Defective Natural Immunity: An Early Manifestation of Human Immunodeficiency Virus Infection

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Summary

Cytotoxicity mediated by natural killer (NK) and lymphokine-activated killer (LAK) cells may be of significance in host defense against viral infections. This study included 347 patients infected with human immunodeficiency syndrome virus (HIV) type 1 and 110 controls. The NK cell activity, either unstimulated or stimulated with interferon-α (IFN-α) or interleukin-2 (IL-2), and the LAK cell activity were suppressed in patients, but the NK/LAK cell activity did not differ between patients with AIDS and patients without AIDS. However, the IFN-α–stimulated NK cell activity and LAK cell activity were reduced in patients with symptoms of HIV disease (CDCIV) when compared with asymptomatic patients (CDCII+III). When the data were analyzed by multiple linear regression, the percentage of $CD4^+$ cells had a positive effect on these two parameters in patients without AIDS, whereas the percentage of $CD4^+$ cells had no significant effect on unstimulated and IL-2–stimulated NK cell activity in these patients. In controls and AIDS patients, the percentage of $CD4^+$ cells had no effect on NK/LAK cell activity in multiple linear models. The total number of $CD16^+$ cells was low in patients compared to controls, whereas the percentages of $CD16^+$, $CD56^+$, and $CD16^+CD56^+$ were either normal or elevated. Therefore, the decrease in NK cell subpopulations did not contribute to the observed depression in NK/LAK cell activity in vitro. It is concluded that natural immunity is suppressed in HIV-seropositive patients primarily because of a qualitative defect of the NK/LAK cells. This qualitative defect includes a reduced responsiveness to IFN-α, which is progressive until the onset of symptoms, and possibly related to the loss of $CD4^+$ cells.

Infection with HIV type 1 causes progressive immunosuppression (1–3). This includes suppression of the natural immunity mediated by NK cells (4–12) and their in vitro counterparts, LAK cells (13–17).

NK cells are $CD3^-$ large granular lymphocytes that express characteristic NK cell markers such as CD16 and CD56; a subpopulation expresses CD8 (18). They mediate non–MHC-restricted cytotoxicity and offer potential resistance to viral opportunistic infections mediated by EBV, CMV, herpes simplex, and herpes zoster virus (19). They also mediate cytotoxic activity against malignant cells and may mediate resistance against AIDS-associated tumors such as Kaposi's sarcoma (18). The in vitro–generated LAK cells show a broader range of non–MHC-restricted target killing (20).

The clinical significance and the mechanism of HIV-induced loss of natural immunity are not fully understood. The impaired NK cell activity is observed on the clonal level and is related to defects both at the target and postbinding levels (5). It is not clear whether the suppressed NK and LAK cell activity in HIV-seropositive subjects is caused by mainly low percentage, altered phenotypic appearance, or decreased function of NK cells (6, 10, 12).

The aim of this study was by use of a large study material to test whether NK and LAK cell activity were related to (*a*) expression of NK cell related cell markers; (*b*) prognostic markers of HIV infection; and (*c*) clinical outcome (judged by occurrence of AIDS and presence of herpes virus infections and malignancy).

Materials and Methods

Patients. 347 HIV-infected patients agreed to participate in the study; 308 (86%) were male and the median age was 39 yr (quartiles = 33–47 yr). Of all the patients, 266 were homo- or bisexual males, 54 were infected through heterosexual contact, 9 were drug addicts, 6 were hemophiliacs, 10 were infected by blood transfusions, and 9 had unknown risk factors (7 patients had more than one risk factor). 110 (32%) patients had AIDS, AIDS-defining symptoms were *Pneumocystis carinii* pneumonia (PCP) in 55 patients, candidadiasis of the esophagus, trachea, bronchi, or lungs in 26, Kaposi's

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sarcoma in 26, toxoplasmosis of the brain in 19, cytomegalovirus infection in 11, HIV wasting syndrome in 8, disseminated atypical mycobacteriosis in 6, and other AIDS-defining events in 23. Patients were further subclassified by use of The Center for Disease Control (CDC)[1] system of classification. 132 asymptomatic patients were classified into CDC group II and CDC group III (CDCII+III), whereas 215 patients with symptoms related to HIV infection were classified to CDC group IV (CDCIV). A control group of 110 HIV-seronegative subjects (89% males) not at risk for HIV infection with a median age of 39 yr (quartiles = 33–49 yr) was used for comparison.

Blood Sampling and Isolation of Blood Mononuclear Cells (BMNC). 60 ml of blood was drawn from each subject and BMNC were isolated by density gradient centrifugation (Lymphoprep; Nyegaard, Oslo, Norway) on LeucoSep tubes (Greiner, Frickenhausen, Germany) and washed three times in medium RPMI 1640 (GIBCO BRL, Gaithersburg, MD). Cells were frozen in freezing medium (50% RPMI, 30% FCS [GIBCO BRL], 20% DMSO [Bie & Berntsen, Rødovre, Denmark]) and were kept in liquid nitrogen until thawed for analysis.

Some patients had so few cells that it was impossible to analyze all of the immune markers.

Determination of NK Cell Activity. NK cell activity was measured using K562 target cells in a ^{51}Cr release assay. BMNC were thawed and incubated for 1 h at 37°C with: (*a*) medium; (*b*) 10^3 IU/ml IFN-α (kindly provided by Dr. Robert Jordal, The Blood Bank, Copenhagen County Hospital, Gentofte, Denmark); (*c*) 20 IU/ml IL-2 (Boehringer Mannheim GmbH, Mannheim, Germany). Triplicates of 100 μl effector cells in their incubation medium and 100 μl target cells (10^5 cells per ml) were incubated in microtiter plates for 4 h at 37°C. Unstimulated effector cells were added in different concentrations, E/T ratios of 100:1, 50:1, 25:1, and 12.5/, whereas IL-2- and IFN-α–stimulated effector cells were used at an E/T ratio of 50:1. The plates were centrifuged for 10 min; 100 μl supernatant was transferred to new tubes and radioactivity was determined. Spontaneous release was determined by incubation of 100 μl target cells with 100 μl medium and maximum release by incubation of 100 μl target cells plus 100 μl medium with 10% Triton X-100. Percentage of ^{51}Cr release (NK cell activity) was determined by the equation

$$\text{percent of lysis} = \frac{(\text{test}-\text{spontaneous})\text{cpm}}{(\text{maximum}-\text{spontaneous})\text{cpm}} \times 100,$$

and was given as mean of triplicates. For subjects with NK cell activity >5% (214 patients, 91 controls) lytic units (LU_{15}) were calculated. One LU_{15} is the number of effector cells required to achieve 15% cytotoxicity of K562 as derived from a titration curve of twofold serial dilutions of effector cells. One $LU_{15}/10^6$ $CD16^+$ cells is the number of LU_{15} in 10^6 $CD16^+$ cells. In each assay, three preparations of cryopreserved BMNC obtained from buffy coats were also analyzed. Whenever two out of three control preparations were outside their normal range (mean ± 2 SD), the assay was rejected. On average, ^{51}Cr spontaneous release never exceeded 15% and was 8% of maximum release. Three controls and six patients (three non-AIDS, three AIDS) had NK cell activity measured by use of fresh and frozen BMNC of the same blood sample. Frozen cells had in general a slightly reduced cytotoxic activity; the effect differed between assays, whereas the relative decrease was similar in patients and controls (data not shown).

[1] *Abbreviations used in this paper:* BMNC, blood mononuclear cells; CDC, Center for Disease Control; LU_{15}; lytic units.

Determination of LAK Cell Activity. After thawing, BMNC were incubated with 6,000 IU/ml IL-2 (Proleukin; Eurocetus B.V., Amsterdam, The Netherlands) for 48 h at 37°C. LAK activity was measured in a ^{51}Cr release assay using DAUDI target cells. 100 μl LAK cell suspension and 100 μl target cells (2×10^4 cells per ml) were added to each well in microtitre plates. LAK cells were added in different concentrations giving E/T ratios of 50:1, 25:1, 12.5:1, and 6.25:1. Otherwise the ^{51}Cr release assay was performed as described for the NK cell assay. Cryopreserved control preparations were made and used as already described. On average, the spontaneous release of ^{51}Cr never exceeded 25% and was 11% of maximum release.

Clinical Chemical Tests. Leukocyte, lymphocyte, and neutrocyte concentrations were determined using a cell counter (Technicon H.1; Miles Inc., Tarrytown, NY). Antibodies to HIV were assessed using an ELISA and confirmed by the Western blot method. HIV-p24 antigen was detected with a sandwich-type immunoassay that used murine mAb (anti-HIV core antigen) coated onto microwell strips (Coulter Corp., Hialeah, FL). Serum β_2-microglobulin concentrations were measured with a quantitative competitive enzyme (Pharmacia Diagnostics, Piscataway, NJ).

FACS® Analysis. PE-conjugated mAbs IgG1, Leu-2a (CD8), Leu-M3 (CD14), and Leu-19 (CD56) (Becton Dickinson and Co., Mountain View, CA) and fluorescein-conjugated mAbs IgG1, Leu-4 (CD3), Leu-2 (CD4), and Leu-11a (CD16) (Becton Dickinson) were used. Frozen mononuclear cells were thawed and washed twice in PBS with 2% FCS. 10^5 cells were resuspended in 100 μl PBS containing FCS and incubated for 30 min at 5°C with 10 μl of one or two mAbs. Labeled cells were washed three times and analyzed by flow cytometry using a FACStar® (Becton Dickinson). The mononuclear cells were incubated with antibodies to CD16 in combination with CD8 and CD16 in combination with CD56. PE- and fluorescein-conjugated mouse IgG1 was used as a negative control. Lymphocytes were distinguished from monocytes by their forward vs right angle light scatter. The monocyte-specific reagent CD14 was used to test for monocyte contamination of lymphocytes. On average, the proportion of $CD14^+$ cells within the lymphocyte gate was 2.6% for the patient group and 1.7% for controls. In patients, fresh and frozen BMNC were independently analyzed for expression of CD4. Three controls and three patients had a complete FACS® (Becton Dickinson) analysis performed on fresh and frozen cells. Freezing did not change the composition of lymphocyte subsets in neither group (data not shown).

Statistical Analysis. Since the data did not have a normal distribution, they are presented as medians, 1. and 3. quartiles. Groups were compared by rank sum tests with $P = 0.05$ as the significance level. Initially, NK/LAK cell activity was compared between groups at an E/T ratio of 50:1, and only if this was significant, other E/T ratios were compared. Correlations and multiple linear regression were used to examine relations between different markers within each group. BMDP (Los Angeles, CA) and SYSTAT (Evanston, IL) statistical software were used.

Results

NK and LAK Cell Activity. Unstimulated NK cell activity expressed at E/T ratios of 100:1, 50:1, 25:1, or 12.5:1 was suppressed in the patients compared to controls ($P < 10^{-9}$ in all E/T ratios; Fig. 1). Unexpectedly, no difference was found between patients with AIDS and patients without AIDS (P = 0.9 at an E/T ratio of 50:1; Fig. 1). Furthermore, there was no difference between CDCII+III patients and CDCIV patients (P = 0.2 at an E/T ratio of 50:1; Table 1) or between

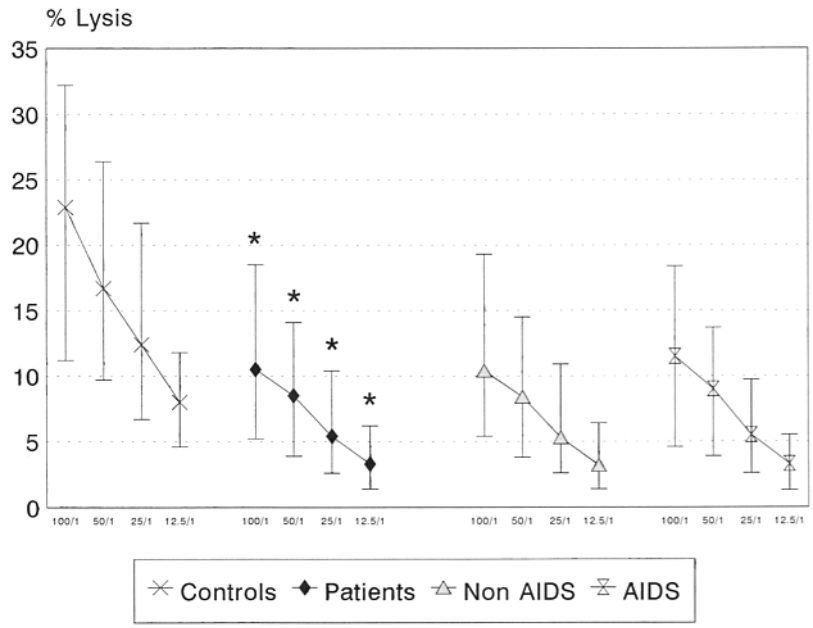

Figure 1. NK cell activity. Percentage of Cr release of K562 target cells at E/T ratios 100:1, 50:1, 25:1, and 12.5:1. Controls, n = 98; patients, n = 287; non-AIDS, n = 213; AIDS, n = 74. *Significantly different from controls ($P < 10^{-9}$).

patients who had and had not developed Kaposi's sarcoma, lymphoma, or herpes virus infections (data not shown).

The LAK cell activity was lower in patients than in controls ($P < 10^{-9}$ in all E/T ratios), but no difference was found between patients with AIDS and patients without AIDS (P = 1 at an E/T ratio of 50:1; Fig. 2). Patients in CDCIV had lower LAK cell activity in E/T ratio 50:1 and E/T ratio 25:1 compared to patients in CDCII+III (Table 1). NK cell activity stimulated by IL-2 or IFN-α was reduced in patients compared to controls, whereas patients with AIDS had similar activity as patients without AIDS (Table 2). It also appears from Table 2 that the NK cell activity evaluated by percentage of lysis of target cells was boosted with IL-2 to the same extend in patients and controls, whereas there was an impaired

Table 1. *Comparison of NK Cell and LAK Cell Activity between CDC Groups*

	CDCII + III (n = 132)	CDCIV (n = 215)	Comparison of groups
NK cell activity (percentage of lysis) Unstimulated (E/T = 50:1)	9 (4–16)	8 (4–13)	P = 0.2
NK cell activity (Percentage of lysis) IL-2 stimulated (E/T = 50:1)	16 (7–25)	13 (6–22)	P = 0.08
Difference (Stimulated–unstimulated)	5 (0–11)	3 (1–9)	P = 0.3
NK cell activity (percentage of lysis) IFN-α stimulated (E/T = 50:1)	17 (7–25)	12 (6–19)	P = 0.02
Difference (Stimulated–unstimulated)	6 (2–10)	3 (0–7)	P <0.0001
LAK cell activity (percentage of lysis) (E/T) = 50:1	33 (19–46)	24 (12–42)	P = 0.02
LAK cell activity (percentage of lysis) (E/T) = 25:1	24 (12–42)	22 (9–34)	P = 0.05

Median, quartiles, and P values are shown.

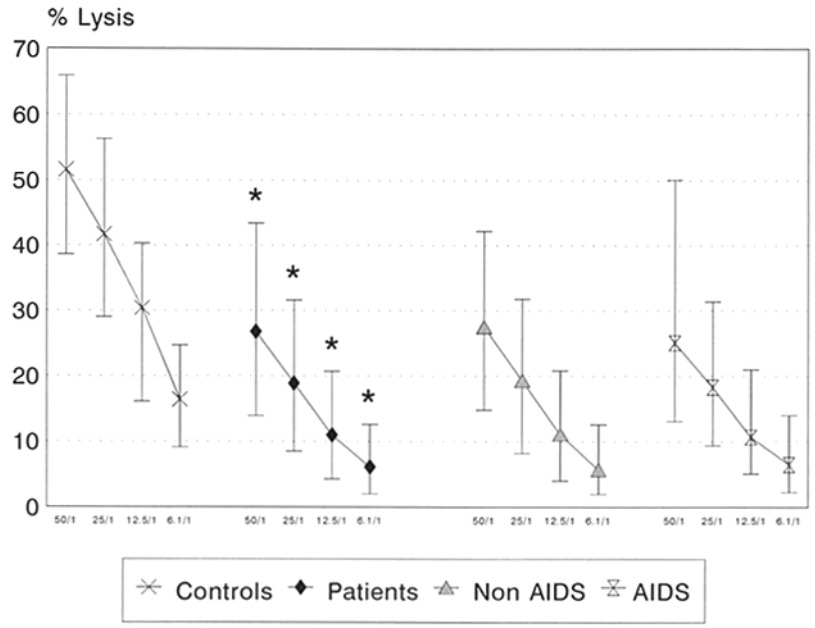

Figure 2. LAK cell activity. Percentage of Cr release of K562 target cells at E/T ratios 50:1, 25:1, 12.5:1, and 6.25:1. Controls, $n = 94$; patients, $n = 258$; non-AIDS, $n = 201$; AIDS, $n = 57$. *Significantly different from controls ($P < 10^{-9}$).

IFN-α boosting of NK cell activity in the patients. Patients in CDCIV had reduced IFN-α–stimulated NK cell activity when compared to patients in CDCII+III, whereas the IL-2–stimulated NK cell activity did not differ between these two subgroups (Table 1).

Lymphocyte Subpopulations. The percentages of $CD16^+$ and $CD16^+CD56^+$ cells did not differ between patients and controls ($P = 0.3$ vs $P = 0.2$), but the percentages of $CD56^+$ and $CD16^+CD8^+$ NK cell subtypes were higher in patients compared to controls ($P < 0.0001$). The percentage of $CD16^+CD8^+$ cells did not differ between patients with AIDS and patients without AIDS ($P = 0.3$), but the percentages of all other NK cell subtypes were higher in the AIDS group compared to the non-AIDS group ($P < 0.0001$).

The total concentrations of $CD16^+$ and $CD16^+56^+$ cells were lower in patients compared to controls ($P = 0.0007$ vs $P = 0.0003$), whereas the total concentration of $CD56^+$ cells did not differ between patients and controls ($P = 1.0$) and the concentration of $CD16^+CD8^+$ cells was elevated in the patient group ($P = 0.0004$). The total concentrations of all NK cell subsets were suppressed in the AIDS group compared to the non-AIDS group (Table 3).

The percentage and absolute number of $CD8^+$ cells were elevated in patients compared to controls. There was a slight increase in the percentage of $CD8^+$ cells in the AIDS group compared to the non-AIDS group, whereas the total number of $CD8^+$ cells was twofold increased in the non-AIDS group compared to the AIDS group (Table 3). The percentage of $CD3^+$ cells did not differ between patients and controls, whereas the total concentration of $CD3^+$ cells was lower in

Table 2. *Analysis of IL-2– and IFN-α–stimulated NK Cell Activity*

	(C) $n = 97$	(P) $n = 237$	(N) $n = 195$	(A) $n = 42$	C vs P	N vs A
NK cell activity (percentage of lysis)	25	14	13	16	$P < 0.0001$	$P = 0.09$
(IL-2 stimulated)	(11–36)	(6–23)	(6–22)	(8–26)		
Difference	5	4	4	5	$P = 0.61$	$P = 0.21$
(Stimulated–unstimulated)	(0–13)	(1–10)	(0–10)	(1–12)		
NK cell activity (percentage of lysis)	33	13	13	15	$P < 0.0001$	$P = 0.06$
(IFN-α stimulated)	(21–47)	(6–24)	(6–24)	(11–19)		
Difference	15	4	5	3	$P < 0.0001$	$P = 0.11$
(Stimulated–unstimulated)	(9–21)	(1–8)	(1–9)	(0–6)		

Median, quartiles, and P values are shown. All NK/LAK cell activities are at E/T ratio 50:1. C, controls; P, patients; N, non-AIDS; A, AIDS.

Table 3. *Analysis of Lymphocyte Subpopulations*

	C (n = 106)	P (n = 325)	N (n = 223)	A (n = 102)	Comparison of groups: C vs P	Comparison of groups: N vs A	Correlation to percent of CD4‡ (P only)
CD 16⁺							
Percentage	9.1	10.0	9.3	12.0	P = 0.3	P = 0.006	P = 0.002
	(6.6–13.7)	(6.6–15.9)	(6.1–14.9)	(8.0–17.5)			r = −0.17
Concentration	179	120	149	90	P = 0.0007	P <0.0001	P = 0.1
(cells per mm³)	(103–271)	(73–230)	(89–258)	(44–155)			r = 0.08
CD56⁺							
Percentage	6.1	8.9	8.3	10.7	P <0.0001	P = 0.0003	P = 0.001
	(4.1–9.7)	(5.7–14.1)	(5.4–12.9)	(7.4–17.3)			r = −0.26
Concentration	115	114	129	74	P = 1.0	P = 0.0001	P = 0.9
(cells per mm³)	(69–169)	(55–208)	(73–220)	(36–190)			r = 0.01
CD16⁺CD56⁺							
Percentage	2.4	2.9	2.5	3.6	P = 0.2	P = 0.002	P = 0.008
	(1.1–4.4)	(1.2–5.2)	(1.0–4.9)	(1.7–6.4)			r = −0.15
Concentration	41	33	38	23	P = 0.003	P = 0.006	P = 0.4
(cells per mm³)	(21–88)	(13–71)	(17–75)	(9–61)			r = 0.05
CD16⁺CD8⁺							
Percentage	0.7	1.6	1.5	1.7	P <0.0001	P = 0.3	P = 0.003
	(0.3–1.4)	(0.9–2.9)	(0.9–2.7)	(0.9–3.4)			r = −0.17
Concentration	13	21	23	12	P = 0.0004	P <0.0001	P = 0.6
(cells per mm³)	(5–25)	(9–40)	(13–47)	(5–29)			r = 0.03
CD8⁺							
Percentage	22.2	42.6	40.9	46.9	P <0.0001	P = 0.02	P = 0.001
	(17.5–26.8)	(33.1–53.1)	(33.0–50.7)	(33.9–57.0)			r = −0.21
Concentration	386	531	633	310	P <0.0001	P <0.0001	P = 0.2
(cells per mm³)	(255–562)	(346–831)	(434–915)	(157–556)			r = 0.08
CD3⁺							
Percentage	62.0	62.8	64.8	56.4	P = 0.6	P = 0.0001	P = 0.001
	(50.2–69.5)	(51.8–71.9)	(54.0–72.4)	(45.0–69.4)			r = 0.30
Concentration	1,077	810	1,002	390	P <0.0001	P <0.0001	P <0.001
(cells per mm³)	(826–1421)	(468–1236)	(681–1364)	(230–630)			r = 0.305
CD4⁺*							
Percentage	37.9	13.7	17.9	3.7	P <0.0001	P <0.0001	P <0.0001
	(31.0–42.2)	(4.5–23.0)	(11.9–25.0)	(2.1–8.5)			r = 0.88
Concentration	677	190	286	34	P <0.0001	P <0.0001	P <0.0001
(cells per mm³)	(496–925)	(46–363)	(170–462)	(11–66)			r = 0.75

continued

Table 3. *Continued*

	C (n = 106)	P (n = 325)	N (n = 223)	A (n = 102)	Comparison of groups: C vs P	Comparison of groups: N vs A	Correlation to percent of CD4‡ (P only)
CD4+‡							
Percentage	ND	14.0 (4.5–23.0)	18.0 (13.0–26.0)	3.0 (1.0–7.5)	ND	P <0.0001	ND
Concentration (cells per mm³)	ND	200 (38–369)	286 (180–456)	21 (7–54)	ND	P <0.0001	ND

Median, quartiles, P values, and correlation coefficients (r) are shown.
* Measured in frozen BMNC.
‡ Measured in fresh BMNC.

the patient group. The percentage and concentration of CD3+ cells were lower in the AIDS group compared to the non-AIDS group (Table 3). The percentage and concentration of CD4+ cells were, as expected, reduced in the AIDS group compared to the non-AIDS group, and it appears that the freezing procedure did not influence the CD4 count in either of the patient groups (Table 3). The differences in lymphocyte subpopulations between AIDS and non-AIDS patients were also found when patients in CDCII+III were compared with patients in CDCIV, except that there were

Table 4. *Correlation between NK and LAK Cell Activity and Lymphocyte Subsets in Peripheral Blood*

		% of CD16+ cells	% of CD56+ cells	% of CD16+CD56+ cells	% of CD16+CD8+ cells	% of CD8+ cells	% of CD4+* cells
NK cell activity (unstimulated)	(C)	P <0.001	p = 0.006	P <0.001	P = 0.9	P = 0.06	P = 0.002
		r = 0.39	r = 0.27	r = 0.36	r = −0.01	r = −0.19	r = −0.31
	(P)	P <0.001	P <0.001	P <0.001	P = 0.3	P = 0.1	P = 0.9
		r = 0.23	r = 0.33	r = 0.40	r = 0.06	r = −0.09	r = 0.00
NK cell activity (IL-2 stimulated)	(C)	P = 0.001	P = 0.03	P <0.001	P = 0.2	P = 0.007	P = 0.004
		r = 0.33	r = 0.22	r = 0.36	r = −0.12	r = −0.27	r = −0.29
	(P)	P <0.001	P <0.001	P <0.001	P = 0.2	P = 0.06	P = 0.4
		r = 0.25	r = 0.34	r = 0.41	r = 0.08	r = −0.12	r = 0.05
NK cell activity (IFN-α stimulated)	(C)	P <0.001	P = 0.02	P <0.001	P = 1.0	P = 0.008	P = 0.006
		r = 0.43	r = 0.23	r = 0.37	r = 0.00	r = −0.27	r = −0.28
	(P)	P <0.001	P <0.001	P <0.001	P = 0.1	P <0.001	P = 0.03
		r = 0.26	r = 0.36	r = 0.44	r = 0.11	r = −0.22	r = 0.15
LAK cell activity	(C)	P = 0.2	P = 0.4	P = 0.4	P = 0.6	P = 0.9	P = 0.1
		r = 0.14	r = 0.08	r = 0.08	r = 0.04	r = −0.01	r = −0.16
	(P)	P = 0.06	P <0.001	P <0.001	P = 0.1	P = 0.3	P = 0.2
		r = 0.12	r = 0.29	r = 0.29	r = −0.14	r = −0.07	r = 0.08

Results of correlation analyses P values and correlation coefficients (r) are shown. All NK/LAK cell activities are at an E/T ratio 50:1.
* Measured in fresh BMNC for patients and in frozen BMNC in controls.

Table 5. *Correlation between NK and LAK Cell Activity and HIV Antigen Titer, β_2-Microglobulin, IgG, IgA, and IgM in Plasma of HIV-infected Patients*

	HIV antigen titer	β_2-Microglobulin	IgG	IgA	IgM
NK cell activity (unstimulated)	$P = 0.8$ $r = 0.01$	$P = 0.4$ $r = -0.06$	$P = 0.4$ $r = -0.06$	$P = 0.2$ $r = 0.07$	$P = 0.9$ $r = 0.01$
NK cell activity (IL-2 stimulated)	$P = 0.9$ $r = 0.00$	$P = 0.2$ $r = -0.08$	$P = 0.2$ $r = -0.09$	$P = 0.7$ $r = 0.02$	$P = 0.9$ $r = 0.01$
NK cell activity (IFN-α stimulated)	$P = 0.7$ $r = -0.04$	$P = 0.002$ $r = -0.21$	$P = 0.1$ $r = -0.10$	$P = 0.4$ $r = -0.06$	$P = 1.0$ $r = 0.00$
LAK cell activity	$P = 0.4$ $r = -0.05$	$P = 0.002$ $r = -0.19$	$P = 0.1$ $r = -0.10$	$P = 0.4$ $r = -0.05$	$P = 0.7$ $r = -0.02$

Results of correlation analyses P values and correlation coefficients (r) are shown.
ND, not done. All NK/LAK cell activities are at E/T ratio 50:1.

no differences in concentrations of $CD56^+$ and $CD16^+$ $CD56^+$ cells between these two patient groups (data not shown).

Correlation Analysis. For patients, an inverse correlation was found between the percentage of $CD4^+$ cells and percentages of all NK cell subtypes and $CD8^+$ cells, whereas there was no correlation between the percentage of $CD4^+$ cells and the total number of any NK cell subtypes or $CD8^+$ cells, (Table 3) and no correlation between the percentage of $CD4^+$ cells and the proportions of $CD16^+$ NK cells coexpressing CD8 or CD56 (data not shown). The percentage of $CD4^+$ cells correlated to percentage and total number of $CD3^+$ cells (Table 3).

To examine the mechanisms behind the suppressed NK and LAK cell activity in patients, the unstimulated NK cell activity E/T ratio 50:1, the IL-2– and IFN-α–stimulated NK cell activity, and the LAK cell activity E/T ratio 50:1 were correlated to proportions of the different NK cell subsets ($CD16^+$, $CD56^+$, $CD56^+CD16^+$, $CD16^+CD8^+$) for patients and controls (Table 4). A positive correlation was found between the unstimulated as well as the IL-2– and IFN-α–stimulated NK cell activity and the percentages of $CD16^+$, $CD56^+$, and $CD16^+CD56^+$ cells in both groups, whereas there was no correlation to the percentage of $CD16^+CD8^+$ cells in either group. In the controls, the LAK cell activity did not correlate to any of the NK cell subsets. In the patients, the LAK cell activity positively correlated to the percentages of $CD56^+$ cells and $CD16^+CD56^+$ cells (Table 4).

In patients, a weak positive correlation was found between IFN-α–stimulated NK cell activity and the percentage of $CD4^+$ cells ($r = 0.15$), whereas the unstimulated NK cell activity E/T ratio 50:1, the IL-2–stimulated NK cell activity, and the LAK cell activity E/T ratio 50:1 did not correlate to the percentage of $CD4^+$ cells (Table 4). The relationships between NK/LAK cell activity and the percentage of $CD4^+$ cells were analyzed separately in patients with AIDS and patients without AIDS. In non-AIDS patients, the IFN-α–stimulated NK cell activity did correlate with the percentage of $CD4^+$ cells ($r = 0.19$, $P = 0.009$); this correlation, however, was not found in AIDS patients (data not shown). The unstimulated and IL-2–stimulated NK cell activity and the LAK cell activity did not correlate to the percentage of $CD4^+$ cells in neither group (data not shown). The unstimulated NK cell activity per NK cell ($LU_{15}/10^6$ $CD16^+$ cells and $LU_{15}/10^6$ $CD56^+$ cells) did not correlate with the percentage of $CD4^+$ cells in patients (data not shown). In contradiction to this, a negative correlation was found between the NK cell activity, either unstimulated or stimulated by IL-2 or IFN-α, and the percentage of $CD4^+$ cells in controls, whereas no correlation was found between LAK cell activity and the percentage of $CD4^+$ cells (Table 4).

The NK cell activity, either unstimulated or stimulated with IL-2 or IFN-α, and the LAK cell activity did not correlate to the HIV antigen titer, IgG, IgA, or IgM in patients (Table 5). A weak inverse correlation was found between β_2-microglobulin titer and IFN-α–stimulated NK cell activity or LAK cell activity ($r = -0.21$ vs $r = -0.19$; Table 5).

The relationship of NK/LAK cell activity with other parameters was further analyzed in patients with AIDS and patients without AIDS, as well as in controls, by means of multiple linear regression. The square roots of the unstimulated NK cell activity (E/T ratio 50:1), the IL-2– and IFN-α–stimulated NK cell activity, and the LAK cell activity (E/T ratio 50:1) were included in the models as dependent parameters in patients and controls. The square roots of the dependent parameters were used to obtain normal distribution of the

Table 6. *Analysis of NK/LAK Cell Activity by Multiple Linear Regression*

Independent variable		Dependent variables	Coefficients of dependent variables	*P* values of dependent variables
NK cell activity (unstimulated)	(C)	% of $CD4^+$ cells	−0.028 ± 0.018	0.1
		% of $CD16^+$ cells	0.069 ± 0.025	0.007
		% of $CD56^+$ cells	0.030 ± 0.040	0.5
	(N)	% of $CD4^+$ cells	0.018 ± 0.010	0.08
		% of $CD16^+$ cells	0.014 ± 0.014	0.2
		% of $CD56^+$ cells	0.071 ± 0.015	0.00001
	(A)	% of $CD4^+$ cells	−0.006 ± 0.027	0.8
		% of $CD16^+$ cells	−0.040 ± 0.022	0.07
		% of $CD56^+$ cells	0.058 ± 0.025	0.02
NK cell activity (IL-2 stimulated)	(C)	% of $CD4^+$ cells	−0.032 ± 0.023	0.2
		% of $CD16^+$ cells	0.072 ± 0.031	0.02
		% of $CD56^+$ cells	0.027 ± 0.052	0.6
	(N)	% of $CD4^+$ cells	0.038 ± 0.014	0.07
		% of $CD16^+$ cells	0.015 ± 0.019	0.4
		% of $CD56^+$ cells	0.078 ± 0.020	0.0002
	(A)	% of $CD4^+$ cells	−0.029 ± 0.041	0.5
		% of $CD16^+$ cells	−0.043 ± 0.032	0.2
		% of $CD56^+$ cells	0.092 ± 0.047	0.06
NK cell activity (IFN-α stimulated)	(C)	% of $CD4^+$ cells	−0.024 ± 0.021	0.3
		% of $CD16^+$ cells	0.097 ± 0.030	0.002
		% of $CD56^+$ cells	0.015 ± 0.050	0.8
	(N)	% of $CD4^+$ cells	0.058 ± 0.014	0.00004
		% of $CD16^+$ cells	0.021 ± 0.019	0.3
		% of $CD56^+$ cells	0.100 ± 0.020	<0.00001
	(A)	% of $CD4^+$ cells	−0.018 ± 0.038	0.6
		% of $CD16^+$ cells	−0.035 ± 0.029	0.2
		% of $CD56^+$ cells	0.067 ± 0.043	0.1
LAK cell activity	(C)	% of $CD4^+$ cells	−0.025 ± 0.019	0.2
		% of $CD16^+$ cells	0.006 ± 0.029	0.8
		% of $CD56^+$ cells	0.019 ± 0.044	0.7
	(N)	% of $CD4^+$ cells	0.038 ± 0.016	0.05
		% of $CD16^+$ cells	−0.026 ± 0.021	0.2
		% of $CD56^+$ cells	0.121 ± 0.023	<0.00001

continued

Table 6. *Continued*

Independent variable		Dependent variables	Coefficients of dependent variables	*P* values of dependent variables
	(A)	% of $CD4^+$ cells	−0.037 ± 0.047	0.4
		% of $CD16^+$ cells	0.000 ± 0.038	1
		% of $CD56^+$ cells	0.013 ± 0.050	0.8

Results of multiple linear regression: parameters included in models, their coefficients ± SE, and *P* values of the parameters in F test are shown. Independent variables were transformed by taking the square root before they were included in the models. All NK/LAK cell activities are at E/T ratio 50:1.

residuals. The dependent variables in each group were the percentages of $CD4^+$, $CD16^+$, and $CD56^+$ cells. In patients, the following additional independent parameters were included in the model: HIV antigen titer, IgA, IgG, IgM, and β_2-microglobulin. These additional dependent parameters were all insignificant in models including the percentages of $CD4^+$, $CD16^+$, and $CD56^+$ cells. It appears from Table 6 that regarding the NK cell activity in controls (unstimulated or stimulated by IL-2 or IFN-α), the percentage of $CD16^+$ cells contributed the most to the variation in cytotoxicity, whereas the percentages of $CD56^+$ and CD4 cells were without significant effect. However, in patients without AIDS, the percentage of $CD56^+$ cells, not the percentage of $CD16^+$ cells, explained the variation in cytotoxicity (Table 6). In patients without AIDS, the percentage of $CD4^+$ cells also had a significant effect on the IFN-α–stimulated NK cell activity and the LAK cell activity (Table 6). In AIDS patients, however, the percentages of $CD4^+$ and $CD16^+$ cells had no influence on NK/LAK cell activity, whereas the percentage of $CD56^+$ cells had a significant effect on the unstimulated NK cell activity, but no influence on the stimulated NK and LAK cell activities (Table 6).

In patients, a strong correlation was found between the LAK cell activity and the NK cell activity, either unstimulated or stimulated with IFN-α or IL-2 ($r = 0.46$, $P < 0.001$, $r = 0.58$, $P < 0.001$, $r = 0.53$, $P < 0.001$, respectively; E/T ratio 50:1), whereas in controls, this correlation was weaker ($r = 0.27$, $P = 0.009$, $r = 0.32$, $P = 0.002$, $r = 0.39$, $P < 0.001$, respectively).

Discussion

Knowledge about the percentual distribution of NK cells among BMNC is important to evaluate the functional in vitro NK cell assay, in which fixed numbers of BMNC are studied. However, knowledge about the total concentration of NK cells is important to evaluate the NK cell activity in vivo. This study shows that in HIV-seropositive patients, the percentages of all NK cell subtypes are elevated or not different from that of controls. Since the fractions of $CD16^+$, $CD56^+$, and $CD16^+56^+$ cells were not decreased, the defective in vitro NK cell activity (lysis per fixed number of BMNC) in HIV-seropositive subjects must be caused by a defective lytic capacity of each NK cell, as suggested by others (5, 8, 11, 21). Pearson correlation analysis revealed that the NK cell activity correlated to the percentages of $CD16^+$, $CD56^+$, and $CD16^+CD56^+$ cells in patients and controls, but not to the $CD16^+CD8^+$ cell subgroup, which is in line with the assumption that the majority of human NK cell activity is mediated by the $CD16^+$, $CD56^+$, and $CD16^+CD56^+$ cells (18). However, through multiple linear regression, it was concluded that in controls, the percentage of $CD16^+$ cells was the only parameter tested with any significant effect on NK cell activity, whereas in patients, the percentage of $CD16^+$ cells had almost no effect on NK cell activity. These results could indicate that a specific defect exists in the $CD16^+$ cell population in subjects with HIV infection. If the cytotoxic function of these cells declines, the importance of the $CD56^+$ cells increases. This could explain the finding that the LAK cell activity correlated to the percentages of $CD56^+$ and $CD16^+CD56^+$ cells only in the patient group. However, others have reported a correlation in LAK cell activity to the percentage of $CD56^+$ cells in HIV-seropositive patients, as well as in healthy HIV-seronegative individuals (13), but since the percentage of $CD56^+$ cells was not decreased in the present study's patients, low fractions of this subpopulation could not explain the depressed in vitro LAK cell activity in HIV infection.

Our findings agree with studies showing normal percentages of $CD16^+$ (10) or $CD56^+$ cells (13), whereas others have shown that the percentages of $CD16^+$ and $CD16^+CD8^+$ NK cells were low in HIV-seropositive subjects (6, 22). The function of NK cells in HIV-seropositive subjects differed from that of controls in their responsiveness to IFN-α. The patients' NK cells were boosted with IFN-α to a lesser degree than cells of controls, in accordance with previous studies (8, 23), whereas IL-2 boosted the NK cell activity to the same degree in both groups, but without restoration of the defective cytotoxic activity in patients as suggested by others (9, 11). It has been suggested that a serum factor contributes to the suppression of the NK and LAK cell functions, but this factor has not been fully identified (16, 24, 25). Our study showed that there was no correlation between

NK cell activity and concentrations of immunoglobulins or HIV antigen in serum.

Some studies have shown that the NK (8) and LAK (16) cell activities in HIV-seropositive subjects decrease with disease progression. Others were unable to show differences in cytotoxicity between AIDS-related complex and AIDS patients (10, 11). Our study showed that NK cell activity, either unstimulated or stimulated, and LAK cell activity did not differ between the AIDS group and the non-AIDS group. Furthermore, the NK and LAK cell activities did not differ between patients with disseminated CMV infection, malignant lymphoma, or Kaposi's sarcoma, compared to those without these AIDS-defining disorders. However, when the CDC classification system was used, it revealed a difference between CDCII+III and CDCIV in IFN-α–stimulated NK cell activity and LAK cell activity. The interpretation of these results is that the defect in these two forms of stimulated cytotoxicity is progressive until the early symptomatic stage of HIV infection, whereas no further progression is seen by the onset of overt AIDS. In line with these observations, there was a positive effect on IFN-α–stimulated NK cell activity and LAK cell activity by the percentage of $CD4^+$ cells in multiple linear regression in patients without AIDS. None of the other classical progression markers, including IgG, IgA, HIV antigen, and β_2-microglobulin were of any importance for NK/LAK cell activity when tested in a model including the percentages of $CD4^+$, $CD16^+$, and $CD56^+$ cells. This, combined with the fact that the effect of the percentage of CD4 cells on NK/LAK cell activity is only seen in patients, indicates that the loss of $CD4^+$ cells could have a direct influence on the decrease in NK/LAK cell activity in HIV infection. The fact that IFN-α–stimulated NK cell activity and LAK cell activity are correlated to the percentage of $CD4^+$ cells and are different between CDCII+III and CDCIV NK cell activity indicates that the defect in HIV disease of these two measures of natural immunity is more specific and of greater importance compared to, e.g., the unstimulated and IL-2–stimulated NK cell activity. Not only the loss of $CD4^+$ cells, but also changes in the function of these cells, could contribute to the defective natural immunity in HIV disease. The important finding of an imbalance in the Th1- and Th2-type responses that contribute to the loss of T cell response to recall antigens and, finally, loss of reactivity to mitogens (26) may be extended to also include a loss of non–MHC-restricted cytotoxic activity.

The finding of a lower $CD16^+$ cell count in patients compared to controls is in accordance with the findings of Mansour et al. (6). However, the finding that the concentration of the $CD16^+CD8^+$ subgroup was elevated in patients compared to controls is in contrast to the findings by Mansour et al. (6), who described a selective depletion of this subgroup. In accordance with previous findings (6, 8) all the NK cell subsets had a higher percentage, but a lower total cell count in the AIDS group compared to the non-AIDS group. The mechanisms behind the loss or decreased production of NK cells are not understood. It has been suggested that HIV can infect NK cells (7, 27, 28), whereas other studies report only nonproductive HIV infection of NK cells (29). Active in vitro HIV infection of NK cells after infection with human herpesvirus 6 has been described (30). The present study shows that the lower NK cell count is determined by a lower total lymphocyte count in the AIDS patients. This might be related to a weakened bone marrow function in AIDS patients (reviewed in 31).

The results presented in this paper have been obtained by the use of frozen cells. Since freezing did not affect the FACS® analysis of lymphocyte subsets, and since it had the same effect on NK cell activity in patients and controls, as well as in AIDS and non-AIDS patients, it is unlikely that the conclusions of this paper are to any major extent influenced by the use of frozen cells.

We conclude that the non–MHC-restricted cytotoxicity is impaired in HIV patients primarily because of a qualitative defect of the NK and LAK cells. This study clearly shows an early defect in NK/LAK cell activity in HIV infection, which for the unstimulated NK cell activity, does not progress by onset of symptoms. However, the defect in IFN-α–stimulated NK and LAK cell activities is, however, progressive until the onset of symptoms, and it is related to the loss of $CD4^+$ cells.

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