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The Majority of Cells Are Superinfected in a Cloned Cell Line That Produces High Levels of Human Immunodeficiency Virus Type 1 Strain MN

DAVID E. OTT,* STEPHEN M. NIGIDA, JR., LOUIS E. HENDERSON, AND LARRY O. ARTHUR

AIDS Vaccine Program, Program Resources Inc./DynCorp, National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, Maryland 21702-1201

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We have isolated seven single-cell clones from an H9 culture infected with human immunodeficiency virus type 1 strain MN so that a stable producer of virus could be obtained. DNAs from these clones were examined by Southern blot analysis and found to contain between one and four proviruses per clone. One of these cell lines, Clone 4, produced high levels of replication-competent virus and contained two proviruses. Southern blot analysis of DNAs from Clone 4 revealed that, after extended culture, some of the cells had acquired additional proviruses, presumably by superinfection. Analysis of Clone 4 single-cell subclones isolated from a late-passage culture found that 14 out of 20 (70%) subclones were reinfected and that 8 out of 20 (40%) were reinfected more than once. Fluorescence-activated cell sorter analysis showed that surface CD4 levels on Clone 4 cells were appropriately down-regulated. Our results indicate that while there is significant interference to superinfection in the Clone 4 culture, it is not absolute and that superinfected cells accumulate in the culture over time in the presence of high virus exposure and extensive cell-to-cell contact. Given our data, it seems likely that superinfection can occur in vivo within the lymphoid reservoirs that harbor human immunodeficiency virus type 1 during the clinically latent period and may contribute to disease progression.

Human immunodeficiency virus type 1 (HIV-1), the etiological agent of AIDS, has been the subject of intense molecular research (3, 36, 47; for a review, see reference 16). In vivo, HIV-1 is highly variable: many different strains of HIV-1 have been isolated from patient samples (40). Furthermore, an infected person often contains a viral population that consists of many variant viruses that have diverged from the initial infecting virus, presumably during viral spread within the individual (5, 20, 53, 64, 65). Therefore, most of the viral isolates from AIDS patients, whether primary or tissue culture adapted, are complex mixtures of an HIV-1 strain consisting of many variant viruses.

Viral heterogeneity in the HIV-1 strain MN [HIV-1(MN)] has been previously characterized by our laboratory: high-pressure liquid chromatography and protein sequence analyses have found at least seven different Gag sequences in HIV-1(MN) virions (26). This finding should be considered as a minimal estimate of the complexity of this HIV-1(MN) culture since these analytical techniques may not detect small populations of variants. Consequentially, laboratory studies of HIV-1(MN) are done on complex mixtures of viruses rather than a single virus type.

Isolation of replication-competent molecular clones of HIV-1 strain IIIB (17) as well as of other HIV-1 strains and the in vitro construction of molecular clones such as pNL4-3 (1) have allowed researchers to focus on and manipulate a single, defined HIV-1 prototype. While molecular clones are powerful tools for molecular biology, biochemical analysis requires considerable amounts of HIV-1. Such amounts can be produced by transfecting molecular clones into tissue culture cells, but this method requires extensive viral replication. Thus, variation can occur during viral spread through the culture and will complicate the analysis of virion preparations made in this way. Additionally, for some prototypic HIV-1 strains, a replication-competent molecular clone has not been isolated.

Production of the virus from uncloned isolates allows HIV-1 to be prepared in amounts that are large enough for viral protein purification and characterization. However, as noted above, these preparations will start out as complex mixtures of viral variants that can sometimes complicate biochemical analyses. Furthermore, the method of large-scale viral production can also lead to increased viral variation: uninfected cells are introduced to the infected culture so that most of the HIV-1 preparation is produced by viral spread. Since reverse transcription is a low-fidelity process (48, 50), the high levels of replication during viral spread can lead to increased variation in the preparation of the virus.

In order to produce more homogeneous preparations of HIV-1(MN) for biochemical study, we isolated single-cell clones from an H9 culture infected with HIV-1(MN). The goal was to isolate a cell line that contains only one or two proviruses and produces high levels of replication-competent virus. Virus preparations from these cloned cultures will have only one or at most two types of viruses as opposed to the many types (at least seven [26]) present in our HIV-1(MN) culture. Thus, a more defined and homogeneous stock of virus can be prepared from these cloned cell lines than can be produced from uncloned HIV-1(MN)-infected cell lines.

Besides limiting the number of viral variants initially present in the culture, the elimination of viral replication is another advantage to this system. The one or two proviruses present in the cloned cells will produce a virus preparation without replication, eliminating the error-prone reverse transcriptase step. Thus, these viral stocks should be homogeneous. Furthermore, as all the cells in the culture express HIV-1, viral interference (25, 35, 54, 58) should eliminate superinfection events that would cause heterogeneity within the culture because of replication errors. In total, this type of HIV-1 culture, with its advantages, should yield consistent, uniform viral preparations.

* Corresponding author. Phone: (301) 846-5986. Fax: (301) 846-5588.

We examined seven HIV-1(MN)-infected cell lines isolated by single-cell cloning. Southern blot analysis of DNAs from these cell lines demonstrated that they had various numbers of proviruses. One of these cell lines, Clone 4, constitutively produced high levels of replication-competent HIV-1(MN) and contained two proviruses. Unexpectedly, we also found that, during extended culture, a majority of the Clone 4 cells acquire new proviruses. Thus, while interference slows the reinfection of Clone 4, it is not an absolute block to superinfection.

MATERIALS AND METHODS

Cell culture. H9 T-lymphoid cells were cultured in RPMI 1640 supplemented with 5% fetal bovine serum, 2 mM glutamine, and 100 U of penicillin per ml and 100 μg of streptomycin per ml (Life Technologies, Gaithersburg, Md.). The cell cloning of the H9 cell lines was accomplished by plating HIV-1(MN)-infected cells from our stock culture into 96-well plates at an average cell density of 0.05 cell per well along with mitomycin (Sigma, St. Louis, Mo.)-treated H9 feeder cells at 2×10^5 cells per well. For the subcloning of Clone 4, we used gamma-irradiated (cumulative dose, 5,000 rads) BS-C-1 African green monkey kidney cells as feeder cells at 1.6×10^4 cells per well. The culture supernatants were analyzed for $p24^{CA}$ as previously described (21).

Genomic DNA isolation. High-molecular-weight DNA from tissue culture cells was harvested by suspending between 10^7 and 10^8 cells in 10 ml of 50 mM Tris-Cl (pH 8.0)–100 mM NaCl–2 mM EDTA and then by lysing them in sodium dodecyl sulfate at a concentration of 0.1%. The protein in the samples was digested with 20 μg of proteinase K (Life Technologies) per ml at 42°C for at least 3 h. After the protein was digested, the DNA samples were extracted with phenol-chloroform (1:1 [vol/vol]) (Life Technologies) and then centrifuged at $3,000 \times g$. The DNA was recovered from the aqueous layer by spooling the DNA on a flamed glass rod after the addition of 2 volumes of ethanol and gentle inversion of the tube. Genomic DNA was dissolved in 50 mM Tris-Cl (pH 8.0)–2 mM EDTA.

Southern blot analysis. For Southern blot analysis, 7.5 μg of genomic DNA was digested with either *Eco*RI, *Bam*HI, *Bsp*EI, or *Ava*I; separated by electrophoresis in a 0.7% agarose gel; and then blotted onto nylon-supported nitrocellulose (BA-S NC; Schleicher and Schuell, Keene, N.H.) as described by Southern (55). The blotted filters were hybridized to the *Spe*I-*Bcl*I *gag* fragment of the HIV-1 pNL4-3 molecular clone (nucleotides 1507 to 2429 [1]) that was random primed labeled with [^{32}P]dCTP (Boehringer Mannheim). Prehybridization and hybridization with formamide and dextran sulfate were carried out as described previously (44). The blots were washed at high stringency with four 250-ml washes of 0.1× SSC (1× SSC is 15 M NaCl plus 0.015 M sodium citrate [pH 7.0])–0.1% sodium dodecyl sulfate at 65°C. The filters were visualized by autoradiography.

FACS analysis. The cells were analyzed for the presence of surface CD3 and CD4 by reaction with a Leu4 phycoerythrin-conjugated monoclonal antibody for CD3 (Becton Dickinson & Co., Mountain View, Calif.) and with Leu3a-Leu3b (Becton Dickinson) or OKT4 or OKT4a (both from Ortho Diagnostics, Raritan, N.J.) fluorescein isothiocyanate-conjugated monoclonal antibodies for CD4. The cells were analyzed on an EPICS Profile fluorescence-activated cell sorter (FACS) (Hialeah, Fla.). Isotype-matched control samples were treated with nonspecific mouse immunoglobulin G1 and G2 antibodies.

RESULTS

Molecular analysis of virion-producing clones. Seven single-cell clones, Clones 4, 25, 52, 80, 90, 114, and 116, were isolated from an H9 culture chronically infected with HIV-1(MN). All of the clones released $p24^{CA}$ and thus were infected with HIV-1; however, only three clones, Clones 4, 114, and 116, produced significant amounts of replication-competent virus as measured by growth on H9 cells (41).

The goal of the cloning was to isolate a producer with only one or two proviruses. To determine the number of proviral sequences in each cell line, high-molecular-weight DNAs from the H9 cell line and from all seven clones were analyzed by *Eco*RI Southern blot analysis. The *Eco*RI restriction endonuclease cuts the HIV-1(MN) proviral genome once at position 5758 (40). Thus, Southern blot analysis with a *gag* probe will reveal each provirus as a separate band since the size of each hybridizing band depends on the distance between the internal *Eco*RI site in the provirus and a unique *Eco*RI site in the 5′ flanking H9 genomic sequences. The results, shown in Fig. 1, revealed that the lanes with DNA from the clones contained various numbers of hybridizing bands by comparison with the H9 control lane, which had no bands. As expected, all of the bands detected by the *gag* probe were larger than the minimal expected 5,758-bp size contributed by the 5′ long terminal repeat to the internal *Eco*RI site in HIV-1(MN) (40). The blot revealed that the DNAs from the clones had differing numbers of bands: lanes with DNAs from Clones 25, 80, and 90 had only one band; the lanes containing DNAs from Clones 4, 52, and 116 had two bands; and the Clone 114 DNA lane had four bands. The Clone 4 DNA lane contained a faint band at 5.7 kb in addition to the two prominent bands at 7.3 and 8.4 kb. The DNAs from the clones were subjected to *Bam*HI Southern blot analysis to confirm the results of the *Eco*RI analysis. Since the *Bam*HI restriction endonuclease does not cut within the genome of HIV-1(MN) (40), the sizes of the restriction fragments detected in this way are determined solely by the flanking DNA. Data from the *Bam*HI analysis confirmed the *Eco*RI Southern blot data, and all of the bands detected had sizes greater than the 9.8-kb unit size (40) for HIV-1(MN) (43), as expected. However, in contrast to the *Eco*RI blot, a faint band was not present in the Clone 4 lane (43). Thus, the combined Southern blot analyses revealed that three of seven clones had only one provirus while the remaining four had more than one provirus.

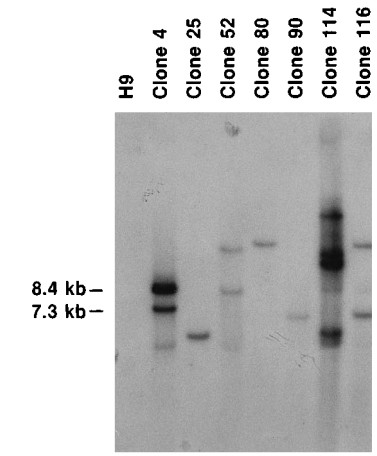

FIG. 1. *Eco*RI Southern blot analysis of DNAs from cloned HIV-1(MN)-infected H9 cell lines. The samples from each cell line analyzed are identified above their respective lanes.

Production of virus from Clone 4. Of the three clones that produced replication-competent virus (Clones 4, 114, and 116), we chose the Clone 4 line to produce virus for further analysis since it had only two proviruses and grew rapidly with good cell viability (41). Clone 4 produced high levels of $p24^{CA}$ in the supernatant. Table 1 summarizes typical data from large-scale productions of Clone 4 and H9 infected with HIV-1(MN). For these preparations, Clone 4 produced 30- to 40-fold more virus than did the H9 cultures alone infected with HIV-1(MN) and produced levels comparable to those of H9 cultures infected with HIV-1(MN) that were refed with an equal number of uninfected H9 cells according to our standard procedure for large-scale virus production. Thus, the Clone 4 cell line clearly achieves the goal of producing constitutively high levels of virus from a defined cell line that contains only two proviruses.

Clone 4 acquires additional proviruses during culture. The current understanding of superinfection interference in HIV-1 (25, 35, 54, 58) predicts that there should be little, if any, reinfection of the Clone 4 cell line; therefore, the culture

TABLE 1. Production of $p24^{CA}$ by HIV-1(MN)-infected cell cultures

Cell culture	$p24^{CA}$ (ng/ml) detected in the culture medium		
	Day 59	Day 69	Day 80
Clone 4	900	800	1,000
H9 infected with HIV-1(MN)[a]	30	20	30
H9 infected with HIV-1(MN) plus uninfected H9[b]	1,300	1,000	700

[a] Uncloned HIV-1(MN)-infected H9 cells.
[b] Uncloned HIV-1(MN)-infected H9 cells cultured with an equal number of uninfected H9 cells.

should be stable. Since reinfection is likely to increase the variability of the virus preparations, we examined DNA from Clone 4 after various periods of time in culture. *Eco*RI Southern blots of DNAs from Clone 4 cells revealed that cells which had been in extended culture displayed a light haze from 5.7 kb (the minimal size for an *Eco*RI proviral locus) to over 20 kb as well as faint bands that were not present in the earlier harvests. Figure 2 shows an example of these data; *Eco*RI Southern blot analysis of DNAs from H9 and Clone 4 cultures harvested at 106, 129, 167, and 593 days after cloning [named Clone 4(106), Clone 4(129), Clone 4(167), and Clone 4(593), respectively] was carried out by hybridizing the DNAs to the *gag* probe. The results clearly show that by comparison with the earliest sample of Clone 4, Clone 4(106), all of the later cultures had additional bands of HIV-1 sequences that appear to increase in number and intensity with time in culture. However, these new bands were less intense than the two original Clone 4 proviral bands, indicating that they were present in a fraction of the cell population. From this blot, it appears that approximately 20% of the cells may have been reinfected. However, this estimate could be too low since there is a haze in the Clone 4 lanes extending from 5.7 kb (the minimal size for the 5′ *Eco*RI fragment) to somewhat over 20 kb that may represent new proviral loci present in a small fraction of the cell population (less than 5%). While these loci are not detectable as discrete bands, in combination, they yield this diffuse signal in the blot.

Subcloning of Clone 4. Subcloning of individual cells from a late-passage Clone 4 culture was accomplished with irradiated

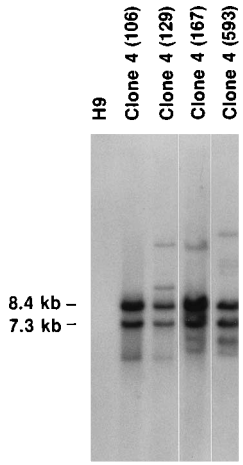

FIG. 2. *Eco*RI Southern blot analysis of DNAs from Clone 4 cultures after extended times in culture. The samples are identified above their respective lanes. The number of days in culture after cloning is indicated in parentheses.

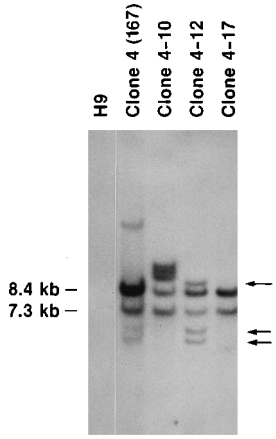

FIG. 3. *Eco*RI Southern blot analysis of DNAs from Clone 4 subclones. The samples are identified above their respective lanes.

$CD4^-$ BS-C African green monkey kidney cells. *Eco*RI Southern blot analysis of DNAs from the resulting cell lines was used to determine the proportion of reinfected cells and the number of proviruses within each reinfected cell in a Clone 4 culture. Twenty Clone 4 subclones were tested for production of high levels of replication-competent virus and were found to have the same properties as the Clone 4 cell line (41). *Eco*RI Southern blot analysis of DNAs from 3 of the 20 subclones (Fig. 3) demonstrated that they all had the characteristic 7.3-kb and 8.4-kb hybridizing bands that are seen in the parental Clone 4 sample. While DNA from one of these subclones, termed Clone 4-17, had only the two original proviral bands, DNAs from two of the other subclones, Clone 4-10 and Clone 4-12, appeared to each have three additional proviruses. The intensities of the new bands are equivalent to the intensities of the two parental Clone 4 bands, with the exception of the 8.4-kb band in the Clone 4-12 lane. This band appears to be twice as intense as the other bands and thus may be two distinct comigrating loci, with one band being the original 8.4-kb band seen in Clone 4 and the other band being a newly acquired band. *Bam*HI Southern blot analysis confirmed that, by comparison with both Clone 4 and Clone 4-17, Clone 4-10 has three new bands and Clone 4-12 has four new bands (43). Thus both Clone 4-10 and Clone 4-12 have acquired multiple new proviruses during culture and contain three and four new bands, respectively.

Clone 4(167) was the culture that was used for the subcloning experiment and is included in Fig. 3 for comparison with the subclones. Therefore, the additional proviral loci that are apparent in the Clone 4(167) population might also be found in the subclone lanes. Comparison of the *Eco*RI Southern blot patterns displayed in the Clone 4-12 and Clone 4(167) lanes (Fig. 3) revealed that these two cultures share two bands lower than 7.3 kb (highlighted by arrows). Therefore, Clone 4-12 seems to be a member of this subpopulation of cells in the Clone 4(167) culture. However, Clone 4-12 was clearly different from the majority of the subpopulation since Clone 4-12 had the additional band at 8.4 kb that comigrates with the uppermost original Clone 4 band and another band at 8.7 kb (highlighted by an arrow) that were not present in Clone 4(167). This pattern indicates that Clone 4-12 may have acquired proviruses on at least two separate occasions: a common event or events early in the growth of this culture produced the common two lower bands and a subsequent event or events produced the two higher comigrating bands that are

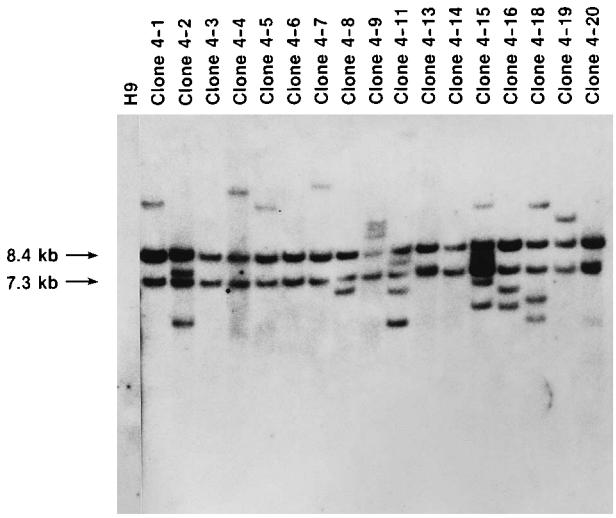

FIG. 4. *Eco*RI Southern blot analysis of DNAs from additional Clone 4 subclones. The samples are identified above their respective lanes.

unique to this clone. These data show that cells in the Clone 4 cultures appear to be susceptible to the acquisition of additional proviruses multiple times during culture.

To broaden our survey of Clone 4 subclones, we examined the remaining 17 subclones by *Eco*RI Southern blot analysis. The results shown in Fig. 4 revealed that the majority of subclones had additional hybridizing bands by comparison with the two original bands found in Clone 4 (the positions marked by 7.3 kb and 8.4 kb on the blot). The sizes of these additional fragments were heterogeneous, showing few common sizes. This absence of common *Eco*RI band sizes indicates that the acquisition of a provirus is an independent event rather than a simple selection for superinfected cells in the culture. Taken together, the data from Fig. 3 and 4 show that 14 out of 20 subclones (70%) contained new proviral sequences. Furthermore, 8 out of 20 subclones (40%) contained more than one additional proviral sequence.

Rate of proviral accumulation. The data above qualitatively suggest that cells in the Clone 4 population acquire proviruses during extended culture at a low rate (reinfection events per cell per generation) and that these events are independent. To test this hypothesis quantitatively, the distribution of subclones that contain one, two, three, or more than three reinfection events (Fig. 3 and 4 and as summarized in Table 2) can be compared with the theoretical Poisson distribution, if it is assumed that these events occur independently at a low rate and accumulate over extended periods of time in culture. The fit between the observed and theoretical results was tested with the chi-square test (67) and is presented in Table 2. The calculated values are not statistically different from the expected values ($P \leq 0.95$ and $P \geq 0.90$), supporting the hypothesis that these reinfections are random and occur at a low rate.

The rate of proviral accumulation can be estimated from the data shown in Fig. 3 and 4 and as summarized in Table 2. From these data, we can conclude that there were 1.55 new proviruses per cell (Table 2, footnote *b*). We estimate that there were 111 generations in the 167 days of Clone 4(167) culture. Therefore, we estimate that the rate of proviral accumulation per cell per generation is 0.014.

Acquired proviruses are full length. We used additional Southern blot analysis to map the proviruses in some of the subclones to determine if the resident viruses were full length.

TABLE 2. Distribution of reinfection events among the Clone 4 subclones

Reinfection event(s)	No. observed[a]	No. expected[b]	Variance (χ^2)[c]
0	6 (0.3)	4.244	0.1712
1	6 (0.3)	6.580	0.007770
2	2 (0.1)	5.098	0.3693
3	4 (0.2)	2.634	0.2689
≥4	2 (0.1)	1.444	0.1483

[a] The fraction of the total number of clones is indicated in parentheses.
[b] The number expected was calculated by determining the theoretical frequency of events, using the Poisson distribution equation [$P_n = (e^{-u}u^n)/(n!)$, where n is the number of reinfection events and u is the number of average events per subclone (31 reinfection events divided by 20 subclones is 1.55)], and multiplying it by the number of observations (20 subclones).
[c] The variance was calculated as $(x_{observed} - x_{expected})^2/x_{expected}$, where x is the number of reinfection events.

Two restriction enzymes, *Bsp*EI, which cuts in the HIV-1(MN) long terminal repeats (at nucleotides 307 and 9423) (40), and *Ava*I, which cuts in the HIV-1(MN) long terminal repeats (at nucleotides 294 and 9399) and internally at position 8917 (40), were used to analyze five clones that contained various numbers of novel proviral loci and the parental Clone 4. The Southern blot (Fig. 5) was probed with a *gag* probe, and it revealed the presence of only one band in all of the DNA samples, with the exception of that in the H9 cell line lane, which had no hybridizing bands. Furthermore, these bands were of the sizes expected for full-length HIV-1(MN): the *Bsp*EI-treated lanes had the expected 9.1-kb size, and the *Ava*I-treated lanes had a 8.6-kb size. Thus, the absence of smaller or larger bands in these Southern blots indicates that these sequences are of the proper length for full-length proviruses and do not contain any gross deletions (we estimate that we could have detected a deletion of >350 bp with these techniques).

CD4 is down-regulated in Clone 4 cells. It has been reported that HIV-1 infection results in a decrease in surface CD4 expression (12, 18, 28, 33, 59). This decrease in HIV-1 surface receptor has been proposed to be a mechanism for HIV viral interference (58). Since the Clone 4 cell line is susceptible to superinfection, it is possible that Clone 4 does not appropriately down-regulate surface CD4. Therefore, FACS analysis using the Leu3a-Leu3b antibody mixture was performed to

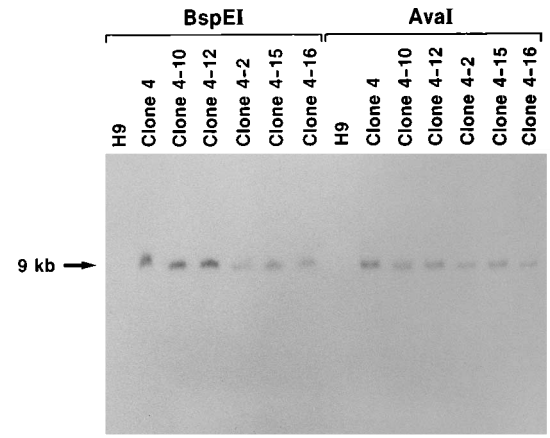

FIG. 5. *Bsp*EI and *Ava*I Southern blot analysis of DNAs from Clone 4 subclones. The samples and the restriction enzymes used for the analysis are identified above the lanes.

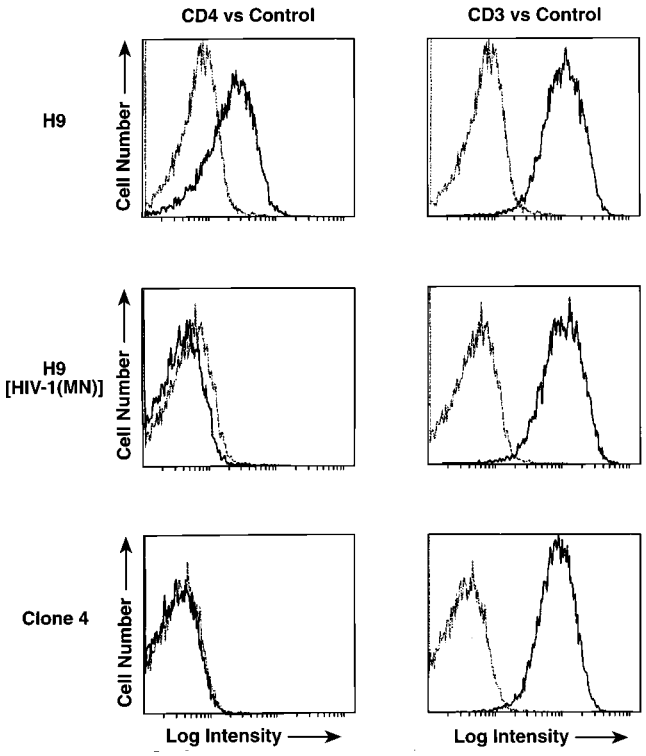

FIG. 6. FACS analysis of Clone 4 cells for surface CD4 expression. The histograms are presented with the cell numbers on the *y* axis and the log fluorescence intensities along the *x* axis. The surface marker detected is indicated above the columns, while the cells examined are labeled on the rows. In each histogram, the isotype-matched control is plotted with a broken line while the data generated by the experimental antibody are plotted with a solid line.

determine the levels of surface CD4 on H9, HIV-1(MN)-infected H9, and Clone 4 cells. The results (Fig. 6) show that the levels of CD4 on both HIV-1(MN)-infected cells and Clone 4 cells were dramatically reduced by comparison with that on the uninfected H9 cells. The surface CD4 staining of the Clone 4 population appeared to be somewhat less than that of the uncloned HIV-1 culture and was indistinguishable from that of the isotype-matched control. This could be a function of the constitutively high production of virus by these cloned cells. While CD4 levels were markedly reduced in the HIV-1-containing cell lines, all three of the cell lines had the same CD3 staining intensity, indicating that the CD4 reduction was specific as expected (28). The CD4 epitope used by the Leu3a antibody component of the Leu3a-Leu3b reagent overlaps the $gp120^{SU}$ binding site, and thus, CD4-Env complexes may not be detected by this antibody (34). The Leu3b epitope has not been determined. To confirm the down-regulation of CD4 on the surface of Clone 4 cells, FACS analysis with the anti-CD4 monoclonal antibodies OKT4, which is not blocked by $gp120^{SU}$ (34, 38), and OKT4a, which is blocked by $gp120^{SU}$ (38, 39), was carried out with similar results (data not shown). Thus, the levels of surface CD4 on Clone 4 cells are appropriately down-regulated.

DISCUSSION

In this report, seven single-cell clones isolated from an HIV-1(MN)-infected H9 cell culture were analyzed by Southern blot analysis. Our data demonstrated that the cell lines which produced little or no replication-competent virus, Clones 25, 80, and 90, had only single proviruses in their genomes. All of the cultures that produced high levels of virus, i.e., Clones 4, 114, and 116, had more than one provirus. Although these data seem to suggest a requirement for the presence of more than one provirus in a cell for efficient production of replication-competent virus, we have recently isolated cell lines with only one provirus that produce high levels of replication-competent virus (42).

Clone 4 was found to contain two proviruses and produced high levels of replication-competent virus; therefore, this clone was chosen for large-scale viral preparations. The yield from these cultures were comparable to those from HIV-1(MN)-infected cells that were refed with uninfected H9 cells. The virus produced from Clone 4 is currently being used for molecular biological and biochemical studies. Cell lines, such as Clone 4, that constitutively produce high levels of replication-competent virus from one or two proviruses are valuable reagents with many important uses. For instance, the properties of Clone 4 should make it ideal for screening of anti-HIV-1 drugs and therapies that affect the assembly stage of replication.

The stability of the number of proviruses in Clone 4 was examined by Southern blot analysis. The data showed that while Clone 4 initially contained only two proviruses, DNA from later passages had acquired new proviral sequences. Acquisition of new proviral sequences was an ongoing process, as DNA from cells at later passages had more novel bands with greater intensities than did that from cells from the earlier passages. To study this observation further, we isolated individual cells from a late-passage Clone 4 culture by single-cell cloning and analyzed the DNAs from these subclones by Southern blot. Examination of DNAs from 20 of the subclones confirmed the presence of new proviral loci in the majority (14 of 20 [70%]) of the cells in the Clone 4(167) culture. Furthermore, 8 of 20 subclones (40%) had more than one additional proviral locus. Common proviral bands in the Southern blots of the subclones were not detected. Thus, the proviruses in the subclones appear to have been acquired independently rather than as the result of selection for superinfected cells in culture.

Clone 4 releases high levels of infectious virus into the supernatant, and it is likely that these virions are responsible for the reinfection events. Similarly, Clones 114 and 116, which both produce infectious virus, also acquire low-intensity bands during culture that are similar to those of the Clone 4 pattern (Fig. 1) (43). Furthermore, Southern blots of DNAs from Clones 25, 80, and 90, which do not produce infectious virus, do not show this reinfection pattern (Fig. 1) (43). These results and the finding that the viruses are generally full length are consistent with the hypothesis that reinfection rather than gene amplification and translocation is responsible for the acquired proviruses.

The use of Clone 4 as a stably infected cloned cell line to produce homogeneous virus preparations seems to be only partially successful. Apparently, any cell culture that produces high levels of infectious HIV-1 may be unstable because of reinfection. In future experiments, to keep reinfection to a minimum, a cloned HIV-1-infected cell line should be kept in culture for only a short period of time.

Resistance to HIV-1 superinfection, viral interference, has been studied previously by several groups (25, 35, 54, 58). In these reports, cells with established infections that were challenged with cell-free HIV inocula were not superinfected. Other groups have reported that latently infected cells which produce extremely low levels of viral proteins can be superinfected at a low level (7, 32, 66). Our data clearly show that HIV-1 viral interference is not an absolute block to superin-

fection in cells that are producing high levels of virus. While a majority of the Clone 4 cells were superinfected after extended culture, 30% of the culture was not reinfected after 167 days in culture (approximately 111 cell divisions), despite the constant exposure of the cells to high levels of virus. This finding strongly suggests that there is a high degree of interference in these cultures since the rate of superinfection appears to be very low in these cultures (we estimate 0.014 reinfection event per cell per generation on the basis of our data).

Our finding of superinfection in this culture system is consistent with the observations on interference with simple retroviruses. Retroviral superinfection interference was originally described by Steck and Rubin for avian viruses (56, 57). In that paper and in subsequent works on murine, feline, and avian retroviruses (49, 52, 61), viral interference has been observed to cause an approximately 1,000-fold reduction of superinfection by comparison with that for uninfected cells. Thus, our data appear to be consistent with these previous observations: the interference block in Clone 4 cells can be overcome by constant, high exposure to the virus.

Our Clone 4 results are unique in that for the first time with either complex or simple retroviruses, a cloned cell line with a defined number of proviruses has been studied for evidence of superinfection in the long-term presence of high virus levels. The data indicate that superinfection is a rare event. Despite the low rate of superinfection, the long duration of viral challenge and the accumulation of reinfections in the culture allow us to detect superinfections with this system. This is a likely reason for the unexpected finding of HIV-1 superinfection events and their presence in the majority of the Clone 4 cells. Therefore, the difference between our findings and those of the previous HIV-1 interference studies appears to be in the two types of experimental approaches.

Another difference between our study and the other HIV-1 interference studies is that Clone 4 cells have many cell-to-cell contacts during culture while the other studies exposed infected cells only to cell-free virus. It has been shown that cell-to-cell contact is a very effective route for infection, with an efficiency that is 100 to 1,000 times greater than that of cell-free exposure to virus (13). Therefore, cell-to-cell contact in the Clone 4 culture is another likely cause for the difference between our results and those from previous cell-free interference studies (25, 35, 54, 58).

Two mechanisms for superinfection interference at the cell surface have been proposed for retroviruses: blockage of the viral receptor(s) by intracellularly produced Env (56, 57) and down-regulation of the viral receptor by viral proteins (by $gp120^{SU}$ [31, 58], Nef [19, 22], and Vpu [62, 63] for HIV-1). In both cases, interference occurs at the cell surface since CD4 receptors are no longer available to the virus. A trivial explanation for the superinfection of Clone 4 cultures is that the proviruses in the cell line are mutants that do not down-regulate the HIV receptor. Our FACS analysis clearly showed that CD4 is significantly and appropriately down-regulated while the T-cell surface marker, CD3, is not affected by HIV-1 infection, as had been expected (28). Therefore, the superinfection of Clone 4 is not due to a significant defect in HIV-1-mediated surface CD4 depletion.

A mechanism for the superinfection of Clone 4 is that the high levels of virus present in the culture can locate and utilize a few remaining receptors on the infected cells that are not blocked by $gp120^{SU}$. Two observations support this as a possible mechanism. First, it has been shown that the efficiency of infection of cells does not correlate with the level of surface CD4 expression: thus, the presence of surface CD4 does not appear to be a limiting step in the infection process (4). Second, levels of surface CD4 that are close to or below the limits of detection are sufficient to render cells infectible by HIV-1 (2). Thus, it is possible for small numbers of surface CD4 molecules on Clone 4 to be undetectable by FACS yet to be sufficient for infection.

It has also been shown that HIV-1 may enter cells that do not express CD4, by means of an alternate receptor (2, 6, 8, 9, 27, 30, 37, 51, 60). Galactosylceramide has recently been demonstrated to function as an HIV-1 receptor on neural and colonic epithelial cell lines (15, 23). While receptors other than CD4 may be used in T lymphocytes, these putative receptors appear to be blocked in infected T cells since they did not allow detectable superinfection events in the previously reported interference studies (25, 35, 54, 58). Therefore, it is unlikely that the superinfection seen in Clone 4 is due solely to the use of an alternate receptor by HIV-1.

A final possible mechanism for these superinfection events is infection without a receptor. The long-term exposure to high levels of virus and extensive cell-to-cell contact could provide for infection without any receptor (46) or by means of a nonspecific fusion receptor that requires cell-to-cell contact to function (24). The level of reinfection by this means may be too low to detect in experiments that use cells with CD4 present on the surface of the cell or when cell-free inocula are used.

The ability of infected cells to be superinfected by HIV-1 under conditions of high levels of virus and multiple cell-to-cell contacts has important implications for HIV-1 variation and ultimately disease progression. It has recently been shown that HIV-1 resides in the infected lymphoid organs during clinical latency (11, 14, 45). These viral reservoirs appear to be the sites at which HIV-1 evolves by variation and becomes increasingly pathogenic to the infected individual. Since these sites contain high levels of virus with multiple cell-to-cell contacts, conditions that are similar to those for Clone 4, it is likely that there is a significant amount of superinfection in these organs. Multiple infections of cells also cause a high rate of recombination (10, 29), which in turn increases the amount of viral variation by allowing the reassortment of the many viral variants. The end result should be an increase in intrahost evolution that is a hallmark of HIV-1 disease progression (5, 20, 53, 64, 65). Thus, superinfection may play a larger role in the host-virus interaction than previously appreciated.

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