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Syncytium Induction in Primary CD4$^+$ T-Cell Lines from Normal Donors by Human Immunodeficiency Virus Type 1 Isolates with Non-Syncytium-Inducing Genotype and Phenotype in MT-2 Cells

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Human immunodeficiency virus type 1 (HIV-1) isolates classified as syncytium-inducing (SI) or non-SI (NSI) in the MT-2 T-cell line exhibit characteristic sequence differences in the V1-V2 and V3 regions of the *env* gene. Seven HIV-1 isolates were phenotyped as NSI or SI in the MT-2 cell line. Unexpectedly, all four NSI viruses induced large syncytia 4 to 8 days postinoculation in a panel of five primary CD4$^+$ T-cell lines (including two clones) generated from the peripheral blood of normal donors by exposure to infectious HIV-1, inactivated HIV-1, or Epstein-Barr virus. The primary T-cell lines yielded neither HIV-1 provirus nor infectious HIV by PCR analysis or exhaustive coculture with phytohemagglutinin-treated blast cells. Three isolates (TC354, PK1, and PK2) were biologically cloned and retained their SI or NSI phenotypes in MT-2 and primary T-cell lines. The biologically cloned provirus DNA was also used to clone and sequence the relevant V2 and V3 regions of the *env* genes. The amino acid sequences of the V2 and V3 regions were characteristic of patterns already reported for the NSI, switch NSI, and SI phenotypes, respectively. This evidence precludes the possibility that these results were due to contamination of the NSI isolates with SI virus. The results unequivocally indicate that HIV-1 isolates with the NSI genotype and phenotype in MT-2 cells may actively induce syncytia in cloned CD4$^+$ T cells in vitro and support the view that direct cytopathic effects may contribute to the steady decline in CD4$^+$ T cells in asymptomatic HIV-1-seropositive patients without detectable SI virus.

A variety of mechanisms have been proposed to explain the depletion of CD4$^+$ T cells which characterizes the progression of asymptomatic human immunodeficiency virus type 1 (HIV-1) infection to AIDS (14, 18, 28). These include a direct cytopathic effect, lysis by HIV-1-specific cytotoxic T cells, an autoimmune response, and apoptosis. A significant role for a more virulent variant of HIV-1 in the pathogenesis of AIDS is an attractive concept (2), and there is now considerable evidence pointing to syncytium induction by HIV-1 isolates in vitro as a significant marker in the progression of HIV-1 infection in seropositive patients (13). Most asymptomatic HIV-1-seropositive patients yielded isolates not inducing syncytia in T-cell lines such as MT-2, and which were referred to as the non-syncytium-inducing (NSI) phenotype (22). Sequential virus isolations revealed the appearance of syncytium-inducing (SI) virus variants in a number of asymptomatic patients, and most significantly, the appearance of SI virus was strongly associated with an increased rate of depletion of CD4$^+$ cells and more rapid development of AIDS (12, 17, 21). However, HIV-1 of the SI phenotype was detected in peripheral blood mononuclear cells (PBMCs) from only approximately 50% of AIDS cases (13). This suggests that the slower progression to AIDS in about half of the seropositive patients is independent of the appearance of SI virus and leaves unexplained the steady decrease in CD4$^+$ T cells. Recently, sequential studies of an infected individual provided evidence that a phenotypic switch from NSI to SI was not a prerequisite for rapid HIV disease progression (4).

The envelope proteins of HIV-1 are known to play an important role in neutralization and target cell tropism (14) as well as in syncytium induction (3, 6, 30). Both the V1-V2 and V3 regions were involved in syncytium induction in MT-2 cells (6, 7), and a detailed analysis of the amino acid sequences of these regions revealed the existence of three groups of HIV-1 variants. These were referred to as SI, switch NSI, and stable NSI (7). The SI variants were characterized by changes to a charged residue at position 11 or 28 in the V3 region that did not occur in the stable and switch NSI variants. In addition, the SI variants had a high level of charged residues at positions 29 to 46 in the hypervariable portion of the V2 region compared with the switch NSI variants. Finally, the stable NSI variants were characterized by an even lower level of charged residues at positions 29 to 46 together with more gaps in the sequence of the V2 region compared with the SI and switch NSI variants.

This report presents evidence that isolates of HIV-1 characterized as having the NSI phenotype in the MT-2 assay and having the stable or switch NSI genotype produced typical syncytia in CD4$^+$ T-cell lines established from PBMCs of normal donors. These results strongly suggest that HIV-1 viruses currently regarded as NSI on the basis of the MT-2 assay may in fact have the capacity to cause syncytia in clones of human CD4$^+$ T cells activated by HIV-1 or other antigens in lymphoid tissue. Such a direct cytopathic effect may contribute significantly to the steady depletion of CD4$^+$ cells leading to AIDS, and the appearance of an SI variant in some patients may accelerate the process.

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MATERIALS AND METHODS

Generation of cell lines. PBMCs were isolated from seronegative donors and stimulated with infectious or inactivated HIV-1 or with Epstein-Barr virus for 6 days in Iscove's medium (GIBCO BRL, Gaithersburg, Md.) supplemented with glutamine, penicillin, streptomycin, and 20% human serum. The cells were then suspended in 0.35% agarose containing Iscove's medium, 20% human serum, recombinant interleukin 2 (Boehringer Mannheim, Mannheim, Germany), and MLA-144 fluid (15). After 6 days of incubation, colonies were harvested into 100 μl of T-cell medium consisting of Iscove's medium supplemented with 20% heat-inactivated fetal calf serum (Moregate Investments, Brisbane, Australia), 10% MLA-144 fluid, and 10 U of recombinant IL-2, together with 5×10^4 γ-irradiated (5,000 rads) umbilical cord blood mononuclear cells (CBMCs) and 0.06 μg of phytohemagglutinin (PHA; Murex Diagnostics Ltd, Dartford, United Kingdom) per ml. All primary T-cell cultures were restimulated weekly with PHA and feeder cells and are referred to in this report as primary T-cell lines because they could be maintained in vitro for up to 15 months. All parameters in the culture system were optimized to achieve maximum cell survival. Two cell lines were cloned by limit dilution.

Cells were used in these experiments when there were sufficient numbers of cells for storage and experimentation, usually 1 to 2 months after colony formation.

Cell lines. The MT-2 cell line was obtained from D. Richman through the AIDS Research and Reference Program, Division of AIDS, National Institute of Allergy and Infections Diseases (8, 9).

The T-cell clone SAS-1 was generated in response to infectious HIV-1 (isolate PK1). Clone WT29A2.7 and T-cell lines PY12B2 and JM04 were generated by exposure to β-propiolactone-inactivated HIV-1 (isolate TC354), and PC5 was generated in response to Epstein-Barr virus. All clones and cell lines tested by flow cytometry had equally high levels of expression of CD4, CD26, and CD29 but were negative for expression of CD45RA (JM04 was not tested for CD29 and CD45RA). The absence of HIV-1 provirus DNA or infectious HIV-1 from all clones and cell lines was confirmed by both PCR for V2 and V3 regions and coculture with PHA-stimulated CBMCs prepared as previously described (25). The HIV-1 specificity of the T-cell lines is currently being examined.

Flow cytometric analysis. T-cell lines were phenotyped by flow cytometry with a panel of fluorescence-labelled monoclonal antibodies: CD4 (Dakopatts A/S, Copenhagen, Denmark); CD8, CD14, and CD16 (Becton Dickinson, San Jose, Calif.); and CD26, CD29, and CD45RA (Coulter, Hialeah, Fla.). For each surface marker, 5×10^5 cells were washed and finally resuspended in 100 μl of buffer (1% fetal calf serum in phosphate-buffered saline [PBS]). Each labelled antibody was added at the manufacturer's recommended concentration and incubated with the cells for 30 min on ice. After being washed with buffer, the cells were fixed in 200 μl of fixative (1% paraformaldehyde in PBS [pH 7.2]) for a minimum of 1 h at 4°C, before analysis with an EPICS 753 flow cytometer (Coulter).

Virus isolates and preparation of pools. HIV-1 isolates PK1 (isolated during primary infection) and PK2 were recovered 70 weeks apart from the same HIV-positive patient in Sydney, Australia. Other isolates (TC354, TC408, TC433, TC440, and TC471) were from five HIV-positive individuals in Brisbane (Centers for Disease Control disease stages: not available, IV.C1, IV.C1, IV.C1, and IV.B, respectively). Virus pools (fourth passage level) were prepared in CBMCs without the use of DEAE-dextran or Polybrene, and aliquots were stored in liquid N_2. Virus titers in CBMCs and PBMCs were determined by the reverse transcriptase (RT) assay (25) and were in the ranges $10^{7.2}$ to 10^8 50% tissue culture infective doses per ml for CBMCs and $10^{6.8}$ to $10^{7.3}$ 50% tissue culture infective doses per ml for PBMCs, reflecting the susceptibility of these cells to HIV-1.

Syncytium formation. The SI or NSI phenotype was defined by infection of MT-2 cells as previously described (13). Syncytia are defined as persisting large multinuclear cells with a diameter greater than 3 normal cell diameters.

T-cell lines were stimulated for 3 to 4 days and then purified by centrifugation over Ficoll-Paque 24 h prior to inoculation. To detect syncytium formation, MT-2 cells or T-cell lines were seeded at 1 to 3×10^5 cells per well in 96-well plates. Triplicate wells were inoculated with HIV-1 at a multiplicity of infection (MOI) of 1 and allowed to adsorb for 1 to 2 h. T-cell medium was then added to make up the volume to 200 μl. Plates were examined daily for the presence of syncytia. RT assays and 50% medium changes were performed at days 4, 7, and 10 of culture. To avoid possible cross-contamination, each virus isolate was tested on a separate tissue culture plate.

RT assay. RT activity was determined as previously described (19). Counts of incorporation of labelled thymidine were adjusted for background and expressed as counts per minute per milliliter of tissue culture fluid (TCF). The mean +2 standard deviations of negative control cultures was 18×10^3 cpm/ml, and RT activity below this value was considered negative. Tests of significance were based on Student's *t* test.

Cloning and sequencing. CBMC cultures infected with the end point dilution of each HIV-1 isolate served as the source of biologically cloned provirus DNA, and the SI or NSI phenotype of each cloned virus was confirmed by subculture in MT-2 and SAS-1 cells. The cloning experiments failed to detect NSI virus in the PK2 isolate, and all three clones tested induced syncytia in MT-2 and SAS-1 cells. DNA was extracted as previously described (23). A PCR was conducted with the nested primers described for the V1-V2 (7) and V3 (24) regions of the *env* gene of HIV-1. The PCR DNA product was purified from agarose gels with BRESAclean (Bresatec Ltd, Adelaide, Australia), cloned into pGEM-T, and confirmed on the basis of size by restriction enzyme analysis in electrophoresis. Sequencing of both DNA strands was carried out and the peptide sequences were determined by the MacVector program.

TABLE 1. Comparison of the syncytium-inducing abilities of three HIV-1 isolates in different T-cell lines[a]

Cell line	Syncytium induction by HIV-1 isolate[b]		
	TC354	PK1	PK2
MT-2	−	−	+
PHA-treated CBMCs	−[c]	−[c]	−[c]
PHA-treated PBMCs	−	−	+
SAS-1	+	+	+
WT29A2.7	+	−	+
JM04	+	−	+
PY12B2	−	−	+
PC5	+	−	+

[a] Cells were inoculated with HIV-1 and monitored for syncytia for 14 days.
[b] Triplicate wells were scored as follows: −, no syncytia; +, syncytia observed. Note that when no HIV-1 isolate was present, no syncytia were induced in any of the cell lines.
[c] No definite syncytia observed.

RESULTS

Syncytium induction in MT-2 cells and T-cell lines from normal donors. The HIV-1 isolate PK2 was characterized as an SI isolate, while TC354 and PK1 were classified as NSI isolates, by the syncytium induction assay in MT-2 cells with comparable MOI. Passage of TC354 and PK1 in MT-2 cells over a period of 20 days failed to detect syncytia, confirming the absence of low levels of contaminating SI virus.

In a first experiment, these three HIV-1 isolates were inoculated into MT-2 cells, PHA-stimulated CBMCs, and five other $CD4^+$ T-cell lines to compare virus replication and syncytium induction by SI and NSI isolates. Surprisingly, the two NSI isolates induced syncytia in some of the $CD4^+$ T-cell lines (Table 1 and Fig. 1). Isolate TC354 induced syncytia in all cell lines tested, with the exception of MT-2 and PY12B2, while PK1 induced syncytia only in SAS-1 cells (Fig. 1). In additional experiments (data not shown), syncytia were observed in PY12B2 and PC5 cells inoculated with TC354 and PK1. Some variation in the level of syncytium formation was observed with the various virus-cell combinations, possibly reflecting minor variations in the condition of the various T-cell lines at different times. However, in this series of experiments, clear-cut patterns in the syncytium response were recorded. SAS-1 was the primary T-cell line most sensitive to induction of syncytia by the TC354 and PK1 isolates, but syncytia were never observed in 16 months of culture of uninfected SAS-1 cells.

Syncytia generally appeared in the primary T-cell lines and clones 4 to 8 days postinoculation. An interesting observation was that in six experiments, the PK2 SI isolate induced syncytia in SAS-1 cells significantly earlier than did the PK1 NSI isolate from the same patient (3.6 days versus 6.6 days; $P < 0.05$).

In the experiment summarized in Fig. 2, the usual high level of RT activity was detected in culture fluid from the control CBMCs ($>10^6$ cpm/ml) and PBMCs ($>5 \times 10^5$ cpm/ml) infected with all three isolates (TC354, PK1, and PK2). Each isolate produced moderate RT activity (between 10^5 and 5×10^5 cpm/ml) in the primary T-cell lines WT29A2.7, SAS-1, JM04, and PY12B2. In contrast, significant RT production was detected in MT-2 cells only when they had been inoculated with the SI isolate PK2, in keeping with the reports of others.

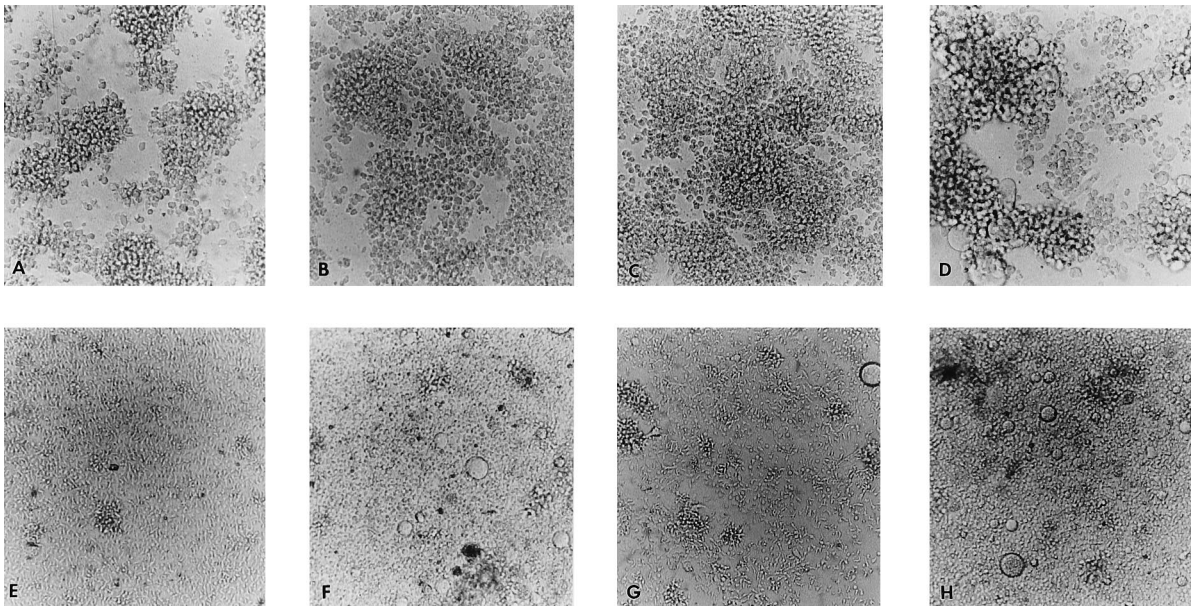

FIG. 1. HIV-1-induced syncytium formation in MT-2 and SAS-1 cells. MT-2 (A, B, C, and D) and SAS-1 (E, F, G, and H) cells were inoculated with isolates of HIV-1 and monitored for syncytium formation. (A and E) Uninfected control. (B and F) TC354. (C and G) PK1. (D and H) PK2. Photomicrographs (×100) were taken 8 days postinoculation.

The replication kinetics of each isolate varied according to the T-cell line inoculated. In MT-2 cells, RT activity and syncytium formation were strongly associated, but in all other cell lines, the presence or absence of syncytia did not correlate with the level of RT activity and virus replication (Table 1 and Fig. 2). As shown in Fig. 2, the level of replication of all three isolates was low in the PC5 cell line, and this was perhaps due to the poor condition of the cells, because in subsequent experiments the PC5 line was able to support HIV-1 replication and produce syncytia.

The study was extended to include four additional HIV-1 isolates: TC408 and TC471 (SI) and TC433 and TC440 (NSI). In this experiment (Fig. 3), all seven isolates were inoculated onto SAS-1 and MT-2 cells, and RT production and syncytium induction were assayed. HIV-1 replication occurred in SAS-1 cells infected with all three SI and four NSI isolates. There was no significant difference in the levels of replication of the three SI viruses in SAS-1 and MT-2 cells ($P > 0.1$), while the four NSI viruses clearly replicated better in the SAS-1 cells than in the MT-2 cells ($P < 0.001$). Only one of the four NSI isolates (TC433) produced a low but significant level of RT activity in MT-2 cells ($P < 0.05$) without the formation of syncytia. Importantly, syncytium formation occurred in all triplicate cultures of SAS-1 cells inoculated with each NSI isolate (TC354, PK1, TC433, and TC440) but in none of the MT-2 cultures, further confirming the results of the earlier experiment. As expected, MT-2 and SAS-1 cultures inoculated with the SI isolates PK2, TC408, and TC471 also developed syncytia. In a repeat experiment, in MT-2 cells the level of replication of isolate TC408 was high while that of TC433 was negligible.

The experiments described above were repeated up to six times, and three additional primary T-cell lines were also examined. The results (data not shown) confirmed that the ability of the NSI isolates to induce syncytium formation in primary T-cell lines within 4 to 8 days of culture is both consistent and reproducible. The possibility that our results with NSI isolates were due simply to contamination with an SI virus was ruled out by the failure of the NSI viruses to produce syncytia in MT-2 cells in 20 days of culture. Blocking of both virus replication and syncytium formation by a monoclonal antibody to CD4 (Leu3a) further confirmed the role of HIV-1.

No correlation was noted between syncytium induction and the expression of the surface markers CD4, CD26, and CD29, which was similar on all primary T-cell lines.

Effect of feeder cells and/or PHA. Maintenance of normal T-cell lines in vitro requires some form of activation, and we investigated whether syncytium induction in SAS-1 cells by NSI HIV-1 isolates may be an artifact related to the culture conditions employed. An experiment was performed in which fresh feeder cells and/or PHA was added to MT-2 cells and to Ficoll-Paque-purified SAS-1 cells immediately before inoculation with HIV-1. The results demonstrated that syncytia were consistently observed in HIV-1-inoculated SAS-1 cells cultured without additional feeder cells or PHA, and no further enhancement by the feeder cells or PHA was detected by microscopy (Table 2). Syncytium formation in MT-2 cells was also not affected, and NSI isolates retained the NSI phenotype in MT-2 cells even in the presence of feeder cells and/or PHA. It is therefore concluded that the induction of syncytia in the SAS-1 primary T-cell line by HIV-1 isolates classified as NSI in the MT-2 assay is not artifactual.

Subculture of HIV-infected SAS-1 TCF to SAS-1 or MT-2 cells. SAS-1 cells inoculated with three HIV-1 isolates (TC354, PK1, and PK2) developed syncytia with each isolate. TCF harvested at days 4, 7, and 10 of culture was passaged into fresh SAS-1 cells, which were monitored over 14 days for syncytium formation. None of the day 4 TCF induced syncytium formation when transferred. However, both day 7 and day 10 TCFs from all three isolates induced syncytia when transferred to fresh SAS-1 cells, indicating successful transmission of syncytium induction in the primary T cells. The time of appearance of syncytia in primary T cells, usually at 4 to 8 days postinoculation, indicated that syncytium induction was associated with virus replication rather than was caused directly by the virus inoculum.

Aliquots of the same TCF described above were also sub-

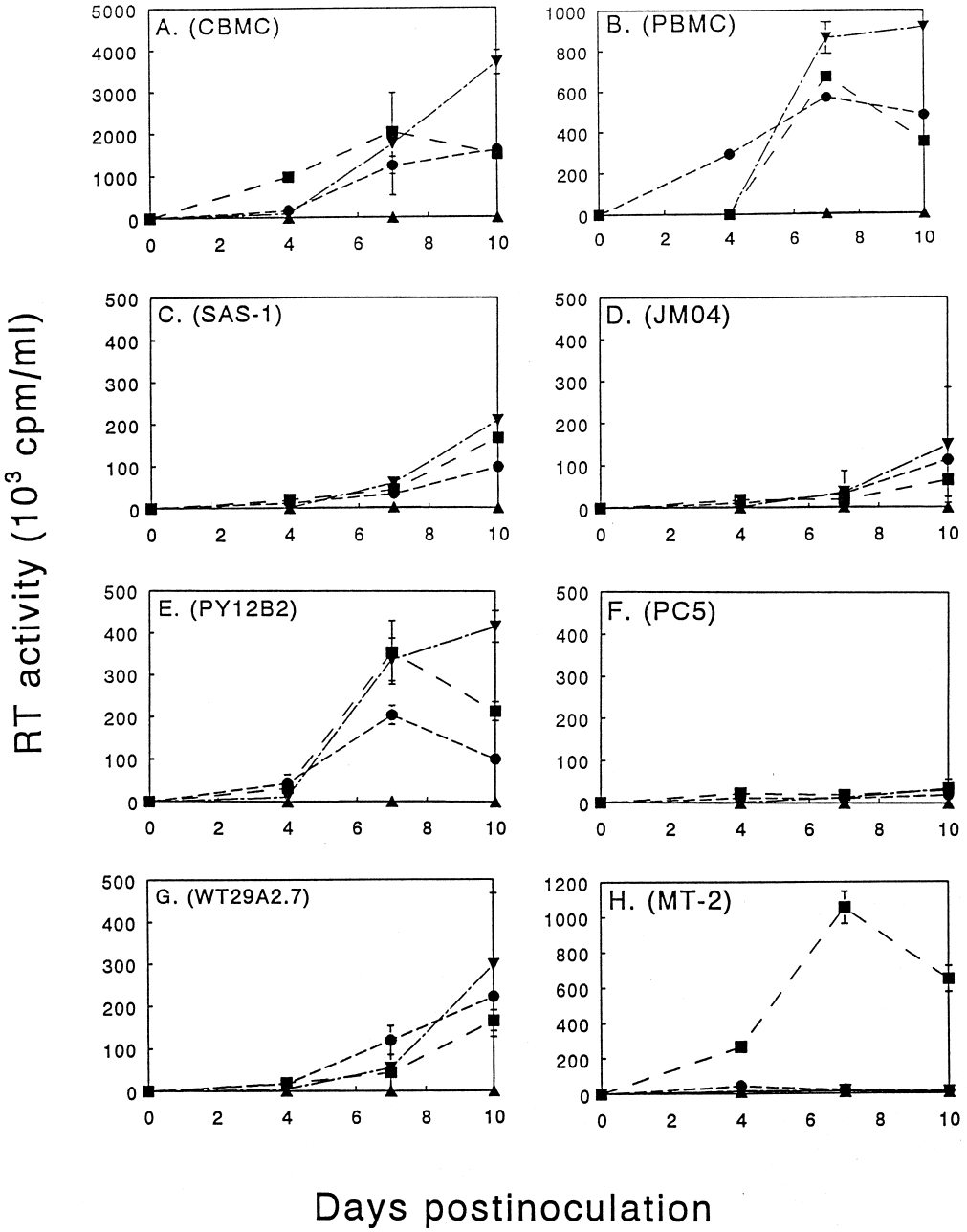

FIG. 2. Comparison of the replication kinetics of three HIV-1 isolates in CBMCs, PBMCs, and other T-cell lines as indicated. Cells were uninfected (▲) or were inoculated at a MOI of 1 with HIV-1 isolate TC354 (●), PK1 (▼), or PK2 (■). Virus replication was assessed by RT activity measured on days 4, 7, and 10 of culture and was expressed as the mean ± standard deviation of triplicate wells.

cultured in MT-2 cells. Unlike the subculture in SAS-1 cells, only the TCF (days 4, 7, and 10) from PK2-infected SAS-1 cells induced syncytium formation on transfer to MT-2 cells. Day 4, 7, and 10 TCFs from TC354- and PK1-infected SAS-1 cells did not induce syncytia in MT-2 cells in the 20-day observation period, in spite of the fact that syncytia were present at the time the TCFs were harvested. These results indicate unequivocally that the syncytia observed in the primary T-cell lines were not induced by a low level of contaminating SI virus in the NSI pools.

Sequence analysis of the V2 and V3 regions. Twelve clones were obtained for the V2 region of each of the TC354, PK1, and PK2 isolates, and the sizes of the inserts were confirmed by restriction enzyme analysis. Three clones from each virus were selected for sequencing, and the results are shown in Table 3. In comparison with the sequence patterns described (7), isolate TC354 clearly fits the NSI pattern, because it had extensive gaps in the hypervariable region, positions 29 to 46, and had an N-linked glycosylation site at position 33 but not at position 39 (arrows in Table 3).

The sequence pattern of the three clones of the PK1 isolate contained an additional charged residue in the 29 to 46 region, had an N-linked glycosylation site in the form of the motif NXT/S at position 39, and had fewer gaps in this region than

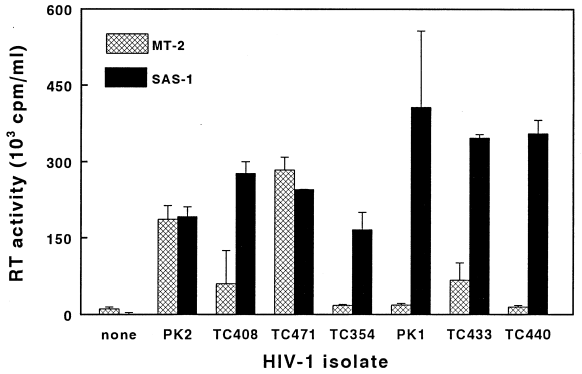

FIG. 3. Replication capacity of SI (PK2, TC408, and TC471) and NSI (TC354, PK1, TC433, and TC440) isolates in MT-2 and SAS-1 cells. HIV-1 isolates were inoculated at a MOI of 1 onto MT-2 or SAS-1 cells. Virus replication was assessed by RT activity measured on days 4, 7, and 10 of culture. Results are peak RT activity expressed as the mean ± standard deviation of triplicate wells of a single experiment.

TC354. The PK1 virus sequence clearly fell into the NSI category, and these differences from the stable NSI genotype categorized it as a switch NSI variant.

The V2 sequence of the PK2 isolate (Table 3) had an NXT/S motif at positions 33 and 39 and was further distinguished from PK1 by the presence of two charged residues in the 29 to 46 region and by showing considerably fewer gaps. Overall, the PK2 V2 region sequence most closely resembled those reported for the SI category.

Analysis of clones of the V3 region (Table 4) showed the presence of a charged amino acid residue (R) in position 11 (arrow), characteristic of the SI genotype, in the sequences of the PK2 isolate only. The sequences of PK1 and PK2, while different, showed distinctive homology in having Q and K in common at positions 28 and 35, respectively, in keeping with their derivation from the same patient.

DISCUSSION

The association of the SI and NSI phenotypes, as assessed by the MT-2 syncytium assay, with patterns of amino acid sequences in the V1-V2 and V3 regions (6, 7) provided exciting new tools for the investigation of HIV-1 infection in patients. The present study was based on seven isolates of HIV-1 from Australian patients which were classified as having the SI or NSI phenotype in MT-2 cells. The most significant finding was that the four isolates with the NSI phenotype, as well as three SI isolates, induced syncytium formation in a panel of five primary $CD4^+$ T-cell lines established from three normal HIV-seronegative donors. Syncytium induction in these normal donor-derived cell lines was consistent and reproducible. SAS-1 cells remained susceptible for over 52 weeks of culture. Two clones of the colony-derived cell lines (SAS-1 and WT29A2.7) produced syncytia, confirming that $CD4^+$ T cells were the target cells, and these NSI isolates were not strictly monocytotropic, as has been sometimes reported (22). Four of the T-cell lines were generated in response to either infectious or inactivated HIV-1, and this is comparable with the exposure of T cells to HIV-1 in infected patients. However, assays by PCR and cocultivation revealed no evidence of HIV-1 in these cell lines. The absence of HIV-1 from the T-cell lines is biologically interesting and may have resulted from either resistance to infection or elimination of the virus genome from the cells (29). It should be noted that syncytia also formed in the PC5 cell line, which was generated in response to Epstein-Barr virus, further reducing the likelihood that syncytium induction was related to generation of the T-cell lines in response to HIV-1.

The possibility that susceptibility to syncytium induction was somehow modulated by the methods involved in generating and maintaining the T-cell lines was also excluded. Although syncytium induction has long been reported in blast mononuclear cultures from normal donors (20), many reports preceded detailed definition of SI and NSI variants. It is generally accepted that NSI isolates may grow to high titer without producing microscopic cytopathology (11, 21). This is believed to be the first report providing unequivocal evidence that isolates with the NSI genotype and phenotype in MT-2 cells actually have the capacity to induce active syncytium formation in primary cloned $CD4^+$ T cells derived from normal donors. In fact, the cell lines of the type used in the present study could well be viewed as a useful model for studies of HIV-1-cell interactions, having more in common with T cells in infected patients than has the MT-2 line, which is transformed by and produces human T-cell leukemia virus type 1.

It was considered essential that the relevant sequences of the HIV-1 isolates employed in this study be compared with those characterized by the Amsterdam group (6, 7). The TC354, PK1, and PK2 isolates were selected for this purpose because they were studied in tissue culture in the greatest detail, and the PK1 and PK2 isolates had additional value in being recovered from the same individual. Biologically cloned provirus DNA of each isolate was used in order to reduce genome variation. In addition, the TCF from each microtiter culture used for sequencing was shown to contain virus exhibiting the appropriate SI or NSI phenotype in MT-2 and SAS-1 cells. The sequence data for the V2 region clearly identify the TC354 and PK1 isolates as NSI and PK2 as SI in terms of the criteria established by the Amsterdam group. It is important in this regard that a recent report (26) found no significant correlation between the V2 sequences and the virus phenotype. The reasons for this apparent discrepancy are not immediately apparent, but clearly further data are required in order to clarify this issue.

Four lines of evidence support the interpretation that the induction of syncytia in the primary T-cell lines was not due to contamination with SI virus. First, no syncytia occurred in 3 weeks of cultivation of TC354 and PK1 in MT-2 cells. Second,

TABLE 2. Effect of feeder cells and/or PHA on HIV-1-induced syncytium formation in MT-2 and SAS-1 cells[a]

HIV-1 isolate	Cell line	Syncytium induction with treatment of cells[b]			
		None	Feeder cells	PHA	Feeder cells and PHA
None	MT-2	−	−	−	−
	SAS-1	−	−	−	−
TC354	MT-2	−	−	−	−
	SAS-1	+	+	+	+
PK1	MT-2	−	−	−	−
	SAS-1	+	+	+	+
PK2	MT-2	+	+	+	+
	SAS-1	+	+	+	+

[a] MT-2 and SAS-1 cells were purified on Ficoll-Paque 24 h prior to inoculation with HIV-1. Feeder cells and/or PHA was added at the time of inoculation with HIV-1 to triplicate wells, which were monitored for syncytia for 14 days.

[b] Wells were scored as follows: −, no syncytia; +, syncytia observed.

TABLE 3. Alignment of V2 sequences of SI and NSI isolates with the consensus sequence[a]

Isolate	1 10 20 30 ↓ ↓40 50
Consensus B	CSFNITTSIRDKVQKEYALFYKLDVVPID•NDNTS••••••••••YRLISC
SI; PK2 (3 of 3 clones)	-------N----M----------I------•NENNTSNRNYTR••••-------
NSI	
PK1 (3 of 3 clones)	-------N--G-M---N-----H-I----••••••••RNYTS•••----N-
TC354 (3 of 3 clones)	-----G-----------F-----I-------•••N-S••••••••••------

[a] For ease of comparison, the consensus B sequence is set as described in reference 7. Similarity is shown as dashes, and gaps are shown as dots.

infection of cultures of the primary T-cell line (SAS-1) by TC354 or PK1 isolates induced syncytia, but the TCF from these cultures did not produce syncytia on subculture in MT-2 cells, as would be expected if the syncytia were due to SI virus contamination. Third, the biologically cloned viruses retained their phenotype in cultures of MT-2 cells and primary T-cell lines. Fourth, the sequencing results with the biologically cloned HIV-1 proviruses confirmed that the TC354 and PK1 isolates fell into the NSI genotype category described by the Amsterdam group. Collectively, this evidence strengthens the interpretation that the TC354 and PK1 isolates are genuine NSI viruses as classified by the MT-2 assay. The reproducible induction of syncytia by these two isolates in five primary T-cell lines and syncytium induction in the SAS-1 line by two other isolates with the NSI phenotype (TC443 and TC440) allow the conclusion that HIV-1 isolates which have the NSI phenotype in MT-2 cells do indeed have the capacity to induce syncytia in primary T-cell lines.

A significant role for SI variants of HIV-1 in the pathogenesis of HIV infection and of AIDS was suggested by the increased rate of loss of $CD4^+$ T cells and the more rapid progression to AIDS following the appearance of virus of the SI phenotype in peripheral blood (12, 17). An increased direct cytopathic effect on $CD4^+$ T cells associated with increased virulence is an attractive hypothesis to explain this acceleration in the progression to AIDS. About half of the AIDS patients tested had no detectable SI variants of HIV-1 in their peripheral blood (13), and this emphasizes the question of what processes are involved in the steady progression to AIDS in these patients without SI virus. A variety of experimental mechanisms, other than direct cytopathic effects, have been described by which HIV-1 may deplete $CD4^+$ T cells in vitro (18, 31). However, there are major difficulties in demonstrating that any of these mechanisms are effective in patients. The present work provides strong evidence that the NSI variants which appear to predominate in asymptomatic HIV-1 infection are in fact capable of depleting the $CD4^+$ T-cell levels by direct cytopathic effects in vitro and may play a similar role in patients.

Recent reports add further support to this view. First, in SCID-hu mice reconstituted with human adult peripheral blood lymphocytes, noncytopathic HIV-1 isolates caused rapid loss of $CD4^+$ T cells (16). In another SCID-hu mouse model with fetal human thymus and liver cells, the JR-CSF NSI virus depleted $CD4^+$ T cells within a few weeks (1); importantly, this model appears not to involve the generation of a cellular immune response, and the $CD4^+$ cell depletion may therefore be a direct cytopathic effect of HIV-1. In contrast, the failure of NSI viruses to deplete thymocytes in SCID-hu mice was also reported (11). The subtle variations in these reports of HIV-1 infection in the SCID-hu mouse model suggest that further clarification is needed. Furthermore, multinucleated giant cells (syncytia) were frequently observed in the brains and lungs of patients with AIDS even though the V3 loop sequences of these HIV variants resembled those of NSI isolates (5). Finally, studies of eight patients revealed the association in one patient of NSI virus with a rapid decrease in $CD4^+$ cell levels, suggesting that the switch from NSI to SI, although common, may not be essential for rapid disease progression. A strong inverse correlation was noted between virus load and the level of $CD4^+$ T cells (4).

The opportunity for infection of $CD4^+$ T cells undergoing clonal expansion in response to HIV-1 or another stimulus must be extremely broad (10, 27), and at some stage the cells must be in optimal condition for HIV-1 replication. Furthermore, it is possible that clonal expansion provides particular conditions, such as close proximity of cells, which may be more conducive to syncytium formation than those existing in vitro. Estimates of the proportion of $CD4^+$ T cells infected with HIV-1 in asymptomatic seropositive patients vary considerably (14), but it is clear that the number of cells at risk is very large (10, 27).

In any particular individual, the basic rate of destruction of infected and perhaps uninfected bystander $CD4^+$ T cells by syncytium formation would be modulated by several factors, including the genetic background of the patient, the complex interplay of relevant immunological factors, and intercurrent infections, which would play a major role in activating T cells. The particular HIV-1 variant involved would be critical, and the appearance of an SI variant may further increase the rate of destruction of $CD4^+$ T cells and accelerate the development of AIDS. If the SI potential of NSI HIV-1 variants demonstrated in the present work should also be expressed in vivo, even at a low level, it may well contribute to the steady decline in $CD4^+$ T-cell numbers over the long term.

TABLE 4. V3 sequence alignment of HIV-1 isolates with the consensus sequence[a]

Isolate	10 ↓ 20 ↓ 30
Consensus B	CTRPNNNTRKSI•HI••GPGRAFYTTGEIIGDIRQAHC
PK2 (6 of 6 clones)	----------R-•--••------L---Q------K---
PK1 (6 of 6 clones)	----------G-•--••-----LF---Q-T----K---
TC354 (5 of 5 clones)	----------G-•--••--------------------

[a] For ease of comparison, the consensus B sequence is set as described in reference 6. Similarity is shown as dashes, and gaps are shown as dots.

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