Differences in Cytopathogenicity and Host Cell Range among Infectious Molecular Clones of Human Immunodeficiency Virus Type 1 Simultaneously Isolated from an Individual

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Human immunodeficiency virus (HIV), the etiologic agent of the acquired immunodeficiency syndrome (AIDS) (2, 14, 26), is known to infect human cells which bear the CD4 surface antigen (23, 28). Such cells include T lymphocytes (2, 14, 26), monocytes and macrophages (15), B lymphocytes (30), and some cells of neuronal or glial origin (4, 8, 48). Infection of human T lymphocytes by HIV in vitro is characterized by massive cell fusion and lysis (2, 14, 26), whereas monocyteid cells appear to be much more resistant to virus-induced cytopathic effects (15). Ablation of the CD4-positive T-helper subset by HIV (17) is theorized to be the principal cause of the immune defects which characterize AIDS (10, 17). In addition, the neurological and gastrointestinal disorders observed in AIDS patients may result from direct HIV infection of cells of the brain and gut (32, 34).

Clearly, HIV possesses a wide host cell range, which may perhaps extend in some cases to non-CD4-bearing cells (4, 39). In vitro studies have shown that HIV isolates possess markedly different biological properties in terms of their ability to replicate and induce cytolysis in different host cell types (1, 7, 9, 24, 37, 45, 50). However, these studies have suffered from the disadvantage that many HIV isolates contain multiple virus genotypes (38, 44), and the observed biological effects could represent the sum of the biological properties of individual variants within a particular isolate. Furthermore, it is likely that such isolates undergo mutation or selection during isolation or propagation in cell culture. The latter is a particular cause for concern in light of the fact that immunodeficiency disease-inducing subtypes of feline leukemia viruses are replication defective and will therefore tend to be selected against during extended passage of virus-infected cells in vitro (31). Selection of HIV subtypes with distinct cellular tropisms during virus isolation in vitro has been observed (4, 15, 24), and new HIV variants have been derived in vitro upon prolonged culture of HIV-infected cells in the presence of anti-HIV antiserum (36).

Understanding of the biology of HIV and its pathogenic effects would benefit from detailed analysis of single genotypic variants of the virus. Although several infectious molecular clones of HIV derived from different patients have been described (11, 25), little attention has been paid to comparative analysis of these clones with respect to the host cell range, replicative potential, or cytopathic effects of progeny viruses. Several recent reports have demonstrated distinct biological properties of multiple HIV isolates derived from individual patients (1, 4, 5, 24, 37, 45); however, these viruses have not yet been cloned, and their structure-function relationships have not been determined. In the present study, HIV type 1 (HIV-1) proviruses were cloned from cellular DNA of CEM cells infected with HIV-1/NIT virus (CEM/NIT) (3, 38) which had previously been shown to contain multiple HIV-1 genotypes (3, 8). The activity of four progeny virus clones was assessed in a variety of cell lines, and each virus clone was found to possess distinct biological properties, including marked differences in cytopathogenicity. Our results demonstrate that HIV-1 genotypes present in an infected individual are heterogeneous both in nucleotide sequence and in biological activity.

MATERIALS AND METHODS

Cells and viruses. CD4-positive T-cell lines used in these studies were obtained as follows: CEM cells (13) were the gift of L. Montagnier; CR-10, the HIV-lysis-resistant subclone of CEM, was previously established in this laboratory (3); HUT-78 cells (16) were received from J. A. Levy; and MT-4 cells (21) were donated by S. Harada. The human monocytoid cell line THP-1 (43) was obtained from the American Type Culture Collection (Rockville, Md.), and the astrocytic cell line U-251MG (47) was the gift of D. D. Bigner. Suspension cell lines were maintained in RPMI 1640.

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medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 5% fetal bovine serum, penicillin G (100 U/ml), and streptomycin (100 μg/ml); the U-251MG cells were grown in Dulbecco modified Eagle medium (GIBCO) supplemented as described above. The NIT isolate of HIV-1 (3, 38) was propagated in CEM or CR-10 cells; the HIV-1sp2 isolate (26) (obtained from J. A. Levy) was propagated in HUT-78 cells. Single genotypic NIT virus clones (see below) were maintained in CEM cells periodically supplemented with uninfected CEM cells if necessary. Virus production was monitored by measuring reverse transcriptase activity in culture supernatants from infected cells (45) and by detecting HIV antigens in cells by using a fixed-cell indirect immunofluorescence assay (3). For infection studies, cell-free supernatants from virus-producing cultures were filtered through 0.45-μm-pore-size Millex-HA filters (Millipore Corp., Bedford, Mass.); the virus was sedimented by centrifugation at 10,000 rpm for 2 h at 4°C in a JA-10 rotor (Beckman Instruments, Inc., Fullerton, Calif.), suspended in medium to give a 100-fold concentration, and tested for reverse transcriptase activity and infectivity on CEM cells. Virus preparations were then standardized to contain 100,000 cpmp of reverse transcriptase activity per ml.

Preparation and analysis of cellular DNA and RNA. Cellular DNA and RNA were prepared by the cesium chloride-guanidinium isothiocyanate method (6) and analyzed by Southern (41) and Northern (RNA) blotting as described previously (3, 8).

Molecular cloning and biological testing of HIV proviruses. Essentially, standard techniques described by Maniatis et al. (29) were followed. Approximately 6 months after initial cocultivation of patient peripheral blood lymphocytes with CEM cells, high-molecular-weight genomic DNA was isolated from NIT virus-producing cells (38) by the cesium chloride-guanidinium isothiocyanate method, digested to completion with XbaI restriction endonuclease, and size fractionated on a 10 to 40% sucrose density gradient. DNA fragments of 9 to 20 kilobases were pooled; a 0.5-μg portion was then ligated to 0.5 μg of Xbal arms of λJ1 (gift of J. I. Mullins) and packaged in vitro by using Gigapack (Stratagene, La Jolla, Calif.). HIV DNA-containing bacteriophage λ plaques were identified by plaque hybridization with a 32P-labeled HIV DNA probe, N1G-G (44). Twenty-one HIV-positive plaques were obtained (from a total of 3 × 106) and plaque purified three times by hybridization. Six clones (NIT-A through -F) were randomly selected for further analysis. Insert DNA was subcloned into the Xbal site of plUC18 and propagated in Escherichia coli JM109. To evaluate the biological activity of HIV-1 clones, purified plasmid DNA was transfected into adherent U-251MG cells by calcium phosphate coprecipitation (18) or into CEM cells by the DEAE-dextran method (27). Five of the six HIV proviruses (NIT-A through -E) gave rise to progeny virus after 3 days, as judged by indirect immunofluorescence staining of transfected cells for HIV antigens and confirmed by virus rescue experiments with MT-4 cells. Clone NIT-F was biologically inactive.

Infectivity studies. For suspension cell cultures, 106 cells were adsorbed overnight with the appropriate HIV clone (100,000 cpmp of reverse transcriptase activity per 106 cells

FIG. 1. Restriction endonuclease map of infectious molecular clones of HIV-1 derived from an individual with lymphadenopathy. All clones contain a full-length HIV-1 provirus (thin lines) flanked by cellular sequences (dark lines). Asterisks denote sites present in NIT-E but missing in other viruses. Restriction enzyme cleavage sites: Ba, BamHI; BgIII; E, EcoRI, H, HindIII; K, KpnI; P, PstI; P, PsI; P, PvuII; S, SacI; Sa, SalI; Sm, SamI; Xb, Xbal; Xh, XhoI. kb, Kilobases; LTR, long terminal repeat.
per ml), washed two times with phosphate-buffered saline, suspended in fresh medium, and seeded in duplicates in 24-well plates at $4 \times 10^5$ cells per 2 ml. Monolayer cells were seeded directly in 24-well plates in triplicate, adsorbed with virus as described above, and supplemented with the appropriate medium. At the designated time intervals, 1-ml portions of suspension cell culture, or the entire well content from the monolayer culture, were removed to determine total cell count, viability, reverse transcriptase activity in culture supernatant, and HIV antigen expression in cells by the indirect immunofluorescence method.

**Standard analytical assays.** Expression of HIV-1-specific proteins in infected cells was determined by indirect immunofluorescence assay, using smears of acetone-fixed cells as described previously (3, 8). HIV-1 replication in infected cells was determined by measuring viral reverse transcriptase activity in 50-fold-concentrated cell culture medium (3, 8). A standard cell fixation and electron microscopy procedure (3) was used to obtain electron micrographs of the progeny HIV-1 particles produced by cells infected with the molecular clones of HIV. The viability of cells infected with HIV-1 clones was determined by the trypan blue exclusion method as described elsewhere (3, 8).

**Chemicals and radiochemicals.** Enzymes for recombinant DNA experiments were purchased from New England BioLabs, Inc. (Beverly, Mass.), and Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). Fluorescein isothiocyanate-conjugated anti-human immunoglobulin G was obtained from Tago Immunochimicals (Burlingame, Calif.). All radiochemicals were obtained from New England Nuclear Corp. (Boston, Mass.), and other chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.).

### Results

Multiple infectious genotypes of HIV-1 in an individual. Restriction endonuclease mapping of the infectious HIV-1 clones obtained by molecular cloning of cellular DNA from NIT virus-producing cell lines CEM/NIT revealed five distinct viral genotypes (NIT-A, -C, -E, -F, and -B/D). Maps of four of these clones (NIT-A, -B, -C, and -D) are shown in Fig. 1. Clones NIT-E and -A appeared to be the most divergent of the four, with clone NIT-E exhibiting four additional restriction sites (KpnI, PstI, HindIII, and BgII) in a 2-kilobase stretch of HIV DNA (including a 3' portion of the env gene and 5' portion of the 3' orf gene) (Fig. 1).

Biological properties of infectious molecular clones of HIV-1. Four of the five infectious HIV DNA clones (NIT-A, -B, -D, and -E) were selected for detailed biological analysis in a variety of human cell lines. DNA from these clones was transfected into U-251MG glial cells, and virus-producing cell lines were established for each clone by adding uninfected CEM cells during culture as required. Electron microscopy and analysis of producer cell DNA and RNA by Southern and Northern blotting, respectively, confirmed that progeny viruses belonged to the HIV-1 family of viruses. An example of such a study for the CEM/NIT-A cell line is shown in Fig. 2.

Biological experiments performed with T-lymphoid cells (CEM, HUT-78, and MT-4), cells of monocytic lineage (THP-1), and the astrocytic cell line U-251MG included evaluation of the following: ability of different clones to infect and replicate in the selected cell lines; kinetics of infection; and ability to aggregate, fuse, and lyse cells.

![FIG. 2. Molecular and morphological analysis of progeny NIT-A virus. U-251MG cells were transfected with NIT-A DNA, and progeny virus was rescued by cocultivation with CEM cells as described in the text. CEM/NIT-A cells were analyzed after the establishment of productive infection as determined by the presence of multinucleated cells and expression of viral antigens.](http://jvi.asm.org/Download)
TABLE 1. Host cell range and biological properties of HIV DNA clones isolated from one individual.

<table>
<thead>
<tr>
<th>Passage</th>
<th>Host Cell Range</th>
<th>Biological Properties</th>
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<tr>
<td>14 days</td>
<td>HIV-1</td>
<td>U-2 OSK (47)</td>
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<tr>
<td>21 days</td>
<td>HIV-2</td>
<td>U-2 OSK (47)</td>
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<tr>
<td>28 days</td>
<td>HIV-3</td>
<td>U-2 OSK (47)</td>
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<tr>
<td>35 days</td>
<td>HIV-4</td>
<td>U-2 OSK (47)</td>
</tr>
<tr>
<td>42 days</td>
<td>HIV-5</td>
<td>U-2 OSK (47)</td>
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Note: The biological properties are determined by the ability of the clones to infect U-2 OSK cells.
FIG. 3. Appearance of various cell cultures after infection with HIV-1 clones of the NIT series and parental strain HIV-1/NIT. (a–e) Morphology of CEM cells at 3 days after exposure to equal amounts of HIV-1 (supernatants totaling \(10^5\) cpm of reverse transcriptase activity per \(10^6\) cells infected) from clones N1T-A (a), N1T-B (b), N1T-D (c), and N1T-E (d) and from HIV-1/NIT virus (e). (f–h) Morphology of MT-4 cells exposed to clones N1T-B (f), N1T-D (g), and N1T-E (h) under the same conditions as described above for CEM cells. Compare the differential effects of N1T-B and N1T-D clones on CEM cells (b and c, respectively) and MT-4 cells (f and g, respectively). (i) Morphology of THP-1 cells of monocytic origin 3 days after exposure to the parental HIV-1/NIT isolate. Magnification, \(\times 100\).

251MG (Table 1, Fig. 3e and i). Clone N1T-A was less effective than HIV-1/NIT in aggregating and fusing CEM cells (Fig. 3a). Clones N1T-B and -D showed only weak cell-agglutinating activity (Fig. 3b and c); however, clone N1T-D, but not clone N1T-B, was able to induce low-level CEM cell fusion (Fig. 3b and c). In contrast, both clones N1T-B and -D showed strong fusogenic activity in MT-4 cells (Table 1, Fig. 3f and g). Clone N1T-E had no effect on CEM aggregation and no cytolytic or fusogenic activity in any of the cell lines tested (Table 1, Fig. 3d and h).

Comparison of N1T-A and N1T-E viral clones. The above data indicated that of the four infectious viral clones tested, N1T-A and N1T-E exhibited the most divergent biological properties. Subsequent analysis of the kinetics of infection and of the cytopathic effects in CEM cells exposed to N1T-A and N1T-E clones, in comparison with the parental HIV-1/NIT virus, confirmed this finding. Clone N1T-A, similar to the parental HIV-1/NIT virus, had rapid kinetics of infection and induced massive cytopathic effects (Fig. 4). In contrast, CEM cells exposed to N1T-E showed early signs of infection, as determined by the detection of viral antigens by immunofluorescence staining, only after 14 days from virus challenge and were fully infected, without cytopathic effects, within 3 to 4 weeks after virus exposure. Chronically infected CEM/N1T-E cells could subsequently be maintained in culture for prolonged periods of time. It is of interest that virus production in CEM/N1T-E cell lines, as measured by the presence of reverse transcriptase activity in culture supernatants, was consistently higher than in those cell lines containing replicating N1T or N1T-A virus (not shown).
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**DISCUSSION**

**Multiple HIV-1 variants within an individual.** This study describes multiple HIV variants derived by molecular cloning from a single parental cytopathic isolate. Previous reports have demonstrated that individuals may be infected with multiple HIV strains (19, 20, 38) and indicated that HIV isolates (which generally include multiple virus genotypes [19, 38]) may possess different capacities for replication in various host cells (1, 4, 5, 9, 24, 37, 45, 50). We now show that the variant molecular clones present in the original HIV-1/N1T isolate have distinguishable genome structures and biological properties, including different host ranges, infection kinetics, replication potential, and cytopathogenicity. The parental isolate appears to represent a sum of certain of these properties (such as the combined host range of each single isolate) but masks some others (such as the low cytopathogenicity of one of the individual variants). Hence, functional analysis of any single HIV isolate must take into consideration the potential contribution (or masking) of individual variants constituting the isolate.

HIV is known to undergo rapid mutational change, particularly in the envelope gene (19, 20, 49). Data presented here highlight this property. Restriction endonuclease mapping of the HIV-1/N1T clones indicates that there is a variation in nucleotide sequence among the different HIVs found in one infected person and that this variation is most marked in the viral env gene. Furthermore, most of the differences in restriction endonuclease patterns among N1T clones seem to be concentrated in the 3' half of the env gene (Fig. 1), the region encoding the gp41 component of the env glycoprotein (35, 46). On the other hand, NIT virus clones had identical restriction endonuclease patterns throughout the entire 5.5-kilobase 5' portion of their genomes. Multiple HIV isolates from individual patients analyzed by Hahn et al. (19, 20) and von Briesen et al. (45) exhibited significant differences in their pol and gag gene maps. The lack of such differences in our HIV-1/N1T clones may indicate that these variants diverged from a common ancestor relatively recently. Analysis of the nucleotide sequences of these variants could confirm this hypothesis. It should also be noted that the cellular sequences which flanked the NIT clones were all different. This suggests that HIV behaves similarly to other known retroviruses (33) in that it does not integrate into cellular DNA in a site-specific manner.

While NIT clones appear to be closely related to each other as judged by their restriction endonuclease maps, they differed significantly in their biological activities. Even clones NIT-A and N1T-B, indistinguishable from each other by restriction endonuclease mapping, had different host cell range and cytopathogenic properties (Table 1, Fig. 3). This observation emphasizes the fact that presumably subtle mutational changes in HIV can lead to significant alterations in biological activity, as has been previously demonstrated for retroviruses which induce immunodeficiency in cats (32a).

**Low cytopathogenicity of NIT-E clone.** Among the four molecular clones of HIV-1 tested, clones NIT-A and NIT-E emerged as being the most highly divergent in their biological properties. Clone NIT-A, similar to the parental isolate, exhibited a wide host range and was highly cytopathic. Clone NIT-E induced neither giant-cell formation nor significant lysis in susceptible cells. The observed low cytopathogenicity of NIT-E was not due to limited infectivity or replication potential of this virus, as chronically infected CEM/NIT-E cell cultures were established in which most of the cells expressed HIV-1 antigens (Fig. 4A) and which replicated NIT-E virus at the same or higher levels than did NIT- or NIT-A-producing cell lines (data not shown). This suggests that the ratio of infectious to noninfectious NIT-E viral particles may be inverted and that defective particles may predominate (which could also account for the slow kinetics of infection with this virus). An alternative possibility is suggested by the fact that the phenotype of NIT-E virus is strongly reminiscent of HIV-1 variants with a mutated or deleted rev gene (12, 42). NIT-E virus differs from NIT-A virus in env, 3' orf, and probably other viral genes also. The possible contribution of such genomic differences to the biological properties of this virus is presently being investigated.

**Implications: role of HIV variants in disease.** It has been hypothesized that HIV variants may be responsible for observed differences in the manifestation of HIV-induced disease in AIDS patients (1, 5, 45). Certainly, some HIV-infected individuals may exhibit immune deficiency in the absence of neurological damage and vice versa, and it is tempting to ascribe this to the evolution of HIV variants with particular tropism and cytopathogenicity for given cell types (34). The rate of disease progression in AIDS may be related to the emergence of especially pathogenic viruses (1, 45). Our data suggest that HIV-1 variants with low and high
cytopathogenicity may coexist in some patients, perhaps reflecting a transition from one viral form to another. Close genetic relationship between the HIV-1/NIT variants may allow us to use the recombinant virus approach (22) to identify the genetic element(s) that controls the cytopathic function of HIV-1.

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LITERATURE CITED