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Homologous Interference Resulting from the Presence of Defective Particles of Human Immunodeficiency Virus Type 1

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Defective particles are naturally occurring virus mutants that lack one or more genes required for viral replication. Such viruses may affect positively or negatively the symptoms of the disease. Thus, it is of great interest to measure the role played by defective particles in the process of human immunodeficiency virus (HIV) infection since accumulating evidence indicates that a great proportion of HIV genomes are defective. We used defective particles produced by two stable cellular clones (UHC-8 and UHC-18) to investigate whether they can affect replication of infectious viral particles generated by a human T-cell line transfected with a molecular HIV-1 clone. Progeny virus harvested from UHC-8 cells has no reverse transcriptase and integrase proteins, while UHC-18 has no reverse transcriptase protein. We demonstrate here that coinoculation of a T-lymphoid cell line and of peripheral blood mononuclear cells with defective and infectious particles leads to a dramatic inhibition of virus replication. Defective particles do not interfere with virus production from proviral DNA. Rather, the inhibition of reinfection events seems to be their mechanism of action. This model closely parallels the in vivo conditions and demonstrates that defective particles may limit the spread of infection and progression of the disease by reducing the yield of infectious virus.

A feature of human immunodeficiency virus (HIV), shared by many RNA viruses, is the enormous genetic heterogeneity (1, 15, 56). This is mainly associated with the high misincorporation rate of the reverse transcriptase enzyme (approximately 10^{-4} per base per cycle), which is similar to rates of some other viral polymerases (18, 23, 39). The HIV genome is less than 10^4 bases long, which means that one misincorporation per genome per replication is occurring. Since this process is not subject to proofreading, misincorporations produced during minus-strand DNA synthesis will persist. A major difference between HIV and other RNA viruses remains in its ability to persist despite a strong immune response. It is therefore plausible to assume that, as for visna virus (42), variants accumulate in vivo. A multitude of phenomena, including deletions, duplications, and recombinational events, also accompany the process of reverse transcription and affect the viral genome. It has therefore been proposed that HIV isolates be referred as quasispecies, i.e., populations of highly related genomes (44). This unique concept was first described by Eigen (21) and later applied to RNA viruses by Holland and coworkers (17, 58). Such genomic heterogeneity is probably advantageous to the virus, allowing it to adapt to the environment and escape immune surveillance. This genomic diversity could also lead to the occurrence of defective virus particles (for a review, see reference 5). Defective particles are produced during the replicative cycles of nearly all animal viruses (32, 33, 48) and were first observed following propagation by undiluted passages of some animal RNA viruses (28, 45, 64). Such particles possess a genome which contains deletions of the infectious virus genome and can then replicate in susceptible cells only when coinfected with fully infectious viruses. In vitro experiments have demonstrated that defective viruses can interfere with the replication of fully infectious particles (for reviews, see references 32 and 48).

Previous studies have determined that as few as 1 per 1,000 to 1 per 10,000 total peripheral blood mononuclear cells (PBMCs) from infected individuals carry HIV-1 DNA (54). The frequency of cells carrying transcriptionally active HIV-1 DNA was demonstrated to be at least 1 to 2 orders of magnitude lower, ranging from 1 per 10^4 to 1 per 10^5 cells (27). These studies have indicated that only few cells are infected with HIV-1 and that a much lower percentage of such cells actively express viruses. If the frequency of cells containing viral DNA is so low and if most of them are transcriptionally silent, this observation might be associated with the fact that many defective particles are produced and that some integrated viral DNA is also defective. Integrated viral DNA (provirus) present in patients with AIDS was investigated, and it was determined that with regard specifically to the *tat* gene, a great proportion was defective (44). The frequency of defective genome present only in the first exon of *tat* was of the order of 10 to 15%. The first exon of *tat* represents 2.3% of the total coding capacity of HIV-1. Assuming that the remaining genome is as defective as the *tat* gene, and there is clear indication that this is the case (23), it is obvious that the great majority of proviruses may be functionally defective. Another report has demonstrated that defective viral genomes represent 42% of PCR-derived clones originating from uncultured brain tissue of a patient with AIDS dementia complex (41). Results from these two molecular studies have indicated that a great proportion of integrated viral DNA in HIV-1-infected individuals is defective. Additional evidence came from a study in which a high frequency of cellular clones, originating from a cell line chronically infected with HIV-1, produced defective particles (7). More recently, levels of circulating HIV-1 RNA in plasma exceeded by an average of 60,000-fold titers of infectious particles as determined by quantitative competitive PCR and endpoint dilution culture, respectively (49). Since biologically replication-competent viral DNA has been isolated, HIV may possess alternative mechanisms to generate

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replication-competent viruses from defective virus particles. Fully infectious viruses isolated from infected individuals may be produced through complementation of defective genomes or recombination between two of them. Genetic homologous recombination is frequently detected in the retrovirus life cycle as a result of the packaging of two RNA molecules in one retrovirus particle (for a review, see reference 31). Recently, replication-competent HIV-1 particles were recovered following genetic recombination between replication-defective virus particles (8, 14, 36).

A number of replication-defective HIV-1 mutants, obtained by site-directed mutagenesis, were shown to interfere very efficiently with the production of infectious viral particles. The generation of such dominant negative variants in either the *gag* (63), *rev* (43), *tat* (24, 47), or *env* (57) gene was demonstrated to exert an inhibitory effect on in vitro replication of infectious virus particles. These molecular studies strongly suggest that defective viruses can modulate replication of fully infectious HIV-1 particles. However, it is important to specify that a low to moderate level of inhibition was achieved in these studies and that cotransfection of molecularly cloned viral DNA represents ideal conditions that do not parallel physiological conditions.

We sought to define a tissue culture system that would approximate the in vivo setting in order to measure the interfering effect on the replication of infectious viruses by using defective particles generated by two different cellular clones chronically infected with HIV-1. We have thus developed a cellular model with which to study regulation of HIV-1 replication by defective viral particles that we believe reflects more accurately virus-virus and virus-cell interactions as they may occur in vivo. This system allowed us to demonstrate that the presence of defective particles dominantly interfered with the replication of infectious viruses in a dose-dependent fashion.

MATERIALS AND METHODS

Cells and DNA. Cells were maintained in complete culture medium made of RPMI 1640 medium supplemented with 10% fetal calf serum, glutamine (2 mM), penicillin G (100 U/ml), and streptomycin (100 μg/ml). The lymphoblastoid $CD4^+$ T-cell line Jurkat E6-1 was obtained from the American Type Culture Collection (Rockville, Md.). OM-10.1, which was cloned from HL-60 promyelocyte cells that had survived an acute HIV-1 infection (10), were provided by the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases (NIAID). PBMCs were isolated from normal healthy donors by centrifugation over Ficoll-Hypaque (Pharmacia). Heparinized blood was kindly provided by the Canadian Red Cross Society (Quebec City, Quebec, Canada). PBMCs were activated with purified phytohemagglutinin (1 μg/10^6 cells) (Wellcome Diagnostics) and were maintained for extended periods in complete culture medium supplemented with 30 U of recombinant human interleukin 2 per ml. This reagent was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID; human interleukin 2 was obtained from Maurice Gately, Hoffmann-La Roche Inc. (38). The cells were passaged 24 h prior to the experiments to ensure that the cells were in the log phase of growth. The T-cell-tropic HIV-1 strain, pHXB2d (22), was used to produce infectious virus particles.

Transfection and production of infectious viral particles. Stocks of infectious virus for clone HXB2d were prepared by transfecting plasmid DNA into the Jurkat E6-1 lymphoblastoid T-cell line. Transfection of pHXB2d linearized with *Xba*I was achieved by electroporation according to a standard protocol (11). Stocks of infectious viral particles were prepared from fresh unfrozen cell-free supernatants originating from transfected Jurkat E6-1 cells. Titration of infectivity was performed by a terminal-dilution microassay using the highly susceptible MT-4 cell line (26). Endpoint titration was carried out in flat-bottomed microtiter wells, using four parallel series of 10-fold dilutions. After 5 to 7 days of incubation, cells from each well were stained for immunofluorescence as described previously (61). Cells were scored as positive or negative, and the 50% tissue culture infective dose was calculated by the method of Reed and Muench (52). Fresh unfrozen clarified supernatants from Jurkat E6-1 cells transfected with pHXB2d were used as a source of infectious particles in coinfection experiments.

Generation of defective viral particles. Defective particles used in these experiments were produced by cellular clones UHC-8 and UHC-18. The premonocytoid cell line U937 was chronically infected with HIV-1_{IIIB}, and several cellular clones carrying defective HIV-1 genomes were generated (7). These cells were kindly provided by Mark A. Wainberg (Lady Davis Institute, Montreal, Quebec, Canada). Particles originating from UHC-8 and UHC-18 cells have been previously demonstrated to be unable to productively infect several cell types (H9, MT-4, U937, and PBMCs) (8). Viral stocks of defective particles were made as follows. Clones UHC-8 and UHC-18 were resuspended in fresh complete culture medium at a concentration of 10^6 cells per ml. The next day, clarified supernatants were harvested, filtered through a 0.45-μm-pore-size membrane, and stored at −85°C in small aliquots until used. The quantitation of defective viral stocks was carried out with the use of a commercial enzyme-linked immunosorbent assay that can detect the main viral core p24 protein (Organon Teknika, Durham, N.C.).

Western immunoblot analysis. Thirty-milliliter portions of cell-free culture supernatants from HIV-1-infected cells (U937-HIV-1_{IIIB}, UHC-8, and UHC-18) were centrifuged at 12,000 rpm for 2 h. The virus pellets were resuspended in 100 μl of radioimmunoprecipitation assay buffer consisting of 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1% deoxycholate, 1% sodium dodecyl sulfate (SDS), and 2 μg of phenylmethylsulfonyl fluoride per ml. The homogenate was vortexed and incubated for 45 min on ice with intermittent mixing. Protein content was determined by the commercial bicinchoninic acid protein assay reagent (Pierce, Rockford, Ill.). Ten micrograms of viral proteins was electrophoresed on an SDS–10% polyacrylamide gel, using the buffer system of Laemmli (37). Thereafter, the gel was transferred to a nitrocellulose sheet and incubated with a mouse anti-HIV-1 reverse transcriptase (p66/p51) or a rabbit anti-HIV-1 integrase (p32) antibody. A second horseradish peroxidase-conjugated antibody, specific for the first antibody, was used. Immunoreactive bands were visualized by using the ECL (enhanced chemiluminescence) detection system (Amersham, Arlington Heights, Ill.). The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID: monoclonal antibody to HIV-1 reverse transcriptase protein (p66/p51) from Epitope Inc. and the antiserum to HIV-1 integrase (residues 1 to 12) from Duane P. Grandgenett.

Monitoring of HIV binding to cells by flow cytometry. Jurkat E6-1 cells (10^5) were centrifuged, resuspended in 100 μl of RPMI 1640 containing 2% fetal calf serum (RPMI-2), and incubated for 30 min at 4°C with increasing amounts of defective particles (5, 50, 500, and 5000 pg of p24) originating from clones UHC-8 and UHC-18. The cells were centrifuged, washed twice in phosphate-buffered saline (PBS), and resuspended in RPMI-2 containing saturating concentrations of the anti-CD4 Leu-3A antibody. After incubation on ice for 30 min, the cells were washed twice in PBS, fixed in 1% (vol/vol) paraformaldehyde, and analyzed by flow cytometry.

HIV-induced cell-cell fusion assay. Cell lines used were washed twice in PBS and resuspended in complete culture medium, and uninfected MT-4 cells (26) were cocultured with UHC-8 or UHC-18 cells at a ratio of 10:1 (uninfected/infected cells) for 24 h at 37°C in a 96-well microtiter plate. Syncytia were visualized with an inverted light microscope. Control cultures consisted of UHC-8 or UHC-18 cells only.

Virus internalization assay. We optimized experimental conditions to measure levels of internalized HIV particles by using a slightly modified version of the technique described previously by Tang and Levy (59). In brief, Jurkat E6-1 cells (5×10^5) were incubated with defective viral particles generated by UHC-8 and UHC-18 cells. After 90 min at 37°C, cells were extensively washed two times with PBS and treated with 25 μg of trypsin (type XII-s; Sigma, St. Louis, Mo.) per ml for 10 min at 37°C to remove any residual uninternalized virus. Digestion was stopped by the addition of complete culture medium. Finally, cells were lysed with Triton X-100 (0.5%, final concentration) and assayed for viral p24.

PCR amplification. Jurkat E6-1 cells (10^5) were washed in PBS and incubated for 12 h at 37°C with HIV-1. Cells were washed twice in PBS and treated with trypsin to remove uninternalized virus particles. Thereafter, DNA was prepared and subjected to PCR analysis as previously described (62). Briefly, 30 cycles of amplification were performed with the ^{32}P-end-labeled M667-M661 primer pair specific for the primer binding site of HIV-1 and a pair of oligonucleotide primers specific for the human β-globin (nucleotides 14 to 33 and 123 to 104) (65). Following amplification, radiolabeled products were resolved on a 6% polyacrylamide gel and visualized by direct autoradiography of the gel.

Coinfection experiments. Samples of 5×10^5 cells (Jurkat E6-1 and PBMCs) were centrifuged and resuspended in 1 ml of complete culture medium in each well of a 24-well plate. The cells were coinoculated with various concentrations of defective particles (0.5, 5, 50, and 500 pg of p24) originating from UHC-8 and UHC-18 cells and a fixed quantity of infectious particles harvested from transfected Jurkat E6-1 cells. The multiplicity of infection (MOI; number of infectious viral particles per target cell) varied from one experiment to the other because the 50% tissue culture infective dose values of fresh unfrozen clarified supernatants from transfected Jurkat E6-1 cells were unknown when the coinfection experiments were initiated. Controls consisted of cells infected with infectious or defective viral particles only. After the initiation of coinfection studies, virus replication was assessed by measuring reverse transcriptase activity in clarified culture supernatants. In all experiments, cells were infected simultaneously with defective and infectious viral particles. Finally, Jurkat E6-1 cells were also coincubated with a fixed amount of infectious particles harvested from

transfected Jurkat E6-1 cells and virus-free supernatants from UHC-8 and UHC-18 cells. In this set of experiments, supernatants from UHC-8 and UHC-18 cells containing 0.5 pg of p24 were subjected to ultracentrifugation (Haerus type HTA 13.8 rotor, 12,000 rpm, 90 min at 4°C) to pellet defective particles prior to initiation of coinoculation experiments.

Interference of viral production in cells latently infected with HIV-1. OM-10.1 cells were centrifuged and resuspended (5×10^5/ml) in 1 ml of complete culture medium in each well of a 24-well plate. Cells were treated with tumor necrosis factor alpha (TNF-α; 20 U/ml) and different concentrations of defective particles (0.5, 5, and 50 pg of p24) generated by UHC-8 and UHC-18 cells. In some experiments, defective particles were added at 24 and 48 h following the initiation of the cultures, and the antiviral drug zidovudine (AZT; 1 and 5 μM) was included in the culture medium. Virus replication was assessed by evaluating the reverse transcriptase activity in clarified supernatant fluids.

Reverse transcriptase assay. Reverse transcriptase activity was monitored by using a previously reported procedure (53), with minor modifications. In brief, enzymatic activity was measured in 50 μl of clarified supernatant fluid to which 10 μl of a solution A (5 mM dithiothreitol, 50 mM KCl, 0.05% Triton X-100) and 40 μl of a solution B {5 mM $MgCl_2$, 0.5 M ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 0.04 mg of poly(rA)-oligo$(dT)_{12-18}$, 3 mCi of [^{3}H]TTP (40 to 70 Ci/mmol)} had been added. After incubation for 1 h at 37°C, samples were precipitated prior filtration onto glass fiber filters by using a cell harvester system (Tomtec). The filters were dried and radioactivity was counted in a liquid scintillation counter (1205/1204 BS Betaplate; Wallac Oy, Turku, Finland). The assays were performed in triplicate.

Cellular proliferation. Samples of 5×10^5 Jurkat E6-1 cells were centrifuged and resuspended in 1 ml of complete culture medium in each well of a 24-well plate. The cells were inoculated with various concentrations of defective particles (0.5, 5, and 50 pg of p24) originating from UHC-8 and UHC-18 cells. After 3 days of incubation, cells were pulsed for 6 h with 1 μCi of [^{3}H]thymidine (specific activity, 6.7 Ci/mmol). Cells were harvested with an automated sample harvester (Tomtec), and radioactivity was monitored in a liquid scintillation counter (1205/1204 BS Betaplate).

Flow cytometry analysis. Levels of surface CD4 molecules were detected by indirect immunofluorescence using a cytofluorimeter. Briefly, 10^5 cells were first incubated with an experimentally determined saturating concentration of an anti-CD4 monoclonal antibody (anti-Leu-3A; Becton Dickinson, Mountain View, Calif.) for 30 min on ice. Samples were washed twice in PBS and incubated for 30 min on ice with a fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G. After two washes with PBS, samples were fixed with 1% (vol/vol) paraformaldehyde and analyzed by a cytofluorimeter.

RESULTS

Characterization of defective particles used in these experiments. To determine whether defective particles could affect replication of infectious viral particles, we used defective viruses generated by cellular clones in order to more closely parallel physiological conditions. UHC-8 and UHC-18 cellular clones, which were initially derived from U937 cells chronically infected with HIV-1, were reported to carry defective HIV-1 genomes that were unable to infect all cell lines tested (8). Virus particles originating from these two cellular clones were demonstrated to be devoid of some specific viral proteins. To further characterize progeny virus produced by these cellular clones, we examined by Western blot analysis the presence of integrase and reverse transcriptase proteins in purified concentrated virus stocks harvested from UHC-8 and UHC-18 cells, using antibodies specific for these proteins. The viral integrase protein (p32) was detected in progeny virus harvested from UHC-18 cells but was absent in viruses originating from UHC-8 cellular clone (Fig. 1A). Therefore, viruses harvested from UHC-8 cells do not carry integrase protein. No reverse transcriptase protein (p66/p51) could be detected by SDS-polyacrylamide gel electrophoresis in progeny virus produced by both UHC-8 and UHC-18 cells (Fig. 1B). These results were confirmed when Western blot analyses were performed with pooled sera from HIV-1-infected individuals containing antibodies to all major viral proteins, including integrase and reverse transcriptase proteins (data not shown). Furthermore, we detected the presence of envelope proteins (gp120 and gp41) in viruses produced by UHC-8 and UHC-18 cells, which is in accord with observations of Boulerice et al. (8).

To test the ability of defective particles to bind to the surface

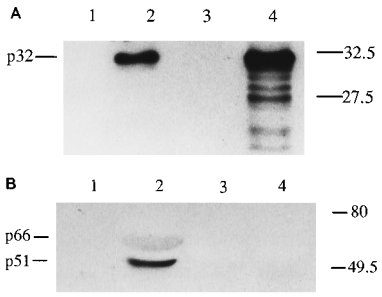

FIG. 1. Western blot analysis of material harvested from clarified supernatants of UHC cellular clones and U937 cells chronically infected with HIV-1. Viral lysates (10 μg) from U937-HIV-IIIB (lanes 2), UHC-8 (lanes 3), and UHC-18 (lanes 4) cells were electrophoresed and immunoblotted with an anti-HIV-1 integrase antiserum (A) or an anti-reverse transcriptase monoclonal antibody (B). The control consisted of whole-cell lysate from uninfected U937 cells (lanes 1). Numbers at the right indicate molecular masses in kilodaltons, while designations at the left indicate the positions of the integrase protein (p32) and reverse transcriptase heterodimer proteins (p66/p51).

CD4, we incubated Jurkat E6-1 cells with increasing concentrations of defective particles prior to the addition of an anti-CD4 antibody that recognizes the gp120 binding site. Results from these studies indicate that viruses harvested from UHC-8 and UHC-18 cells can indeed bind to the CD4 glycoprotein (data not shown).

The fusogenic capacity of HIV-1-producing UHC-8 and UHC-18 cell lines, when cocultured with CD4-expressing cells, was also tested. Results from Fig. 2 reveal that the viral envelope proteins present on the surface of UHC-8 and UHC-18 cells are truly competent in mediating membrane fusion.

We next investigated whether defective viruses can enter susceptible $CD4^+$ cells. For this purpose, Jurkat E6-1 cells were inoculated with defective particles, and entry of viruses into cells was monitored by measuring the intracellular concentration of the viral core p24 protein following elimination of uninternalized virus by trypsin treatment. Under these experimental conditions, the ability of defective particles produced by UHC-8 and UHC-18 cells to enter $CD4^+$ cells was clearly demonstrated (data not shown).

We monitored whether defective particles originating from UHC-8 and UHC-18 cells can perform the reverse transcription process. For this purpose, we inoculated Jurkat E6-1 cells with virus particles harvested from UHC-8 and UHC-18 cells, and PCR analysis was carried out with the use of a primer set that recognizes only full-length or nearly completely synthesized viral DNA. Results from these studies indicated that defective particles generated by UHC-8 and UHC-18 cells were unable to proceed through a complete reverse transcription step (Fig. 3).

Altogether, results from these studies indicate that defective viral particles produced by UHC-8 cells are devoid of integrase and reverse transcriptase proteins, while viruses recovered from UHC-18 cells do not carry reverse transcriptase protein. We also determined that these defective particles could bind to surface CD4 molecules and be internalized into susceptible cells but, as expected, were unable to reverse transcribe their RNA into DNA because of the absence of the reverse transcriptase protein. Therefore, progeny virus harvested from UHC-8 and UHC-18 cells represents an appropriate tool with which to investigate whether defective particles can interfere with replication of infectious viral particles.

Defective particles strongly inhibit replication of infectious viral particles. To evaluate if defective viral particles could restrict replication of infectious viruses, we coinfected the

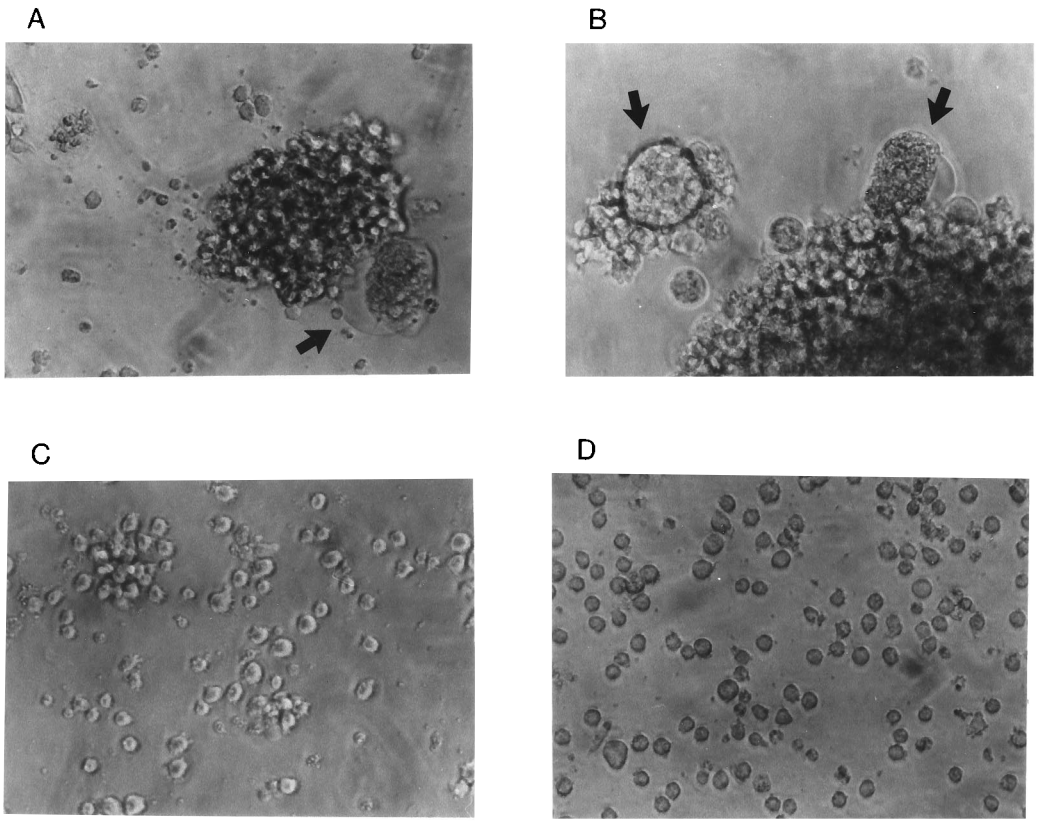

FIG. 2. Phase-contrast micrographs of syncytia formed when uninfected MT-4 cells were mixed at a 10:1 ratio with UHC-8 (A) or UHC-18 (B) cells. The photographs were taken after 48 h. Controls consisted of UHC-8 (C) and UHC-18 (D) cell lines.

human $CD4^+$ T-lymphoblastoid cell line Jurkat E6-1 simultaneously with infectious particles harvested from transfected cells (MOI of 0.016) and different concentrations of defective particles produced by UHC-18 cells. Following coinoculation, virus production was monitored by reverse transcriptase assay of clarified supernatants every 3 to 4 days. A marked inhibition of virus replication was seen when defective particles were present (Fig. 4A). This inhibition was dose dependent since a greater diminution of virus replication was observed with increasing concentrations of defective particles. A less pronounced interfering effect was detected when higher concentrations of infectious particles (MOI of 0.9) were used (Fig. 4B). However, an almost total abrogation of virus replication was detected with the highest concentrations of defective particles (50 and 500 pg of p24) despite the addition of 50-fold more infectious viral particles. The same coinfection studies were performed with defective particles harvested from UHC-8 cells, and a less impressive interfering effect on viral production was observed when the lowest concentrations of defective particles (0.5 and 5 pg of p24) were used (Fig. 5).

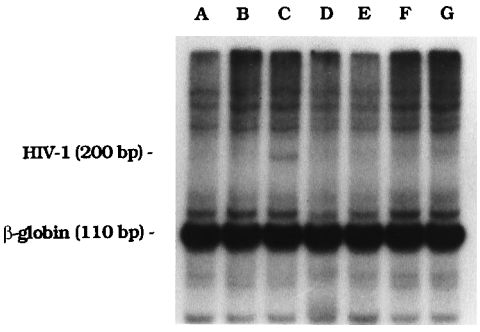

FIG. 3. Reverse transcription process monitored by PCR analysis. PCR amplification was carried out as described in Materials and Methods. Jurkat E6-1 cells were inoculated with progeny virus originating from UHC-8 (100 [lane D] and 1,000 [lane E] pg of p24) or UHC-18 (100 [lane F] and 1,000 [lane G] pg of p24) cells. Uninfected cells (lane A) or Jurkat E6-1 cells infected with $HIV\text{-}1_{IIIB}$ (100 [lane B] and 1,000 [lane C] pg of p24) were used as controls.

To demonstrate that the observed phenomenon was not cell type specific and to more closely parallel in vivo conditions, PBMCs were used as target cells. A significant interference with the replication of virus replication was detected in PBMCs when defective particles harvested from UHC-18 cells were used (Fig. 6). The observed interference in PBMCs is comparable to the diminution of virus replication seen in Jurkat E6-1 cells when the same defective particles, namely, progeny virus produced by UHC-18 cells, were used.

We sought to determine whether the observed interference with viral replication could be associated with a putative soluble factor(s) present in supernatants from UHC-8 and UHC-18 cells. Coinfection experiments were performed with virus-free supernatants from UHC-8 and UHC-18 cells by means of ultracentrifugation. Data from these studies demonstrate that the presence of defective particles from UHC-8 and UHC-18 cells is necessary to inhibit replication of infectious HIV-1 (Fig. 7).

Defective particles interfere with virus replication in latently infected cells. We next attempted to determine whether

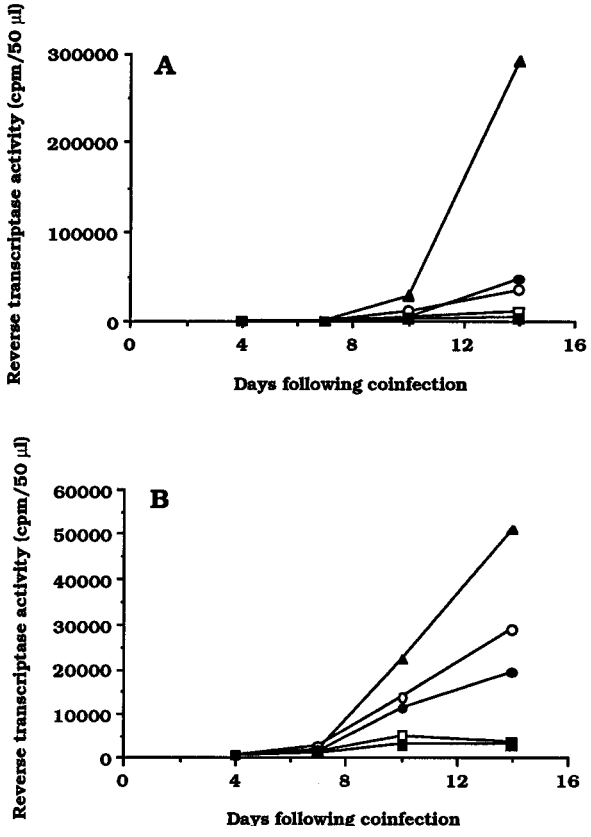

FIG. 4. Coinfection of Jurkat E6-1 cells with infectious viruses and defective particles produced by clone UHC-18. Jurkat E6-1 cells were coinfected with different concentrations of defective particles harvested from clone UHC-18 (0 [▲], 0.5 (○), 5 [●], 50 [□], and 500 [■])] pg of p24) and infectious viruses produced by transfected Jurkat E6-1 cells at an MOI of 0.016 (A) or 0.09 (B). Virus replication was monitored by measuring reverse transcriptase activity in clarified supernatants.

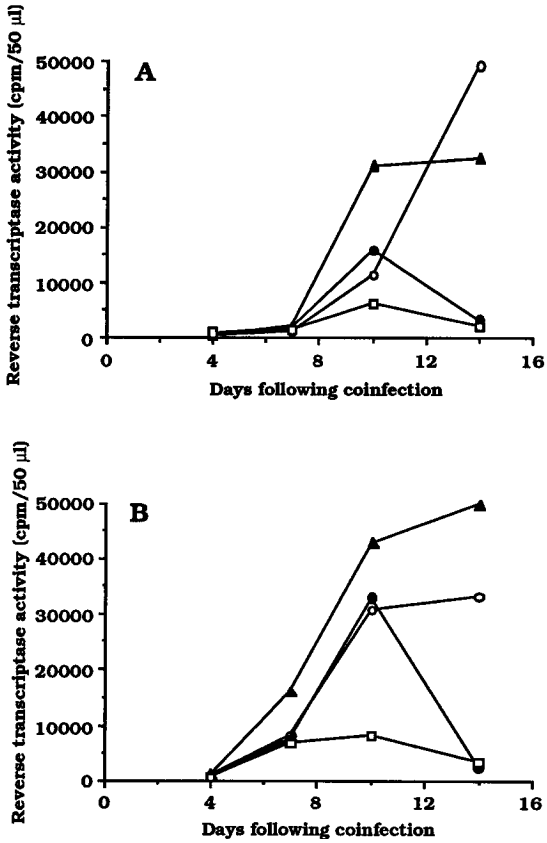

FIG. 5. Coinfection of Jurkat E6-1 cells with infectious viruses and defective particles produced by clone UHC-8. Jurkat E6-1 cells were coinfected with different concentrations of defective particles harvested from clone UHC-8 (0 [▲], 0.5 [○], 5 [●], and 50 [□] pg of p24) and infectious viruses produced by transfected Jurkat E6-1 cells at an MOI of 0.008 (A) or 0.08 (B). Virus replication was monitored by measuring reverse transcriptase activity in clarified supernatants.

defective particles could also negatively affect virus replication in a latently HIV-1-infected cell line. For this purpose, we used the OM-10.1 cell line, which demonstrates minimal constitutive HIV-1 production that can be markedly increased following addition of the cytokine TNF-α (10). Reinfection can occur in these cells since they remain $CD4^+$ for a certain period of time following treatment with TNF-α. No significant decrease in reverse transcriptase activity was seen until 48 h after the addition of defective particles to TNF-α-treated OM-10.1 cells. However, at 72 h after the initiation of the cultures, a moderate interfering effect on virus replication was observed when defective particles harvested from UHC-8 and UHC-18 cells were used (Fig. 8). Thus, it appears that the interference of replication of infectious viral particles mediated by defective viruses was due to inhibition of the reinfection process. Data from these studies indicate that defective particles do not diminish virus replication from integrated viral DNA. To investigate if defective viruses do indeed interfere with reinfection events, defective particles were added at specific time points (24 and 48 h) after treatment of OM-10.1 cells with the cytokine TNF-α. A much more important dose-dependent inhibition of virus replication was detected following the repeated addition of defective particles harvested from UHC-8 and UHC-18 cells (Fig. 9A and B). To further confirm that the mechanism of action of defective particles is to abrogate the reinfection events, the cultures were treated with inhibitory concentrations of the antiretroviral drug AZT. This antiviral drug has no effect on viral DNA but will prevent reinfection since it acts at the level of reverse transcriptase. The inhibition of virus replication when 5 μM AZT was used is comparable to the observed diminution of virus production in TNF-α-treated cells inoculated repeatedly with defective particles (Fig. 9C).

Defective particles do not modulate CD4 expression and cellular proliferation. We investigated whether the basis of diminution of virus replication was associated with a concomitant down-modulation of CD4 expression. We therefore inoculated Jurkat E6-1 cells with different concentrations of defective particles (0.5, 5, and 50 pg of p24) produced by UHC-8 and UHC-18 cells and monitored levels of surface CD4 expression by flow cytometry. No changes was observed over time following the addition of defective particles (Table 1). Therefore, the inhibition of virus replication is not associated with a diminished expression of CD4 on the cell surface mediated by defective particles.

A negative effect on cellular machinery, which would result in a diminished cellular proliferation, represents another possible explanation for the interference with replication of infectious viral particles associated with the presence of defective particles. Thus, we analyzed proliferation of Jurkat E6-1 cells following inoculation with various concentrations of defective particles. No significant changes were detected following the

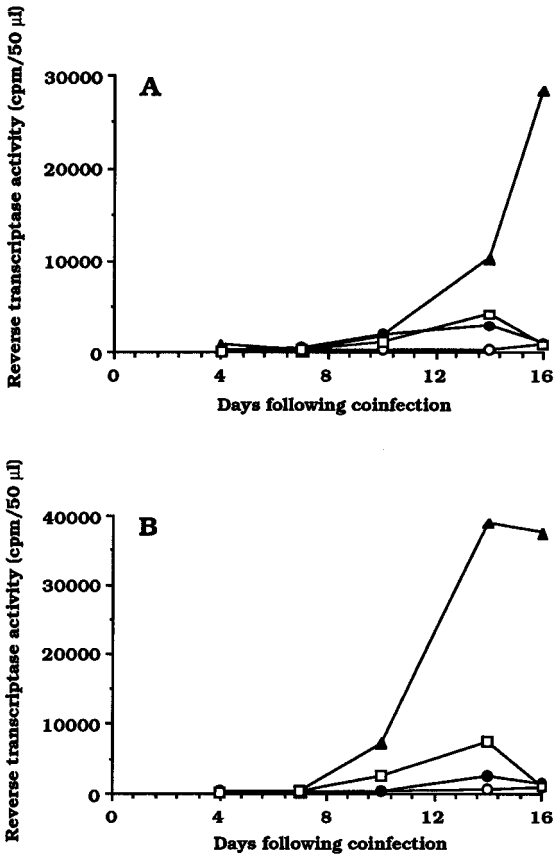

FIG. 6. Coinfection of PBMCs with infectious viruses and defective particles produced by clone UHC-18. PBMCs were coinfected with different concentrations of defective particles harvested from clone UHC-18 (0, [▲], 0.5 [○], 5 [●], and 50 [□] pg of p24) and infectious viruses produced by transfected Jurkat E6-1 cells at an MOI of 0.0014 (A) or 0.014 (B). Virus replication was monitored by measuring reverse transcriptase activity in clarified supernatants.

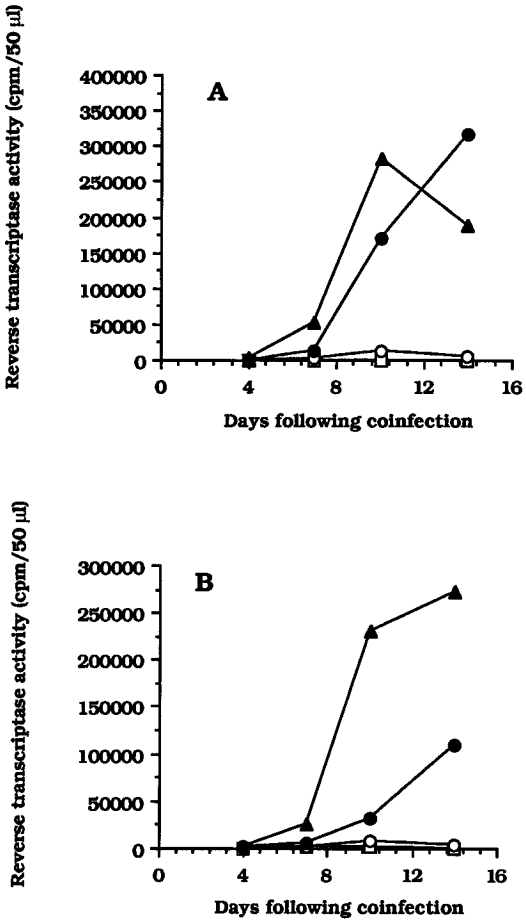

FIG. 7. Coinfection of Jurkat E6-1 cells with infectious viruses and defective particles or virus-free supernatants from clones UHC-8 and UHC-18. Jurkat E6-1 cells were inoculated with infectious viruses produced by transfected Jurkat cells (MOI of 0.012) and defective particles (0.5 pg of p24 [○]) or ultracentrifuged virus-free supernatants (●) from UHC-8 (A) or UHC-18 (B) cells. Controls consisted of Jurkat cells infected with infectious viruses only (▲) and inoculated with defective particles only (□). Virus replication was monitored by measuring reverse transcriptase activity in clarified supernatants.

addition of defective particles, suggesting that defective particles do not affect cellular division (Table 2).

DISCUSSION

This report demonstrates that a strong inhibition of virus replication is achieved when infection is performed in the presence of defective particles. The interference with replication of infectious viruses was seen with the use of defective particles generated by two different cellular clones. Progeny virus harvested from clone UHC-18 does not possess reverse transcriptase protein, and viruses produced by clone UHC-8 are devoid of integrase and reverse transcriptase proteins. Our data further show that these defective viruses can bind to cell surface CD4 glycoprotein and are internalized in susceptible cells. Our results also indicate that inhibition of virus replication was dose dependent and was not cell type specific, since the interfering effect was observed both in a human T-cell line (Jurkat E6-1) and in PBMCs. More importantly, we have determined that defective particles per se were directly responsible for the interfering effect.

Different mechanisms have been demonstrated to be responsible for viral interference mediated by defective particles such as interferon induced by viral infection, competition for cellular receptors, and competition for host metabolic factors. The basis for the strong inhibitory effect on replication of infectious particles mediated by progeny virus generated by UHC-8 and UHC-18 cells remains to be elucidated. However, experiments carried out with a cell line latently infected with HIV-1 indicate that defective particles have no effect on viral DNA already present within infected cells. The same studies also demonstrate that defective viruses negatively affect or abolish reinfection events in a manner similar to that of AZT. This finding is in accord with a previous work which demonstrated that in OM-10.1 cells, AZT led to a reduction in integrated viral DNA probably by a mechanism of inhibition of reinfection events (6). We further investigated the basis for the inhibitory effect mediated by defective particles and determined that neither modulation of CD4 nor changes in cellular proliferation are responsible for the observed phenomenon.

Recently several groups have demonstrated that dominant negative HIV mutants can interfere with replication of wild-type virus. Dominant negative mutants are deficient in one or more of the viral structures that are needed to complete a full replicative cycle. The activities of these molecular constructs are various and can result, for example, in competition for cellular factors that are present in limiting amounts or inhibition of multimerization of viral subunits. The interfering effect

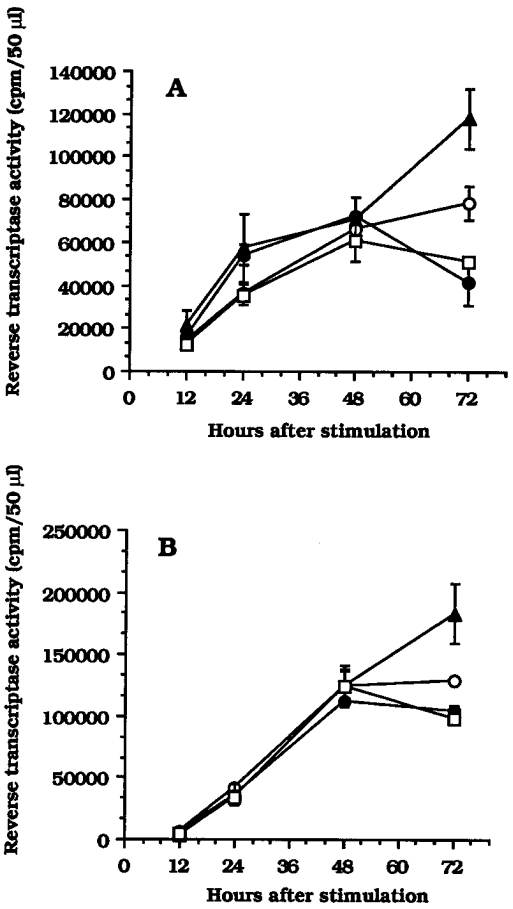

FIG. 8. Inoculation of TNF-α-treated OM-10.1 cells with defective particles originating from clone UHC-18 or UHC-8. OM-10.1 cells were incubated in the presence of TNF-α (20 U/ml) and different concentrations of defective particles (0 [▲], 0.5 [○], 5 [●], and 50 [□] pg of p24) harvested from clone UHC-18 (A) or UHC-8 (B). Virus replication was monitored by measuring reverse transcriptase activity in clarified supernatants.

on virus replication mediated by defective particles harvested from two stable cellular clones found in this study differed in many ways from the inhibitory effect associated with dominant negative viral mutants generated by mutagenesis. Indeed, only a low to moderate level of inhibition that required excessive amounts of negative viral mutants was achieved in these studies. For example, it was recently shown by Steffy and Wong-Staal (57) that a 10-fold excess of transdominant negative *env* mutant of HIV-2 was necessary to achieve a maximal interfering effect on the generation of infectious particles. Trono et al. (63) showed that a 4-fold excess of HIV-1 *gag* mutants was necessary to achieve an effective interference with the replication of infectious particles, while Pearson et al. (47) reported that an 8- to 30-fold molar excess of a *tat* mutant over the wild-type *tat* was required to attain the interfering effect. The weight of p24 per individual HIV particle has been previously estimated to be 1.29×10^{-16} g (9). On the basis of this estimation, we observed a 50% inhibition of virus replication in Jurkat E6-1 cells despite the use of a 110-fold excess of infectious viruses (MOI of 0.9) over defective particles (0.5 pg of p24, which corresponds to 0.008 particle per target). Although defective particles generated by both UHC-8 and UHC-18 cells are able to interfere with the replication of infectious viruses, progeny virus from UHC-18 cells are much

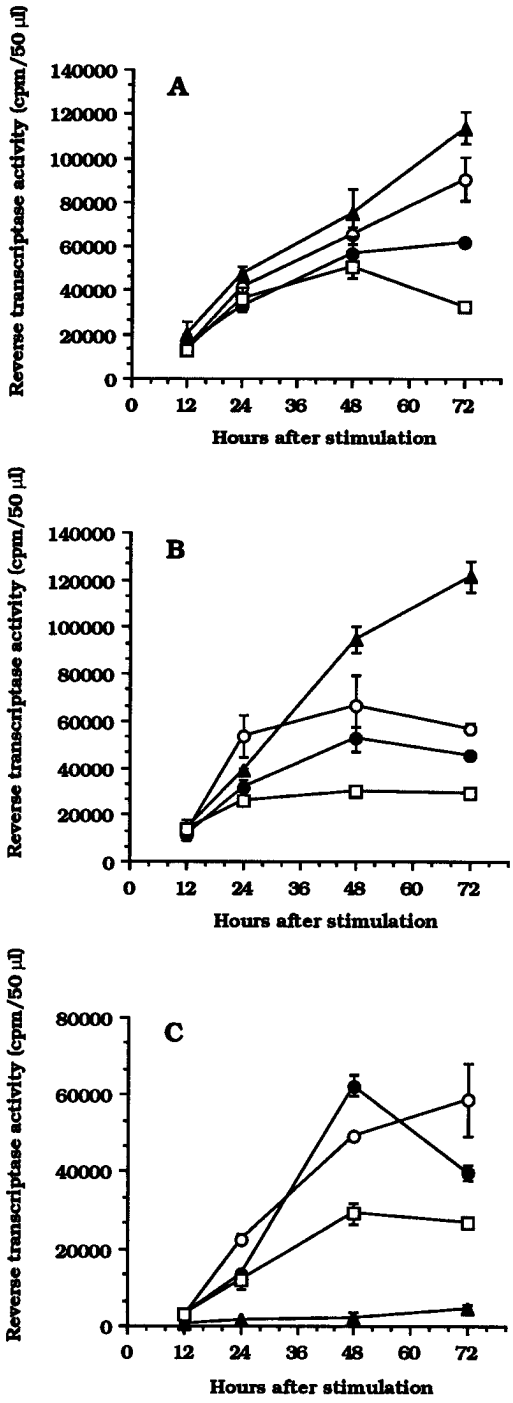

FIG. 9. Inoculation of TNF-α-treated OM-10.1 cells with defective particles originating from clone UHC-18 or UHC-8 or the antiviral drug AZT. OM-10.1 cells were incubated in the presence of TNF-α (20 U/ml) and different concentrations of defective particles (0 [▲], 0.5 [○], 5 [●], and 50 [□] pg of p24) harvested from clone UHC-18 (A) or UHC-8 (B). At 24 and 48 h after initiation of the cultures, similar amounts of defective particles were added. In another set of experiments (C), OM-10.1 cells were either left untreated (▲) or treated with TNF-α (20 U/ml) in the absence (○) or presence of AZT (1 μM [●] and 5 μM [□]). Virus replication was monitored by measuring reverse transcriptase activity in clarified supernatants.

TABLE 1. Flow cytometry analysis of CD4 expression following inoculation with defective particles[a]

Source of defective particles	Days after inoculation	Mean fluorescence value[b]			
		0 pg of p24	0.5 pg of p24	5 pg of p24	50 pg of p24
UHC-18	4	65.11	65.48	64.30	62.52
	7	66.99	66.16	66.32	65.45
	10	68.84	69.87	68.34	69.66
UHC-8	4	62.65	61.80	61.73	60.88
	7	65.53	64.66	63.83	61.64
	10	68.06	66.91	67.04	65.67

[a] CD4 expression was monitored by using the monoclonal anti-CD4 Leu-3A antibody. The number of cells expressing CD4 glycoprotein remained similar throughout the course of the experiment under all experimental conditions.

[b] Mean of three determinations. All standard deviations were within 10% of the mean.

more potent to inhibit virus replication than particles harvested from UHC-8 cells. The fact that viruses from UHC-8 cells are devoid of both reverse transcriptase and integrase proteins, while progeny virus harvested from UHC-18 cells are devoid of reverse transcriptase protein only, may explain the difference in the interfering capability exerted by such defective particles. Indeed, it is possible that coinfection of susceptible cells with defective and infectious particles leads to complementation whereby the deficient protein or gene is supplied in *trans* by the infectious particle. Moreover, this phenomenon is widely accepted as one of the main features of defective particles that can replicate at the expense of infectious viruses in coinfected cells. We believe that complementation between infectious viruses and defective particles from UHC-18 cells is more likely to occur because such viruses require reverse transcriptase protein only, while progeny virus from UHC-8 cells need both integrase and reverse transcriptase proteins to replicate. Complementation between infectious and defective particles originating from UHC-18 cells will lead to a greater spread of defective particles and, consequently, to a more dramatic interfering effect. Another major difference between our system and the use of dominant negative mutants of HIV lies in the fact that cotransfection of molecularly cloned viral DNA does not parallel physiological situations.

The finding that a marked inhibition of virus replication can be reached when susceptible cells are coinfected with both fully

TABLE 2. Proliferation of Jurkat E6-1 cells after addition of defective particles

Concn of defective particles (pg of p24)	[^{3}H]thymidine incorporation (cpm [SD])[a]
From UHC-18	
0	60,127 (4,607)
0.5	54,291 (4,448)
5	65,234 (2,692)
50	64,886 (6,317)
From UHC-8	
0	62,002 (4,908)
0.5	60,283 (4,891)
5	67,827 (3,921)
50	69,799 (6,788)

[a] Mean of three determinations.

infectious and defective HIV-1 particles suggests that this model may be representative of the in vivo situation. The data from our studies may reveal significant implications for the pathogenesis of HIV infection since it has been proposed that defective particles can also modulate in vivo viral infection, based on the interfering ability of defective viruses in cell culture (34). Model experimental systems showed that diseases were affected in a variety of ways. For example, it was reported that lethal encephalitis in young adult mice induced by replication-competent vesicular stomatitis virus could be prevented by purified defective particles (19, 30). Tissues isolated from mice protected with defective particles showed infectivity titers lower than those in animals infected with only infectious virus particles (4, 16, 30, 55). It has been postulated that defective viruses might be responsible for the establishment and maintenance of persistent viral infection (34). Indeed, numerous studies using different viruses such as reovirus, lymphocytic choriomeningitis virus, or Friend spleen focus-forming virus have indicated that defective particles play a direct role in the mechanism of persistence (20, 50). In addition, a cyclic interaction between replication-deficient and fully infectious virus particles whereby infectious viruses would escape inhibition by defective particles has been postulated to occur (32). Experiments performed with vesicular stomatitis virus indicate that a cycling production of infectious virus and replication-defective particles does indeed take place in vivo (12).

Defective retroviruses not only represent deleted versions of the fully infectious virus genome but also can harbor recombinant cellular or viral sequences. It was determined that almost all retroviral oncogenes are present in defective viruses and that helper viruses are required for efficient transmission. Disease associated with defective retroviruses was first reported with a defective strain of Rous sarcoma virus. This replication-defective viral genome was demonstrated to carry an oncogene derived from a cellular gene (25). The association of new disease patterns with the presence of a replication-defective retrovirus, containing scrambled viral genes, has been recently reported by numerous authors. It was first seen with the spleen focus-forming virus, which leads to the proliferation of erythroid precursor cells and, ultimately, to erythroleukemia (40). Severe immunodeficiency diseases in cats and mice were later associated with the presence of replication-defective retrovirus genomes (2, 13, 40, 46). Genes responsible for the erythroleukemia induced by spleen focus-forming virus and for feline immunodeficiency are both derived from *env* sequences (40, 51). Defective retroviruses in murine immunodeficiency were shown to possess, despite deletion of the *pol* and *env* genes, the complete *gag* region (2). Such a severe immunosuppression in mice and cats, induced by the presence of defective retroviruses, raises the question of whether defective HIV genomes can modulate HIV replication and influence disease progression.

It was proposed in 1988 that interfering viruses could be used as a therapeutic tool to interfere with the replication of infectious HIV particles (3). Data from our experiments, which demonstrate that defective particles can greatly reduce the yield of infectious virus, support this strategy. Although this approach seems attractive, it is important to keep in mind that retroviruses possess an extraordinarily high rate of recombination that may give rise to an infectious particle from a virus that was initially defective (60).

In conclusion, our in vitro studies clearly suggest that defective particles can negatively affect replication of infectious viral particles. On the basis of reports which indicate that the great majority of HIV genomes appear to be defective (23, 41, 44, 49), the observed phenomenon of viral interference

induced by defective HIV-1 particles is likely to limit the spread of replication-competent viruses. This may help to explain the observed asymptomatic state that is characterized by a less pronounced rate of virus replication.

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