vif-Negative Human Immunodeficiency Virus Type 1 Persistently Replicates in Primary Macrophages, Producing Attenuated Progeny Virus

Iqbal Chowdhury
St. Luke's/Roosevelt Hospital Center, College of Physicians and Surgeons, Columbia University, New York, New York

Wei Chao
St. Luke's/Roosevelt Hospital Center, College of Physicians and Surgeons, Columbia University, New York, New York

Mary Jane Potash
St. Luke's/Roosevelt Hospital Center, College of Physicians and Surgeons, Columbia University, New York, New York

Pavel Sova
St. Luke's/Roosevelt Hospital Center, College of Physicians and Surgeons, Columbia University, New York, New York

Howard Gendelman
University of Nebraska Medical Center & Nebraska Center for Virology, hegendel@unmc.edu

See next page for additional authors

Follow this and additional works at: http://digitalcommons.unl.edu/virologypub

Part of the Virology Commons

Chowdhury, Iqbal; Chao, Wei; Potash, Mary Jane; Sova, Pavel; Gendelman, Howard; and Volsky, David J., "vif-Negative Human Immunodeficiency Virus Type 1 Persistently Replicates in Primary Macrophages, Producing Attenuated Progeny Virus" (1996). Virology Papers. Paper 98.
http://digitalcommons.unl.edu/virologypub/98

This Article is brought to you for free and open access by the Virology, Nebraska Center for at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in Virology Papers by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.
Authors
Iqbal Chowdhury, Wei Chao, Mary Jane Potash, Pavel Sova, Howard Gendelman, and David J. Volsky
vif-Negative Human Immunodeficiency Virus Type 1 Persistently Replicates in Primary Macrophages, Producing Attenuated Progeny Virus

IQBAL H. CHOWDHURY,1 WEI CHAO,1 MARY JANE POTASH,1 PAVEL SOVA,1 HOWARD E. GENDELMAN,2 AND DAVID J. VOLSKY1*

Molecular Virology Laboratory, St. Luke's/Roosevelt Hospital Center, College of Physicians and Surgeons, Columbia University, New York, New York 10019,1 and Departments of Medicine, Pathology, and Microbiology, University of Nebraska Medical Center, Omaha, Nebraska 68105.

Received 26 October 1995/Accepted 29 April 1996

The vif gene of human immunodeficiency virus type 1 (HIV-1) is required for efficient infection of primary T lymphocytes. In this study, we investigated in detail the role of vif in productive infection of primary monocyte-derived macrophages (MDM). Viruses carrying missense or deletion mutations in vif were constructed on the background of the monocyteotropic recombinant NLHXADA-GP. Using MDM from multiple donors, we found that vif mutants produced in complementing or partially complementing cell lines were approximately 10% as infectious as wild-type virus when assayed for incomplete, complete, and circularized viral DNA molecules by quantitative PCR amplification or for viral core antigen p24 production by enzyme-linked immunosorbent assay. We then determined the structure and infectivity of vif mutant HIV-1 by using MDM exclusively both for virus production and as targets for infection. Biosynthetic labeling and immunoprecipitation analysis of sucrose cushion-purified vif-negative HIV-1 made in MDM revealed that the virus had reduced p24 content compared with wild-type HIV-1. Cell-free MDM-derived vif mutant HIV-1 was infectious in macrophages as determined by the synthesis and maintenance of full-length viral DNA and by the production of particle-associated viral RNA, but its infectivity was approximately 2,500-fold lower than that of wild-type virus whose titer was determined in parallel by measurement of the viral DNA burden. MDM infected with MDM-derived vif-negative HIV-1 were able to transmit the virus to uninfected MDM by cocultivation, confirming the infectiousness of this virus. We conclude that mutations in vif significantly reduce but do not eliminate the capacity of HIV-1 to replicate and produce infectious progeny virus in primary human macrophages.
The studies of the role of vif in macrophages have been confined to the evaluation of viral p24 or reverse transcriptase production (17, 24, 28, 45), with the overall conclusion being that mutations in vif incapacitate HIV-1 in macrophages. The site of this restriction and the progress of HIV-1 infection in macrophages are unknown. In this study, we have investigated the efficiency of vif-negative HIV-1 infection of macrophages, evaluating products of both the early and late stages of the virus life cycle and the synthesis of infectious provirus. In contrast to earlier studies (17, 24, 28, 45), we show that vif-negative HIV-1 can establish stable infection of macrophages and produces both viral DNA and protein. vif-negative HIV-1 produced by macrophages is itself infectious to macrophages, albeit at a low level. We conclude that vif is a modulator but not a requirement of HIV-1 infection of the natural target cells, macrophages. These findings also imply that vif-negative viruses may establish chronic, attenuated infection in vivo.

**MATERIALS AND METHODS**

**Cells.** Human monocytes were isolated by elutriation from blood collected from noninfected donors and were cultured in Dulbecco's modified Eagle's medium with 10% AB human serum and 10% giant cell tumor conditioned medium (Sigma, St. Louis, Mo.), antibiotics, and glutamine. More than 95% of cells were nonspecific esterase positive. Monocytes were allowed to adhere and differentiate to monocyte-derived macrophages (MDM) for 5 to 7 days prior to infection with cell-free HIV-1. The human rhadomyosarcoma cell line RD (41) was cultured as a monolayer in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and antibiotics. The human T-cell leukemia lines SupT1 (37) and CEM (15) were cultured in RPMI 1640 with 5% fetal bovine serum and antibiotics.

**DNA constructions.** To generate macrophage-tropic vif mutant HIV-1, we used plasmids encoding full-length infectious HIV-1; these plasmids have been described previously (35), which carries an intact vif gene; pN1T-E (35), which carries a 35-bp deletion in vif; and pM4, in which both cysteine codons have been changed to leucine codons by site-directed mutagenesis (26). The macrophage-tropic HIV-1 encoding pNL-HXADA-GP provided the proviral cassette and is composed of sequences from NL4-3, HXB-2, and ADA (46). To construct macrophage-tropic vif mutants, we replaced the 621-bp NotI-EcoRI fragment in pGEP with the corresponding fragment from pN1T-A, pN1T-E, or pM4. This produced three new recombinants: AGP, carrying the intact vif coding region; EGP, carrying a deletion in vif; and M4GP, carrying the missense Cys-to-Leu replacements (Fig. 1A). The amino acid sequences of their Vif polypeptides are shown in Fig. 1B.

**HIV-1 stocks and infection.** Virus stocks were prepared by calcium phosphate-mediated transfection (2) of RD cells or by infection of SupT1 cells with RD cell-derived virus. Cell supernatants were clarified by low-speed centrifugation, in infection, MDM were harvested by gentle scraping, counted, and replated as below. For evaluation of transmission by cocultivation 26 days after primary infection, MDM were harvested by gentle scraping, counted, and replated as below. For evaluation of transmission by cocultivation 26 days after primary infection, MDM were harvested by gentle scraping, counted, and replated as below. For evaluation of transmission by cocultivation 26 days after primary infection, MDM were harvested by gentle scraping, counted, and replated as below.

**PCR amplification of viral sequences.** At the time points indicated below, MDM were harvested for PCR by gentle scraping and centrifugation at 200 x g. After two washes in phosphate-buffered saline, dry cell pellets were frozen at -80°C until the completion of the experiment, at which time all the samples were processed in parallel. Cell pellets were prepared by boiling in equal volumes of solution A (10 mM Tris [pH 8.3], 100 mM KCl, 2.5 mM MgCl₂) and solution B (10 mM Tris [pH 8.3], 2.5 mM MgCl₂, 1% Tween, 0.4U 

<table>
<thead>
<tr>
<th>Vif-related protein</th>
<th>EcoRI</th>
<th>Ndel</th>
<th>HpaI</th>
<th>Ag</th>
<th>SphI</th>
<th>SaII</th>
<th>BglII</th>
<th>PvuII</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1TA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N1TE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M4GP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**FIG. 1.** Schematic map of proviral DNA constructions. (A) Map of the parental molecular cloning NLHXA-AGP (solid bars), NIT-A (stipped bars) and NIT-E (hatched bars) and the new recombinants generated from them. (B) Amino acid sequences of Vif deduced from the nucleotide sequences. Dots indicate identity, and dashes indicate deletions.
experiments, in which virus expression ranges widely, after initial amplification and gel analysis as described above, samples with signals above the linear range of the standard curve were diluted and rerun along with other samples. The dilution factor is marked in the figures when appropriate.

Reverse transcriptase PCR for the detection of HIV-1 RNA. Virions were isolated from culture supernatants by centrifugation at 100,000 × g for 1 h at 4°C. RNA was isolated from HIV-1-infected MDM or from virions by using Trizol (Gibco-BRL, Gaithersburg, Md.) as specified by the manufacturer. cDNA synthesis was conducted with the Superscript RT kit (GIBCO-BRL) and the down-stream primer SK39 (33), the gag region was amplified with the primer pair SK38 and SK39 (33), and the PCR product was detected with 32P-labelled probe SK19 (33). The reverse transcriptase PCR product from 2 × 106 cells or from supernatants of 2.5 × 106 cells was loaded per lane.

Metabolic labelling and immunoprecipitation. MDM were infected with AGP or EGP at 0.5 pg per cell for 2 h, labelled with [35S]methionine-[35S]cysteine for 2 h at 16 days postinfection, and harvested or, for pulse-chase experiments, further incubated for 48 h in standard medium before being harvested. To isolate virions, cell supernatants were layered onto 15% sucrose and centrifuged at 100,000 × g at 4°C for 2 h. The pellet was lysed in 20 mM Tris (pH 8.0)–150 mM NaCl–0.2 mM ethylene glycol-bis [aminoethyl ether]-N,N,N,N'-tetraacetic acid (EGTA)–0.2% sodium azide–0.2% sodium dodecyl sulfate (SDS)–0.2% Nonidet P-40–1 mM iodoacetamide–10 μg of aprotinin per ml–10 μg of leupeptin per ml–1 mM peptatin A–1 mM phenylmethylsulfonyl fluoride and frozen at −80°C until analysis. Immunoprecipitation was performed under standard conditions with the mouse monoclonal antibody AG3.0, kindly provided by J. Allan, which binds to p55gag, p24, and processing intermediates. Briefly, lysates were pre-cleared by incubation with mouse immunoglobulin G and then exposed to AG3.0 for 2 to 3 h at 4°C, and antibody-bound material was collected with protein G-Sepharose (Pharmacia, Piscataway, N.J.) by centrifugation at 10,000 × g for 2.5 min. The supernatant was discarded, the beads were washed in 20 mM Tris (pH 8.0)–150 mM NaCl–0.2% Nonidet P-40 three times, and the bound material was eluted from the beads with electrophoresis sample buffer and was frozen at −80°C until use. Samples were electrophoresed in 12% polyacrylamide containing SDS, fixed, dried, and exposed to X-ray film by standard methods (2).

Extracellular virions of 4 × 106 AGP-infected cells and 1.6 × 106 EGP-infected cells were loaded per lane.

RESULTS

vif mutant HIV-1 produced in permissive or semipermissive cells persistently infects macrophages. For the studies described here, we reconstructed the macrophage-tropic HIV-1 clone NLHXADA-GP (46) to generate three isogenic infectious molecular clones carrying different vif regions (Fig. 1). AGP carries the intact vif gene of NIT-A (35); EGP carries the vif deletion of NIT-E and encodes a truncated nonfunctional Vif (35); and M4GP carries the missense vif mutant of M4 in which both cysteines are replaced, yielding a full-length nonfunctional Vif (26). The three viral clones produced progeny virus after DNA transfection into RD cells (data not shown) and caused similarly high levels of infection of SupT1 cells, which are susceptible to both T-cell-tropic and macrophage-tropic HIV-1 strains (7). Typical infection profiles of AGP, EGP, and M4GP in SupT1 cells, obtained by measuring the kinetics of viral p24 antigen accumulation in culture supernatants, are shown in Fig. 2.

Viral stocks produced by transfection into RD cells or by infection of SupT1 cells were standardized by their p24 content and were used for infection of MDM. Representative infection kinetics for AGP, EGP, and M4GP, monitored by the levels of p24 antigen in culture supernatants, are shown in Fig. 3. SupT1-derived AGP exhibited a typical macrophage-tropic infection phenotype (7), with p24 production peaking 12 to 15 days after infection at about 100 ng of p24 per ml (Fig. 3A). Infection with SupT1-derived vif mutants was slower than that with AGP, but both mutants reached high levels of p24 production 15 to 30 days after virus infection (Fig. 3A). In multiple experiments, the peak p24 production by SupT1-derived vif mutants in MDM was 30 to 100% of that of AGP (data not shown). The RD-derived vif mutants also infected MDM, as determined by the presence of p24 antigen in culture supernatants, but to lower levels than the SupT1-derived mutants did (Fig. 3B). The average peak p24 production by these mutants versus wt virus, based on results from nine independent infections, was 10% (Table 1). In all cases, mutant virus infection in MDM was persistent, lasting for at least 30 days of observation (Fig. 3). The observed difference in p24 production by SupT1- and RD-derived vif mutants in MDM is consistent with previously reported differences in phenotypic complementation of the vif defect by tumor cell lines (6, 16, 36, 40, 45). In the next series of experiments, we used RD cell-derived viruses to minimize the effects of host cell environment on our stocks of vif-negative virus. We conclude that in contrast to previously published studies (17, 24, 28, 45), vif-negative mutants constructed on the background of a macrophage-tropic NLHXADA-GP clone can infect macrophages, resulting in low but persistent production of p24 core antigen.

Efficient viral DNA synthesis during infection of macrophages with vif-negative HIV-1 produced in RD cells. We next determined the progress of vif mutant HIV-1 infection in MDM by quantitative PCR for the detection of different forms of viral DNA. We and others have shown previously that inefficient infection of T cells with vif-negative virus correlates with inefficient synthesis of viral DNA (6, 11, 40, 45). MDM infected with wt and vif-negative viruses produced in RD cells were evaluated over the course of infection for levels of the initial reverse transcript strong-stop DNA (18), full-length viral DNA, and 2-LTR circular DNA, which represents viral DNA present in the cell nucleus (21, 25). The results are presented as autoradiograms of amplified DNA (Fig. 4A and 5A) and as plots of DNA copy numbers estimated from the autoradiograms (Fig. 4B and C and Fig. 5B, respectively). As shown in Fig. 4A and 5A, both wt virus-infected and vif-negative virus-infected MDM contained all three viral DNA forms; however, the kinetics of accumulation of each form differed between wt and mutant infections (Fig. 4B and C and 5B). MDM infected with any of the three viruses contained about 600 to 900 copies of strong-stop DNA per 10,000 cells 1 day after infection (Fig. 4B). This indicates both that the mutant and wt viruses entered MDM and initiated viral DNA synthesis equally well and that infectious titers of these viruses as measured by the products of
first cycle viral DNA synthesis were similar. Although strong-stop DNA increased 30-fold in wt virus-infected cells, from 700 copies on day 1 to a peak of 22,000 copies on day 6, in the same period incomplete DNA increased only from 900 to 3,000 copies for EGP-infected cells and from 600 to 800 copies for M4GP-infected cells (Fig. 4B). Starting 6 days after infection, the level of full-length DNA was also consistently higher (by about 10-fold) in wt-infected MDM than in vif mutant-infected cells (Fig. 4C), consistent with greater p24 production (Fig. 3).

As expected, 2-LTR circular DNA was detected later in infection than was linear DNA, but the significant difference in DNA levels between wt and mutant viruses was maintained (Fig. 5B). These data demonstrate that RD-derived vif-negative viruses can enter macrophages and complete all the stages of the viral replicative cycle through DNA entry into the nucleus but that the overall efficiency of viral DNA synthesis is about 10-fold lower than that of wt virus at the same dose. There is no evidence that reverse transcription is initiated and then aborted in vif-negative HIV-1-infected MDM.

Previous studies of vif-negative HIV-1 infection in macrophages were usually performed with low doses of virus (17, 24, 28, 45), which may explain the negative results obtained. We therefore determined the dose response of macrophages to infection with vif mutant HIV-1 collected from RD cells after transfection with AGP or EGP DNA. MDM were infected with 0.5, 0.1, or 0.05 pg of p24 of either virus per cell and were harvested for PCR 10 days after infection (Fig. 6). As estimated from the standard curve in Fig. 6, MDM infected by EGP at 0.5 pg of p24 per cell contained roughly 1,000 copies of viral DNA per 20,000 cells. Reducing the dose of EGP to 0.1 or 0.05 pg of p24 per cell reduced the DNA burden to 50 to 100 copies of viral DNA per 20,000 cells, but viral DNA was clearly detectable in EGP-infected MDM. No viral p24 was detected in culture supernatants in these systems (data not shown). In contrast, MDM infected by AGP at 0.05 pg of p24 per cell contained about 2,500 HIV-1 DNA copies per 20,000 cells, a difference of 50 to 100-fold compared with the number of copies in MDM infected by EGP at the same dose (Fig. 6). These findings indicate that a low dose of vif mutant virus is infectious to macrophages, as determined by synthesis of viral DNA, and that macrophages are infected by vif mutant HIV-1 in a dose-dependent manner.

To confirm that DNA synthesized by vif mutant HIV-1-infected MDM maintains the vif mutant genotype and that revertants were not generated during propagation of the virus, we infected MDM with vif mutant viruses EGP or M4GP derived from either RD cells or SupT1 cells, amplified viral DNA from the vif region with primers flanking the 35-bp deletion in EGP vif (35) (see also the schematic representation of the EGP genome in Fig. 1), and used two different probes to demonstrate the presence of the vif deletion mutant on one hand and the absence of wt viral DNA on the other (Fig. 7). In the first procedure, we used probe VF5282, which hybridizes to a region shared by the deletion mutant (EGP), M4GP, and AGP (Fig. 7A). Because of the 35-bp deletion in EGP, its product in this region is 306 bp rather than 341 bp in AGP and M4GP. No 341-bp DNA product was detected in EGP-infected macrophages (Fig. 7A), indicating a lack of significant contamination by wt virus in these infections. In the other approach, we used probe VF5161, which hybridizes to a region in vif deleted from EGP (Fig. 7B). This approach permits

<table>
<thead>
<tr>
<th>Expt. no.</th>
<th>HIV-1 p24 antigen level (pg/ml) in:</th>
<th>p24 level (% of wt) in:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AGP</td>
<td>EGP</td>
</tr>
<tr>
<td>1</td>
<td>27,445</td>
<td>1,559</td>
</tr>
<tr>
<td>2</td>
<td>19,990</td>
<td>1,840</td>
</tr>
<tr>
<td>3</td>
<td>37,500</td>
<td>2,920</td>
</tr>
<tr>
<td>4</td>
<td>68,700</td>
<td>3,055</td>
</tr>
<tr>
<td>5</td>
<td>108,700</td>
<td>11,190</td>
</tr>
<tr>
<td>6</td>
<td>25,800</td>
<td>6,000</td>
</tr>
<tr>
<td>7</td>
<td>84,800</td>
<td>15,790</td>
</tr>
<tr>
<td>8</td>
<td>55,000</td>
<td>4,570</td>
</tr>
<tr>
<td>9</td>
<td>45,400</td>
<td>2,020</td>
</tr>
</tbody>
</table>

* MDM from nine different donors were infected by AGP, EGP, or M4GP, and virus replication was monitored by measurement of extracellular p24 levels. Peak values, attained on days 10 to 21 after infection, depending upon the donor, are shown. wt, wild-type virus (AGP).
direct detection of revertant or contaminating wt viral DNA. As shown in Fig. 7B, EGP-infected macrophages contained no viral DNA hybridizing with the VF5161 probe, whereas strong signals were obtained in lysates from cells infected with GP and M4GP, both of which carry a full-length vif gene. DNA produced by M4GP-infected MDM is not distinguishable from that produced by AGP-infected cells by either of the methods used here. We conclude that our vif-negative viral stocks do not contain cryptic wt virus revertants and that the infectivity data presented here reflect exclusively the biological activity of vif mutant virus in macrophages.

**vif mutant HIV-1 completes the virus replicative cycle in macrophages by producing sedimentable viral particles.** To determine whether p24 detected in culture supernatants of vif mutant-infected MDM was newly synthesized and was contained in virions, we performed protein analyses. MDM were infected with GP (the parental vif-positive, macrophage-tropic virus clone), AGP, or EGP; the cells were metabolically labelled; and extracellular particles were purified from cell supernatants by sedimentation through sucrose cushions and analyzed by immunoprecipitation with anti-Gag antibodies and gel electrophoresis (Fig. 8). After 20 h of labeling, MDM infected by wt virus infected produced sedimentable material that contained high levels of newly synthesized p24. EGP-infected cells produced about 10-fold less particle-associated p24 (Fig. 8). As expected, a chase for 48 h reduced the levels of particle-associated p24, but the AGP/EGP p24 ratio remained the same (Fig. 8). Thus, the lifetimes of particle-associated wt and vif mutant p24 are similar. We conclude that MDM infected with vif mutant macrophage-tropic HIV-1 produce sedimentable particles similarly to wt virus but with lower efficiency.

**FIG. 4.** Synthesis of viral DNA by MDM infected with wt or vif mutant HIV-1 produced in permissive cells. MDM were infected at 0.5 pg of p24 per cell with AGP (A), EGP (E), or M4GP (M) isolated from RD cells. The cells were incubated at 4°C for 1 h (day 0) or at 37°C for the times indicated and harvested for PCR as described in Materials and Methods. (A) Lysates of 10,000 cells or the indicated dilution of 10,000 cells were subjected to PCR for β-globin, strong-stop, or full-length viral DNA as described in Materials and Methods. (B and C) Autoradiograms were subjected to densitometry, and the copy number per 10,000 cells of strong-stop DNA (B) or full-length viral DNA (C) was calculated.
The progeny virus produced by vif mutant HIV-1-infected macrophages can be rescued by infection of complementing cells. To determine whether vif mutant HIV-1 produced by MDM is infectious, we first attempted to infect permissive cells which complement the vif defect and permit the spread of mutant virus (16, 40, 45). AGP and EGP produced by infected MDM were used to infect permissive SupT1 cells at approximately 0.1 pg per cell, and infection was monitored by measurement of extracellular p24 (Fig. 9). Both viruses replicated in SupT1 cells, although EGP infection lagged behind AGP infection (Fig. 9). This contrasts with the infection of SupT1 cells by RD-derived AGP and EGP (Fig. 2) and probably reflects the inherent defect in infectiousness of vif-negative HIV-1 produced by noncomplementing cells like MDM (6, 14, 16, 36, 40, 42, 45). Thus, vif-negative virus produced in MDM during primary infection contains infectious viral particles which can be rescued by culture in permissive SupT1 cells.

vif mutant HIV-1 produced in macrophages is infectious but highly attenuated in primary macrophages. We next analyzed the infectivity and life cycle of the MDM-derived vif mutant in its natural host cells, macrophages. We previously reported that analogous vif mutant lymphotropic HIV-1 virions produced by PBL have defective Gag protein composition, which compromises their infectivity, but the biological activity of such viruses was not fully defined (36). Others reported that vif mutants made in PBL are noninfectious in PBL (11). In the experiments described below, we took advantage of the greater productivity of vif-negative, macrophage-tropic virus in MDM (Fig. 3 to 5) and the ability to maintain MDM in culture for longer periods than PBL could be maintained. We infected MDM with vif mutant M4GP produced by MDM and monitored infection by measuring p24 production (Fig. 10). In this and the following experiments, the target MDM presumably are unable to complement vif defects, and thus they offer a neutral cellular milieu in which to test the biological activity of MDM-derived vif mutants. MDM were infected in parallel with M4GP at 0.5 pg of p24 per cell and fivefold dilutions of AGP as indicated; all virus stocks were produced in MDM (Fig. 10). M4GP-infected cells had no detectable p24 in culture supernatants at the time of the expected peak of infection (2 to 3 weeks). The initial p24 readings probably represented gradual shedding of cell-adsorbed virus. In contrast, MDM infected with the lowest dose of AGP tested, 0.004 pg of p24 per cell, still produced significant amounts of p24 at the peak of infection. Thus, on the basis of p24 production, the MDM-derived vif mutant suffered a decline of at least 2 orders of magnitude.
in its ability to productively infect macrophages compared with similarly prepared wt virus. These experiments did not exclude a total loss of infectivity of this "second-generation" vif mutant virus in MDM.

To determine whether M4GP derived from MDM was capable of entering and synthesizing viral DNA in noncomplementing cells, the infections described in the previous section were repeated with DNase-treated viruses and viral DNA levels were evaluated by quantitative PCR (Fig. 11). MDM infected with M4GP at 0.5 pg of p24 per cell clearly synthesized strong-stop viral DNA 24 h after infection but at a level of only about 50 DNA copies per 10,000 cells, similar to that achieved with 0.02 pg of p24 of wt virus per cell (Fig. 11) and about 10-fold less than that with RD-derived M4GP 24 h after primary infection at the same dose (Fig. 4). This result indicates that macrophage-derived M4GP can enter MDM and initiate viral DNA synthesis, albeit with significantly lower efficiency than that of the same virus produced by RD cells. Full-length HIV-1 DNA was barely detectable at 24 h in M4GP-infected cells (fewer than 10 copies per 10,000 cells), but both strong-stop and complete DNA levels increased in these cells to clearly detectable amounts during the subsequent 25 days of culture (Fig. 11B and C). Quantitation of full-length viral DNA copy numbers in infected cells by densitometry (Fig. 11D) showed that on day 16, which was the peak of infection with low doses of wt virus (Fig. 10), MDM infected with M4GP at 0.5 pg of p24 per cell contained 20 DNA copies per 10,000 cells and MDM infected with AGP at a 125-fold-lower dose contained 400 copies per 10,000 cells. By day 26 after infection, the number of full-length DNA copies in wt virus-infected cells was similar to or smaller than on day 16 whereas it doubled in M4GP-infected cells (Fig. 11D). As measured by its ability to synthesize full-length viral DNA in macrophages 16 days after infection, the biological titer of the macrophage-derived vif mutant was about 2,500, or at least 3 orders of magnitude lower than that of wt virus.

To determine whether the vif-negative DNA made in macrophages was expressed, infected cells and their supernatants were investigated for the presence of viral RNA. At 26 days after AGP or EGP infection, total cellular RNA or RNA present in high-speed centrifugation-derived pellets of culture supernatants was extracted, treated with DNase, subjected to reverse transcription, and amplified by PCR for the gag region (Fig. 12). Cells and sedimented culture supernatants from both AGP- and EGP-infected macrophages contained HIV-1 RNA, indicating that viral DNA in these cells was expressed and at least some of it was secreted to the culture medium as sedimentable particles.

As the most sensitive test of HIV-1 transmissibility, MDM infected by MDM-produced EGP were cocultivated with a large excess of uninfected MDM 26 days after primary infection. Transmission was observed by detection of 12 pg of p24 per ml of culture supernatant 28 days after cocultivation. Taken together, these analyses indicate that vif-negative HIV-1 made in macrophages is highly attenuated but that it can establish a slowly spreading infection in its natural host cells.
DISCUSSION

We report here that mutations in \textit{vif} reduce but do not eliminate the capacity of HIV-1 to replicate and produce infectious progeny virus in primary human macrophages. These results establish Vif as a potent facilitator but not an absolute requirement of HIV-1 infection of macrophages. Vif is therefore the only auxiliary gene product of HIV-1 required for efficient replication in both natural target cell types, PBL (1, 11, 17, 24, 28, 36, 45), and macrophages, suggesting that it may play a pivotal role in HIV-1 replication during natural infection of humans.

Our conclusions are based on measurement of viral DNA, protein, or RNA from cells infected by \textit{vif} mutants derived from cell lines or directly from infected macrophages. These results differ from most of the previously published data concerning the extent of impairment of replication of \textit{vif} mutant HIV-1 in macrophages (17, 24, 28, 45). One study in which wt virus infection was poor showed only a sixfold reduction in protein production in MDM infected by \textit{vif} mutant HIV-1 derived from Cos cells (45), entirely consistent with our findings reported in Table 1. Two other reports of systems optimized for HIV-1 protein production in MDM (17, 24) and one of less quantitation by virus rescue (28) indicate a total block to virus replication by \textit{vif} mutations. In the former studies, input virus doses were probably too small to permit analysis of virus spread from infected to uninfected MDM. We have elaborated upon this scheme by isolating \textit{vif} mutant HIV-1 from infected macrophages and then testing its infectivity in MDM in terms of dose response, thereby permitting reliable estimates of the extent of its defects. As we show in Fig. 10, the production of extracellular viral p24 is undetectable in this format, indicating a profound block to the synthesis and export of fully mature virions; these results are entirely consistent with those of the previous studies. However, we also analyzed the stages of \textit{vif}-negative HIV-1 replication preceding virus export and found that \textit{vif} mutant HIV-1 produced by MDM is able to complete viral DNA synthesis (Fig. 11), transcribe viral RNA, and export it in the form of sedimentable particles (Fig. 12), albeit at a lower efficiency than wt virus does. Moreover, the \textit{vif} defect does not eliminate the production of infectious virus, as shown by the ability of EGP-infected MDM to transmit virus by cocultivation. Some of the first studies of \textit{vif}-negative HIV-1 indicated that the virus was more transmissible by cocultivation than by cell-free virus infection (13, 42), an observation that we confirm here with virus derived from and replicating in natural target cells.

Using MDM as targets for infection, we localized the site of attenuation of \textit{vif}-negative replication by quantitation of incomplete, complete, and nuclear forms of viral DNA (Fig. 4 and 5). The ratio of strong-stop to full-length linear viral DNA is close to 1 at the peak of infection by \textit{vif}-negative HIV-1, indicating that reverse transcription is completed efficiently. However, there is a 10-fold reduction in all forms of viral DNA in \textit{vif}-negative virus-infected cells compared with wt virus-infected cells when using virus prepared from partially complementing RD cells. Since the ratio of full-length viral DNA to nuclear DNA in \textit{vif}-negative virus- and wt virus-infected cells is similar, localization of DNA to the nucleus is unaffected by \textit{vif} mutations (Fig. 5). The 10-fold reduction in the level of strong-
FIG. 12. Synthesis of viral RNA by MDM infected with wt or vif mutant HIV-1 derived from MDM. RNA was isolated from cells or from sedimented cell supernatants (virus) and was subjected to reverse transcriptase PCR for HIV-1 of stop DNA. Suggests that the major limitation to the infectivity of vif-negative virions is their ability to enter cells, uncoat, or initiate reverse transcription. These defects are consistent with aberrations in envelope glycoprotein display on particles (34), in virion architecture (6, 22), or in Gag protein composition (11, 36), all of which have been attributed to the absence of Vif during HIV-1 production in nonpermissive cells.

Defects in vif-negative viruses can be masked by cell-dependent complementation during virus replication, and the degree of complementation differs among cells (6, 16, 45). Complementation is revealed by the differences in the biological activity of vif-negative virus produced by different cells during infection of noncomplementing cells like MDM. RD cell-derived vif mutant HIV-1 produced complete and circularized forms of viral DNA and viral p24, all at approximately 10% of the level produced by wt virus in MDM (Table 1; Fig. 3 and 4). However, MDM infected by the same dose (in p24) of viruses produced by MDM were unable to synthesize p24 (Fig. 10) and synthesized approximately 2,500-fold less viral DNA than did wt virus (Fig. 11). Thus, RD cells partially replace vif function and produce a virus only moderately (10-fold) defective.

In contrast, MDM synthesize virions which are attenuated in infectivity, virions we infer to be representative of vif defects with little interference from cellular factors. On the basis of these findings and previous studies from this laboratory (36) and others (6, 16), we believe that the differences in infectivity of RD- and MDM-derived viruses may be attributed to differences in capsid structure. In this view, both cells produce heterogeneous vif-negative virions, a minority of virions carry only p24, and it is this minority which is infectious. Studies with suboptimal doses of an inhibitor of the HIV-1 protease indicate that a minor component of unprocessed Gag renders HIV-1 particles noninfectious (23). Previous studies from this laboratory illustrate that vif-negative particles produced by PBL carry various amounts of unprocessed Gag and a small amount of mature p24 but are severely impaired in infectivity (36).

Unlike studies of other retroviruses, the initial work on HIV-1 was conducted most often in transformed cells, which provided the original designation of certain genes as accessory (19). Recent studies (1, 3, 11, 17, 24, 28, 45) including our own (36), have refined these definitions by investigating the virus life cycle in primary lymphocytes and macrophages and have revealed a greater role for accessory genes than was first proposed. On the basis of the degree of HIV-1 attenuation by ablation of vif estimated here (3 to 4 orders of magnitude), the major role of vif in HIV-1 replication in both macrophages and lymphocytes in culture, and its maintenance in lentiviruses (30) and in HIV-1-infected persons (39), we propose that vif is a critical determinant of HIV-1 virulence during natural infection of humans. Consistent with this proposition, a long-term survivor of HIV-1 infection was shown recently to harbor virus carrying multiple mutations in accessory genes, including vif (27), and one of these mutations was the missense cysteine replacement described first by this laboratory (26). This work indicates that vif mutant viruses persist in vivo and are not pathogenic. The results reported here provide a potential molecular basis for this chronic, avirulent infection.

ACKNOWLEDGMENTS

We thank Galina Bentsman for excellent technical assistance, Linda Peters for typing the manuscript, J. Allan for gifts of monoclonal antibody, and M. Simm for illuminating discussions.

This work was supported by grants HL43629 and AI35466 from the Public Health Service.

REFERENCES


