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In Vitro Infection of Natural Killer Cells with Different Human Immunodeficiency Virus Type 1 Isolates

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Natural killer (NK) cells are a discrete subset of leukocytes, distinct from T and B lymphocytes. NK cells mediate spontaneous non-MHC-restricted killing of a wide variety of target cells without prior sensitization and appear to be involved in initial protection against certain viral infections. Depressed NK cell-mediated cytotoxicity, one of the many immunological defects observed in AIDS patients, may contribute to secondary virus infections. Here we report that clonal and purified polyclonal populations of NK cells, which expressed neither surface CD4 nor CD4 mRNA, were susceptible to infection with various isolates of human immunodeficiency virus type 1 (HIV-1). Viral replication was demonstrated by detection of p24 antigen intracellularly and in culture supernatants, by the presence of HIV DNA within infected cells, and by the ability of supernatants derived from HIV-infected NK cells to infect peripheral blood mononuclear cells or $CD4^+$ cell lines. Infection of NK cells was not blocked by anti-CD4 or anti-FcγRIII monoclonal antibodies. NK cells from HIV-infected and uninfected cultures were similar in their ability to lyse three different target cells. Considerable numbers of cells died in HIV-infected NK cell cultures. These results suggest that loss of NK cells in AIDS patients is a direct effect of HIV infection but that reduced NK cell function involves another mechanism. The possibility that NK cells serve as a potential reservoir for HIV-1 must be considered.

Human immunodeficiency virus (HIV), the causative agent of AIDS, can devastate the immune system. Although the pathogenesis of AIDS is clearly related to the tropism of the virus for $CD4^+$ T lymphocytes (13, 22, 26, 37, 38, 46), a wide variety of cell types, all bearing CD4, can be infected by HIV type 1 (HIV-1) (reviewed in reference 43). However, there is mounting evidence that $CD4^-$ cells are also susceptible to HIV-1 infection in vivo and in vitro (7), raising the possibility that HIV can enter cells via a receptor other than the CD4 glycoprotein. Recent studies have shown that HIV-1 infection of some $CD4^+$ cells can occur via a CD4-independent mechanism (11, 24, 51). Taken together, these studies extended the original host range of HIV-1 and raised the possibility that multiple receptors for HIV-1 exist.

The nature and role of humoral and cellular immune mechanisms against HIV have not been fully characterized. Natural killer (NK) cells represent a discrete leukocyte subset distinct from T and B lymphocytes (reviewed in reference 48). NK cells have been identified in peripheral blood as a population of T-cell-receptor-negative cells that express CD16 (FcγRIII, a low-affinity receptor for the Fc fragment of immunoglobulin G [IgG]) and CD56 (NKH1) cell surface markers. NK cells mediate two forms of cytotoxicity: (i) lysis of tumor cells and virus-infected cells in a non-MHC-restricted manner and without prior sensitization; (ii) through CD16, NK cells can lyse IgG antibody-coated target cells; this mechanism is known as antibody-dependent cellular cytotoxicity. NK cells may provide the first line of defense against certain viruses before activation of specific humoral and cellular immune mechanisms. Decreased NK cell activity has been reported in patients with a variety of different diseases (48). Several studies have shown a correlation between low levels of NK cell activity and the severity of viral infections in immunocompromised hosts. For example, in immunosuppressed recipients of a bone marrow transplant, a correlation exists between fatal cytomegalovirus (CMV) infection and failure to develop NK cell and cytotoxic T-lymphocyte-mediated cytotoxicity (40). NK cell-mediated antibody-dependent cellular cytotoxicity may play an important role in the pathogenesis of HIV-1 infection (49); however, conflicting data have appeared concerning NK cell activity in AIDS patients (reviewed in reference 5), although in most studies decreased NK cell-mediated cytotoxicity was observed. Reported differences among studies may be attributed to different patient populations, different target cells, or different assay conditions. Nevertheless, impaired NK cell activity is one of the many immunological defects observed in patients with AIDS and related syndromes, and its cause remains unclear.

We show for the first time in this report that purified NK cells, which neither bear the CD4 antigen on their surface nor contain CD4 mRNA, can be productively infected in vitro by various HIV-1 isolates.

MATERIALS AND METHODS

NK cells. All experiments were performed on clonal and polyclonal populations of human peripheral blood NK cells. Polyclonal peripheral blood NK cells were prepared by a method similar to that previously described by Perussia et al. (32). Briefly, peripheral blood mononuclear cells (PBMC) from healthy adult donors who were seronegative for hepatitis B virus and HIV were isolated by centrifugation over Ficoll-Hypaque. Monocytes were partially depleted by two cycles of adherence on plastic. Peripheral blood lymphocytes (2.5×10^5/ml in RPMI 1640 [Flow Laboratories, Inc., McLean, Va.]) containing 10% fetal bovine serum [FBS]

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[Hyclone Laboratories, Logan, Utah]) were then cocultured with 50-Gy-irradiated, RPMI 8866 B-lymphoblastoid cells at a ratio of 5:1 for 10 days at 37°C. Viable cells were collected, and NK cells were enriched by density gradient centrifugation after sensitization of the lymphocytes with a mixture of anti-T monoclonal antibodies (MAbs) B36.1 (anti-CD5, IgG2b) (35) and OKT3 (anti-CD3 purchased from the American Type Culture Collection, Rockville, Md.) and antimonocyte B52.1 (anti-CD14, IgM) (14), followed by indirect rosetting with $CrCl_3$-treated goat anti-mouse Ig-coated sheep erythrocytes. Enriched NK cell preparations were further depleted of minimal numbers of contaminating T or B cells and monocytes by two cycles of antibody-plus-complement-mediated lysis as described previously (9). The following antibodies were used: OKT4 (anti-CD4 from the American Type Culture Collection) for depletion of $CD4^+$ cells, B1 (anti-CD20; Coulter Immunology, Hialeah, Fla.), and B52.1 for depletion of monocytes. Viable cells were collected by centrifugation over Ficoll-Hypaque.

The generation of NK clone 3.3 has been previously reported by Kornbluth et al. (23).

Virus. Culture supernatants of the following HIV-1 isolates were used: IIIB, grown in HUT78 cells (39); SF2, formerly called ARV-2 (10), grown in Sup-T1 cells; SF162 (21), grown in phytohemagglutin-interleukin-2 (PHA-IL-2)-stimulated PBMC (kindly provided by J. A. Levy, University of California San Francisco); and WMJ1 (20), grown in Sup-T1 cells (kindly provided by J. A. Hoxie, University of Pennsylvania); as well as five fresh isolates from pediatric AIDS patients seen at the Children's Hospital of Philadelphia (M1, M2, M3, 43NA, and 44NA). M1, M2, and M3 were isolated from monocytes, and 43NA and 44NA were isolated from T cells. The detailed cellular tropisms of these isolates are still under investigation. All fresh isolates were used at passage 1. Supernatants were cleared of cells by centrifugation, filtered through 0.45-μm-pore-size filters, and stored in liquid nitrogen until use. Cells and supernatants were tested routinely for mycoplasma contamination and found to be negative. Virus stocks were titered for p24 core antigen production by an antigen capture assay (Coulter Immunology) and by tissue culture infective dose titrations with Sup-T1 cells, HUT78 cells, or PHA-IL-2-stimulated PBMC.

Surface marker analysis. NK cell populations were analyzed for surface markers by flow cytometry as previously described (9). Fluorescein isothiocyanate conjugates of the following MAbs were used: B1; B33.1 (anti-HLA-DR nonpolymorphic determinant; kindly provided by G. Trinchieri, The Wistar Institute) (35); OKT3 and B36.1 for detection of T cells; B52.1; B73.1 (anti-CD16 antigen) (31, 33); 3G8, which recognizes a different epitope of the CD16 molecule (17); and B159.1 (anti-CD56, provided by G. Trinchieri). The CD4 antigen was detected by indirect immunofluorescence with the Leu-3a (Becton Dickinson, Mountain View, Calif.) and OKT4 anti-CD4 MAbs. Irrelevant isotype-matched MAbs (Becton Dickinson) were used as controls. To exclude the possibility of T-cell outgrowth in the polyclonal peripheral blood NK cells, we analyzed IL-2-cultured cells for the presence of T cells after 10 days in culture. Cells were analyzed with a Cytofluorograph System 50 H connected to a 2150 data handling system (Ortho Diagnostic Systems Inc., Westwood, Mass.). Forward and right-angle light scatter was used to establish appropriate gates on the cells, excluding nonviable cells. The fluorescence distribution of 5,000 to 10,000 cells was accumulated for analysis. Cells were considered positive when their fluorescence intensity exceeded the threshold at which 99% of cells stained with control MAb had lower fluorescence intensity.

Detection of CD4 mRNA by Northern (RNA) blot analysis. Total RNA from the clonal and polyclonal populations of human NK cells was extracted by the method previously described by Favaloro et al. (16). RNA (10 μg) was separated on a 1% agarose–0.75 M formaldehyde gel and transferred to nitrocellulose (Optibind; Schleicher and Schuell, Inc., Keene, N.H.). Prehybridization was done at 42°C for 1 h in 50% formamide (vol/vol)–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt solution–0.1% sodium dodecyl sulfate (SDS)–100 μg of sonicated denatured salmon testes DNA per ml. Hybridization was done at 42°C for 24 h with 1.5×10^6 cpm of ^{32}P-labeled CD4 probe per ml. The probe used for the detection of the CD4 mRNA was the full-length 3.0-kb cDNA, pT4B (kindly provided by A. Srinivasan, The Wistar Institute) (27). Washing was done at room temperature for 15 min (three times, 5 min each) with 2× SSC–0.1% SDS and then twice at 42°C (30 min each) with 2× SSC–0.1% SDS. The membrane was dried and exposed for autoradiography at −70°C to an XRP-5 (Kodak) X-ray film and an intensifying screen. β-Actin probe was used to verify the integrity of the RNA samples.

Cell culture and virus infection. Polyclonal populations of purified NK cells from peripheral blood were incubated at 37°C in RPMI 1640–10% FBS plus 100 U of IL-2 (Pharmacia Inc., Piscataway, N.J.) per ml. NK clone 3.3 was maintained in RPMI 1640 containing 15% FBS plus 15% IL-2-conditioned medium (Lymphocult T-LF; Biotest, Denville, N.J.) with biweekly feeding and splitting back to a density of 3×10^5 cells per ml. Polyclonal and monoclonal NK cells (2×10^6 to 3×10^6) in 15-ml conical plastic centrifuge tubes were inoculated with different cell-free HIV isolates at a multiplicity of infection of 0.01 50% tissue culture infective dose. After 3 to 4 h of incubation at 37°C, cells were washed five to six times with serum-free RPMI. The final wash was done in 1.5 ml of medium and saved for p24 antigen determinations. Cells were resuspended in culture medium and incubated for an additional 18 h. The cells were then washed twice and maintained in 25-cm^2 tissue culture flasks or in 12-well tissue culture plates.

Detection of HIV infection. At different time intervals, culture supernatants and cells were harvested for p24 core antigen determinations by an antigen capture assay (Coulter Immunology). Cells were washed three times in phosphate-buffered saline (PBS) and lysed. Assays were done according to the manufacturer's instructions. The A_{450} cutoff value for each p24 assay was calculated by using the mean of results obtained with cells and supernatants of uninfected control cultures adjusted according to the manufacturer. Cutoff values ranged from 0.120 to 0.160. The percentage of infected cells was determined by indirect immunofluorescence, using MAb to p24 core protein (Du Pont Co., Wilmington, Del.) and heat-inactivated serum from an adult patient with AIDS. Cells were washed and fixed in suspension with 95% methanol for 90 min at −70°C. Cells were then washed with PBS containing 10% goat serum and incubated with a 1:10 dilution of MAb to p24 or with a 1:50 dilution of the HIV-seropositive serum for 1 h at 37°C. Cells were washed twice in a large volume of PBS–10% goat serum and 0.05% Tween and incubated for 1 h with a 1:80 dilution of fluorescein isothiocyanate-goat $F(ab')_2$ anti-mouse Ig (Organon Teknika Corp., Durham, N.C.) or with a 1:50 dilution of fluorescein isothiocyanate-goat $F(ab')_2$ anti-human IgG (Organon Teknika) for 45 min at 37°C. After extensive washing,

cells were analyzed by flow cytometry and examined by fluorescence microscopy.

Cell viability. Cell viability was determined by the trypan blue dye exclusion method. HIV-infected and uninfected polyclonal and clonal populations of NK cells were counted every 3 to 4 days, and the percent cell viability was determined.

Detection of HIV DNA in infected NK cells. DNA from uninfected and infected NK cells was extracted by the method previously described by Blin and Stafford (3), digested with the restriction endonuclease *Sac*I, and electrophoresed in a 1% agarose gel. The digests were transferred to a nitrocellulose membrane and hybridized to a nick-translated ^{32}P-labeled full-length 9.0-kb HIV probe derived from the clone HXB2 (41), kindly provided by P. Reddy, The Wistar Institute. Prehybridization was done at 55°C for 1 h as described above for detection of CD4 mRNA. Hybridization was then done overnight at 55°C as described above with 10^6 cpm of the ^{32}P-labeled probe per ml. The membrane was washed at 55°C three times for a total of 90 min in 2× SSC-0.1% SDS and exposed for autoradiography at −70°C to an XRP-5 (Kodak) X-ray film and an intensifying screen.

Blocking experiments. Polyclonal peripheral blood NK cells and clone 3.3 cells at a concentration of 5×10^5 cells per ml were incubated for 1 h at 37°C with 25 μg of MAb 3G8 (anti-FcγRIII) or 10 μg of MAb Leu-3a detecting the CD4 molecule and which has been shown to block HIV replication in CD4$^+$ cells (46). The cells were then inoculated with HIV-1 isolate IIIB or SF162 and incubated for an additional 2 h. The cells were washed (four times) with serum-free medium, the appropriate MAb was added at the same concentration as previously, and the cells were incubated at 37°C. At days 4 and 10, cells and supernatants were harvested for p24 antigen determinations.

Syncytium assays. Filtered cell-free supernatants from HIV-IIIB-infected NK clone 3.3 cells, containing approximately 0.8 to 1.5 ng of p24 antigen, were added to the indicator cells. Four different indicator cells were used: PHA-IL-2-stimulated PBMC from healthy donors; two CD4$^+$ T cell lines, HUT78 and MT-4; and the monoblastoid cell line U937. In parallel, the same indicator cells were infected with supernatant from HUT78 cells chronically infected with IIIB. HIV production was monitored at various time postinfection by the appearance of p24 antigen in the supernatants and in the cells. Cells were checked daily for cytopathic effects (CPE) and syncytia formation.

NK cell-mediated cytotoxicity assays. Cytotoxicity assays with K562 cells, CMV-infected fibroblasts (CMV-FS4), and HUT78 cells chronically infected with IIIB (IIIB-HUT78) were performed as described previously (9). Briefly, uninfected and HIV-1-infected polyclonal peripheral blood and clone 3.3 NK cells were used as effector cells. NK cells infected with different HIV strains (IIIB, SF2, and WMJ1) as described above were harvested 8 to 10 days after HIV infection, washed twice, and kept for 2 h in IL-2-free RPMI medium before use. Cells were then washed one more time and used in cytotoxicity assays. CMV-FS4 were prepared as previously described (1). Briefly, human embryonic foreskin fibroblasts (FS4 strain, kindly provided by J. Vilcèk, New York Medical Center, New York, N.Y.) were maintained in Eagle modified minimal essential medium (Flow Laboratories) supplemented with 10% heat-inactivated FBS, 2% vitamins, and 2 mM glutamine. FS4 cells were used at passage 16 to 24. Monolayers of FS4 cells in 75-cm^2 flasks were infected with human CMV strain AD-169 at a multiplicity of infection of ~0.1. When 90% of cells exhibited CPE, the monolayers were trypsinized, and the cells were washed and frozen at −190°C until use. K562 cells passaged in RPMI 1640 medium supplemented with 5% heat-inactivated FBS, 2% vitamins, and 2 mM glutamine were cryopreserved as described above. IIIB-HUT78, maintained in RPMI 1640 containing 10% FBS, was used as previously described (2). Cryopreserved targets were thawed rapidly, washed twice, and resuspended in 0.2 ml of Hanks balanced salt solution. $Na_2{}^{51}CrO_4$ (50 mCi of 407 mCi/mg; ICN Radiochemicals, Irvine, Calif.) was added to 1×10^6 to 2×10^6 cells. After 1 h of incubation at 37°C in 5% CO_2 with gentle shaking every 15 min, the cells were washed three times and then resuspended in RPMI 1640 with 10% FBS at a concentration of 5×10^4 cells per ml. In preliminary experiments, effector-to-target cell (E:T) ratios of 50:1 to 1:1 were tested, and curves were obtained for percent specific lysis versus E:T ratios. E:T ratios of 6:1 for K562 cells and 25:1 for CMV-FS4 and IIIB-HUT78 targets gave levels of cytotoxicity on the linear portion of respective curves and were used in subsequent experiments. A total of 5×10^3 target cells and 3×10^4 or 1.25×10^5 effector cells were added to round-bottom microtiter wells (Linbro Chemical Co., Hamden, Conn.). All determinations were done in triplicate. Plates were centrifuged at $100 \times g$ for 5 min and then incubated for 4 h (K562) or 18 h (CMV-FS4 and IIIB-HUT78) at 37°C in 5% CO_2. After incubation, 0.1 ml of supernatant was collected and its radioactivity was counted in a gamma counter 4000 (Beckman Instruments, Inc., Fullerton, Calif.). Percent ^{51}Cr release was calculated by using the following formula: percent ^{51}Cr release = 100 × [(cpm experimental − cpm spontaneous)]/[(cpm total − cpm spontaneous)], where spontaneous release was that obtained from target cells incubated with medium alone, total release was that obtained from target cells incubated with 1% Triton X-100, and cpm is counts per minute.

TABLE 1. Phenotypic characterization of NK cells[a]

MAbs used	% Positive cells	
	NK clone 3.3	Polyclonal NK cells[b]
CD3	0	<1
CD4	0	0
CD5	0	<1
CD14	0	0
CD16	>98	>92
CD20	0	0
CD56	>92	>95
HLA-DR	>98	>96

[a] Results are expressed as percentage of positive cells as determined by cytofluorographic analysis. Isotype-matched control MAb stained less than 1% of cells. Values shown are representative of four to eight separate experiments. Standard deviations were within 2% of mean values (not shown).

[b] Polyclonal populations of peripheral blood NK cells were prepared as described in Materials and Methods. Polyclonal peripheral blood NK cells were analyzed by flow cytofluorometry for the presence of T cells after 10 days of culture in IL-2; none were detected (0% CD3, 0% CD4, <1% CD5).

RESULTS

Characterization of NK cells: phenotype and CD4 mRNA analysis. Table 1 shows the cell surface characteristics of the NK cells used in this study. Polyclonal populations of human peripheral blood NK cells were purified as previously described (32), followed by antibody-plus-complement-mediated lysis with MAbs to B and T lymphocytes and mono-

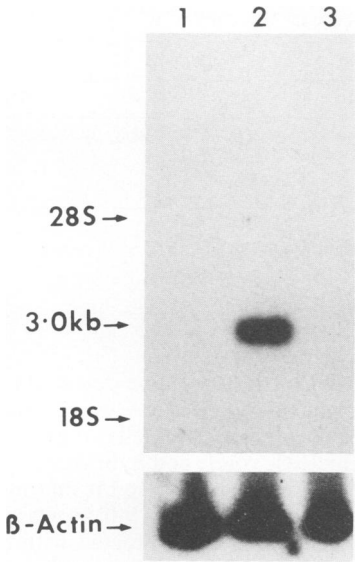

FIG. 1. Northern blot analysis of expression of CD4-specific mRNA in NK cells. Total RNA (10 μg) was electrophoresed through a 1% agarose-formaldehyde gel, transferred to nitrocellulose, and hybridized with a nick-translated full-length 3.0-kb CD4 cDNA as described in Materials and Methods. Lanes: 1, NK clone 3.3; 2, Sup-T1 ($CD4^+$) cells; 3, polyclonal peripheral blood NK cells. The relative migrations of 28S and 18S rRNAs are indicated. Hybridization with a β-actin probe was done to verify the integrity of the RNA samples and to show that approximately similar amounts of RNA were loaded in each well.

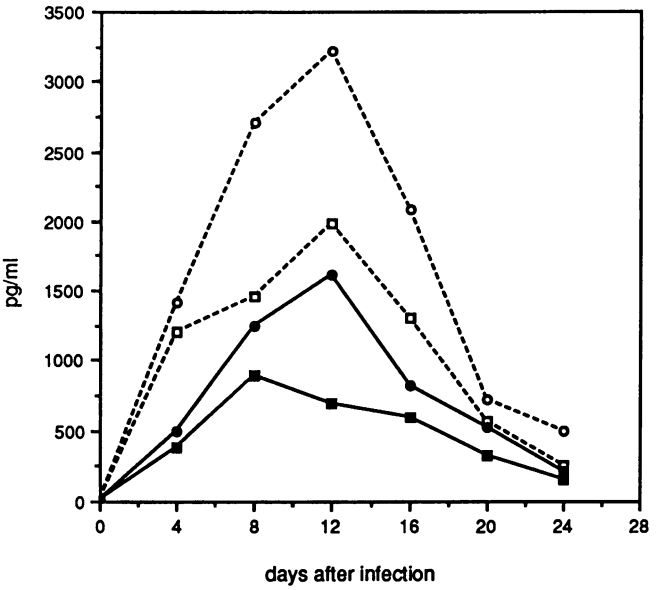

FIG. 2. Kinetics of appearance of p24 antigen in NK clone 3.3 cultures after infection with isolate IIIB or SF162. Cells were infected with IIIB or SF162 as described in Materials and Methods. Cultures were monitored at 4-day intervals for p24 antigen production in the culture supernatant (extracellular) and in the cell lysates (intracellular). The lower limit of detection of HIV p24 antigen was 20 ± 8 pg/ml. Symbols: □, cell-associated SF162; ■, cell-free SF162; ○, cell-associated IIIB; ●, cell-free IIIB.

cytes to ensure complete B-cell, T-cell, and monocyte depletion. Clonal and short-term-cultured polyclonal populations of NK cells expressed CD16 and CD56 as well as HLA-DR, in agreement with previous reports (23, 32). CD3, CD4, CD5, and CD14 antigens were not detected on clonal or polyclonal NK cells. In addition, when polyclonal NK cells were characterized after an additional 10 days of incubation in IL-2-containing medium, $CD4^+$ cells were still not detected. Although we were unable to detect CD4 by flow cytometry, NK cells were also examined for the presence of CD4 mRNA. Total RNA extracted from NK cells was probed for CD4 mRNA by Northern blot analysis. While neither NK clone 3.3 (lane 1) nor polyclonal NK cells (lane 3) expressed any detectable CD4 mRNA (Fig. 1), $CD4^+$ Sup-T1 cells (lane 2) expressed a 3.0-kb transcript. Taken together, these results ruled out minor contamination of polyclonal NK cells with T cells.

HIV infection of NK cells. Multiple HIV-1 isolates displaying different patterns of cellular tropism were examined for their ability to replicate in NK cells. In each experiment, the last wash after infection was tested for residual virus by p24 antigen assay, and each was shown to contain less than 20 to 30 pg/ml. At different time intervals after infection, cell-free culture supernatants and cells were tested for p24 antigen content. Figures 2 and 3 show the kinetics of appearance of p24 antigen in clone 3.3 and polyclonal NK cells, respectively. Strain IIIB as well as strains SF2 and WMJ1 (not shown) consistently yielded higher p24 levels compared with strain SF162. Intracellular p24 levels were higher than extracellular levels throughout the experimental period, with the highest p24 levels occurring at days 8 to 12 after infection. With polyclonal NK cells, the p24 levels decreased starting at days 12 to 16 and reached low levels at day 24. With IIIB-infected NK clone 3.3 cells, p24 levels fell starting on day 12 but were still 250 to 500 pg/ml at 24 days. M1, M2, and M3, isolated from the monocytes of children with AIDS, failed to replicate in clone 3.3 cells (Table 2). p24

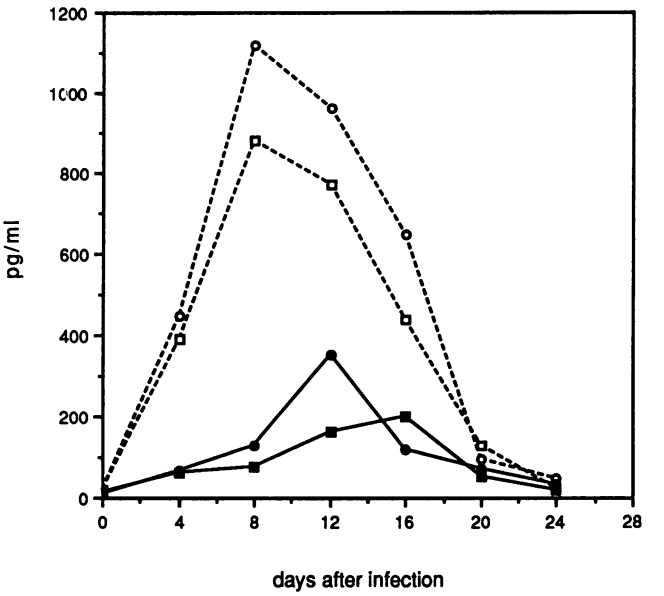

FIG. 3. Kinetics of p24 antigen production with polyclonal NK cells. Polyclonal NK cells prepared as described in Materials and Methods were infected with strain IIIB or SF162. Cultures were monitored at the indicated time points for p24 antigen production as described in the legend to Fig. 2. Symbols: □, cell-associated SF162; ■, cell-free SF162; ○, cell-associated IIIB; ●, cell-free IIIB.

TABLE 2. Ability of different strains of HIV to replicate in NK cells[a]

Cells	HIV isolate[b]								
	IIIB	SF2	SF162	WMJ1	M1	M2	M3	43NA	44NA
Polyclonal NK	++	++	++	++	ND[c]	ND	ND	+	++
Clone 3.3	+++	+++	++	+++	−	−	−	+	++
PBMC	++++	++++	++++	++++	++	++	++	++	++

[a] NK cells were infected as described in Materials and Methods.
[b] p24 antigen production was determined in supernatants and in cells. Maximal peak titers are indicated as follows: −, <15 to 30 pg/ml; +, <200 pg/ml; ++, <2,000 pg/ml; +++, >2,000 pg/ml; ++++, ≥10 ng/ml.
[c] ND, Not determined.

antigen was never detected in these cells or their supernatants for up to 20 days after infection. Two isolates recovered from monocyte-depleted T lymphocytes of children with AIDS (43NA and 44NA) replicated in NK cells. Syncytia were not observed in these HIV-infected cultures at any time during the experimental period. NK clone 3.3 cells survived HIV infection with isolate IIIB, as well as with SF2 and SF162 (data not shown), to a greater extent than did polyclonal NK cells (Fig. 4). By 24 days postinfection, >90% of HIV-infected polyclonal NK cells were nonviable, compared with 50% of clone 3.3 NK cells. Strains IIIB, SF2, and SF162 also inhibited transiently the growth of those NK clone 3.3 cells which survived the infection (data not shown).

DNA analysis of HIV-infected NK cells. We next determined whether HIV DNA could be detected in infected NK cells. High-molecular-weight DNA was extracted from uninfected and HIV-infected NK cells, digested with the restriction endonuclease *Sac*I, and hybridized to a nick-translated ^{32}P-labeled HIV probe derived from the clone HXB2. A unique band of approximately 9.0 kb was detected with DNA from NK clone 3.3 cells infected with SF162 or IIIB (Fig. 5, lanes 3 and 4, respectively), while no bands were detected with DNA obtained from uninfected clone 3.3 cells (lane 1) or with DNA extracted 2 h postinfection from NK 3.3 cells infected with SF162 (lane 2) or IIIB (lane 5).

Detection of viral antigen in infected NK cells. To determine the proportion of cells expressing viral proteins, we analyzed IIIB-infected NK clone 3.3 cells at 8 to 10 days postinfection by cytofluorometry with HIV-specific MAb to p24 and

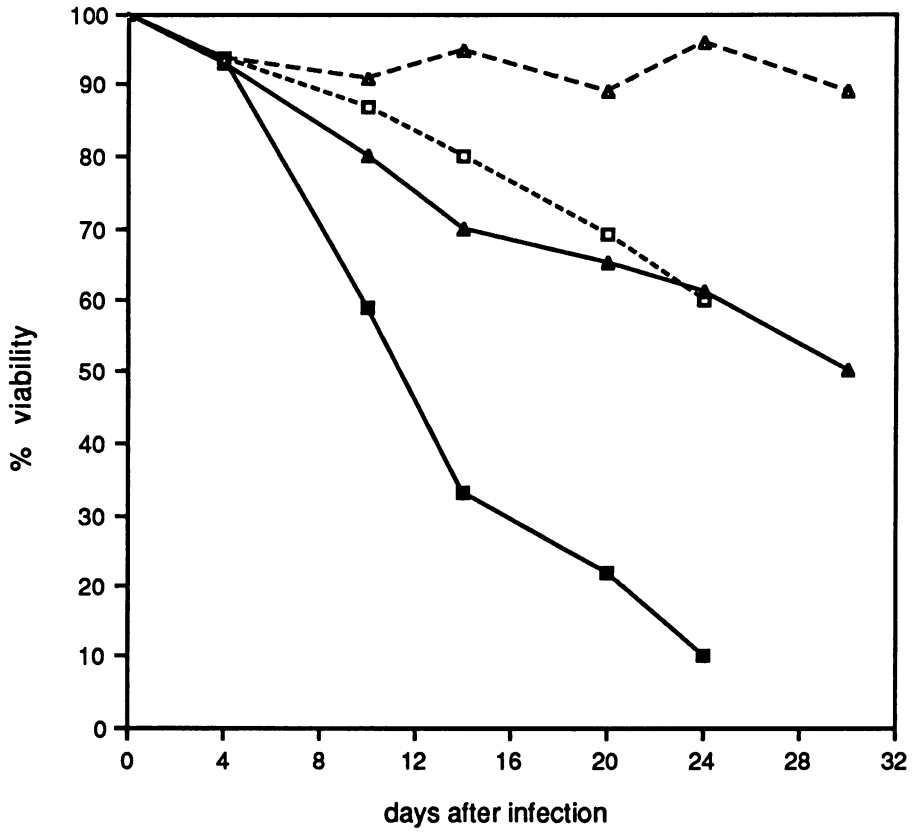

FIG. 4. Viability of HIV-infected NK cells. Clonal and polyclonal populations of NK cells were infected with strain IIIB as described in Materials and Methods. Cell viability was determined by trypan blue dye exclusion every 4 days. Symbols: ■, polyclonal HIV-infected NK cells; □, polyclonal NK cells; ▲, HIV-infected NK 3.3 cells; ▲, NK 3.3 cells.

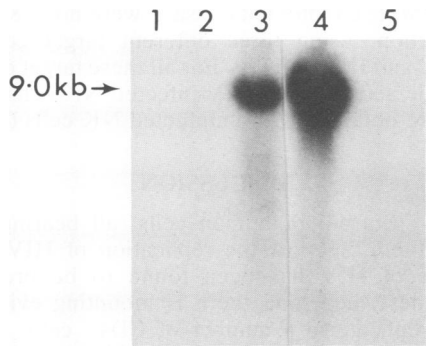

FIG. 5. Southern blot analysis of HIV DNA in NK clone 3.3. High-molecular-weight DNA was extracted from 2×10^6 to 3×10^6 uninfected and infected NK clone 3.3 cells. DNA (20 μg) was digested with *Sac*I, electrophoresed, transferred, and hybridized to a nick-translated 9.0-kb HXB2 probe as described in Materials and Methods. Lanes: 1, uninfected NK clone 3.3 cell; 2, SF162-infected clone 3.3, 1 h postinfection; 3, SF162-infected clone 3.3, 10 days postinfection; 4, IIIB-infected clone 3.3, 10 days postinfection; 5, IIIB-infected clone 3.3, 1 h after infection.

HIV-seropositive human serum. A total of 10 to 20% of infected cells expressed HIV p24 antigen, whereas with the seropositive serum viral antigens were detected in 20% of cells (Fig. 6). As a positive control, 70 to 80% of IIIB-HUT78 cells expressed HIV antigen, in agreement with our previous results (2). Irrelevant isotype-matched MAb and a seronegative human serum did not react with HIV-infected NK cells.

Effect of anti-CD4 and anti-CD16 MAbs on infection of NK cells. To determine the ability of anti-CD4 MAb (Leu-3a) or anti-CD16 MAb (3G8) to inhibit HIV infection, we performed blocking experiments. When NK cells were incubated with Leu-3a or isotype-matched control MAb and then infected with strain IIIB or SF162, no inhibition of virus was observed as determined by p24 antigen production (Table 3). When MAb 3G8 was used, incomplete blocking was detected at day 4 postinfection, while at day 8 no blocking was seen (0 to 5%). When PHA-IL-2-stimulated PBMC were incubated with Leu-3a, 75 to 90% inhibition of infection was detected, while little or no inhibition was observed when 3G8 was used (Table 3).

Syncytium assays. We next assessed the infectivity of

TABLE 3. Blocking experiments with anti-CD4 and anti-CD16 MAbs[a]

Cells	Virus	Percent inhibition of p24 antigen in the presence of MAbs					
		Control MAb		CD4 (Leu-3a)		CD16 (3G8)	
		Day 4	Day 8	Day 4	Day 8	Day 4	Day 8
Clone 3.3	IIIB	0	0	0	0	10 ± 8	5 ± 3
Clone 3.3	SF162	0	0	0	0	7 ± 6	0
Polyclonal	IIIB	0	0	0	0	6 ± 4	3 ± 2
Polyclonal	SF162	0	0	0	0	0	0
PBMC	IIIB	0	0	75 ± 5	80 ± 10	5 ± 3	0

[a] Control isotype-matched MAb (10 μg), Leu-3a (10 μg), and 3G8 (25 μg) were preincubated with polyclonal NK cells and NK 3.3 cells for 60 min at 37°C. The cells were then inoculated with HIV-IIIB or HIV-SF162 for 2 h and washed, and fresh medium containing the same MAb was added. At days 4 and 10, supernatants and cells were tested for p24 antigen. Mean ± standard deviation of three experiments.

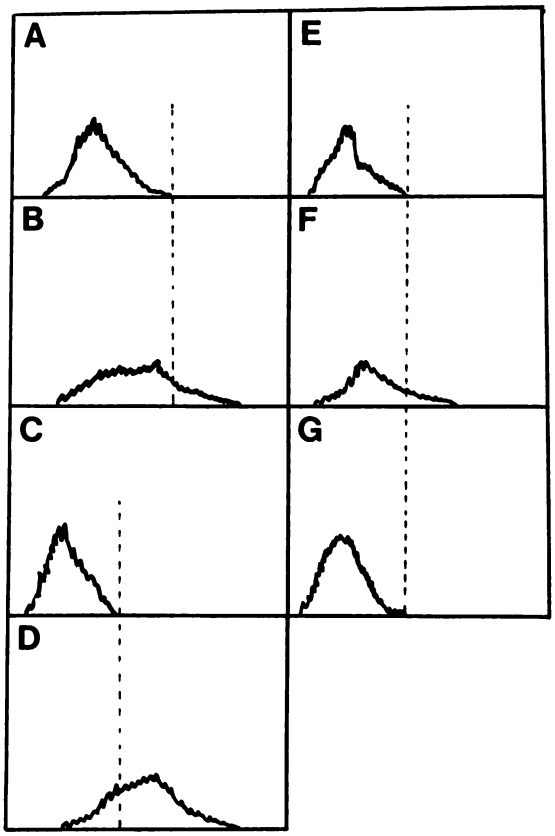

FIG. 6. Cytofluorographic analysis of HIV-infected NK clone 3.3 cells. NK clone 3.3 cells were infected with HIV-IIIB. Ten to 12 days later, cells were fixed and stained with an MAb to p24 as well as HIV-seropositive human serum. (A) IIIB-infected NK clone 3.3 cells plus seronegative human serum; (B) IIIB-infected NK clone 3.3 cells plus HIV seropositive human serum; (C) HUT78 cells chronically infected with IIIB plus seronegative human serum; (D) HUT78 cells chronically infected with IIIB plus HIV-seropositive serum; (E) uninfected NK clone 3.3 cells plus anti-p24 MAb; (F) IIIB-infected clone 3.3 cells plus anti-p24 MAb; (G) IIIB-infected clone 3.3 cells plus isotype-matched control MAb. Figure is plotted as log fluoresence intensity versus cell number.

supernatants derived from NK clone 3.3 cells infected with IIIB. Filtered cell-free supernatant, containing approximately 0.8 to 1.5 ng of p24 antigen, was added to uninfected indicator cells. In parallel, the same cells were incubated with supernatant generated from HUT78 cells chronically infected with IIIB. Supernatants from IIIB-infected NK clone 3.3 cells induced multinucleated giant cell formation and p24 antigen production in PHA-IL-2-stimulated PBMC (Table 4). Giant cells were evident by day 7 after infection. In contrast, HUT78 and MT-4 cells exposed to supernatants died without forming giant cells. Supernatants derived from IIIB-infected NK clone 3.3 cells were also able to infect U937 cells as evidenced by p24 antigen production. This infection was followed by giant cell formation and cell death (Fig. 7). Infection of U937 cells with IIIB generated from chronically infected HUT78 cells did not induce any morphological changes or cell death but resulted in higher p24 antigen levels (data not shown).

Effects of HIV infection on NK cell activity. To determine whether HIV infection had an effect on NK cell activity, we infected polyclonal and clonal NK cells with strain IIIB,

TABLE 4. Infectivity of cell-free supernatant from HIV-IIIB-infected NK clone 3.3 cells

Indicator cell[a]	CPE[b]	p24 production (ng/ml)[c]
PHA-IL-2-stimulated PBMC	G, D	1.5–2.0
U937	G, D	0.5–1.0
HUT78	D	0.5–1.0
MT-4	D	0.4–0.8

[a] Uninfected PBMC, HUT78, MT-4, and U937 cells (10^6) were incubated with filtered cell-free supernatant derived from HIV-IIIB-infected NK 3.3 cells as described in Materials and Methods.
[b] G, Giant cell formation; D, cell death.
[c] p24 antigen production was determined in supernatants; concentrations detected on day 10 after infection are shown.

SF2, or WMJ1. Cytotoxicity assays were done 8 to 10 days after infection, using three different target cells: K562, CMV-FS4, and IIIB-HUT78. For all these target cells, levels of cytotoxic activity with HIV-infected NK cells were similar to those obtained with uninfected NK cells (Table 5).

DISCUSSION

A wide spectrum of human cells, all bearing the CD4 surface protein, support the replication of HIV (43). The host range of HIV has been found to be broader than originally described, and there is mounting evidence that HIV can replicate in a number of $CD4^-$ cells: glial cells, neuronal cells, colorectal cells, muscle cells, endothelial cells, fibroblasts, and rhabdomyosarcoma cells (7). Apparently, HIV-1 infection of these cells occurs via a CD4-

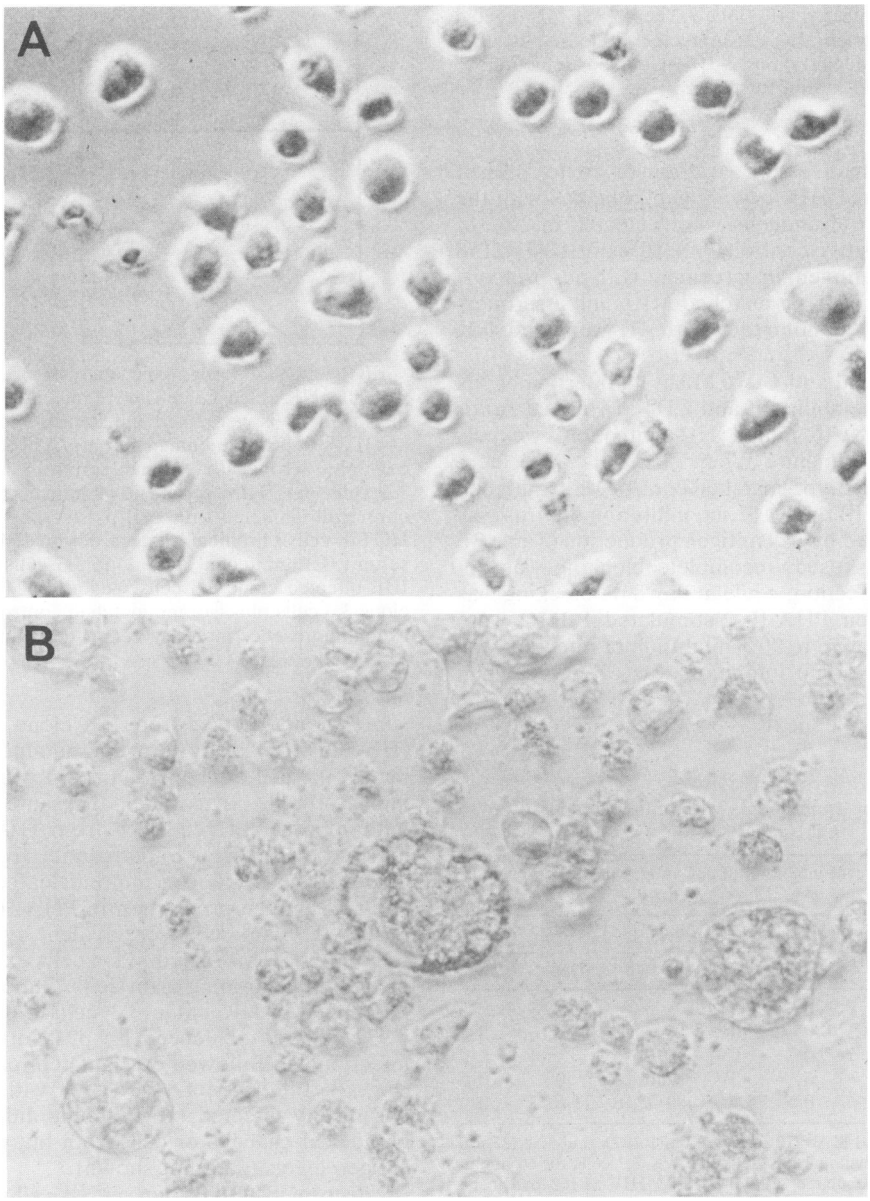

FIG. 7. CPE of supernatant derived from IIIB-infected NK clone 3.3 cells. (A) U937 cells incubated with supernatant from uninfected NK clone 3.3 cells; (B) U937 cells incubated with supernatant derived from IIIB-infected 3.3 cells. Magnification, ×400.

TABLE 5. Effect of HIV infection on NK cell-mediated lysis of three different target cells[a]

NK cells[a]	Percent ^{51}Cr release with different target cells[b]		
	K562	CMV-FS4	IIIB-HUT78
Clone 3.3			
Uninfected	73 ± 2	56 ± 5	69 ± 4
IIIB infected	76 ± 3	52 ± 3	75 ± 6
SF2 infected	72 ± 3	49 ± 3	ND[c]
WMJ1 infected	73 ± 2	53 ± 4	ND
Polyclonal NK			
Uninfected	70 ± 3	63 ± 5	75 ± 11
IIIB infected	75 ± 6	72 ± 8	72 ± 5
SF2 infected	73 ± 4	ND	ND
WMJ1 infected	75 ± 5	ND	ND

[a] Clonal and polyclonal populations of NK cells, infected as described in Materials and Methods, were used as effector cells at 8 to 10 days after infection. Results shown are mean percent specific lysis ± standard error of four to eight different experiments.

[b] Target cells were prepared as described in Materials and Methods. E:T ratios were 6:1 for K562 and 25:1 for CMV-FS4 and IIIB-HUT78.

[c] ND, Not determined.

independent mechanism. Recent studies have also suggested the existence of a CD4-independent mechanism of viral entry in CD4-positive cells (11, 24, 51).

Ruscetti et al. (45) reported that enriched large granular lymphocytes that were stimulated with IL-2 in vitro could be infected with HIV. However, large granular lymphocyte morphology is not a unique characteristic of NK cells, and the large granular lymphocyte preparations used by Ruscetti et al., although enriched for NK cells, also contained ≈20% T lymphocytes. Robinson et al. (44) reported coexpression of CD16 and HIV antigens in HIV-infected, IL-2-stimulated PBMC. They concluded that NK cells could be infected with HIV in vitro. In both these studies, the cell populations infected with HIV were phenotypically heterogeneous. Since in healthy donors approximately 5 to 10% $CD3^+$ T cells coexpress CD16 (25), identification of NK cells on the basis of the detection of CD16 alone must be interpreted with caution.

The availability of methods for growing peripheral blood NK cells in short-term culture and the availability of NK cell clones provided us with an opportunity to study the interaction of NK cells with HIV using homogeneous cell preparations. Our results indicate that purified populations of NK cells are susceptible to HIV infection. It is unlikely that contaminating $CD4^+$ cells in polyclonal NK cell preparations were responsible for HIV replication, since CD4 mRNA was not detected in polyclonal NK cell preparations, and $CD4^+$ cells could not be detected by flow cytometry even after such preparations were cultured in IL-2-containing media for 10 days. The inability to detect p24 antigen in the final wash after infection excluded the possibility that residual input virus was responsible for subsequent detection of p24 antigen. In addition, when NK cells were inoculated with heat-inactivated HIV, levels of p24 antigen in culture supernatant and cells were negligible (data not shown). We could also detect productive infection of NK 3.3 cells with the IIIB strain when a multiplicity of infection as low as 0.001 was used (data not shown). The detection of HIV DNA in NK cells infected with IIIB and SF162, by Southern analysis, confirmed that NK cells were infected. HIV DNA was also detected in infected NK cells by the polymerase chain reaction with long terminal repeat *gag* primers (data not shown).

Infected clone 3.3 cells consistently produced higher levels of p24 antigen than did infected polyclonal NK cells. These differences may depend on the relative proliferative activity and degree of activation, which are higher for clone 3.3 cells than for polyclonal NK cells. We observed decreased p24 antigen levels beginning at days 12 to 16 after infection, and this decrease was more dramatic with the polyclonal population of NK cells. These observations correlated with differences in the CPE induced by HIV in these NK cell populations. IIIB as well as SF2 and SF162 (data not shown) induced extensive CPE in polyclonal NK cells, with 90% cell death after 22 to 24 days, while 40 to 50% of NK clone 3.3 cells died during this time period. After more than 90 days in culture, the infection of NK 3.3 cells by IIIB became persistent, with detectable levels of p24 antigen (0.3 to 1.5 ng/ml) in the intracellular and extracellular compartments (8a).

The infection of NK cells was highly cell associated, and only a subpopulation expressed HIV antigens by immunofluorescence. Similarly, Fauci and colleagues (15a) reported that only a small proportion of $CD3^+$ $CD8^+$ T cells from peripheral blood lymphocytes of healthy donors were infected in vitro with HIV-1.

Productive infection of NK cells was not limited to one isolate of HIV-1, but the levels of viral production in NK cells, as measured by p24 antigen determinations, were consistently lower than those observed when PHA-IL-2-stimulated PBMC were infected with the same HIV strains (IIIB, SF2, SF162, and WMJ1). Strain IIIB, as well as strains SF2 and WMJ1 (data not shown), replicated to a greater extent in NK cells than did strain SF162. Strains IIIB, SF2, and WMJ1 were isolated from PBMC of patients with AIDS, while SF162 was isolated from the brain of a patient with AIDS. Two viruses isolated from lymphocytes of children with AIDS were able to infect NK cells. Three isolates from monocytes of children with AIDS failed to infect NK cells. Since these five isolates have not been fully characterized, it remains to be determined whether NK cells are differentially permissive to lymphocyte-tropic or monocyte-tropic strains of HIV-1.

Resting NK cells do not express CD4 (48), and in the present study, polyclonal and clonal NK cell populations did not express surface CD4 or CD4 mRNA. In addition, HIV infection could not be blocked by anti-CD4 MAb (Leu-3a). Three classes of FcγRs (FcγRI, FcγRII, and FcγRIII) have been identified on human leukocytes (48). The large majority of NK cells bear only FcγRIII (CD16), a low-affinity receptor that binds IgG in immune complexes with soluble or insoluble antigen (48). Different MAbs to CD16 have been described which react with different determinants (34, 48). Homsy and colleagues (19) reported that one MAb to FcγRIII receptor (MAb 3G8) could block antibody-dependent enhancement of HIV infection of human macrophages. In our experiments, treatment of NK cells with MAb 3G8 failed to block HIV infection. Thus, neither CD4 nor the epitope of the FcγRIII recognized by 3G8 seem to be involved in viral entry. However, additional MAbs reacting with other CD16 epitopes need to be tested. Participation of CD16 cannot be totally excluded, as it may be an epitope-dependent phenomenon analogous to that observed with anti-CD4 MAbs. In addition, further studies will be required to define the role of FcγRIII in the enhancement of HIV-1 infection.

Infectious virus was recovered from cell-free supernatants

of HIV-infected NK cells. Surprisingly, cell-free virus derived from NK clone 3.3 cells infected with HIV-IIIB induced giant cell formation when U937 cells were used as indicator cells. In contrast, infection of U937 cells with HIV-IIIB was reported to be noncytopathic, with no morphological changes or cell death (12). One possible explanation for this is that lymphokines produced by HIV-infected NK cells are involved in giant cell formation. The occurrence of this effect, several days after incubation of U937 cells with cell-free viral inoculum, argues against such a mechanism. Of note, addition of exogenous recombinant human tumor necrosis factor alpha to HIV-infected U937 cells did not induce giant cell formation (8a).

Depressed NK cell-mediated cytotoxicity has been detected in patients with persistent generalized lymphadenopathy or AIDS (5). In drug abusers and drug abusers with persistent generalized lymphadenopathy or AIDS, NK cell activity, as well as antibody-dependent cellular cytotoxicity, were reduced (15, 30, 36, 42). Some investigators have monitored the NK cell subsets in patients with AIDS and related diseases, Vuillier and colleagues (50) reported a selective relative and absolute depletion of low-density cells bearing the phenotype $CD16^+$ $CD8^+$. Since possible functional differences between $CD16^+$ $CD8^+$ and $CD16^+$ $CD8^-$ NK cells have not been reported, the importance of this depletion is difficult to interpret. Murayama and colleagues (29) have noted a decrease in the number of $CD16^+$ cells in men in the late stage of asymptomatic HIV infection. Cai et al. (6) reported that absolute $CD16^+$ cell counts were not significantly reduced in men with early HIV infection but were significantly lower in men with frank AIDS. The same investigators reported that the progressive decline of NK cell activity during HIV infection was independent of the number of circulating $CD16^+$ cells.

We investigated the effect of HIV infection on NK cell-mediated lysis. Since the mechanisms involved in killing of virus-infected targets might be different from those involved in lysis of K562 cells, we used K562 cells and two virus-infected cells, CMV-FS4 and IIIB-HUT78 cells, as targets. We detected no differences in the magnitude of cytotoxicity mediated by HIV-infected and uninfected NK cells at any of the E:T ratios tested (data not shown). Since only 10 to 20% of infected NK cells expressed HIV antigen in our experiments, we cannot conclude that HIV has no direct effect on NK cell activity, since the NK cell activity detected may have been mediated by uninfected NK cells. In contrast, Robinson et al. (44) reported significant suppression of the cytotoxic activity of IL-2-stimulated PBMC as early as 3 days postinfection with the LAV strain of HIV. One major difference between our study and that of Robinson et al. was the source of NK cells. The suppression noted by Robinson et al. (44) with heterogeneous cell populations may have been due to suppressive factors released by non-NK cells rather than the direct effect of HIV on NK cell function. Soluble HIV proteins might be responsible for reduced NK cell activity in HIV-infected patients. In this regard, two investigations have reported that different synthetic peptides depress NK cell function in vitro (8, 18). Harris et al. (18), using the synthetic peptide CKS-17, which is homologous to a highly conserved region of the p15E envelope of numerous retroviruses, showed decreased cytolytic activity of fresh and activated human NK cells, although NK cell-target cell conjugate formation was not affected. Cauda et al. (8) reported an inhibition of NK cell activity using two synthetic peptides (sequences 735 to 752 and 846 to 860) corresponding to HIV transmembrane glycoprotein gp41. This inhibition did not appear to result from an inhibition of effector-target cell conjugate formation. In both these studies, the reduction in NK cell activity was attributable to a block in the postbinding stage of lysis. Interestingly, other investigators have shown that after binding to K562 target cells, NK cells from patients with AIDS fail to release NK cytotoxic factor (4) or to polarize tubulin (47).

Opportunistic viral infections, which contribute to progression of disease in AIDS patients, may also contribute to the decreased NK cell-mediated cytotoxicity in HIV-infected patients. Severe CMV infection, in particular, has been associated with decreased NK cell activity in recipients of bone marrow transplants (40). It is therefore possible that infection with multiple immunosuppressive viruses contributes to the immunological dysfunction including depressed NK cell-mediated cytotoxicity. AIDS patients develop several severe immunological defects, and it is difficult at present to define the role of NK cell dysfunction in the pathogenesis of the disease. Depressed NK cell-mediated cytotoxicity in AIDS patients is probably due to several different mechanisms reflecting the complexity of NK cell regulation in vivo. An important issue raised by our studies is whether NK cells are infected in vivo. Administration of IL-2-activated NK cells for the treatment of HIV infection, as suggested by Melder et al. (28), might increase the number of cells in which HIV can replicate and thus be harmful.

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