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Antibody-Dependent Cellular Cytotoxicity via Humoral Immune Epitope of Nef Protein Expressed on Cell Surface[1]

Takeshi Yamada,[2]* Nobukazu Watanabe,† Tetsuya Nakamura,‡ and Aikichi Iwamoto[3]*‡

Antibodies against various proteins of HIV type 1 (HIV-1) can be detected in HIV-1-infected individuals. We previously reported that the level of Ab response against one Nef epitope is correlated with HIV-1 disease progression. To elucidate the mechanism for this correlation, we examined Ab-dependent cellular cytotoxicity (ADCC) against target cells expressing Nef. We observed efficient cytotoxicity against Nef-expressing target cells in the presence of patient plasma and PBMCs. This ADCC activity was correlated with the dilution of plasma from HIV-1-infected patients. Addition of a specific synthetic peptide (peptide 31: FLKEKGGLE) corresponding to the Nef epitope reduced cell lysis to ~50%. These results suggest that PBMCs of HIV-1-infected patients may exert ADCC via anti-Nef Abs in the patients' own plasma and serve as a mechanism used by the immune system to regulate HIV-1 replication. *The Journal of Immunology,* **2004, 172: 2401–2406.**

Highly active antiretroviral therapy dramatically suppresses HIV-1 replication and has thereby contributed to decrease the incidence of AIDS-related opportunistic infections and subsequent mortality (1, 2). However, elimination of HIV-1 from infected individuals has not yet been achieved by highly active antiretroviral therapy alone (3–5). Therefore, the development of different therapeutic approaches is mandatory.

Ab-dependent cellular cytotoxicity (ADCC)[4] as well as CTL play an important role in protective immunity against viral infections (6, 7). ADCC can inhibit viral replication and cell-to-cell infection by killing HIV-1-infected cells before maturation of virus particles (8, 9). Therefore, ADCC activity could benefit the prevention of disease progression. In early studies, Rook et al. (10) and Ljunggren et al. (11) demonstrated that sera from HIV-1-infected individuals were able to mediate ADCC against HIV-1-infected T cells, and there was a positive correlation between ADCC activity and disease progression. When HIV-1-infected cells produce virus particles, viral envelope glycoproteins are abundantly exposed to the cell surface through the plasma membrane. In fact, ADCC via Abs against gp120 or gp41, HIV-1 envelope protein, has been well documented (12–20). It has been described that gp120 or gp120/41-specific ADCC correlates with rate of disease progression (19, 21). But, in contrast, ADCC via envelope proteins could potentially kill the uninfected $CD4^+$ T cells with free viral envelopes on their surface, and therefore ADCC could contribute to depletion of $CD4^+$ T cells and AIDS pathogenesis (22, 23). In addition, gp120 is prone to high frequency of mutations; thereby, viral escape mutants may evolve easily (24–26). In view of these disadvantages, envelope proteins appear to be unsuitable as targets for ADCC against the progression of disease in HIV-1-infected patients. Conserved proteins may be better targets if one considers ADCC as a durable therapeutic weapon against HIV-1. With regard to this, Gag and Pol are very conserved proteins, and if their epitopes were expressed on the cell surface, these proteins could be good candidates for specific ADCC. Rook et al. (10) described that Ab reactivity with the p24 (Gag) protein of patient's serum correlates inversely with disease progression. It has been reported that Gag proteins are expressed on the cell surface (27, 28); nevertheless, the inductions of ADCC via Gag have never been succeeded (29). And, furthermore, there has been no evidence that Pol proteins are expressed on the HIV-1-infected cells; therefore, Pol Ags could not be exposed to the extracellular enviroment as ADCC target. Thus, the contribution of other HIV-1 proteins except envelope proteins to ADCC has remained unclear.

Nef protein is an HIV-1 accessory protein with important roles for pathogenesis of HIV-1 infection (30–35). Nef protein is partially expressed on the surface of HIV-1-infected cells (36–38). We previously reported that highly conserved amino acid residues (FLKEKGGLE) are expressed on the surface of HIV-1-infected cells. The peptide residues served as an epitope for Ab response, and the plasma level of the Abs against the epitope was correlated with HIV-1 disease progression (39, 40). To elucidate the mechanism of this correlation, we studied ADCC activities using patients' peripheral mononuclear cells (PBMCs) and a patient's plasma, which contained high amount of anti-Nef Abs. We also analyzed characteristics of patients' NK cells that should be the key player in ADCC against virus-induced target cells.

Materials and Methods

Cells

Five HIV-1-infected subjects whose PBMCs were used as effector cells for the ADCC assay are listed in Table I. PBMCs were freshly isolated by centrifuging heparinized blood over Ficoll-Hypaque (Meneki-seibutsuken, Gunma, Japan). PBMCs were counted and adjusted to the concentration of 2×10^6 cells/ml in RPMI 1640 medium supplemented with 10% heat-inactivated FCS (RPMI 10). A portion of the cells was used for phenotypic analysis using flow cytometry. For the flow cytometric analysis of NK

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[4] Abbreviations used in this paper: ADCC, Ab-dependent cellular cytotoxicity; LTNP, long-term nonprogressor.

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Table I. *Patient profiles*

Patient	Age	Sex	$CD4^+$ Count (cells/μl)	$CD8^+$ Count (cells/μl)	NK Cell Count (cells/μl)	% NK Cell in FBMC	HIV RNA (copies/ml)[a]	Antiretroviral Drugs[b]
P1	37	M	754	996	155	8.0	<400	d4T + 3TC + NFV
P2	32	M	63	214	20	3.7	770	d4T + 3TC + NFV
P3	45	M	204	620	220	12.6	<400	AZT + ddC + IDV
P4	37	M	638	1034	102	5.7	<400	d4T + 3TC + NFV
P5	35	M	372	877	73	5.0	2200	AZT + ddC + IDV

[a] Amplicor HIV monitor test (Roche Diagnostics Systems, Somerville, NJ).
[b] AZT, zidovudine; d4T, stavudine; 3TC, lamivudine; ddC, zalcitabine; NFV, nelfinavir; IDV, indinavir.

cells, PBMC samples from another 40 HIV-1-positive subjects and 16 uninfected donors were included in this study.

For the ADCC assay, we used CEM-NK^R cells that were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health from J. Corbeil (41). Nef proteins were expressed in these cells by using a recombinant Sendai virus system, which has been shown to express large amounts of heterologous recombinant proteins in 24 h after infection in suspension cells (42). CEM-NK^R cells were infected with SeV-Nef to express HIV-1 (NL43 strain) Nef proteins or wild SeV at a multiplicity of infection of 10 for 1 h at 37°C, as previously described (43), and cultured for 24 h in RPMI 10. These cells were designated CEM-NK^R-Nef or CEM-NK^R-mock cells, respectively.

Subjects and reagents

For ADCC assay, we used the plasmas from long-term nonprogressor 2, 5, and 6 (LTNP 2, 5, and 6), whose characterization was published previously (39). $Na_2[^{51}Cr]O_4$ was obtained from NEN Life Science Products (Boston, MA). mAbs N901 (NKH-1) (anti-CD56; FITC conjugated) and 3G8 (anti-CD16; PE) were obtained from Coulter (Miami, FL). mAbs SJ25C1 (anti-CD19; PerCP) and SK7 (anti-CD3; allophycocyanin) were obtained from BD Immunocytometry Systems (San Jose, CA). mAb δG9 (anti-perforin) was a generous gift of E. Podack (University of Miami, Miami, FL). δG9 was conjugated with FITC in our laboratory. Nine-mer peptide 31 (=FLKEKGGLE) and control peptide (=GGGGGGGGG) were synthesized using a Multipin peptide synthesis kit (Chiron Mitotopes, Clayton, Victoria, Australia). The yields were analyzed by gas-liquid chromatography to confirm the correct synthesis.

Immunofluorescent staining

For analysis of Sendai virus-infected CEM-NK^R cells, cells (10^5) were centrifuged over silan-coating glass coverslips (DAKO, Carpenteria, CA), fixed with 2% paraformaldehyde in PBS for 5 min, blocked with BlockAce (Snow-Brand, Tokyo, Japan) for 30 min, and incubated for 1 h with plasma of LTNP 5 1/2.5 diluted in PBS. Then cells were incubated for 30 min with FITC-conjugated goat anti-human Igs (IgG, IgA, and IgM) $F(ab')_2$ (BioSource International, Camarillo, CA) after wash with PBS, and were mounted in 85% glycerol, 10 mM of Tris-HCl (pH 8), and 5% *n*-propylgallate. These stained cells were inspected with a confocal microscope (MRC 1024; Bio-Rad, Hercules, CA).

ADCC assays

ADCC assays were performed in 200 μl, total volume. Patient plasma used in the ADCC assay was incubated for 30 min at 56°C to inactivate the complement system. Plasmas from randomly selected healthy donors were used as control. A total of 1×10^6 target cells was labeled by incubation with medium containing $Na_2[^{51}Cr]O_4$ (0.5 mCi/ml) at 37°C for 1 h. Cells were washed three times with plain RPMI 1640 medium and resuspended in RPMI 10 at 2×10^5 cells/ml. A total of 50 μl of resuspended cells was added to each well of a 96-well microtiter plate (U bottom). Then, 50 μl of heat-inactivated healthy or patient's plasma diluted to 1/2.5 (thus, final concentration equals to 10^{-1} of original in 200 μl, total volume) in RPMI 10 was added to the plate before incubating for 30 min at 37°C. For the dilution assay of plasma, final concentration of plasma was adjusted to 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} of original with RPMI 10, respectively. After incubation, either 100 μl of patients' PBMCs (2×10^6 cells/ml) (for sample count), 100 μl of RPMI 10 containing 2% Triton solution (for maximum count), or 100 μl of RPMI 10 (for spontaneous release count) was added to each well. The mixtures of reaction were incubated at 37°C in a humidified 5% CO_2 atmosphere for 4 h as in previous reports (8, 41). A total of 100 μl of supernatant was collected from each well, and γ emission was counted using a gamma counter. The percentage of dead cells was calculated using the following formula: cell death (%) = 100 × (sample count − spontaneous release)/(maximum count − spontaneous release).

Blocking of ADCC by peptide 31

After diluted plasma was added with 0, 10, or 100 μg/ml peptide 31 (=FLKEKGGLE) or 100 μg/ml of control peptide (=GGGGGGGGG), 50 μl of the solution was added to resuspended target cells. ADCC assay was performed as above.

Flow cytometric analysis

For analysis of NK cell subsets, we used the following Ab combinations: 1) FITC-conjugated anti-CD56, PE anti-CD16, PerCP anti-CD19, allophycocyanin anti-CD3; 2) FITC anti-perforin, PE anti-CD56, PE anti-CD16, PerCP anti-CD19, allophycocyanin anti-CD3. For phenotypic analysis of NK cells, PBMCs were suspended in 50 μl of culture medium, and stained with Ab combination 1, for 20 min on ice. After incubation, cells were washed twice with cold PBS. Cells were resuspended in 200 μl of PBS containing 0.5% formaldehyde. For intracellular staining of perforin, cells were stained with Ab combination 2 (without anti-perforin Ab) for 20 min. After incubation, cells were washed twice with cold PBS, and resuspended in 100 μl of PBS. After addition of 100 μl of 4% formaldehyde and incubation for 20 min at room temperature, cells were pelleted and supernatants were removed. Cells were washed once with PBS/0.5% BSA/1 mM of sodium azide (PBS/BSA/azide buffer), and resuspended in 150 μl of permeabilization buffer (PBS/BSA/azide buffer containing 0.5% saponin). After pipetting gently to mix and incubating for 10 min at room temperature, cells were pelleted and supernatant was removed. A total of 25 μl of permeabilization buffer containing the appropriate amount of Abs against intracellular perforin was added to the cell pellets and incubated at room temperature for 30 min in the dark. Cells were washed once with 0.5 ml of permeabilization buffer and once with 1 ml of PBS/BSA/azide buffer. Finally, cells were suspended in 200 μl of PBS/BSA/azide buffer. All samples were kept at 4°C and protected from light until analysis on the flow cytometer.

Six-parameter flow cytometric analysis was done on a FACSCalibur flow cytometer (BD Immunocytometry Systems) using CellQuest software (BD Immunocytometry Systems) with FITC, PE, PerCP, and allophycocyanin as the four fluorescent parameters. FlowJow software (Tree Star, San Carlos, CA) was used to make configurations. Light scatter gates were designed to include only lymphocytes, and up to 100,000 events in this gate were collected. The absolute lymphocyte count was determined from the complete blood count. The number of NK cells per microliter of whole blood was calculated by multiplying the fraction of lymphocytes that were $CD16^+$ or $CD56^+$ by the absolute lymphocyte per microliter of blood. For analysis and display of statistical comparisons, we used JMP software for the Apple Macintosh (SAS Institute, Cary, NC). Comparisons of distributions were performed by the nonparametric two-sample Wilcoxon rank test.

Results

Nef protein expression on the cell surface infected with SeV-Nef

LTNP 5 in the previous study had a high titer of the Abs against peptide 31 (39). When CEM-NK^R-Nef cells fixed with paraformaldehyde were stained with diluted plasma from healthy donor or LTNP 5, and FITC-conjugated anti-human Ig secondary Abs, positive fluorescent signals were given on the surface of CEM-NK^R-Nef cells by plasma from LTNP 5, but not from a healthy donor

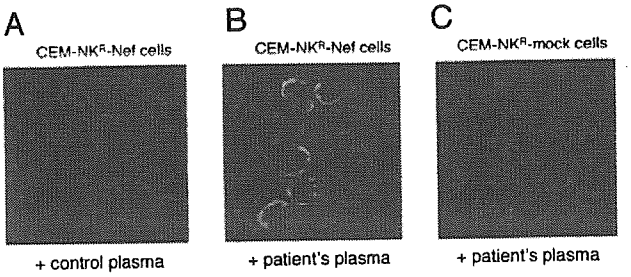

FIGURE 1. Immunological staining of CEM-NK^R cells infected with SeV-Nef (CEM-NK^R-Nef cells). Cells were stained with 1/2.5 diluted plasma and FITC-conjugated anti-human Ig secondary Abs. The stained cells were observed by confocal microscopy. *A*, CEM-NK^R-Nef cells stained with plasma of a healthy donor. *B*, CEM-NK^R-Nef cells stained with plasma from LTNP 5. *C*, CEM-NK^R-mock cells stained with plasma from LTNP 5.

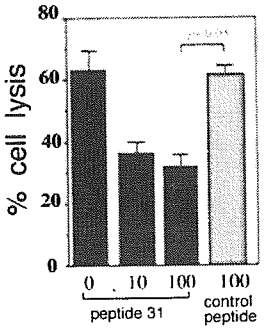

FIGURE 3. Inhibition of ADCC by peptide 31. Percentage of cell lysis by PBMCs of P3 was examined by ADCC assay in the presence of peptide 31 (■) or control peptide (hatched column) at an E:T ratio of 20:1. Data are shown as the mean of triplicate determinations (bars represent SDs). There is a significant difference between peptide 31 and control peptide at the concentration of 100 μg/ml (Student's *t* test, $p < 0.05$).

(Fig. 1, *A* and *B*). Plasma from LTNP 5 did not recognize proteins on the cell surface of CEM-NK^R-mock cell (Fig. 1*C*).

ADCC assay

An ADCC assay was conducted using plasma from LTNPs (LTNP 2, 5, and 6) (39) and PBMCs of either a healthy volunteer or from a patient 1–5 whose profiles are provided in Table I. As shown in Fig. 2*A*, CEM-NK^R-Nef incubated with plasma of LTNP 5 (final concentration, 10^{-1} of original) was efficiently lysed with PBMCs of a healthy volunteer at an E:T ratio of 20:1 (mean percentage of cell lysis, 58%) and 50:1 (66%). When the E:T ratio was lowered to 5:1, percentage of cell lysis decreased to 30% (Fig. 2*A*). The plasmas from LTNP 2, 5, and 6 (final concentration, 10^{-1} of original) induced ADCC activity via Nef, and the plasma of LTNP 6 indicated lower activity compared with that of LTNP 2 or LTNP 5 (Fig. 2*B*). Cytotoxic activity against CEM-NK^R-Nef was observed when PBMCs of five HIV-1-infected patients (p1–5) were used as effector cells at an E:T ratio of 20:1 (Fig. 2*C*). This cytotoxicity was specific to plasma of HIV-1-infected patients, because cell lysis was less than 10% when plasma from a healthy donor was used instead of patient plasma (Fig. 2*C*). In addition, the observation that dilution of patient plasma reduced the percentage of CEM-NK^R-Nef cell lysis (Fig. 2*D*) also suggested that lysis was mediated by the Ab in the plasma. To examine whether the cell lysis is specific to Nef, we added synthetic peptide 31 to the mixture of ^{51}Cr-labeled CEM-NK^R-Nef, PBMCs of patient 3, and LTNP 5 plasma at an E:T ratio of 20:1. Addition of 10 or 100 μg/ml peptide 31 decreased the percentage of cell lysis by 42 or 48% when compared with cell lysis without peptide 31, respectively, whereas addition of 100 μg/ml of control peptide did not show any effect on cytotoxicity (Fig. 3).

NK cells of HIV-1-infected patients

We analyzed NK cells in the peripheral blood using flow cytometry. NK cells were defined as CD3$^-$, CD19$^-$, CD16$^+$, or CD56$^+$ lymphocyte (44). PBMCs from 41 HIV-1-infected patients and 16 healthy donors were examined. There was a significant difference between HIV-1-infected patients and normal controls in total counts of NK cells (mean ± SD = 131 ± 85 and 198 ± 87 cells/μl, respectively, $p = 0.014$) (Fig. 4*A*). When HIV-1-infected individuals were divided into two groups by CD4$^+$ T cell counts (CD4 ≥ 200 or CD4 < 200 cells/μl), there was no significant difference between these two groups in absolute counts of NK cells (CD4 ≥ 200 and CD4 < 200 cells/μl; mean ± SD = 125 ± 94 and 142 ± 82 cells/μl, respectively, $p = 0.643$). For the functional analysis of NK cells, we next examined the expression of intracellular perforin in NK cells of HIV-1-infected patients. As shown in Fig. 4*B*, there was no significant difference between HIV-1-infected patients and healthy controls in frequency of perforin high-positive cell (%) of total NK cells (CD4 ≥ 200, CD4 < 200 cells/μl, and healthy controls; mean ± SD = 83 ± 12, 90 ± 6, and

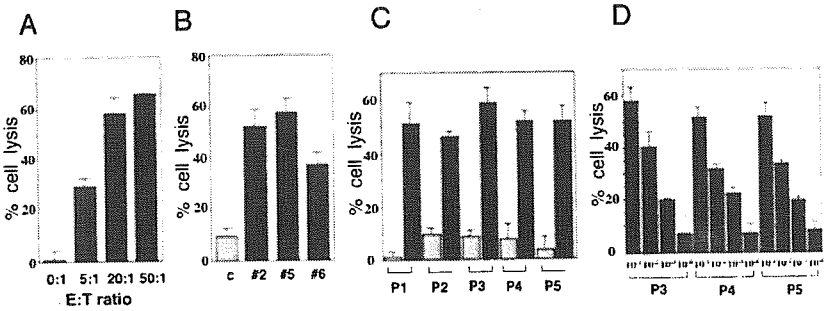

FIGURE 2. ADCC assay using diluted plasma, PBMCs, and radiorabeled CEM-NK^R-Nef. The values are given as percentage of specific cell lysis = 100 × (sample count − spontaneous release)/(maximum count − spontaneous release). *A*, Various E:T ratio with healthy donor PBMCs in the presence of plasma from LTNP 5. *B*, Plasma from a healthy donor (hatched column) or LTNPs (LTNP 2, 5, and 6) (■) at an E:T ratio of 20:1 with healthy donor PBMCs. *C*, PBMCs from five patients (P1-P5, Table I) at an E:T ratio of 20:1 in the presence of either plasma from a healthy donor (hatched column) or LTNP 5 (■) in *C*. *D*, Plasma Ab titration. Percentage of cell lysis by PBMCs from patient P3, P4, or P5 was examined with serially diluted plasma from LTNP 5 at an E:T ratio of 20:1. The values along the *x*-axis represent final concentration, 10^{-1}~10^{-4} of original plasma. Data are shown as the mean of triplicate determinations (bars represent SDs).

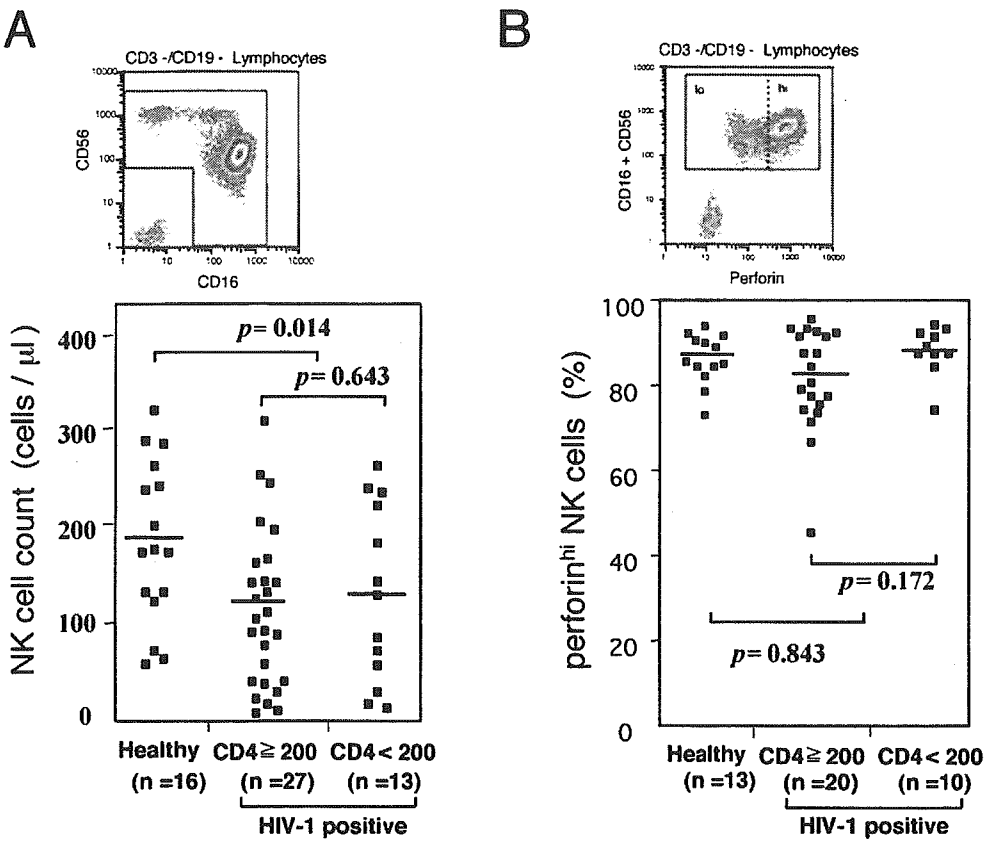

FIGURE 4. Flow cytometric analysis of NK cells. NK cells were defined by $CD3^-$, $CD19^-$, $CD16^+$, or $CD56^+$ expression. *Upper panels*, Show flow cytometry profiles gated on $CD3^-$ and $CD19^-$ lymphocytes. NK cells were gated by red filled line. *A*, *Lower panel*, Comparison of NK cell counts was conducted between 16 healthy donors and 40 HIV-1-positive individuals. *B*, *Upper panel*, NK cells are distingushed between perforin high/positive (hi) and low (lo) populations by red dotted line. *Lower panel*, Frequency of perforin high-positive cells (%) of total NK cells for each donor was calculated. Comparison was conducted between 13 healthy donors and 30 HIV-1-positive individuals. Median values are shown as bars.

88 ± 6%, respectively), suggesting that NK cells in HIV-1-infected patients were as functionally active as those in non-HIV-1-infected individuals.

Discussion

In a previous report, we showed that the progression of disease in HIV-1-infected patients was correlated with Ab titers against peptide 31 (39). In an effort to elucidate the mechanism for this correlation, we studied the role of ADCC against peptide 31 in this study. The interaction between plasma Abs of LTNP 5 and Nef proteins was specific (Fig. 1). We showed that PBMCs from HIV-1-infected donors as well as healthy donors could exert specific ADCC against the cells expressing Nef protein (CEM-NK^R-Nef cells) with patient's plasma even in the face of less than normal NK cell count (Table 1; Fig. 2, *A*, *B*, and *C*). Thus, the ADCC activity may contribute to the elimination of HIV-1-infected cells in vivo. Because ADCC activity is dependent on the titer of plasma Ab (Fig. 2*D*), the lower activity of LTNP 6 (Fig. 2*B*) could be attributed to the lower titer of Ab against Nef epitope compared with LTNP 2 or 5, based on our previous data (39). The ADCC activity was inhibited up to ~50% by peptide 31 compared with control peptide (Fig. 3), suggesting that specific Abs against peptide 31 may contribute substantially to eliminate the HIV-1-infected cells. However, other Nef-derived peptides may also contribute to the residual 50% activity as epitopes we have not yet isolated. It was previously shown that selective down-regulation of MHC class I molecules protects HIV-1-infected cells from CTL and NK cells (45–49). In contrast, ADCC via Abs against the conserved cell surface HIV-1 epitopes such as peptide 31 may be an alternative armor against HIV-1 infection.

Although percentages of NK cells varied in the five patients examined (3.7~12.6%) (Table I), they showed almost the same levels of ADCC activity (Fig. 2*C*). This result may be due to the high E:T ratio that we used in the cytotoxicity assay (Fig. 2*A*); however, it is possible that ADCC activity may be retained until late in the clinical stage, as previously reported (50, 51). Flow cytometric analysis revealed a reduction of total NK cell counts in HIV-1-infected individuals, similar to the previous reports (52, 53) (Fig. 4*A*). There was no significant difference between the two groups of HIV-1-positive patients (CD4 ≥ 200 cells/μl and CD4 < 200 cells/μl); therefore, NK cells appear to be retained even late in the disease progression. With regard to Nef epitope expressing on the cell surface, we previously documented that HIV-1-infected cells were lysed by the combination of rabbit polyclonal Abs against peptide 31 and rabbit complements (39). Thus, we speculate that the level of Nef expression could be sufficient for the induction of ADCC via Nef epitope on the cell surface. However, it could be too difficult to estimate ADCC via Nef epitope with HIV-1-infected cells and patient's plasma because of the existence of abundant anti-envelope Abs as well as anti-Nef Abs in the plasma from HIV-1-infected patient.

We and others showed that HIV-1-specific CD8 T cells contain less perforin (54–56). NK cells may function as better effector cells in the HIV-1-infected individuals. Although the number of

NK cells was lower in HIV-1-infected patients than healthy controls, NK cells retained the high expression of perforin until late in the clinical course (Fig. 4*B*). Rukavina et al. (57) demonstrated that perforin expression significantly correlates with NK cytotoxicity against K562 cells. The fact that LTNPs had higher anti-peptide 31 Abs than progressors may indicate that ADCC against conserved cell surface HIV-1 epitopes such as peptide 31 may have favorable influence on the clinical course. Finally, therapeutic intervention that contributes to raise specific Ab levels against the conserved cell surface HIV-1 epitopes may prove to have a clinical benefit.

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