

# Infection of SK-N-MC Cells, a CD4-Negative Neuroblastoma Cell Line, with Primary Human Immunodeficiency Virus Type 1 Isolates

JANET M. HAROUSE<sup>1,2,3</sup> AND FRANCISCO GONZÁLEZ-SCARANO<sup>2,3\*</sup>

*Graduate Group in Molecular Biology<sup>1</sup> and Departments of Neurology<sup>2</sup> and Microbiology,<sup>3</sup> University of Pennsylvania Medical Center, Philadelphia, Pennsylvania 19104-6146*

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**Most studies looking at CD4-independent infection have used laboratory strains or their respective molecular clones. To determine whether primary human immunodeficiency virus type 1 isolates could infect CD4-negative cells, we obtained a panel of 23 clinical isolates and characterized the early steps of the viral life cycle in SK-N-MC cells, a CD4-negative, galactosylceramide-positive neuroblastoma cell line. Eight of 23 isolates established a nonproductive infection; entry and postentry restrictions were noted in the others. We were unable to correlate the infectivity for SK-N-MC cells with established biological phenotypes, such as syncytium induction, or with genetic classifications, suggesting that pantropism is an independent biological variable.**

Human immunodeficiency virus type 1 (HIV-1) viral load is primarily due to infection of CD4-positive cells, specifically T lymphocytes and cells of monocyte/macrophage lineage, and the presence of CD4 is the principal determinant of cellular tropism. However, experimental evidence suggests that expression of the CD4 molecule is not the sole factor determining viral entry (7, 14); expression of human CD4 on the surfaces of nonprimate cells does not render these cells susceptible to HIV-1 infection (10, 28). Additionally, CD4-independent HIV-1 infection has been demonstrated *in vitro* in several human cell lines and in primary human cell cultures (6, 8, 9, 19, 23, 36, 38, 40). These observations indicate that other cell surface molecules function as coreceptors in the presence of the CD4 molecule or as alternative receptors in the absence of CD4.

Two closely related glycosphingolipids, galactosylceramide (GalCer) and 3'-sulfo-galactosylceramide (GalS), have been proposed as alternative cellular receptors for HIV-1 infection in some CD4-negative cell lines (15, 17). In these studies antibodies against GalCer inhibited HIV-1 entry and infection in neural and colonic epithelial cell lines. Concomitantly, purified recombinant gp120 (rgp120) bound to GalCer-GalS with specificity in enzyme-linked immunosorbent assays (ELISA), thin-layer chromatography assays, and liposome flotation assays, demonstrating a stable interaction between these molecules (3, 12, 15, 17, 27). Experiments with monoclonal antibodies (MAbs) and with recombinant viruses indicate that the envelope regions involved in GalCer-GalS entry encompass the V3, V4, and V5 domains and suggest that a specific conformation of the oligomeric virion-associated envelope protein is necessary for GalCer-GalS-mediated entry (12, 18, 22, 41).

GalCer is a major component of the plasma membranes of many neural cells, and it is commonly used as a cell surface marker for oligodendrocytes of the central nervous system and Schwann cells of the peripheral nervous system (32, 33). In addition to the nervous system, these glycolipids are also present on colonic and other intestinal epithelial cells and in

sperm cells (20, 24). Antibodies against lactosylceramide-II<sup>3</sup>-sulfate, a glycolipid closely related to sulfatide (GalS), blocked entry of HIV-1 into vaginal and colonic epithelial cells (16), hinting at a role for glycolipid-mediated entry in the dissemination of HIV-1.

Most studies investigating the mechanism of GalCer-GalS-mediated HIV-1 infection have used laboratory-adapted strains or their respective molecular clones, which are not representative of the viral populations found *in vivo*. In this study we asked whether primary HIV-1 isolates (PHI) derived from diverse genetic and biologic classifications (1, 11, 39, 42) could infect CD4-negative, GalCer-GalS-positive SK-N-MC cells. Furthermore, we used PCR in combination with oligonucleotide primers that amplify DNA species associated with specific stages of reverse transcription to analyze the viral life cycles of PHI in SK-N-MC cells.

**Entry of PHI into SK-N-MC cells.** Although all HIV-1 isolates can enter and initiate viral replication in CD4-positive primary cells, only a subset of strains have been studied in CD4-independent infections. To assay infectivity of PHI in SK-N-MC cells, we obtained a panel of 23 clinical HIV-1 isolates (Table 1) and screened them for their ability to enter GalCer-GalS-positive cells by PCR to detect newly synthesized viral DNA. SK-N-MC cells were inoculated with equivalent doses of cell-free virus (equilibrated by viral p24<sup>gag</sup> antigen in the supernatant), and cellular DNA was extracted and subjected to PCR analysis. SK-N-MC/CD4, an SK-N-MC cell line constitutively expressing CD4, was used as a control for all experiments (18). Detection of viral DNA with primer pair LTR1-LTR2 (35) indicated viral entry and the completion of the first strand switch of reverse transcription (Table 2) (29, 37). As illustrated in Fig. 1 and summarized in Table 1, 14 of 23 PHI could initiate reverse transcription in SK-N-MC cells. Entry of virus BR/93/029 was only occasionally detected in the SK-N-MC cells, and this isolate was considered to be restricted in entry-level events. With these primers, apparent differences in the amplified products may have been due to the rates of efficiency of entry and of reverse transcription initiation for each of the isolates. The abilities of primary isolates to enter and initiate reverse transcription in SK-N-MC cells did not correlate with other known tropism phenotypes nor with the replication kinetics or cytopathology of CD4-positive cells.

\* Corresponding author. Mailing address: Department of Neurology, University of Pennsylvania, Clinical Research Building, 415 Curie Blvd., Philadelphia, PA 19104-6146. Phone: (215) 662-3389. Fax: (215) 573-2029. Electronic mail address: Scarano@mail.med.upenn.edu.

TABLE 1. Detection of viral DNA by PCR and results of cocultivation in SK-N-MC and SK-N-MC/CD4 cells

HIV-1 isolate	Clade	Phenotype <sup>a</sup>	Test result with cell type:			
			SK-N-MC		SK-N-MC/CD4	
			Viral DNA (LTR) <sup>b</sup>	Virus rescue <sup>c</sup>	Viral DNA (LTR)	Virus rescue
RW/92/009	A	NSI	+	0.3	+	8.9
BR/92/021	B	SI	+	14.2	+	19.8
UG/92/021	D	SI	+	25.7	+	30.8
UG/92/029	A	SI	+	2.8	+	17.0
26-6884	B	NSI	+	0.4	+	17.1
AJ-4331	B	NSI	+	0.4	+	4.6
AJ-3879	B	NSI	+	0.1	+	3.7
AQ-5945	B	NSI	+	0.2	+	35.8
BR/92/025	C	SI	+	0.0	+	12.8
UG/92/035	D	NSI	+	0.0	+	12.9
AD-8874	B	NSI	+	0.0	+	22.8
AL-6244	B	NSI	+	0.0	+	21.7
AM-3107	B	NSI	+	0.0	+	30.3
AQ-6809	B	NSI	+	0.0	+	18.4
BR/93/029 <sup>d</sup>	D	SI	-	0.0	+	10.7
THA/92/022	E	NSI	-	0.0	+	11.5
THA/92/026	B	NSI	-	0.0	+	18.0
AQ-4697	B	NSI	-	0.0	+	21.7
SUMA	B	NSI	-	0.0	-	0.0
HOBR	B	NSI	-	0.0	-	0.0
FASH	B	NSI	-	0.0	-	0.0
WEAU	B	NSI	-	0.0	-	0.0
AD6	B	NSI	-	0.0	-	0.0

<sup>a</sup> SI, syncytium inducing; NSI, non-syncytium inducing.

<sup>b</sup> Viral DNAs were detected by PCR with LTR primers (Table 2).

<sup>c</sup> Cells were cocultivated with PBMC, and levels of p24<sup>gag</sup> in the supernatants were measured by antigen-capture ELISA 10 to 14 days later. Values are in nanograms per milliliter, and 0.0 is <0.012, the limit of the assay.

<sup>d</sup> HIV-1-specific DNAs were detected in two of seven experiments.

Furthermore, despite the presence of a functional CD4 molecule, SK-N-MC/CD4 cells were refractile to entry by a subset of isolates (Table 1), suggesting that additional cellular factors modulate entry of some isolates into SK-N-MC cells. As a control for the integrity of the target DNA, all samples were independently subjected to PCR analysis with primers that amplified the  $\beta$ -globin gene (data not shown).

**Infection of SK-N-MC cells with PHI can be inhibited by anti-GalCer MAb.** To determine whether infection by PHI could be inhibited by an antibody directed against GalCer, we preincubated cells with R-MAb, a MAb that inhibits entry of HIV-1 into GalCer-GalS-positive cells (2, 18, 40). As shown in Fig. 2 for five representative isolates, R-MAB inhibited the entry of PHI. In separate experiments cell cultures pretreated with either R-MAB or control MAb were cocultivated with peripheral blood mononuclear cells (PBMC) 72 h after infec-

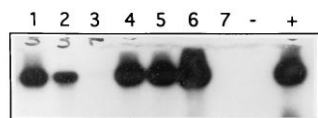


FIG. 1. SK-N-MC cells were infected with five representative primary HIV-1 isolates, and DNAs were isolated 18 h later (18), amplified with the LTR1-LTR2 (LTR1+, 5'-ACAAGCTAGTACCAGTTGAGCC-3'; LTR2-, 5'-GCACACA CTAAGCAAGCACTC-3') primer pair, and probed with a  $\gamma$ -<sup>32</sup>P-labeled internal oligonucleotide (Table 2) (35). Preincubation with AZT inhibited the synthesis of viral DNA. Lanes: 1, UG/92/021; 2, UG/92/029; 3, THA/92/026; 4, UG/92/035; 5, BR/92/025; 6, HxB2; 7, HxB2-AZT pretreatment; -, no DNA; +, positive PCR control.

tion and the culture supernatants were tested for the production of viral protein. Pretreatment of SK-N-MC cells with R-MAB reduced the amounts of viral p24<sup>gag</sup> antigen produced; however, SK-N-MC cells pretreated with a control MAB could be stimulated to produce viral p24<sup>gag</sup> antigen, depending on the isolate used for infection (see below and data not shown). R-MAB inhibited the PCR signal from all 14 isolates that initiated reverse transcription in the SK-N-MC cells (data not shown).

**Production of viral antigen in SK-N-MC cells.** The SK-N-MC cell line produces low levels of viral antigen when infected with laboratory-adapted HIV-1 strains, although the cells harbor proviral DNA that can be stimulated to produce infectious virus by treatment with exogenous factors or by cocultivation with CD4-positive cells (17, 26). To determine whether SK-N-MC cells could be stimulated to produce viral antigen when infected with primary isolates, we cocultivated SK-N-MC or SK-N-MC/CD4 cells with PBMC 72 h after virus exposure and determined the concentrations of p24<sup>gag</sup> antigen in the culture supernatants 10 to 14 days later (Table 1). Eight of the 14 isolates that were able to initiate reverse transcription in the SK-N-MC cells also produced measurable amounts of p24<sup>gag</sup> antigen (at least a 30-fold increase above the zidovudine [AZT] control value). Conversely, 6 of 14 PHI that initiated reverse transcription in the SK-N-MC cells gave negative results in the cocultivation, indicating a potential postentry block to viral replication. These results indicated that GalCer-GalS-mediated infection can be restricted at multiple points in the viral replication cycle.

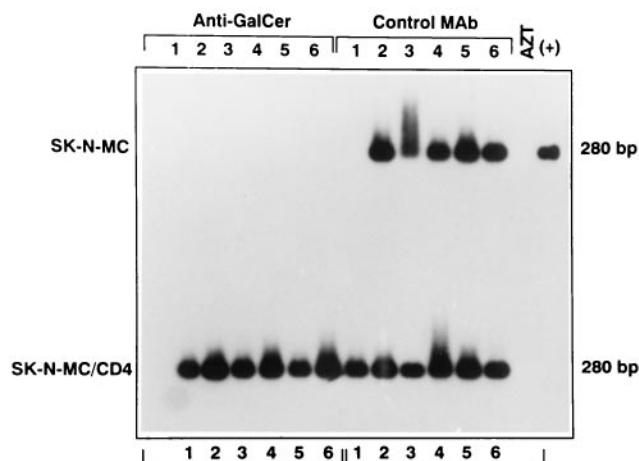


FIG. 2. Infections of SK-N-MC or SK-N-MC/CD4 cells with five representative primary isolates were performed with or without pretreatment with R-MAb, a MAb directed against GalCer-GalS (2, 33). DNAs were isolated 18 h after infection and amplified with primer pair GAG04 (5'-CATICTATTGTTCTG-3') (30, 31). Infections of SK-N-MC cells by PHI were blocked by R-MAb but not by a control MAb. Lanes: 1, THA/92/022; 2, RW/92/009; 3, BR/93/029; 4, UG/92/021; 5, BR/92/025; 6, HxB2; AZT, cells pretreated with AZT and then infected with HxB2; (+), positive PCR control. Molecular size is noted at the right.

**Reverse transcription in SK-N-MC cells.** To identify potential postentry steps that are delayed in the SK-N-MC cells, we utilized primer pairs that amplify DNA species associated with distinct stages of reverse transcription (Table 2). Eighteen hours after infection, cellular DNA was subjected to thermocycling with primer pair GAG04-GAG06 (30), which amplifies proviral DNA at an intermediate stage of reverse transcription (Table 2). While a PCR product was obtained from each of the 14 PHI that initiated reverse transcription, there was considerable heterogeneity in the levels of intensity of the signals (Fig. 3). In fact, those isolates that were negative by cocultivation had decreased PCR product. As the GAG04 and GAG06 primers were designed to recognize viral DNA from primary isolates with equal levels of efficiency (30, 31), this variability likely reflects differences in the amount of HIV-1 DNA in each sample and is not the consequence of differential priming of the target DNA sequence.

**Detection of viral DNA within the nucleus.** Concurrent with reverse transcription, viral DNA is transported into the cell nucleus as part of a nucleoprotein complex. There, HIV-1 DNA exists as several species: integrated linear or covalently closed circular forms with one or two tandem long terminal repeats (LTRs) (4). These circular DNAs are a hallmark of HIV-1 nuclear transport and can be used as surrogate markers for this stage of the replicative cycle (5). DNAs were isolated

TABLE 2. Amplification of DNA species associated with stages of reverse transcription

Primer set	Probe	Type of DNA amplified:			
		Early	Intermediate	Late	Two-LTR circles
LTR1-LTR2	U3	+	+	+	-
GAG04-GAG06	<i>gag</i>	+	+	-	-
GAG04-U5+	<i>gag</i>	+	+	+	-
U5+-R-	U3	-	-	-	+

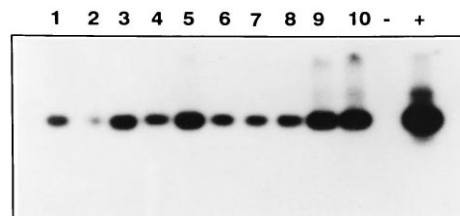


FIG. 3. SK-N-MC cells were infected with PHI, and DNAs were isolated 18 h later, PCR amplified for 25 cycles with the GAG04-GAG06 primer pair, and probed with a  $\gamma$ -<sup>32</sup>P-labeled internal oligonucleotide (30, 31). Differences in the amounts of amplified product among the isolates were detected at the intermediate stage of *gag* DNA synthesis. Lanes: 1, AL-6244; 2, AM-3107; 3, AJ-4331; 4, AD-8874; 5, AQ-5945; 6, AQ-6809; 7, UG/92/035; 8, BR/92/025; 9, BR/92/021; 10, RW/92/009; -, cells pretreated with AZT and then infected with HxB2; +, HxB2 positive control.

18 or 24 h after infection with the six PHI that were negative in the cocultivation assay and amplified with primer pair U5+-R- (34), which recognizes the two-LTR circles (Table 2). We could not detect the circularized two-LTR viral DNA in the SK-N-MC cells (Fig. 4), although it was clearly present in the SK-N-MC/CD4 cells. Similar results were obtained with DNAs isolated 24 h after infection (data not shown). As expected, for those PHI in which proviral integration was assumed from the cocultivation results, two-LTR circles were easily detected by PCR (data not shown).

**Discussion.** These results demonstrate that 8 of 23 PHI from different clades were able to infect SK-N-MC cells, as defined by their ability to rescue provirus by cocultivation. Although those isolates that showed the highest level of p24<sup>gag</sup> production after cocultivation had a syncytium-inducing phenotype (Table 1), non-syncytium-inducing isolates were also fully capable of infecting these CD4-negative cells. Specifically, these isolates completed reverse transcription, entered the nucleus, and produced viral antigen when stimulated with PBMC. These results demonstrate that primary isolates with only limited PBMC passage in vitro can enter SK-N-MC cells with GalCer-GalS as receptor molecules.

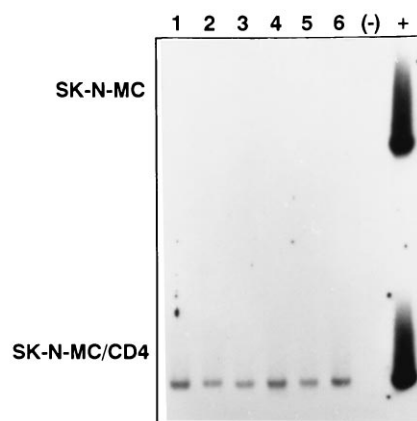


FIG. 4. SK-N-MC or SK-N-MC/CD4 cells were infected with the six PHI that did not produce viral antigen (Table 1), and DNAs were isolated 18 h later, PCR amplified with primer pair U5+ (5'-GTCTGTTGTGTGACTCTGGT-3')-R- (5'-GAGGCTTAAGCAGTGGGTTTC-3'), and probed with a  $\gamma$ -<sup>32</sup>P-labeled internal oligonucleotide (34). We could not detect two-LTR nuclear DNA in the SK-N-MC cells, although nucleus-associated viral DNA species were easily detected in the SK-N-MC/CD4 cells. Lanes: 1, BR/92/025; 2, UG/92/035; 3, AL-6244; 4, AM-3107; 5, AD-8874; 6, AQ-6809; (-), cells pretreated with AZT and then infected with HxB2; +, HxB2 positive control.

Five of the 15 isolates did not infect the SK-N-MC cells nor the control SK-N-MC/CD4 cells, and we would speculate that they are incapable of infecting most continuous cell lines. Among the remaining 10 isolates that did not produce viral antigen in SK-N-MC cells, 6 caused an abortive infection in which cytoplasmic DNAs could be easily detected 24 h after infection but in which nuclear transport, and presumably proviral integration, did not proceed to completion. As expected, results of cocultivation with PBMC were negative for these isolates.

The intracellular events of the retroviral life cycle have been extensively characterized, and host cell-specific restrictions have been proposed for both murine and human cells (7, 21). While it is possible that unique cellular factors are necessary for the nuclear transport of some but not all isolates in SK-N-MC cells, results with the SK-N-MC/CD4 cells, which were derived from the same parental stock, indicate that the differences noted in the infectability of SK-N-MC cells are likely to map elsewhere. The SK-N-MC/CD4 cell line is quantitatively more efficient at HIV-1 entry than the SK-N-MC cell line, suggesting that higher amounts of virus are necessary for entry into the nucleus (18). Thus, the ability of a PHI to complete the retroviral life cycle in SK-N-MC cells appears to be determined at early stages of the virus cycle, including binding, fusion, and initiation of reverse transcription. Most likely, the inhibition of nuclear entry for some PHI is the culmination of a series of inefficient viral events that results in an abortive infection. In this regard, the *gag-pol* region of the HIV-1 genome has been recently determined to alter replication in GalCer-positive colonic epithelial cells (13), supporting a model for multiple levels of viral restriction in GalCer-GalS-positive cells.

To date, the role of infection of CD4-negative cells *in vivo* is uncertain, although recent studies have reconsidered the potential importance of this area in the pathogenesis of HIV dementia. GalCer is abundant within the nervous system, while GalS is more prevalent in the intestinal tract. Additionally, GalS and a similar glycosphingolipid, sulfo-lactosylceramide, were found to be expressed in cervical and vaginal epithelial cells (16). Although the principal routes of HIV transmission are sexual, the mechanisms of cellular transmission are not clear (25). Determining whether glycolipid-mediated infection occurs *in vivo* will help in the understanding of HIV pathogenesis.

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