HIV-1 Macrophage Tropism Is Determined at Multiple Levels of the Viral Replication Cycle

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Abstract

The ability of HIV-1 to infect macrophages is thought to be essential in AIDS pathogenesis. We tested the ability of 19 primary virus isolates to infect monocyte-derived macrophages (MDM) from different donors. Two HIV-1 isolates were able to establish a productive infection in MDM from all donors tested, whereas eight completely lacked this capacity. Next to these isolates with extreme phenotypes, 50% of the primary isolates under study displayed an intermediate phenotype. These intermediate macrophage-tropic isolates established a productive infection in MDM from some but not all donors tested. PCR analysis demonstrated that the capacity to replicate in MDM could be determined at the previously described level of virus entry. However, for intermediate macrophage-tropic isolates replication was abrogated at the level of reverse transcription. Entry of highly macrophage-tropic isolates resulted in efficient completion of the reverse transcription process, whereas entry of intermediate macrophage-tropic isolates did not. Our experiments indicate that primary HIV-1 isolates may differ in their dependency on cellular factors required for reverse transcription in MDM. Differences in susceptibility of MDM for in vitro HIV-1 infection suggest variation in the availability of these cellular factors between MDM from different individuals. (*J. Clin. Invest.* 1994. 94:1806–1814.) Key words: reverse transcription • monocyte • CD4 • AIDS • virus entry

Introduction

HIV-1 macrophage-tropism is thought to be essential in AIDS pathogenesis. Infection of macrophages in addition to T-lymphocytes enables virus spread to a variety of tissues in which HIV-1–infected macrophages may constitute a reservoir for the virus (1–5). The predominance of macrophage-tropic HIV-1 isolates during the stable asymptomatic period (6) and their presence throughout all stages of infection (7, 8) is suggestive of their importance in viral persistence. Moreover, HIV-1 infec-

tion of macrophages may contribute to immunological deterioration, finally resulting in the development of AIDS (9, 10).

The third variable domain (V3)[1] of the envelope molecule gp120 has been shown to be an important determinant of viral phenotype. Minor amino acid substitutions in V3 affect macrophage tropism (11–16), syncytium inducing capacity (17, 18), and infectivity (16, 18) of certain virus isolates. However, V3 appeared not to be the only determinant for HIV-1 phenotype variability. Studies with chimeric viruses revealed involvement of other parts of the viral genome, such as the second variable domain (V2) of gp120 (19, 20), tat (21), vif (22), or vpr (23), highly dependent on the virus strain used.

Whereas early studies indicated that restriction of HIV-1 tropism is determined at the level of entry (13, 24–26), viral requirements independent of envelope function are suggestive for involvement of other steps of the viral replication cycle as well. Indeed, recent data suggest that efficient virus–cell membrane fusion does not necessarily lead to efficient infection of macrophages (27). The exact mechanism that lead to abortive infection of macrophages however, has not yet been defined.

We and others previously described differences between HIV-1 isolates with respect to their ability to productively infect monocyte-derived macrophages (MDM) and differences between MDM from different donors to support a productive HIV-1 infection (7, 28). Here, using clinical virus isolates and MDM from different seronegative donors, we investigated distinct steps of the viral replication cycle that might determine the outcome of HIV-1 infection. We demonstrate that next to the previously described level of virus entry, establishment of a productive HIV-1 infection in MDM can be restricted at the level of reverse transcription of the viral genome. In addition, our data show that the differential HIV-1 susceptibility of MDM from different individuals is determined at this post-entry level, pointing to differences in availability of cellular requirements that may be essential for HIV-1 replication.

Methods

Cell isolation and culture. Monocytes were prepared from PBMCs from plasmapherese donors as described previously (29). All donors were adult males, aged 32 to 49, and were tested negative for HIV, HTLV, CMV, and hepatitis A, B, and C viruses. In brief, PBMCs were obtained from citrated venous blood by isolation on a Percoll density gradient and then enriched for monocytes by centrifugal elutriation. Adherent cells after centrifugal elutriation were routinely over 95% pure monocytes as determined by nonspecific esterase staining. Monocytes were cultured at a cell concentration of 10^6 per ml in endotoxin-free (30)

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1. *Abbreviations used in this paper:* MDM, monocyte-derived macrophages; RT, reverse transcriptase; V3, third variable domain of the HIV-1 envelope gp120 molecule.

Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% pooled human serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were maintained at 37°C in a humidified atmosphere supplemented with 5% CO_2. Cultures were kept for four weeks and the medium was changed once a week.

Viruses. Laboratory strains HIV-Ba-L and HIV-IIIB were obtained from M. Popovic and R.C. Gallo (5, 31). Isolate ACH-172.Ba-L was obtained from cells present in a broncho alveolar lavage from a patient with Kaposi Sarcoma (6). All other isolates were obtained from PBMC of asymptomatic individuals and individuals with AIDS as described previously (7, 32, 33). Virus stocks were grown in 3 d PHA (1 μg/ml) stimulated PBL except for HIV-Ba-L and ACH-172.Ba-L which were grown on MDM. Cell-free supernatants were stored at -80°C. Amounts of virus in stocks were determined by measurement of p24 protein content in a p24 antigen capture ELISA (34) and reverse transcriptase (RT) activity.

Cell-free inoculation of MDM. Monocytes were cultured for 5 d before inoculation to obtain MDM. Inocula with equal RT activity and p24 content were DNaseI (200 ng/ml RQ1 DNase; Promega, Madison, WI) treated for 30 min at 37°C in medium containing 6 mM $MgCl_2$ and filtered through a 0.22-μm filter. Before inoculation, part of the cells were incubated for 30 min at 37°C in the presence of 10 μM zidovudine which prevents reverse transcription, to discriminate newly synthesized DNA from DNA present in the inoculum. Cells were incubated for 24 h with virus (10^6 cpm RT activity/10^6 MDM) after which the cells were carefully washed. 5×10^6 MDM were used for DNA isolation and 10^6 were cultured for 4 wk. Once a week, medium was changed and twice a week p24 antigen production was measured.

DNA isolation. Total DNA was isolated from MDM by proteinase K treatment in the presence of 0.5% SDS, followed by extraction with phenol-chloroform and precipitation with 2-propanol. DNA was quantified by Ethidium-bromide staining after agarose gel electrophoresis and 250 ng DNA was used for PCR. HIV-1 DNA standards used to quantify viral DNA were prepared by dilution of 8E5 cells (35) in uninfected PBMC. DNA isolation and PCR analysis of standards was performed in parallel with the samples.

PCR analysis. For all PCR primer-sets, $MgCl_2$ concentration and thermo-cycling were optimized. We used a two step nested PCR amplifying a conserved pol fragment as described previously (36). Primers used in the first step: pol-D: 5'-TTA.GTC.AGT.GCT.GGA.ATC.AGG-3' (positions relative to the HxB2D proviral genome: nucleotides 4447–4467) and pol-F: 5'-GCT.ACA.TGA.ACT.GCT.ACC.AGG-3' (nucleotide positions 4199–4219). In the second step: pol-B: 5'-TTA.ACC.T-GC.CAC.CTG.TAG.TAG.C-3' (nucleotide positions 4410–4429) and pol-E: 5'-ATG.TGT.ACA.ATC.TAG.TTG.CC-3' (nucleotide positions 4305–4335). Amplification cycles of the first PCR: 5' 95°C once, 1'30'' 95°C, 2' 50°C, 2' 72°C, repeated 25 times, followed by extra 5' extension at 72°C and subsequent cooling to 4°C. Amplification cycles of the nested PCR: 5' 95°C, 2'30'' 25°C, 1'30'' 72°C once, 1'30'' 95°C, 1' 37°C, 2' 72°C, repeated 25 times, followed by extra 5' extension at 72°C and subsequent cooling to 4°C. PCR products were separated on 1.5% agarose gels, blotted on Genescreen membranes and hybridized with α32P-dATP end-labeled oligonucleotide pol-C 5'-GCC.ATG.-CAT.GGA.CAA.GTA.GAC.TGT.AGT.CCA.GG-3' (nucleotide positions 4373–4404) as described below.

Reverse transcription was monitored with primers M667–AA55 in the presence of 2 mM $MgCl_2$, M667–M661 in the presence of 3 mM $MgCl_2$ as described previously (37) and with the first pol primer-set in the presence of 3 mM $MgCl_2$ (locations of the primer-sets in the HIV-1 genome are depicted in Fig. 5). As a control for the general efficiency of PCR amplification of the DNAs, all DNAs were subjected to PCR analysis with primer-set GP1-GP2 (5'-ATA.AGC.TTG.CTG.CTC.CT-G.CTG.CCA.AGC.C-3', 5'-TAG.AAT.TCA.GCA.TTG.TCC.TGC.AG-C.CAG.CG-3', respectively) amplifying part of the human platelet glycoprotein Ibα gene (38) or primer-set CD4A-CD4B (5'-AAG.CT-G.AAT.GAT.CGC.GCT.GAC.TCA.AGA-3', 5'-ACT.ATC.CTG.GA-G.CTC.CAG.CTG-3', respectively) amplifying part of the human CD4 gene (10). For quantification, PCR products were directly spotted

		MACROPHAGE DONOR					
VIRUS ISOLATE	**SI/NSI**	**A**	**B**	**C**	**D**	**E**	**F**
ACH-172.BA-L	NSI	7	7	11	7	11	11
HIV-BA-L	NSI	7	7	11	11	11	11
AMS-44	NSI	16	28	16	21		
ACH-63.11	NSI	28		16		21	
ACH-15.9	NSI	28	28	28			
AMS-24	NSI	16	28				
HIV-IIIB	SI			16			
AMS-117	NSI		28				
ACH-168.1	NSI		28				
AMS-37	SI	16					
ACH-479.7	SI	16					
AMS-169.2	NSI						
AMS-167.2	NSI						
AMS-105	NSI						
ACH-320.2A.5	SI						
AMS-258	SI						
ACH-16.1	SI						
ACH-168.7	SI						
AMS-42	SI						

Figure 1. Ability of 19 HIV-1 isolates to establish a productive infection in MDM from six seronegative donors. 5-d cultured MDM were infected with cell-free virus (5.10^5 cpm of RT activity). Virus-production in MDM cultures was measured repeatedly in a p24 core antigen-capture ELISA. Cultures were considered positive when at least two sequential samples gave values more than three times the background extinction value. Grey shaded boxes indicate p24 positive samples, white boxes p24 negative samples. First day of virus detection is indicated.

on Genescreen membranes in parallel with amplimers from DNA standards using a slot-blot apparatus (GIBCO BRL, Gaithersburg, MD) and hybridized with α32P-dATP end-labeled oligonucleotides LTR-B (5'-GCA.CTC.AAG.GCA.AGC.TTT.ATT.GAG.GC-3') for M667–AA55 and M667–M661 amplified fragments, pol-C for polB-polF amplified fragments, GP3 (5'-CCT.CAC.TCA.GCT.GAA.CC-3') for GP1-GP2 amplified fragments and CD4C (5'-CAA.GGT.CAG.GGT.CAG.GCT.-CTG.CCC.CTG.AAG.CAG-3') for CD4A-CD4B amplified fragments. Since all PCR reactions resulted in uniform bands after hybridization (signals of bands of unexpected length were below 5% of total hybridization signals, see Fig. 6 C), slot-blots rather than southern blots were used for practical reasons. Hybridization to end-labeled oligonucleotides was performed overnight at 42°C in buffer consisting of 3× SSPE, 2× Denhardt's reagent, 1%SDS, 2 mg/ml yeast RNA, 0.15 mg/ml denatured fragmented salmon sperm DNA and filters were subsequently washed two times 15 min with 2× SSPE, 1% SDS at 42°C. Dependent on the specific activity of the probes, exposure to x-ray films was performed 2–16 h at -70°C with intensifying screens.

Results

Replication of HIV-1 isolates in MDM from different donors. We and others previously described differences between primary HIV-1 isolates with respect to their ability to productively infect MDM (7, 28). Here, we characterized a set of 2 laboratory strains and 17 clinical HIV-1 isolates obtained from 16 seropositive individuals for their ability to replicate in MDM from 6 seronegative individuals (Fig. 1). Each isolate was

A

B

C

D
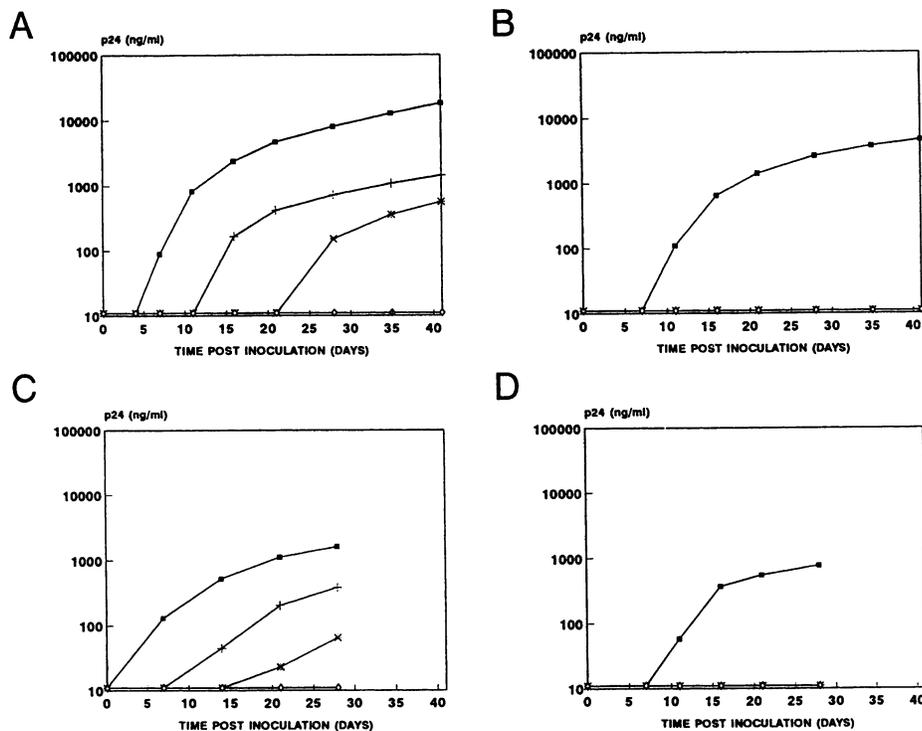
Figure 2. Kinetics of replication of HIV-1 variants in MDM from different donors. MDM from donors A (*A* and *C*) and F (*B* and *D*) were inoculated with cell-free virus (5.10^5 cpm of RT activity) of isolates ACH-172.Ba-L(■), Ams-24(+), ACH-15.9(*), and Ams-42(◊) after which virus production was measured with a p24 antigen capture ELISA. MDM were isolated from PBMC obtained from the two donors in December 1989 (*A* and *B*) and May 1992 (*C* and *D*).

tested on the same MDM preparation. MDM were inoculated with equal doses of cell-free virus (5×10^5 cpm RT activity per 10^6 MDM). Inocula were standardized on basis of RT activity rather than $TCID_{50}$ or MOI, since the latter methods for quantification are strongly biased by the susceptibility of the target cells on which the titer is determined (6). Virus production was measured twice a week, for a period of 4 wk using a p24 antigen capture ELISA (34).

With respect to their capacity to establish a productive infection in MDM, a spectrum of phenotypically distinct HIV-1 isolates was observed, ranging from non–macrophage-tropic isolates to highly macrophage-tropic isolates. Highly macrophage-tropic isolates HIV-Ba-L and ACH-172.Ba-L replicated in MDM regardless the MDM-donor used. Eight isolates did not replicate in MDM from any of the donors tested and may therefore be considered non–macrophage-tropic. Next to these isolates with extreme phenotypes, nine isolates displayed an intermediate phenotype. These intermediate macrophage-tropic isolates could replicate in MDM from some but not all donors tested. In PHA-stimulated PBMC all virus isolates represented in Fig. 1 replicated very efficiently, indicating all isolates are replication competent (data not shown).

Second, a spectrum of HIV-1 susceptibility of MDM was observed, ranging from relatively susceptible MDM from donor A to insusceptible MDM from donor F (respectively supporting replication of 42 to 10 percent of the isolates tested). In Fig. 2 replication kinetics of four isolates in MDM from donors A (Fig. 2 *A*) and F (Fig. 2 *B*) are shown. The macrophage-tropic isolate ACH-172.Ba-L replicated with comparable kinetics in MDM from donors A and F. Intermediate macrophage-tropic isolates Ams-24 and ACH-15.9 did not replicate in MDM from donor F, and with delayed and lower virus production in MDM from donor A as compared to ACH-172.Ba-L. In general, as compared with intermediate macrophage-tropic isolates, p24 an-

tigen production by macrophage-tropic isolates was observed earlier and reached higher levels. Moreover, in relatively susceptible MDM p24 antigen production was generally earlier detectable and higher than in less susceptible MDM.

The experiment shown in Fig. 2, *A* and *B* was repeated with MDM isolated from the same donors ~ 2.5 yr later. Replication kinetics of the four distinct HIV-1 isolates was comparable in MDM obtained in December 1989 (Fig. 2, *A* and *B*) and May 1992 (Fig. 2, *C* and *D*). These experiments indicated that susceptibility of the MDM to HIV-1 infection was relatively constant over time, suggesting that susceptibility is a stable characteristic of the donor rather than a temporal property. The seronegative status of the plasmapherese donors for HIV, HTLV, CMV, and Hepatitus A, B, and C viruses rules out a role for these viruses to act as co-factors for in-vitro HIV-1 susceptibility of MDM. However, other unidentified viruses may still play a role. We also found no correlation between in vitro susceptibility of the MDM and age or gender of the donor.

Post-entry determinants for productive HIV-1 infection of MDM. Differences in tropism as observed between macrophage-tropic and non–macrophage-tropic isolates might be determined at the level of virus entry, as has previously been demonstrated (26). Establishment of a productive infection by intermediate macrophage-tropic isolates in MDM from susceptible donors suggested that these isolates express envelope proteins capable of fusion, allowing entry into MDM. Therefore, limitations of intermediate macrophage-tropic isolates to replicate in less susceptible MDM are suggestive for restriction at a post-entry level.

We next tested at which level of the viral replication cycle establishment of a productive infection in MDM was restricted. MDM from three different seronegative donors were cell-free inoculated with equal doses (10^5 cpm RT activity/10^6 MDM) of seven virus isolates. Selection of the virus isolates was based

A

HIV-1 COPY NUMBERS   0   2   5   10   100   1000

PolD-PolF

NESTED POL

CD4A-CD4B

B

ACH-172.Ba-L   HIV-Ba-L   AMS-24   AMS-42   AMS-37   ACH-15.9   HIV-IIIB

MDM-1

Zidovudine   + - + - + - + - + - + - + -
p24   - + - + - - - - - - - - - +

Pol 125bp

MDM-2

Zidovudine   + - + - + - + - + - + - + -
p24   - + - + - - - - - - - - - -

Pol 125bp

MDM-3

Zidovudine   + - + - + - + - + - + - + -
p24   - + - + - - - - - - - - - -

Pol 125bp
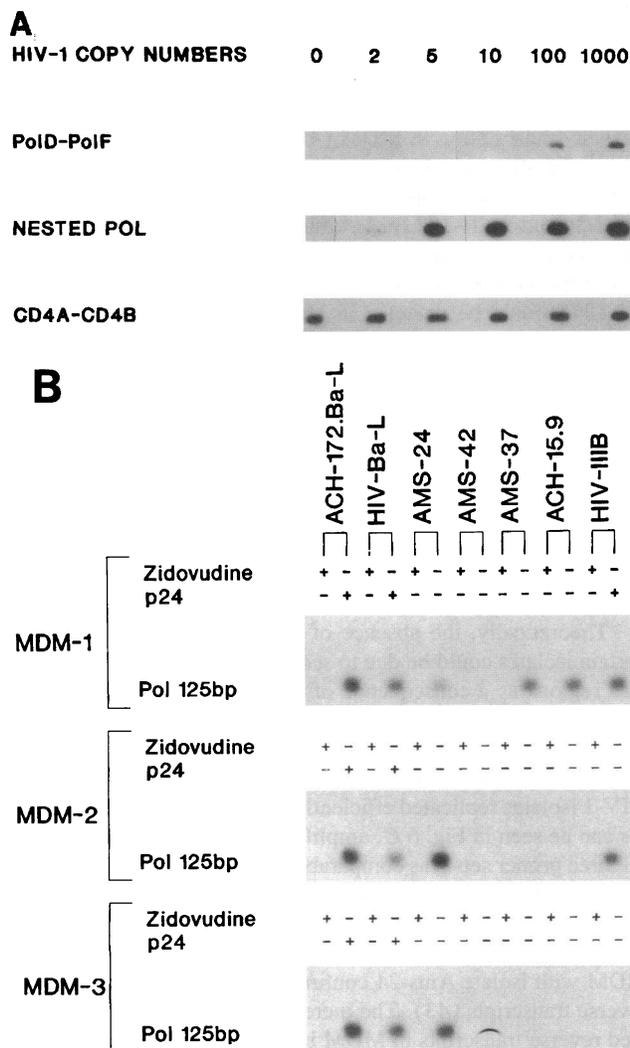
Figure 3. (*A*) PCR analysis on DNA obtained from serial dilutions of 8E5 cells in uninfected PBMC. The first PCR (polD-polF) amplifies proviral DNA in the linear range as determined by densitometric scanning. The nested PCR (polB-polE) reaches plateau levels of amplification in samples containing 5 or more copies of proviral DNA and detects a minimum of two copies. Primer-set CD4A-CD4B was used to amplify part of the human CD4 gene as a control for DNA input. (*B*) Nested PCR analysis for the presence of proviral DNA in MDM from three different seronegative donors, 24 h after cell-free inoculation with seven virus isolates. Inocula were treated with DNaseI to detect only newly synthesized proviral DNA. Zidovudine-treated MDM were used as controls for DNaseI treatment and PCR contamination. Ability to establish a productive infection as measured with a p24 core antigen-capture ELISA is indicated. Southern blots display the amplified 125-bp pol fragment.

on their capacity to replicate in MDM as depicted in Fig. 1: two macrophage-tropic isolates HIV-Ba-L and ACH-172.Ba-L; four intermediate macrophage-tropic isolates Ams-24, ACH-15.9, HIV-IIIB, and Ams-37, and a non–macrophage-tropic isolate Ams-42. After 24 h, cells were washed and total DNA was isolated and used in a highly sensitive PCR with nested primer sets spanning the pol region of HIV-1 (36) (Fig. 3 *B*). Parallel cultures were kept for 4 wk to monitor p24 production. For validation of nested pol PCR analysis, see Fig. 3 *A* (Note

that intensity of the bands after nested PCR do not reflect differences in copy numbers in the original samples since amplification is continued to plateau levels). All viral inocula were treated with DNaseI to ascertain only newly synthesized DNA would be detected in this experiment. As a control for efficacy of DNaseI treatment duplicate cell samples were incubated for 30 min at 37°C in the presence of 10 μM zidovudine before inoculation, which prevents reverse transcription. Zidovudine-treatment of MDM before inoculation indeed resulted in the complete absence of proviral DNA in this PCR analysis, indicating that all proviral DNA in Fig. 3 was newly synthesized. This also excluded the possibility that our PCR data were obscured by proviral DNA carried in virions (39, 40). Detection of proviral DNA upon inoculation of MDM in this experiment thus would provide conclusive evidence for virus entry.

Inoculation with HIV-Ba-L and ACH-172.Ba-L resulted in positive PCR and high p24 production in MDM from all three donors thus confirming the highly macrophage-tropic characteristics of these isolates. Upon inoculation of MDM from the three donors with isolate Ams-42, PCR analysis remained negative and p24 production could not be detected, indicating that an early step of the replication cycle was blocked. Upon inoculation with isolate Ams-24 proviral DNA could be detected in MDM from all three donors but establishment of a productive infection as determined with a p24 antigen capture ELISA was not observed. PCR analysis upon exposure to HIV-IIIB, Ams-37, and ACH-15.9 resulted in positive PCR signals in MDM-cultures from 1 or 2 out of 3 donors tested. Subsequent establishment of a productive infection however, was only observed in MDM from donor two inoculated with laboratory strain HIV-IIIb, confirming the intermediate macrophage tropic capacity of this virus (28). Detection of proviral DNA upon inoculation with the four intermediate macrophage-tropic isolates indicated their ability of entry, uncoating, and (at least partial) reverse transcription in MDM. Since establishment of a productive infection could not be demonstrated, replication of these isolates in MDM seemed to be blocked at a post-entry level.

Differential macrophage-tropism is not caused by quantitative differences in viral inocula. To exclude that results from Fig. 3 might be explained by differences in viral inoculum size, we performed a comparable experiment as presented in Fig. 3 but used 1:1, 1:10, 1:100, and 1:1,000 dilutions of DNaseI treated virus stocks with equal RT activity and p24 content from ACH-172.Ba-L, Ams-24 and ACH-15.9 to inoculate highly susceptible MDM (donor A, Fig. 1). For the detection of proviral DNA, again the nested PCR was used. P24 production was monitored in parallel samples. The absence of PCR products in MDM treated with zidovudine before inoculation again pointed to efficient DNaseI treatment. Inoculation with 1,000-fold diluted virus-stocks from Ams-24 and the macrophage-tropic isolate ACH.172.Ba-L resulted in PCR negative MDM cultures 24 h after inoculation, whereas exposure to 1,000-fold diluted stocks from the intermediate macrophage-tropic isolate ACH-15.9 still remained PCR-positive (Fig. 4). MDM inoculated with a 1:10 diluted inoculum of Ams-24 and ACH-15.9 did not result in virus-production, whereas inoculation with 1:10 dilution of ACH-172.Ba-L still resulted in establishment of productive infection. These findings thus exclude quantitative differences in inocula as an explanation for differential macrophage-tropism of HIV-1 isolates.

Macrophage tropism of HIV-1 can be determined at the level of reverse transcription. Next, the process of reverse tran-

	ACH-15.9					AMS-24					ACH-172.Ba-L				
Inoculum	1:1000	1:100	1:10	1:1	1:1	1:1000	1:100	1:10	1:1	1:1	1:1000	1:100	1:10	1:1	1:1
Zidovudine	–	–	–	–	+	–	–	–	–	+	–	–	–	–	+
p24	–	–	–	+	–	–	–	–	–	+	–	–	+	+	–

Pol 125bp
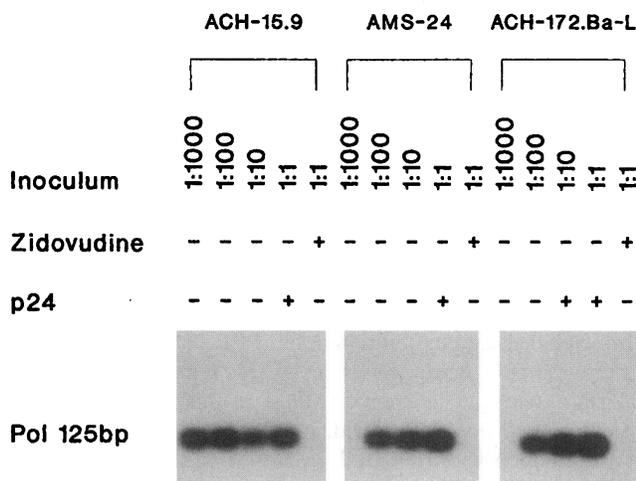
Figure 4. Nested PCR analysis for the presence of proviral DNA in MDM, 24 h after inoculation with diluted DNaseI treated virus-stocks from four established virus isolates. Zidovudine-treated MDM inoculated with undiluted virus-stocks were used as controls for DNaseI treatment and PCR contamination. Ability to establish a productive infection as measured with a p24 core antigen-capture ELISA is indicated. Southern blots display the amplified 125-bp pol fragment.

scription, which is one of the first steps of the viral replication cycle after entry, was monitored. For this purpose, a semi-quantitative PCR analysis was performed with previously described primer sets that discriminate between proviral DNA species synthesized early and late during reverse transcription (37). The reverse transcription process and the location of the primer-sets used in this experiment are illustrated in Fig. 5. DNA-standards (8E5 cells containing 1 copy of proviral DNA per cell [35] were diluted in uninfected PBMC) were processed in parallel with DNA samples to allow comparison of copy numbers of the (incomplete) reverse transcripts. As a control for the general efficiency of PCR amplification, all DNAs were subjected to PCR amplification of the human platelet glycoprotein Ibα gene (38). PCR analysis as used in this experiment was optimized for $MgCl_2$ concentration and thermocycling and allows amplification in the linear range as determined by densitometric scanning (data not shown).

MDM were inoculated with 10^4 cpm of RT activity/10^6 MDM from virus isolates that displayed large differences in macrophage-tropic capacity (Fig. 1, ACH-172.Ba-L, Ams-24, ACH-15.9, and Ams-42) and DNA was isolated 1 and 7 d after inoculation. Parallel cultures were kept for 4 wk and monitored for virus production twice a week using a p24 antigen capture ELISA (34). Inoculation of zidovudine-treated MDM with the four virus stocks resulted in extremely low (Ams-42) or undetectable levels of proviral DNA at day 1, indicating that proviral DNA detected in this experiment was newly synthesized (Fig. 6 B).

Primer-set M667-AA55 (37), used for amplification of early reverse transcripts, resulted in strong signals at 1 d after inoculation in MDM exposed to isolates Ams-24 and ACH-172.Ba-L, indicative for efficient entry and initiation of reverse transcription. MDM inoculated with ACH-15.9 revealed only a moderate signal with this primer set, whereas in cells exposed to the non–macrophage-tropic isolate Ams-42 only very few early reverse transcription products could be detected, pointing to less efficient entry or reverse transcription initiation for these

two isolates. Analysis with primer-sets polD-polF and M667–M661 revealed only low levels of elongated and absence of complete products of reverse transcription respectively at 1 d after inoculation, confirming the relatively slow progression of reverse transcription in MDM (41, 42).

At 7 d after inoculation, reverse transcription throughout the pol-region was accomplished in MDM inoculated with isolates Ams-24 and ACH-172.Ba-L, which indicates that the first template switch event and first strand DNA synthesis in the reverse transcription process were completed. In contrast, no pol proviral DNA could be detected in MDM exposed to Ams-42 and ACH-15.9, as might be expected from the low copy numbers of early reverse transcripts 1 d after inoculation.

Despite comparable copy numbers of initiated reverse transcription products 1 d after inoculation with isolates Ams-24 and ACH-172.Ba-L, efficient completion of reverse transcription at day 7 was observed in cells exposed to ACH-172.Ba-L only, indicating lower efficiency of reverse transcription for the intermediate macrophage-tropic isolate Ams-24. In agreement, productive infection was only observed in MDM cultures inoculated with ACH-172.Ba-L (data not shown).

Theoretically, the absence of specific RT transcripts from certain isolates could be due to sequence variation in the amplified regions as a consequence of which primers for PCR may not be able to anneal. To exclude this possibility, we performed PCR analysis for proviral DNA in PHA-stimulated PBMCs, productively infected with the different HIV-1 isolates. All 4 HIV-1 isolates replicated efficiently in PHA stimulated PBMCs. As can be seen in Fig. 6 C, amplification of proviral DNA with all three primer sets was comparable for the four HIV-1 isolates, excluding sequence variation in the amplified regions as explanation for the results in Fig. 6 A.

The decrease in copy numbers in time upon inoculation of MDM with isolate Ams-24 confirmed instability of incomplete reverse transcripts (43). The increase in copy numbers of initiated reverse transcripts in MDM inoculated with ACH-172.Ba-L probably resulted from reinfection and consequently continuous initiation of reverse transcription.

Similar results were obtained with intermediate susceptible MDM from two additional donors (data not shown). Moreover, a 10-fold increased inoculum did not change the results, confirming that qualitative rather than quantitative differences in inocula are important (Fig. 4).

From these data we conclude that reverse transcription is an important determinant for replication of primary HIV-1 isolates in MDM. Intermediate macrophage-tropic isolates, comprising 50% of all primary HIV-1 isolates, may enter MDM efficiently, but lack the ability to complete reverse transcription in MDM from some donors.

Discussion

Until now, molecular determinants for HIV-1 T cell line and macrophage-tropism have been investigated by using molecular clones from established HIV-1 isolates. In most cases, tropism was reported to be determined by the viral envelope gene (11–16), at the level of virus entry (13, 24–26). Here, using primary HIV-1 isolates, we demonstrate that differences in macrophage-tropism as observed between absolute macrophage-tropic and non–macrophage-tropic isolates indeed might be determined at the level of virus entry. However, 50% of the primary HIV-1 isolates tested replicated in MDM from some donors

REVERSE TRANSCRIPTION
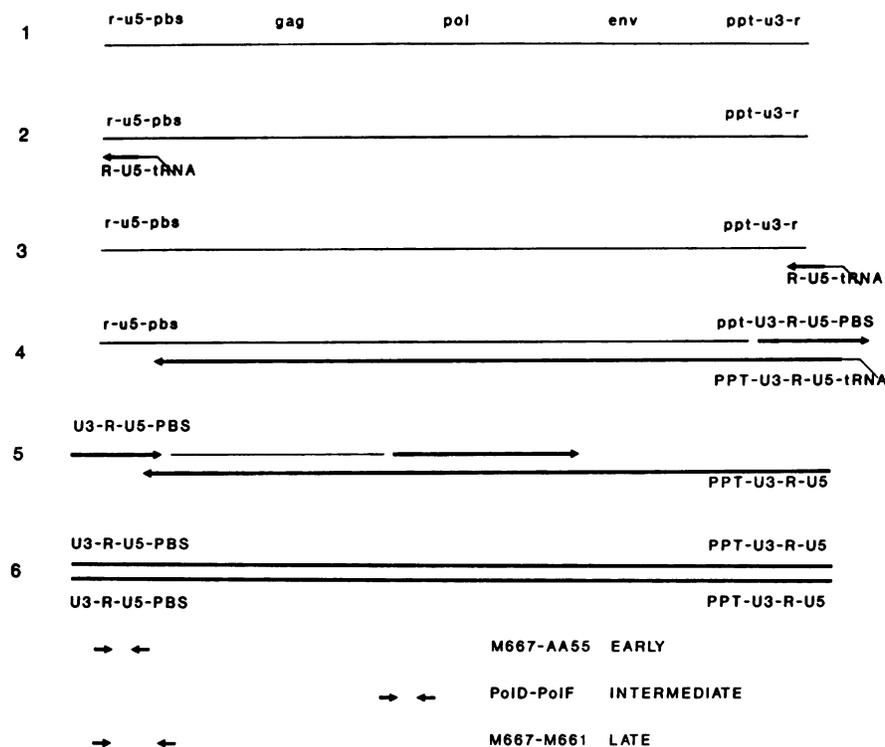
Figure 5. Schematic presentation of the reverse transcription process. Relative positions of the primer sets used for monitoring reverse transcription in the HIV-1 genome are indicated at the bottom of the diagram. Thin lines and small letters represent viral RNA whereas thick lines and capital letters represent proviral DNA. (*2*) A tRNA molecule primes cDNA synthesis of the unique 5' region (U5) to the end of R (repeat). (*3*) The R-U5 cDNA undergoes the first template switching event and anneals to the 3' R region. (*4*) R-U5 serves to prime synthesis of the minus strand proviral DNA and a second priming event at the 3' end of the genome enables synthesis of plus strand U3-R-U5-PBS cDNA. (*5*) After synthesis of minus strand DNA is completed throughout the PBS, a second template switching event occurs in which U3-R-U5-PBS hybridizes to the minus strand PBS. (*6*) DNA synthesis proceeds until full length proviral DNA with two LTRs is synthesized. Primer set M667-AA55 was designed for amplification of minus strand DNA early after RT initiation. Primer set PolD-PolF was used for detection of minus strand proviral DNA that underwent the first template switching event. Primer set M667-M661 was designed for amplification of proviral DNA after the second template switching event and subsequent polymerization had occurred. Adapted from (37, 54).

only. These intermediate macrophage-tropic isolates displayed delayed replication kinetics compared with highly macrophage-tropic isolates, even in highly susceptible MDM. Productive infection in MDM from some donors indicate that intermediate macrophage-tropic isolates express envelope proteins capable of fusion, allowing entry into macrophages. Moreover, by using PCR analysis we demonstrated efficient entry and initiation of reverse transcription without establishment of productive infection upon inoculation with intermediate macrophage-tropic isolates. Thus, post-entry determinants play an important role in macrophage-tropism of HIV-1, as has also been suggested by others (27, 44).

With PCR analysis, we indeed showed that for intermediate macrophage-tropic isolates, post-entry events are critical for establishment of productive infection in MDM and revealed the process of reverse transcription as the level at which macrophage-tropism can be abrogated. With the panel of primary isolates with variable capacity to replicate in macrophages, we demonstrated that highly macrophage-tropic isolates complete the process of reverse transcription more efficiently than intermediate macrophage-tropic isolates. Our data suggest that highly macrophage-tropic isolates are less dependent on certain, so far unknown, cellular factors required for reverse transcription than intermediate macrophage-tropic isolates.

Since only a limited number of primer sets was used in this study, we could not determine whether either overall reverse transcription or defined steps of reverse transcription may be impaired for intermediate macrophage-tropic isolates. It is tempting to speculate that the activity of the RT enzyme and possible existence of strong stops for reverse transcription in the viral RNA or first strand DNA may be the viral determinants

for efficient replication in MDM. In highly susceptible MDM, the incompetence to complete reverse transcription of certain virus isolates may be overcome by cellular factors.

In our experiments, differences in macrophage-tropism were not merely a reflection of virus titers as demonstrated with the diluted virus stocks (Fig. 4). Using supernatants from PBMC cultures containing short-term passaged full length molecular clones of HIV-1 (25) we obtained comparable results as with the virus isolates (data not shown), thus excluding the possibility that a macrophage-tropic variant within the virus isolates accounted for the viral DNA synthesis in some of our experiments.

The differences in HIV-1 susceptibility between MDM from different donors for intermediate macrophage-tropic isolates point to differential availability of certain cellular factors required for HIV-1 replication between individuals. Theoretically, differences in susceptibility might be determined at the level of virus entry due to polymorphism or differential expression of the CD4 molecule or other membrane-proteins that may be involved in HIV-1 fusion and entry (45). However, so far there is no evidence for polymorphism in the viral receptor that can account for the variation in susceptibility. Moreover, we demonstrated that differences in susceptibility are determined at a post-entry level (Figs. 3 and 6).

In earlier studies it has been shown that in resting T cells reverse transcription is the limiting step for virus replication. Activation or proliferation of T cells resulted in enhanced completion of reverse transcription (43, 46, 47). A similar situation may exist in MDM since activation (48, 49), differentiation (50), or proliferation of MDM (51) results in enhanced establishment of productive HIV-1 infection in MDM. Indeed, we found that reverse transcription of HIV-1 only occurred in

A

DNA STANDARDS

Ams-42   ACH-15.9   Ams-24   ACH-172.Ba-L

HIV-1 COPY NUMBERS   0   10   100   1000

DAYS POST INOCULATION   1   7   1   7   1   7   1   7

M667-AA55

PolD-PolF

M667-M661

GP1-GP2

B

Ams-42   ACH-15.9   Ams-24   ACH-172.Ba-L

M667-AA55

PolD-PolF

M667-M661

GP1-GP2

C

HIV-1 COPY NUMBERS

0   10   100   1000   AMS-42   ACH-15.9   AMS-24   ACH-172.Ba-L

M667-AA55   139 bp →

PolD-PolF   269 bp →

M667-M661   197 bp →
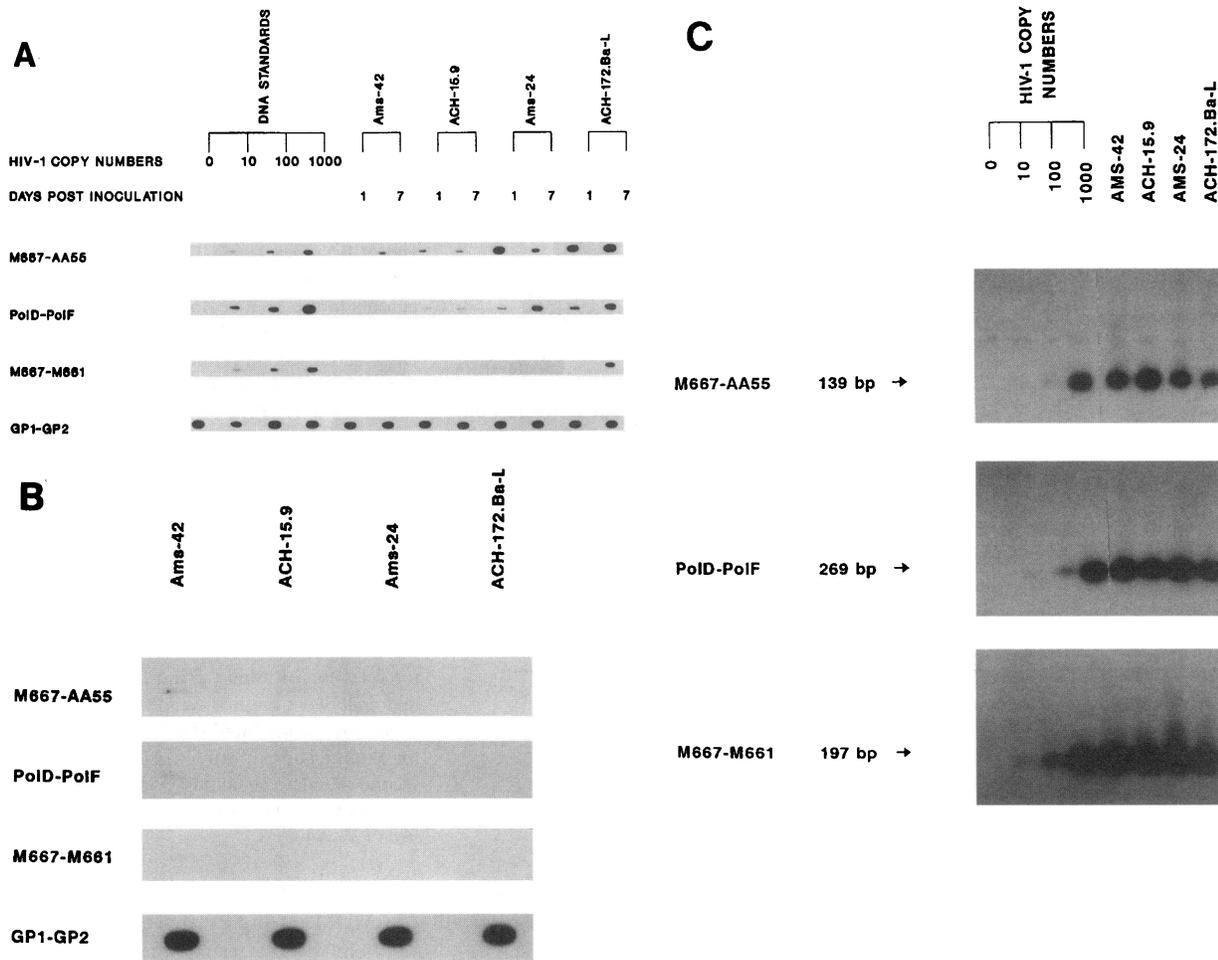
Figure 6. (*A*) PCR analysis for the presence of different proviral intermediates during reverse transcription in MDM 1 and 7 d after inoculation with DNaseI treated virus stocks from four HIV-1 isolates. Slot-blots display amplimers from samples and DNA standards. Only isolate ACH-172.Ba-1 was capable of establishing a productive infection in this experiment. Primer sets M667-AA55, PolD-PolE, and M667-M661 amplify early, intermediate and late reverse transcription products respectively. Primer-set GP1-GP2 was used to amplify part of the human glycoprotein Ibα as a control for DNA input. (*B*) Control PCR analysis 1 day post inoculation in zidovudine treated MDM. (*C*) PCR analysis of proviral DNA from PHA stimulated PBMC, productively infected (day 14) with the four HIV-1 isolates.

MDM with proliferative potential, apparently dependent on certain cellular factors present in the G1/S phase of the cell cycle (Schuitemaker, H., N. A. Kootstra, R. A. M. Fouchier, A. B. Van 't Wout, B. Hooibrink, and F. Miedema, manuscript submitted for publication). MDM from different individuals may vary in their in vitro activation or differentiation state or in their proliferative capacity, which may contribute to differential HIV-1 susceptibility. The nucleotide pool or other cellular components required for efficient reverse transcription may be rate-limiting in less susceptible MDM (41). This variation in cellular factors may be due to stable, genetic differences between MDM donors or temporal differences, for instance related to the degree of immune activation. The MDM donors used in this study are all male blood donors of comparable age, tested negative for hepatitis A, B, and C, CMV, HTLV, and HIV-1, suggesting that differences in gender, age, or one of these viruses that may act as cofactors for HIV-1 infection do not account for differential susceptibility. Our observation that repetitive use of MDM from different donors displayed identical susceptibility to HIV-1 infection over a 2.5-yr period (Fig. 2) suggests that stable possibly genetic differences between MDM donors may be an important determinant for HIV-1 susceptibility.

Our results demonstrate variable capacity of HIV-1 isolates to establish infection in primary macrophages and variable susceptibility of macrophages from different individuals to HIV-1 infection. These observations may be of clinical interest since in early HIV-1 infection the predominant viruses are macrophage-tropic, suggesting that macrophages are the first target cells upon virus-transmission (6, 52). This indicates that both the presence of macrophage-tropic HIV-1 variants in the inoculum and high susceptibility of the macrophages in the recipient may be advantageous for the virus to efficiently establish persistent infection. The susceptibility of an individual for infection upon HIV-1 exposure therefore may be in part determined by the susceptibility of this individual's macrophages in addition to immunological parameters (53).

Acknowledgments

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