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Killing of primary CD4+ T cells by non-syncytium-inducing macrophage-tropic human immunodeficiency virus type 1

(AIDS/CD4+ T lymphocytes)

XIAOFANG YU*†, MARY FRAN MCLANE*, LEE RATNER‡, WILLIAM O'BRIEN§, RONALD COLLMAN¶, MAX ESSEX*, AND TUN-HOU LEE*‖

*Department of Cancer Biology, Harvard School of Public Health, Boston, MA 02115; ‡Department of Medicine and Molecular Microbiology, Washington University School of Medicine, St. Louis, MO 63110; §Division of Infectious Diseases, Department of Medicine, West Los Angeles Veterans Affairs Medical Center, University of California School of Medicine, Los Angeles, CA 90073; and ¶Pulmonary and Critical Care Division, Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA 19104

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ABSTRACT **Understanding the mechanism by which human immunodeficiency virus type 1 (HIV-1) kills CD4+ T lymphocytes is important to the development of therapeutic and prophylactic strategies. Recent studies have indicated that, in some cases, progression to AIDS is associated with the appearance of syncytium-inducing, T cell line-tropic HIV-1 variants. Nevertheless, approximately 50% of subjects with AIDS harbor only non-syncytium-inducing, macrophage-tropic (NSI-M) variants of HIV-1. In most asymptomatic patients, NSI-M HIV-1 isolates are the predominant virus type found. We report here that cytopathicity of NSI-M HIV-1 for primary CD4+ T lymphocytes can be directly detected *in vitro*. The extent of CD4+ T-cell killing was not completely correlated with the rate of viral replication, suggesting that other characteristics of HIV-1 contribute to its cytopathicity. Our findings suggest that: (*i*) direct killing by NSI-M HIV-1 may contribute to CD4+ T-lymphocyte depletion *in vivo*, and (*ii*) the determinants of HIV-1 cytopathicity for CD4+ T lymphocytes and cell tropism or syncytia-forming ability are not necessarily tightly linked.**

Human immunodeficiency virus type 1 (HIV-1) isolates have been grouped phenotypically into either non-syncytium-inducing, macrophage-tropic (NSI-M) or syncytium-inducing, T cell line-tropic (SI-T) classes (1–8). NSI-M viruses replicate efficiently in primary monocyte-derived macrophages (MDM) but not in established human CD4+ T cell lines. In contrast, SI-T HIV-1 do not replicate efficiently in MDM but replicate efficiently in a wide range of established human CD4+ T cell lines where they generate multinucleated giant cells.

The NSI-M class of HIV-1 is predominant in asymptomatic carriers (9–14) and can be detected in all stages of HIV-1 infection (15). In contrast, the SI-T class of HIV-1 is more often isolated from AIDS patients (9–14). A shift from the NSI-M phenotype to the SI-T phenotype has been linked to an accelerated depletion of CD4+ T cells *in vivo* and to a rapid progression to AIDS (9–14, 16, 17). Connor *et al.* recently observed significant killing of CD4+ T lymphocytes by SI-T HIV-1 *in vitro* and reported that switching from NSI-M HIV-1 to SI-T HIV-1 over time is correlated with the development of AIDS (16).

The SI-T phenotype is most readily and frequently detected in the MT-2 cell line (18). Syncytium induction in peripheral blood mononuclear cells correlated with replication in MT-2 cells in 96% of uncloned HIV-1 isolates and 100% of cloned HIV-1 isolates (18). The killing of human primary CD4+ T lymphocytes by SI-T HIV-1 can be directly demonstrated *in vitro* (16, 19). Such a finding not only is compatible with the epidemiologic link between this class of HIV-1 and advanced HIV-related disease but also suggests that *in vivo* auxiliary factors are not necessary for the killing of CD4+ T lymphocytes by SI-T HIV-1 (16, 19).

The role of NSI-M HIV-1 in AIDS pathogenesis is not well understood. Since this class of HIV-1 exists at all stages of disease and is frequently isolated from brain tissues of patients with AIDS dementia, it is considered to play a major role in the persistence of HIV-1 infection (7, 11, 15) and in the pathogenesis of AIDS dementia (6, 20). NSI-M HIV-1 are also thought to play a major role in virus transmission. Newly infected individuals harbor primarily NSI-M HIV-1 (21–24). When donors harbor both NSI-M and SI-T HIV-1 variants, the NSI-M viruses appear to be preferentially transmitted to the recipients (23).

Mosier *et al.* recently suggested that NSI-M HIV-1 can have a significant role in progression of HIV-related disease (25). While they reported that NSI-M HIV-1 lacked cytopathicity for CD4+ T lymphocytes *in vitro*, efficient killing of CD4+ T lymphocytes could be demonstrated in the human peripheral blood lymphocyte–severe combined immunodeficiency (hu-PBL-SCID) model. This finding suggests that killing of CD4+ T lymphocytes by NSI-M HIV-1 is dependent upon one or more as-yet-undefined auxiliary *in vivo* factors. Since killing of CD4+ T lymphocytes by SI-T HIV-1 appears to occur in the absence of auxiliary *in vivo* factors (16, 19), the finding of Mosier *et al.* suggests different cytopathic mechanisms for these two classes of HIV-1.

In the present study, we used three-color flow cytometric analysis to quantitate the killing of primary human CD4+ T lymphocytes by various NSI-M and SI-T HIV-1 strains. We report here that NSI-M HIV-1, like SI-T HIV-1, can induce direct and efficient killing of primary CD4+ T lymphocytes.

MATERIALS AND METHODS

Cells. Peripheral blood mononuclear cells from HIV-1 seronegative individuals were purified by the Ficoll/Hypaque method. Macrophages were depleted by attachment to tissue culture flasks in RPMI 1640 medium containing 5% (vol/vol) human serum and 15% (vol/vol) fetal bovine serum for 48 hr. Suspension cells (PBL) were stimulated with 10 μg of phytohemagglutinin A (PHA) per ml (Sigma) for 72

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Abbreviations: HIV-1, human immunodeficiency virus type 1; MDM, monocyte-derived macrophages; NSI-M, non-syncytium-inducing, macrophage-tropic; SI-T, syncytium-inducing, T-cell line-tropic; RT, reverse transcriptase; PHA, phytohemagglutinin A; PBL, peripheral blood lymphocytes.

†Present address: Department of Immunology and Infectious Diseases, Johns Hopkins School of Hygiene and Public Health, Baltimore, MD 21205.
‖To whom reprint requests should be addressed.

hr and were maintained at a density of $<1 \times 10^6$ cells per ml in RPMI 1640 with 20% fetal bovine serum, 20 units of recombinant human interleukin 2 (Collaborative Research) per ml, 100 units of penicillin per ml, and 100 μg of streptomycin per ml. MDM were isolated by the plastic adherence method and maintained in RPMI 1640 with 10% fetal bovine serum, 100 units of penicillin per ml, and 100 μg of streptomycin per ml. MT-2 cells were obtained from the National Institutes of Health and were maintained at a density of $<1 \times 10^6$ per ml in RPMI 1640 with 15% fetal bovine serum, 100 units of penicillin per ml, and 100 μg of streptomycin per ml.

Viruses and Infection. All viruses were propagated in PHA-stimulated peripheral blood mononuclear cells. Two nanograms of each cell-free virus were used to infect 5×10^6 MT-2 cells, 5×10^6 PBL, and 2×10^6 MDM. Cells were exposed to HIV-1 for 14 hr at 37°C, washed twice with phosphate-buffered saline (GIBCO/BRL), and resuspended in fresh complete media. The strains of virus and sources are as follows: IIIB, MN, Ba-L, and ADA, which were obtained from the AIDS Research and Reference Reagent Program, National Institutes of Health, Bethesda, and 89.6, which has previously been described.

p24 and RT Assay. Culture supernatants were obtained by removing cells and cell debris after centrifugation at 3500 rpm for 15 min (DuPont RT-6000). For the p24 assay, sequential 1:9 dilutions of culture supernatant were prepared and analyzed as suggested by the manufacturer. For the reverse transcriptase (RT) assay, 1 ml of supernatant was mixed with 0.5 ml of 30% (wt/vol) polyethylene glycol/0.5 M NaCl at 4°C overnight. The samples were centrifuged at 2500 rpm for 45 min (DuPont RT-6000). Pellets were dissolved in 0.1 ml of RT lysis buffer [1% (vol/vol) Triton X-100/20 mM Tris·HCl, pH 7.5/60 mM KCl/1 mM dithiothreitol/30% (wt/vol) glycerol], and 10 μl of each sample was used for the RT assay as described (26).

Flow Cytometric Analysis of Primary CD4+ T Lymphocyte Killing. At indicated time points after infection, 10^5 PBL in 0.1 ml of culture medium were simultaneously stained with 20 μl of PerCP-conjugated monoclonal antibody against CD3, 20 μl of fluorescent isothiocyanate-conjugated monoclonal antibody against CD4, and 20 μl of phycoerythrin-conjugated monoclonal antibody against CD8 (Becton Dickinson) at 4°C for 60 min. Cells were washed twice with phosphate-buffered saline and fixed with 200 μl of 2% formaldehyde in phosphate-buffered saline for 2 hr. Viable cell surface markers were analyzed by three-color flow cytometry (FACScan; Becton Dickinson).

RESULTS AND DISCUSSION

To study the cytopathicity of HIV-1 for primary $CD4^+$ T lymphocytes, PBL were stimulated with PHA for 3 days and subsequently infected with different strains of HIV-1. The percentages of $CD4^+$ and $CD8^+$ T cells in the culture were monitored periodically by three-color flow cytometry using fluorescence-conjugated monoclonal antibodies to CD4, CD8, and CD3. Two weeks after stimulation with PHA, >90% of the cells in mock-infected cultures were $CD3^+$ T lymphocytes and <1% of the cells expressed CD14, a surface marker of peripheral blood monocytes (unpublished data).

Killing of primary $CD4^+$ T lymphocytes by IIIB or MN strains of HIV-1 was readily scored in this assay (Fig. 1). The IIIB and MN viruses are characteristic of SI-T HIV-1, as they replicate in several established T cell lines, including Sup-T1 (26). Moreover, as summarized in Table 1, they form syncytia in MT-2, an indicator cell line proposed by Koot *et al.* for identifying SI-T HIV-1 (18). In the IIIB-infected PBL culture from this donor, the percentage of viable $CD4^+$ T cells began to decline 2 weeks after infection (Fig. 1 *Left*). By 21 days after infection, the percentage of $CD4^+$ T cells in the culture decreased to <2.5% (Fig. 1 *Left*). A similar decrease in percentage of viable $CD4^+$ T cells was also observed in the MN-infected culture 21 days after infection (Fig. 1 *Left*).

Killing of primary $CD4^+$ T lymphocytes by IIIB or MN viruses was also detected in this assay with PBL from a different donor. Intersample variation was evaluated by using duplicate cultures; the intersample variation for mock- and different virus-infected cultures was limited (Fig. 1 *Right*). In the IIIB-infected PBL culture from the second donor, the percentage of viable $CD4^+$ T cells began to decline 1 week after infection (Fig. 1 *Right*). By 14 days after infection, the percentage of $CD4^+$ T cells in the culture decreased to <1.4% (Fig. 1*B*). A similar decrease in the percentage of viable $CD4^+$ T cells was also observed in the MN-infected culture 21 days after infection (Fig. 1 *Right*). Despite some interdonor variation, the sharp decline of $CD4^+$ T cells, which was observed in repeated experiments with the IIIB- or MN-infected primary cultures, never occurred in mock-infected cultures (Fig. 1 and unpublished results).

The decreased percentage of $CD4^+$ T cells in the IIIB- or MN-infected primary cultures could be due to depletion of $CD4^+$ T cells or down-regulation of CD4 molecules. If it were caused by a down-regulation of CD4 molecules, a significant increase of $CD3^+/CD4^-$ T cells would have been expected. In fact, no such increase was detected by three-color flow cytometry (unpublished data). It appears that virus killing caused a reduction in the absolute number of $CD4^+$ T cells, thereby decreasing the proportion of $CD4^+$ T cells in the IIIB- or MN-infected primary cultures. Since $CD8^+$ T cells were not killed by the viruses studied, IIIB- or MN-infected cultures had an increased proportion of $CD8^+$ T cells, which accounted for the majority of viable cells 21 days after infection (Fig. 1).

NSI-M HIV-1 are known to replicate in peripheral blood mononuclear cells and PBL (1–4). However, cytopathicity for fresh $CD4^+$ T cells by NSI-M HIV-1 has not been evaluated in the assay described above. To address this issue, we analyzed three different primary NSI-M HIV-1 isolates in this study. Isolates 89.6 (27), Ba-L (7), and ADA (5) are clinical isolates with known nucleotide sequences. 89.6 is a molecularly cloned macrophage-tropic isolate from the peripheral blood of an AIDS patient that does not replicate in Sup-T1 or CEM cell lines (27). Ba-L was isolated from primary cultures of lung tissue from a child with AIDS (7). ADA was obtained by cocultivation of peripheral blood mononuclear cells from an HIV-1-infected individual with uninfected primary monocytes (5).

All of the NSI-M viruses replicated in MDM and macrophage-depleted PBL (Table 1). None of the NSI-M viruses replicated in MT-2 cells, nor did any induce syncytia in this cell line. These NSI-M viruses showed significant cytopathicity for fresh $CD4^+$ T lymphocytes. Fig. 1 *Left* shows that the percentage of viable $CD4^+$ T cells in Ba-L-infected PBL cultures began to decline 2 weeks after infection compared with the mock-infected culture. $CD4^+$ T-cell depletion in Ba-L-infected cultures was more obvious 21 days after infection, although the decrease was less profound than that in IIIB- or MN-infected cultures. The decrease in $CD4^+$ T lymphocytes in the 89.6-infected culture was more pronounced than that in Ba-L-infected cultures (Fig. 1 *Left*).

Killing of primary $CD4^+$ T lymphocytes by the three NSI-M viruses could also be demonstrated by using different donor PBLs. Fig. 1 *Right* shows that in the 89.6- and Ba-L-infected PBL cultures, the percentage of viable $CD4^+$ T cells began to decline 1 week after infection compared with the mock-infected culture. $CD4^+$ T-cell depletion in the 89.6- or Ba-L-infected cultures was more obvious 14 and 21 days after infection, although less profound in Ba-L-infected cultures than that in IIIB- or MN-infected cultures (Fig. 1 *Right*). A decrease in $CD4^+$ T lymphocytes in ADA-infected cultures

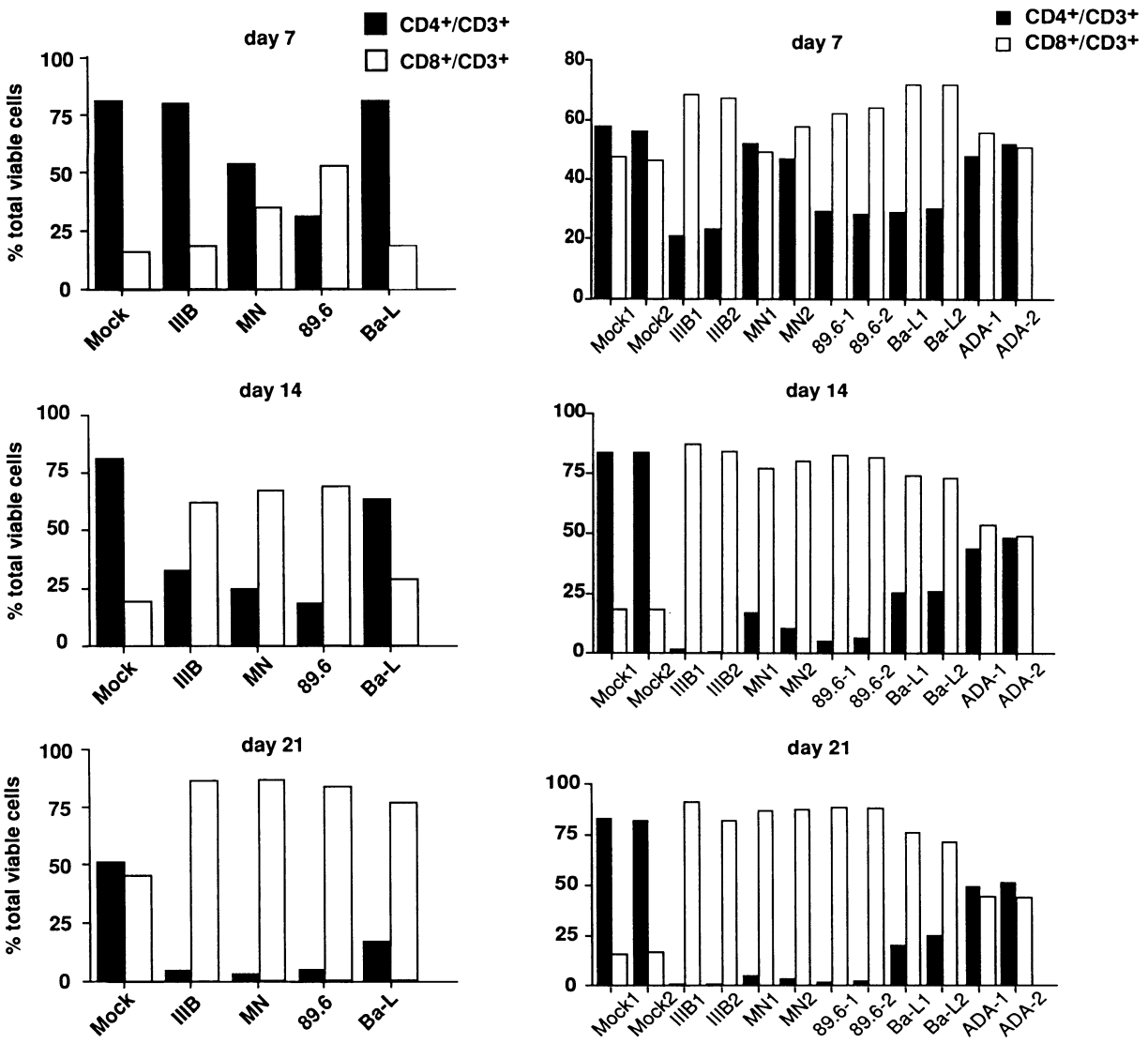

FIG. 1. (*Left*) Percentages of $CD4^+/CD3^+$ and $CD8^+/CD3^+$ T cells in PBL from one donor mock-infected or infected with HIV-1 strains IIIB, MN, 89.6, and Ba-L and monitored by three-color flow cytometry at different time points after HIV-1 infection. (*Right*) Percentages of $CD4^+/CD3^+$ and $CD8^+/CD3^+$ T cells in PBL from another donor mock-infected or infected with HIV-1 strains IIIB, MN, 89.6, Ba-L, and ADA and monitored by three-color flow cytometry at different time points after HIV-1 infection. Results from duplicate infected cultures are shown.

was also observed, although less pronounced than that in 89.6- or Ba-L-infected cultures (Fig. 1 *Right*).

To evaluate the relationship of virus replication and killing of $CD4^+$ T lymphocytes, the kinetics of virus replication were studied. The kinetics of virus replication and the levels of virus production for IIIB, 89.6, and Ba-L were comparable throughout the study period (Fig. 2 *Upper*). They reached a peak level of p24 7 days after infection, 300 ng/ml, which gradually declined for the rest of the follow-up period (Fig. 2 *Upper*). Despite comparable levels of virus replication for IIIB, 89.6, and Ba-L, killing of $CD4^+$ T lymphocytes in Ba-L-infected cultures was less complete than that in IIIB- and 89.6-infected cultures 21 days after infection (Fig. 1 *Right*). ADA replicated at comparable or higher levels compared to MN at all time points of infection (Fig. 2 *Lower*). However, killing of $CD4^+$ T lymphocytes in ADA-infected cultures was less efficient compared with that in MN-infected cultures (Fig. 1 *Right*). These results suggest that the ability to kill $CD4^+$ T cells by HIV-1 is not absolutely determined by the rate of virus replication.

Table 1. Virus replication as monitored by syncytium formation, virion-associated RT activity, and level of p24 in the culture supernatant

Virus	Cells		
	MT-2	MDM	PBL
Ba-L	−	+	+
ADA	−	+	+
89.6	−	+	+
MN	+	−	+
IIIB	+	−	+

MT-2 cells were monitored for syncytium formation and virion-associated RT activity. +, Peak RT activity $> 5 \times 10^5$ cpm/ml and formation of multinucleated giant cells; −, background level of RT activity. Virus replication in MDM and PBL was monitored by the level of p24 in the culture supernatants. +, Peak levels > 10 ng/ml; −, levels < 20 pg/ml. Viruses harvested from the PBL cultures maintained the same phenotypes as the original virus stocks.

Immunodeficiency in AIDS patients is caused by the depletion and dysfunction of $CD4^+$ T cells (28). SI-T HIV-1 have been linked to disease progression in AIDS patients (9–16). However, the emergence of SI-T HIV-1 has been observed in only 50% of patients who develop AIDS (17, 29). In the remaining patients, only NSI-M viruses have been detected. Furthermore, the $CD4^+$ T lymphocyte count con-

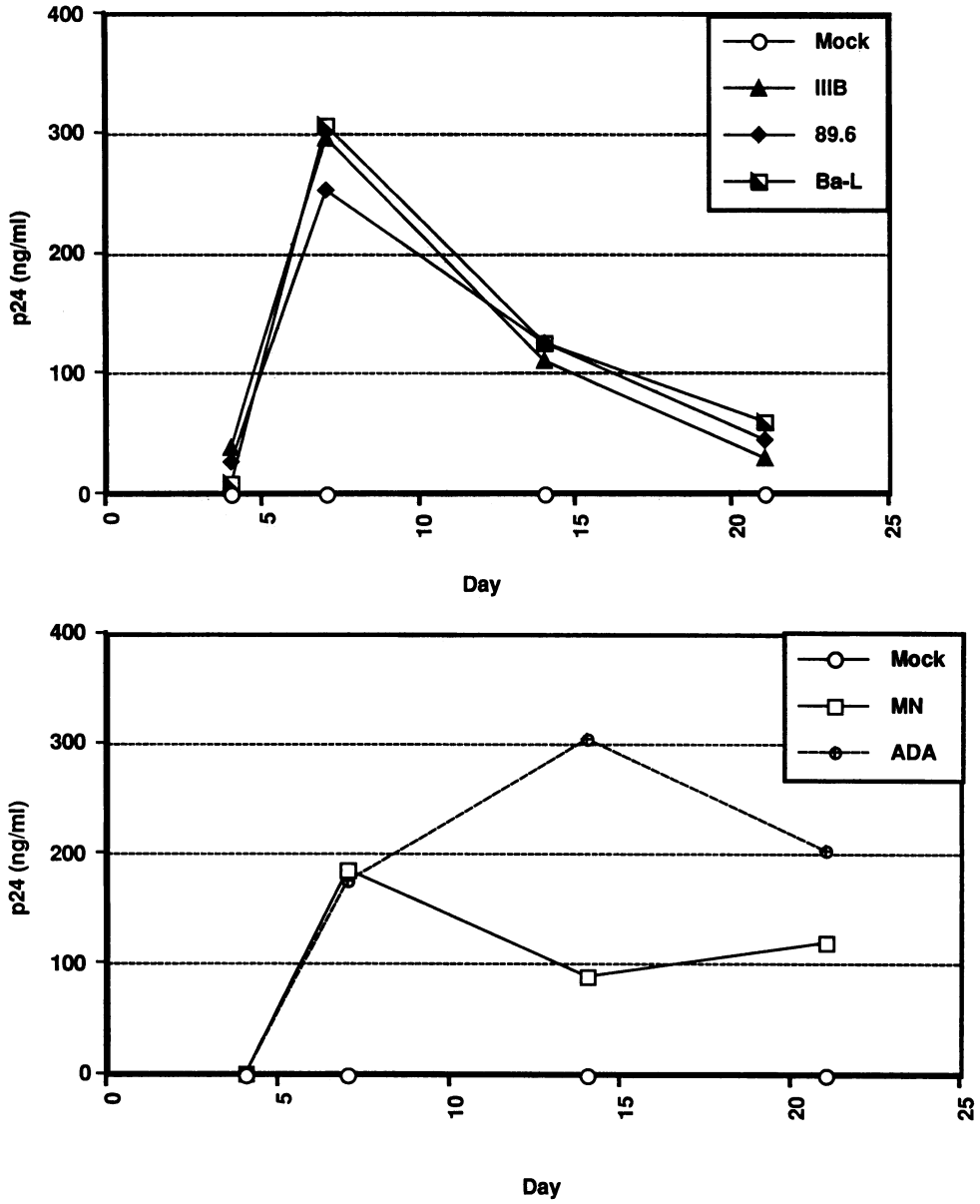

FIG. 2. (*Upper*) Kinetics of virus replication in mock-infected and IIIB-, 89.6-, and Ba-L-infected PBL cultures. Virus replication was monitored by the production of secreted p24 on postinfection days 4, 7, 14, and 21. (*Lower*) Kinetics of virus replication in mock-infected and MN- and ADA-infected PBL cultures. Virus replication was monitored by the production of secreted p24 on postinfection days 4, 7, 14, and 21.

tinued to drop in the majority of HIV-1-infected individuals during the asymptomatic stage, even when NSI-M HIV-1 are predominant. Our study demonstrates that NSI-M viruses can induce efficient killing of primary $CD4^+$ T lymphocytes. These viruses, which are not detected by the conventional syncytium-forming assay using MT-2 cells, may account for the depletion of $CD4^+$ T cells in the majority of asymptomatic individuals and a rapid progression to AIDS in some HIV-1 infected patients. The assay used in this study allows the direct measurement of killing of fresh $CD4^+$ T cells without the use of the human PBL–severe combined immunodeficiency model. When combined with other assays such as the syncytium-forming assay, this assay has the potential to increase the sensitivity of detecting virulent strains of HIV-1 that are more directly linked to the pathogenesis of AIDS.

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