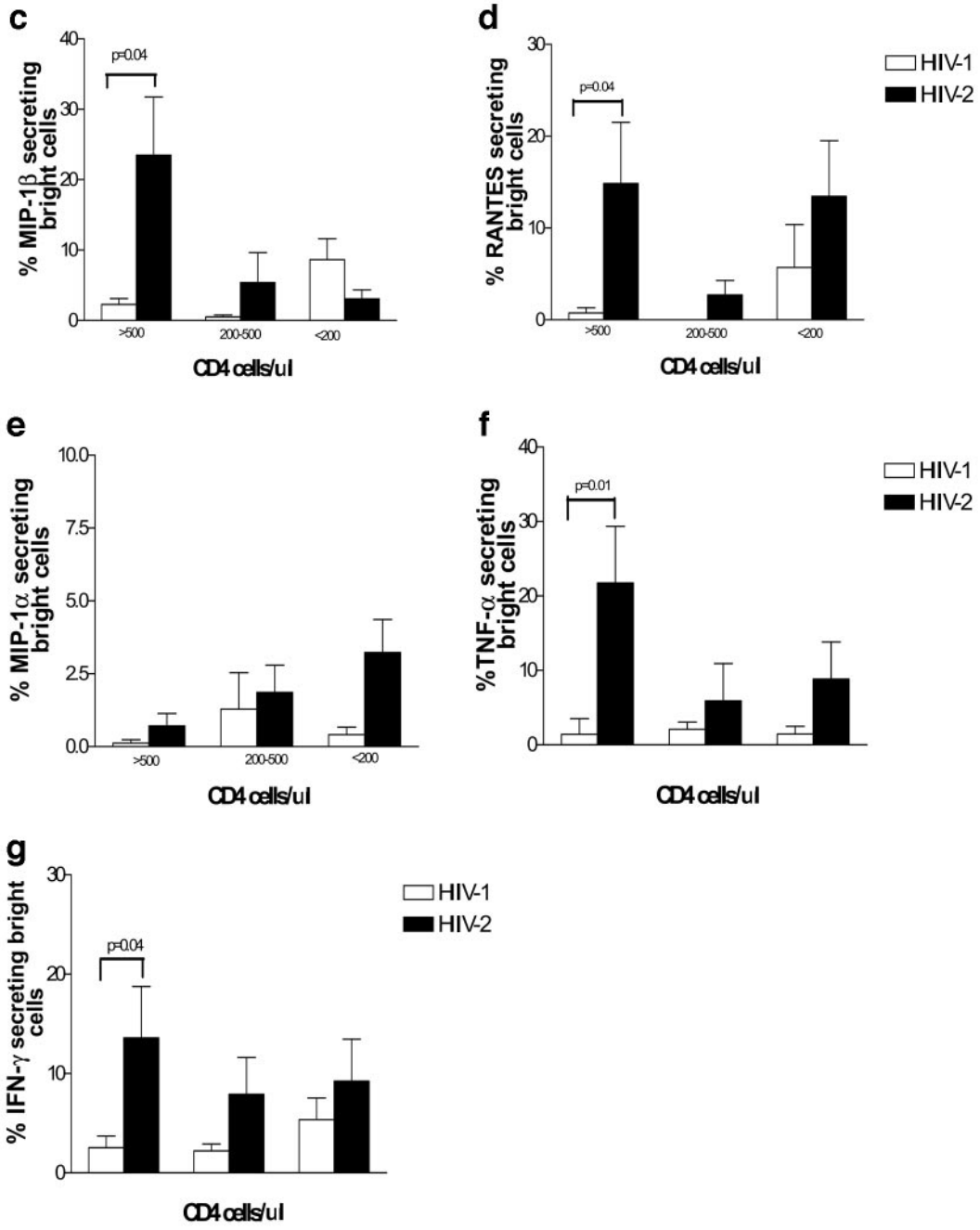

FIG. 3—*Continued.*

Our results indicated that NK CD56^{bright} cells mediated the increased secretion of CC chemokines and proinflammatory cytokines in HIV-2 infection. Secretion of CC chemokines by NK cells from HIV-1-infected patients, evaluated by enzyme-linked immunosorbent assay, has been found to be similar to that from seronegative individuals and could suppress HIV-1 replication in vitro (17, 25). We showed similar levels of IFN-γ secretion by NK cells in the two infections when measured by ELISPOT, but intracellular cytokine staining showed that the NK CD56^{bright} subpopulation had significantly higher levels in HIV-2 infection. Perhaps the intracellular cytokine assay was more specific than the ELISPOT assay.

The NK subtypes have different functions: CD56^{bright} cells produce cytokine/chemokines and CD56^{dim} cells have cytotoxic functions (8). There is evidence that as HIV disease advances, there is a marked reduction in the number of the chemokine- and cytokine-producing NK cells (12). This decrease in cell phenotypes was not clear in our study, but we noted lower levels of both chemokine and cytokine secretion in HIV-1- and HIV-2-infected individuals with low CD4$^+$-T-cell

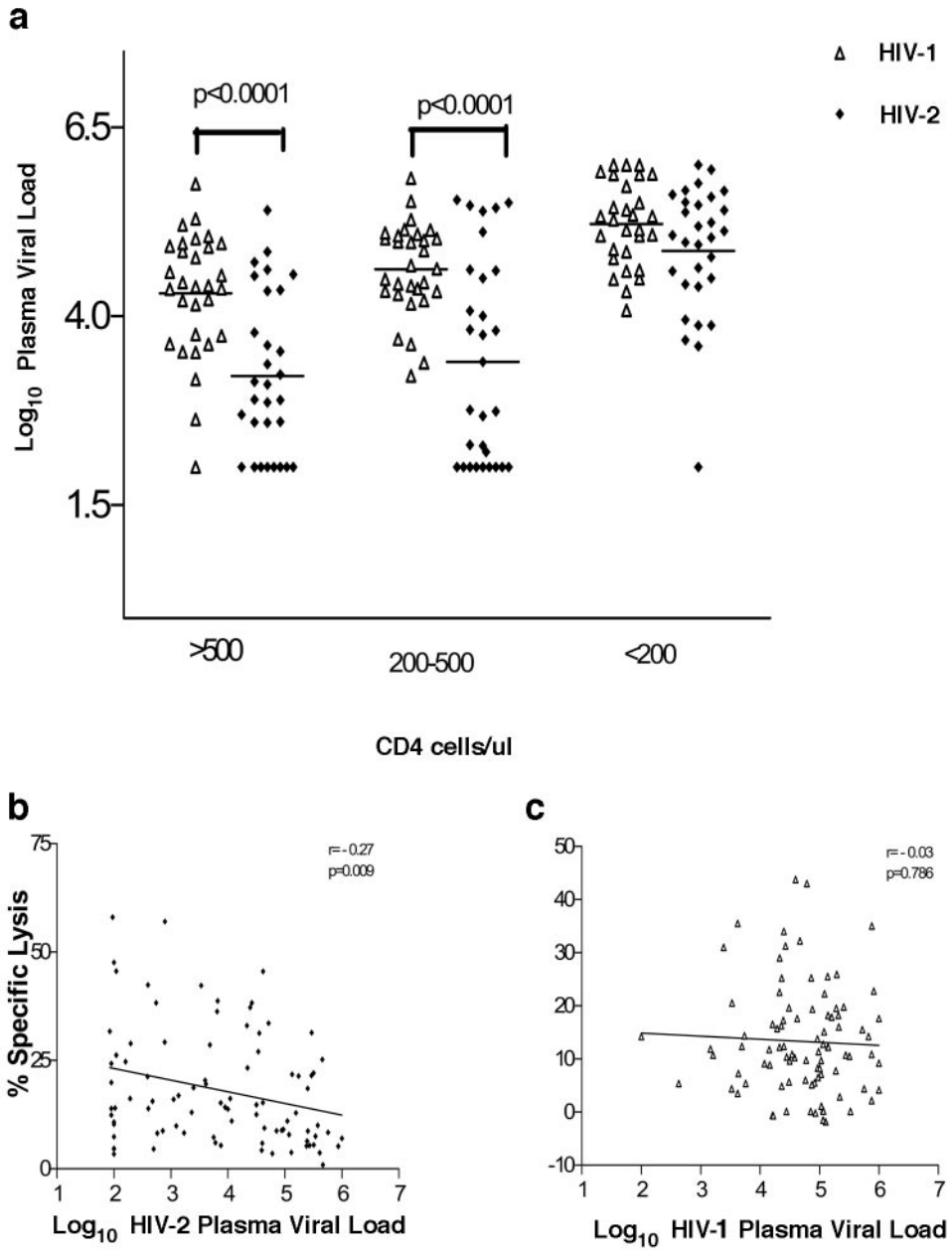

FIG. 4. Plasma viral load and its relationship with specific lysis of NK cells in HIV-1- and HIV-2-infected subjects. Plasma viral load was log transformed and that of the three categories of $CD4^+$ T cells is shown (a). The mean plasma viral load is represented as a horizontal line. Correlations between NK lysis and plasma viral load of HIV-2 and HIV-1 subjects are shown in b and c, respectively.

counts, though this was not obvious in HIV-1 subjects, where secretions remained low or increased nonsignificantly. An exception to the observed differences in frequencies of chemokine-secreting cells was MIP-1α, which to our surprise was low and tended to increase in HIV-2 with low CD4 counts. It is possible that this particular chemokine maybe influenced by the state of HIV-2 replication akin to what is observed with respiratory syncytial virus, where production of MIP-1α required ongoing viral replication (11).

In general, the observed lower levels of NK function at lower CD4 T-cell counts may occur because IL-15 production from monocytes, which is known to be essential for the function of $CD56^{bright}$ subpopulations, is affected as disease progresses (7). Furthermore, increased NK activity has long been observed when T cells exhibit overlapping functions such as increased production of cytokines (34). NK cells are stimulated by dendritic cell-derived IL-12 to produce IFN-γ, which in turn is a decisive factor in T helper type 1 development that is essential for long-term control of many pathogens (27). Thus, HIV-mediated $CD4^+$-T-cell destruction may compromise T lymphocyte immune functions, rendering NK cells nonfunctional. Our recent work has shown that $CD4^+$-T-cell function

is preserved in HIV-2 donors with a normal $CD4^+$-T-cell count (9) and this in turn may contribute to strong NK activity in these donors. Thus, as $CD4^+$-T-cell levels decline the NK cell function would also decline, as both functions are interdependent.

In summary, we provide evidence that in the asymptomatic phase of infection, NK function is better in HIV-2- than in HIV-1-infected subjects. Whether this innate response truly suppresses viral replication or whether function is better as a consequence of the lower level of viremia found in asymptomatic HIV-2 subjects remains to be determined. If the former is true, the presence of a consistently higher level of activity in asymptomatic HIV-2 infection has implications for the design of vaccine and immune-based therapies by enhancing NK cell function to control HIV disease.

ACKNOWLEDGMENTS

We extend our appreciation to Bakary Sanneh, Mamadi Njie, Yayah Singhateh, and Ramu Sarge Njie for laboratory assistance and Abraham Alabi and Steve Kaye for advice received on viral load measurements. We thank Akum Aveika and the field staff for access to patients' data and patients' follow-up visits. Thanks also to Sarah Crozier and David Jeffries for assisting in statistical analysis of the data. We thank Tumani Corrah, Director of the MRC Unit, for his support.

Our appreciation is extended to the MRC Head Office, London, United Kingdom, for funding this project.

REFERENCES

1. **Ahmad, A., and J. Menezes.** 1995. Positive correlation between the natural killer and gp 120/41-specific antibody-dependent cellular cytotoxic effector functions in HIV-infected individuals. J. Acquir. Immune Defic. Syndr. Hum. Retroviruses **10:**115–119.
2. **Ahmad, R., S. T. Sindhu, E. Toma, R. Morisset, J. Vincelette, J. Menezes, and A. Ahmad.** 2001. Evidence for a correlation between antibody-dependent cellular cytotoxicity-mediating anti-HIV-1 antibodies and prognostic predictors of HIV infection. J. Clin. Immunol. **21:**227–233.
3. **Berry, N., K. Ariyoshi, S. Jaffar, S. Sabally, T. Corrah, R. Tedder, and H. Whittle.** 1998. Low peripheral blood viral HIV-2 RNA in individuals with high CD4 percentage differentiates HIV-2 from HIV-1 infection. J. Hum. Virol. **1:**457–468.
4. **Biron, C. A., K. B. Nguyen, G. C. Pien, L. P. Cousens, and T. P. Salazar-Mather.** 1999. Natural killer cells in antiviral defense: function and regulation by innate cytokines. Annu. Rev. Immunol. **17:**189–220.
5. **Bonaparte, M. I., and E. Barker.** 2003. Inability of natural killer cells to destroy autologous HIV-infected T lymphocytes. AIDS **17:**487–494.
6. **Bruunsgaard, H., C. Pedersen, P. Skinhoj, and B. K. Pedersen.** 1997. Clinical progression of HIV infection: role of NK cells. Scand. J. Immunol. **46:**91–95.
7. **Carson, W. E., J. G. Giri, M. J. Lindemann, M. L. Linett, M. Ahdieh, R. Paxton, D. Anderson, J. Eisenmann, K. Grabstein, and M. A. Caligiuri.** 1994. Interleukin (IL) 15 is a novel cytokine that activates human natural killer cells via components of the IL-2 receptor. J. Exp. Med. **180:**1395–1403.
8. **Cooper, M. A., T. A. Fehniger, and M. A. Caligiuri.** 2001. The biology of human natural killer-cell subsets. Trends Immunol. **22:**633–640.
9. **Duvall, M. G., J. A. Dong, T. Brenchley, et al.** Delayed disease progression in HIV-2 infection is associated with preserved HIV-specific $CD4^+$ T-cell help. Submitted for publication.
10. **Fehniger, T. A., G. Herbein, H. Yu, M. I. Para, Z. P. Bernstein, W. A. O'Brien, and M. A. Caligiuri.** 1998. Natural killer cells from HIV-1+ patients produce C-C chemokines and inhibit HIV-1 infection. J. Immunol. **161:**6433–6438.
11. **Harrison, A. M., C. A. Bonville, H. F. Rosenberg, and J. B. Domachowske.** 1999. Respiratory syncytial virus-induced chemokine expression in the lower airways: eosinophil recruitment and degranulation. Am. J. Respir. Crit. Care Med. **159:**1918–1924.
12. **Hu, P. F., L. E. Hultin, P. Hultin, M. A. Hausner, K. Hirji, A. Jewett, B. Bonavida, R. Detels, and J. V. Giorgi.** 1995. Natural killer cell immunodeficiency in HIV disease is manifest by profoundly decreased numbers of CD16+CD56+ cells and expansion of a population of CD16dimCD56- cells with low lytic activity. J. Acquir. Immune Defic. Syndr. Hum. Retroviruses **10:**331–340.
13. **Hultstrom, A. L., G. Bratt, A. Cosma, V. Erfle, B. Wahren, and E. Carbone.** 2004. Autologous cytotoxicity of natural killer cells derived from HIV-infected patients. Immunol. Lett. **91:**155–158.
14. **Jaffar, S., A. Wilkins, P. T. Ngom, S. Sabally, T. Corrah, J. E. Bangali, M. Rolfe, and H. C. Whittle.** 1997. Rate of decline of percentage $CD4^+$ cells is faster in HIV-1 than in HIV-2 infection. J. Acquir. Immune Defic. Syndr. Hum. Retroviruses **16:**327–332.
15. **Jaye, A., R. Sarge-Njie, M. Schim van der Loeff, J. Todd, A. Alabi, S. Sabally, T. Corrah, and H. Whittle.** 2004. No differences in cellular immune responses between asymptomatic HIV type 1- and type 2-infected Gambian patients. J. Infect. Dis. **189:**498–505.
16. **Khatri, V. P., T. A. Fehniger, R. A. Baiocchi, F. Yu, M. H. Shah, D. S. Schiller, M. Gould, R. T. Gazzinelli, Z. P. Bernstein, and M. A. Caligiuri.** 1998. Ultra low dose interleukin-2 therapy promotes a type 1 cytokine profile in vivo in patients with AIDS and AIDS-associated malignancies. J. Clin. Investig. **101:**1373–1378.
17. **Kottilil, S., T. W. Chun, S. Moir, S. Liu, M. McLaughlin, C. W. Hallahan, F. Maldarelli, L. Corey, and A. S. Fauci.** 2003. Innate immunity in human immunodeficiency virus infection: effect of viremia on natural killer cell function. J. Infect. Dis. **187:**1038–1045.
18. **Landay, A. L., C. E. Mackewicz, and J. A. Levy.** 1993. An activated CD8+ T-cell phenotype correlates with anti-HIV activity and asymptomatic clinical status. Clin. Immunol. Immunopathol. **69:**106–116.
19. **Lowin, B., M. Hahne, C. Mattmann, and J. Tschopp.** 1994. Cytolytic T-cell cytotoxicity is mediated through perforin and Fas lytic pathways. Nature **370:**650–652.
20. **Marlink, R., P. Kanki, I. Thior, K. Travers, G. Eisen, T. Siby, I. Traore, C. C. Hsieh, M. C. Dia, E. H. Gueye, et al.** 1994. Reduced rate of disease development after HIV-2 infection as compared to HIV-1. Science **265:**1587–1590.
21. **Mavilio, D., J. Benjamin, M. Daucher, G. Lombardo, S. Kottilil, M. A. Planta, E. Marcenaro, C. Bottino, L. Moretta, A. Moretta, and A. S. Fauci.** 2003. Natural killer cells in HIV-1 infection: dichotomous effects of viremia on inhibitory and activating receptors and their functional correlates. Proc. Natl. Acad. Sci. USA **100:**15011–15016.
22. **Meier, U. C., R. E. Owen, E. Taylor, A. Worth, N. Naoumov, C. Willberg, K. Tang, P. Newton, P. Pellegrino, I. Williams, P. Klenerman, and P. Borrow.** 2005. Shared alterations in NK cell frequency, phenotype, and function in chronic human immunodeficiency virus and hepatitis C virus infections. J. Virol. **79:**12365–12374.
23. **Odehouri, K., K. M. De Cock, J. W. Krebs, J. Moreau, M. Rayfield, J. B. McCormick, G. Schochetman, R. Bretton, G. Bretton, D. Ouattara, et al.** 1989. HIV-1 and HIV-2 infection associated with AIDS in Abidjan, Cote d'Ivoire. AIDS **3:**509–512.
24. **O'Donovan, D., K. Ariyoshi, P. Milligan, M. Ota, L. Yamuah, R. Sarge-Njie, and H. Whittle.** 2000. Maternal plasma viral RNA levels determine marked differences in mother-to-child transmission rates of HIV-1 and HIV-2 in The Gambia. MRC/Gambia Government/University College London Medical School working group on mother-child transmission of HIV. AIDS **14:**441–448.
25. **Oliva, A., A. L. Kinter, M. Vaccarezza, A. Rubbert, A. Catanzaro, S. Moir, J. Monaco, L. Ehler, S. Mizell, R. Jackson, Y. Li, J. W. Romano, and A. S. Fauci.** 1998. Natural killer cells from human immunodeficiency virus (HIV)-infected individuals are an important source of CC chemokines and suppress HIV-1 entry and replication in vitro. J. Clin. Investig. **102:**223–231.
26. **Ratcliffe, L. T., P. T. Lukey, C. R. MacKenzie, and S. R. Ress.** 1994. Reduced NK activity correlates with active disease in HIV- patients with multidrug-resistant pulmonary tuberculosis. Clin. Exp. Immunol. **97:**373–379.
27. **Scharton, T. M., and P. Scott.** 1993. Natural killer cells are a source of interferon gamma that drives differentiation of $CD4^+$-T-cell subsets and induces early resistance to Leishmania major in mice. J. Exp. Med. **178:**567–577.
28. **Scharton-Kersten, T. M., and A. Sher.** 1997. Role of natural killer cells in innate resistance to protozoan infections. Curr. Opin. Immunol. **9:**44–51.
29. **Schim van der Loeff, M. F., and P. Aaby.** 1999. Towards a better understanding of the epidemiology of HIV-2. AIDS **13**(Suppl. A)**:**S69–84.
30. **Schim van der Loeff, M. F., S. Jaffar, A. A. Aveika, S. Sabally, T. Corrah, E. Harding, A. Alabi, A. Bayang, K. Ariyoshi, and H. C. Whittle.** 2002. Mortality of HIV-1, HIV-2 and HIV-1/HIV-2 dually infected patients in a clinic-based cohort in The Gambia. AIDS **16:**1775–1783.
31. **Schramm, B., M. L. Penn, E. H. Palacios, R. M. Grant, F. Kirchhoff, and M. A. Goldsmith.** 2000. Cytopathicity of human immunodeficiency virus type 2 (HIV-2) in human lymphoid tissue is coreceptor dependent and comparable to that of HIV-1. J. Virol. **74:**9594–9600.
32. **Scott-Algara, D., and P. Paul.** 2002. NK cells and HIV infection: lessons from other viruses. Curr. Mol. Med. **2:**757–768.
33. **Scott-Algara, D., L. X. Truong, P. Versmisse, A. David, T. T. Luong, N. V. Nguyen, I. Theodorou, F. Barre-Sinoussi, and G. Pancino.** 2003. Cutting edge: increased NK cell activity in HIV-1-exposed but uninfected Vietnamese intravascular drug users. J. Immunol. **171:**5663–5667.
34. **Trinchieri, G., and D. Santoli.** 1978. Anti-viral activity induced by culturing lymphocytes with tumor-derived or virus-transformed cells. Enhancement of

human natural killer cell activity by interferon and antagonistic inhibition of susceptibility of target cells to lysis. J. Exp. Med. **147:**1314–1333.
35. **van der Ende, M. E., M. Schutten, T. D. Ly, R. A. Gruters, and A. D. Osterhaus.** 1996. HIV-2 infection in 12 European residents: virus characteristics and disease progression. AIDS **10:**1649–1655.
36. **Weber, K., D. Meyer, V. Grosse, M. Stoll, R. E. Schmidt, and H. Heiken.** 2000. Reconstitution of NK cell activity in HIV-1-infected individuals receiving antiretroviral therapy. Immunobiology **202:**172–178.
37. **Whittle, H., J. Morris, J. Todd, T. Corrah, S. Sabally, J. Bangali, P. T. Ngom, M. Rolfe, and A. Wilkins.** 1994. HIV-2-infected patients survive longer than HIV-1-infected patients. AIDS **8:**1617–1620.
38. **Zheng, N. N., N. B. Kiviat, P. S. Sow, S. E. Hawes, A. Wilson, H. Diallo-Agne, C. W. Critchlow, G. S. Gottlieb, L. Musey, and M. J. McElrath.** 2004. Comparison of human immunodeficiency virus (HIV)-specific T-cell responses in HIV-1- and HIV-2-infected individuals in Senegal. J. Virol. **78:** 13934–13942.