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Antisense Phosphorothioate Oligodeoxynucleotides Targeted to the *vpr* Gene Inhibit Human Immunodeficiency Virus Type 1 Replication in Primary Human Macrophages

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The replication of human immunodeficiency viruses (HIV) in human macrophages is influenced by genetic determinants which have been mapped predominantly to the viral envelope. However, in HIV-2, the *vpr* gene has also been suggested as an important modulator of viral expression in human macrophages. We synthesized five antisense phosphorothioate oligodeoxynucleotides complementary to the *vpr* mRNA of HIV-1_{Ba-L}, a highly macrophage-tropic viral strain, and measured their effect on HIV-1_{Ba-L} replication in primary human macrophages. All of the oligodeoxynucleotides displayed some level of non-sequence-specific inhibition of viral replication; however, only the antisense one had an additional effect on viral production in primary macrophages. Of the five antisense oligodeoxynucleotides tested, only one did not show any additional effect on viral production, whereas all the others inhibited viral replication to a similar degree (70 to 100%). Variation in the degree of inhibition was observed by using five different donors of human primary macrophages. The phosphorothioate oligonucleotides, targeted to the initiating methionine of the Vpr protein, had an inhibitory effect at both 20 and 10 μM only when the size was increased from 24 to 27 bases. Thus, HIV-1 replication in human macrophages is modulated by the expression of the *vpr* gene, and it is conceivable that *vpr* antisense oligodeoxynucleotides could be used in combination with antisense oligodeoxynucleotides against other HIV-1 regulatory genes to better control viral expression in human macrophages.

Human immunodeficiency virus type 1 (HIV-1) (3, 32), the etiological agent of AIDS (10), can be isolated from both T cells and macrophages of infected individuals (13, 32). HIV-1 replication in both cell types has been demonstrated in vivo, and infection of macrophages is thought to be an important pathogenic step in the induction of dementia and other central nervous system ailments often observed in AIDS patients (1, 12, 13, 18, 21, 22, 41). Furthermore, HIV-1 can remain in a latent state in macrophages and this cell type might represent an important virus reservoir in the tissues of infected individuals. Selection of viruses with tropism for one cell type or the other often occurs in vitro, indicating that each HIV-1 strain is composed of a collection of microvariant genotypes with different biological features, such as the capability of infecting different target cells (2, 4, 8). Both T cells and macrophages express the CD4 molecule, the major receptor for HIV-1 (6). Several studies aimed to address the issue of HIV-1 macrophage tropism have revealed the existence of specific genetic determinants in the viral envelope which influence viral replication in human macrophages, including the HIV-1 V3 region (19, 31, 43, 45). However, evidence is accumulating that macrophage tropism has multiple determinants, and it is likely that both cellular and viral factor(s) influence viral replication after entry. In addition to genes that are indispensable for viral replication, such as *tat* and *rev*, HIV-1 and its relatives, HIV-2 and simian immunodeficiency virus, carry other genes (i.e., *vpr*, *vpu*, *vpx*, *vif*, and *nef*), the function of which is still uncertain. Previous findings indicate that some of these genes may play a role in the mode of viral transmission and the extent of viral replication. For example, the HIV-1 and HIV-2 Vif proteins are indispensable for viral infection and replication in primary human cells and in some cell lines but not in others (9, 20, 29, 36, 37, 40) and the HIV-2/simian immunodeficiency virus Vpx protein positively influences viral growth in primary peripheral blood monocytes (16, 26, 42, 47). More specifically, the HIV-2 *vpr* gene, which has a counterpart in HIV-1 (46), has been shown to modulate viral replication in primary human macrophages (17) but not in T cells (7, 17, 40). To investigate whether this feature is also common to the *vpr* gene of HIV-1, we synthesized phosphorothioate oligodeoxynucleotides directed against the *vpr* open reading frame and tested their ability to inhibit HIV-1_{Ba-L} replication in primary human macrophages. The open reading frame of the HIV-1_{MN} isolate (23, 32) encoding a putative Vpr protein of 96 amino acids was chosen as a reference for Fig. 1. Most of the synthesized phosphorothioate oligodeoxynucleotides are depicted in the lower part of the figure. Oligodeoxynucleotides 9 and 9a (antisense oligodeoxynucleotides 24 and 27 bases long, respectively) were targeted to the AUG of the HIV-1 Vpr mRNA. Their nucleotide sequence is highly conserved among different HIV-1 isolates and was derived from HIV-1_{MN} (15, 33), a prevalent virus in the western world. The remaining antisense oligodeoxynucleotides (no. 1, 2, and 3) were 27 bases long and were derived from the DNA sequence of the HIV-1_{Ba-L} isolate (12). Oligodeoxynucleotides 9, 9a, and 1 overlap with the sequence encoding the C-terminal portion of the Vif protein (Fig. 1). In contrast, oligodeoxynucleotides 2 and 3 were targeted at the genomic region presumably encoding only the Vpr protein (Fig. 1). The control oligodeoxynucleotide no. 4 (sense) was derived from the complementary strand of no. 3, whereas the control oligodeoxynucleotides 10 and 10a were antisense as were no. 9 and 9a, respectively, but with opposite 5′–3′ orientation.

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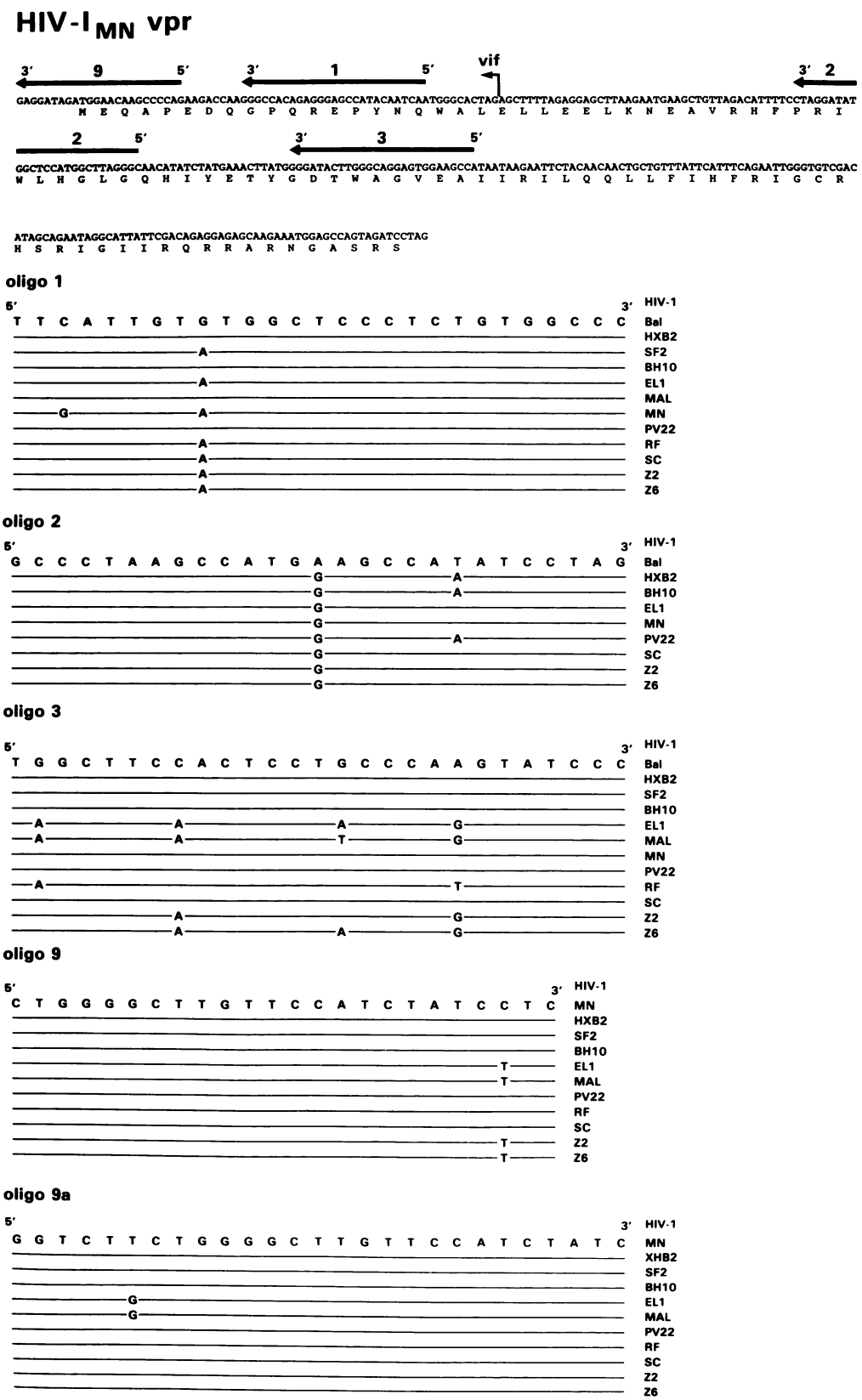
HIV-I MN vpr
3′ 9 5′
3′ 1 5′
vif
3′ 2
GAGGATAGATGGAACAAGCCCCAGAAGACCAAGGGCCACAGAGGGAGCCATACAATCAATGGGCACTAGAGCTTTTAGAGGAGCTTAAGAATGAAGCTGTTAGACATTTTCCTAGGATAT
M E Q A P E D Q G P Q R E P Y N Q W A L E L L E E L K N E A V R H F P R I
2 5′
3′ 3 5′
GGCTCCATGGCTTAGGGCAACATATCTATGAAACTTATGGGGATACTTGGGCAGGAGTGGAAGCCATAATAAGAATTCTACAACAACTGCTGTTTATTCATTTCAGAATTGGGTGTCGAC
W L H G L G Q H I Y E T Y G D T W A G V E A I I R I L Q Q L L F I H F R I G C R
ATAGCAGAATAGGCATTATTCGACAGAGGAGAGCAAGAAATGGAGCCAGTAGATCCTAG
H S R I G I I R Q R R A R N G A S R S
oligo 1
5′ 3′ HIV-1
T T C A T T G T G T G G C T C C C T C T G T G G C C C Bal
HXB2
SF2
BH10
EL1
MAL
MN
PV22
RF
SC
Z2
Z6
oligo 2
5′ 3′ HIV-1
G C C C T A A G C C A T G A A G C C A T A T C C T A G Bal
HXB2
BH10
EL1
MN
PV22
SC
Z2
Z6
oligo 3
5′ 3′ HIV-1
T G G C T T C C A C T C C T G C C C A A G T A T C C C Bal
HXB2
SF2
BH10
EL1
MAL
MN
PV22
RF
SC
Z2
Z6
oligo 9
5′ 3′ HIV-1
C T G G G G C T T G T T C C A T C T A T C C T C MN
HXB2
SF2
BH10
EL1
MAL
PV22
RF
SC
Z2
Z6
oligo 9a
5′ 3′ HIV-1
G G T C T T C T G G G G C T T G T T C C A T C T A T C MN
XHB2
SF2
BH10
EL1
MAL
PV22
RF
SC
Z2
Z6

FIG. 1. DNA and amino acid sequence of the HIV-1_{MN} *vpr* gene. The DNA sequence of oligodeoxynucleotides 1, 2, 3, and 4 was derived from the HIV-1_{Ba-L} DNA sequence, whereas oligodeoxynucleotides 9, 9a, 10, and 10a were obtained from the genomic sequence of a biologically active molecular clone of the HIV-1_{MN} strain (23). The sequence of the antisense oligodeoxynucleotides and their genetic variability are presented in the lower part of the figure. Number 4 is the complementary strand of number 3 and has the following sequence: 5′ GGG ATA CTT GGG CAG GAG TGG AAG CCA 3′. Oligodeoxynucleotides 10 and 10a have the following sequences: 10, 5′ CTC CTA TCT ACC TTG TTC GGG GTC 3′; 10a, 5′ CTC CTA TCT ACC TTG TTC GGG GTC TTC 3′. The end of the *vif* open reading frame is indicated with an arrow. All of the phosphorothioate oligodeoxynucleotides were purchased from Syntecell (Rockville, Md.) and displayed a high degree of purity on acrylamide gel electrophoresis.

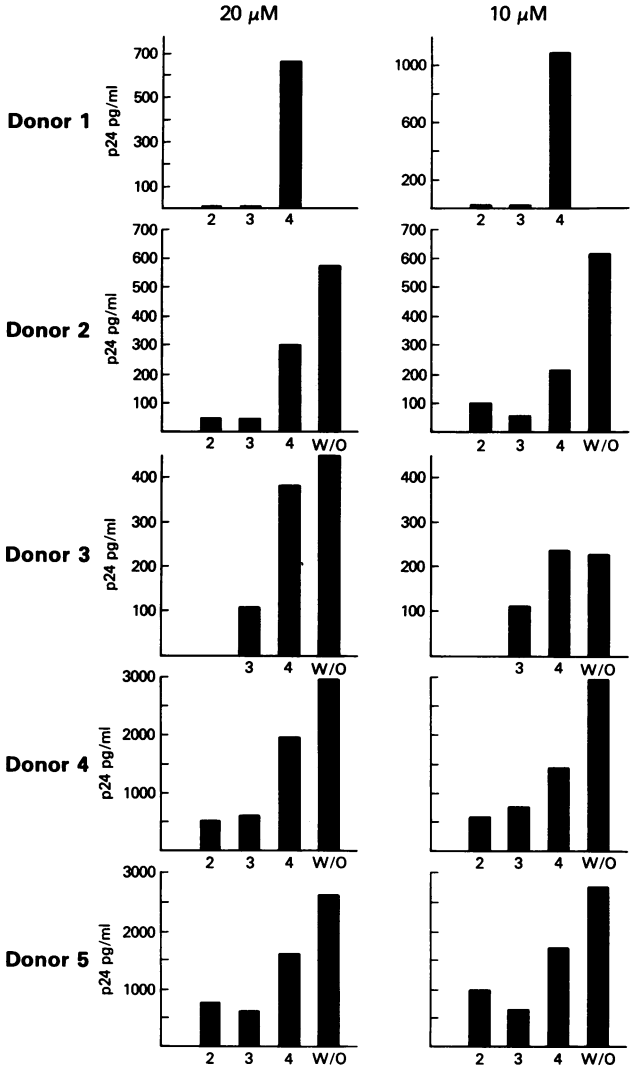

FIG. 2. Inhibition of viral replication induced by the antisense *vpr* phosphorothioate oligodeoxynucleotides. Monocyte-derived macrophages were prepared from the peripheral blood of five healthy HIV-1 seronegative human volunteers and purified by adherence. The same stock of frozen HIV-1_{Ba-L} was used in all experiments. Unfractionated mononuclear cells were seeded in 96-well plates (Costar, Cambridge, Mass.) at a concentration of 10^7/ml in the presence of recombinant granulocyte-macrophage colony-stimulating factor (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) at 50 IU/ml. Every 3 days, one-half of the medium was removed from the wells together with one-half of the nonadherent-cell population and replaced with fresh medium containing 50 IU of granulocyte-macrophage colony-stimulating factor per ml. At the 14th day nonadherent lymphoid cells were completely removed from the wells by twice washing the adherent monolayer with sterile phosphate-buffered saline. The adherent cells were tested by immunofluorescence, and more than 95% consistently expressed CD4 and CD11b macrophage-associate markers, as tested by monoclonal antibodies Leu M3 and Mol (Becton Dickinson, Mountain View, Calif.). HIV-1_{Ba-L} was grown in primary monocyte-derived macrophages cultured as adherent monolayers in 75-cm_2 flasks (Costar, Cambridge, Mass.). The virus yield of these cultures was tested repeatedly by reverse transcriptase assay over the course of 2 to 3 weeks postinfection, and supernatants containing the highest reverse transcriptase levels (about 150,000 cpm/ml) were stored as viral stocks in liquid nitrogen. Monolayer macrophages were incubated for 2 h at 37°C with 50,000 cpm of the viral stock per ml. After infection the cells were washed twice with phosphate-buffered saline and then 100 μl of complete medium, containing 20 μM, 10 μM, or none of the phosphorothioate oligodeoxynucleotide, was added. The culture supernatant was collected biweekly and replaced with fresh medium containing the appropriate phosphorothioate oligodeoxynucleotide. The antigen capture test was done with an enzyme-linked immunosorbent assay commercial kit (Organon Teknika, Durham, N.C.), by following the instructions of the manufacturer. The number under each column refers to the phosphorothioate oligodeoxynucleotide present in the cell culture; W/O refers to the parallel infected macrophage in the absence of phosphorothioate oligodeoxynucleotide (the W/O data for donor 1 was not available). The concentration of phosphorothioate oligodeoxynucleotide used is indicated at the top of the figure, and on the *y* axis the amount of viral p24 measured at day 15 in the culture medium is presented.

Monocyte-derived macrophages were prepared from the peripheral blood of healthy human volunteers and enriched by adherence on plastic. Macrophage monolayers, cultured for 14 days, were exposed to HIV-1_{Ba-L} for 2 h. After extensive washings, the macrophages were cultured in the presence of either 20 or 10 μM of phosphorothioate oligodeoxynucleotide. The culture supernatants were replaced with fresh medium containing the same initial concentration of oligodeoxynucleotide every 3 to 4 days, and most cultures were maintained for 21 days. Each culture was monitored for virus production by measuring the amount of p24 Gag protein present in the supernatant fluids. In some experiments the phosphorothioate oligodeoxynucleotides were added 22 h before infection, kept during the infection period, and removed thereafter. When the antisense and the control phosphorothioate oligodeoxynucleotide were used to pretreat cells, invariably we observed total abolishment of viral infection (data not shown), indicating a non-sequence-specific effect of phosphorothioate oligodeoxynucleotides on viral infectivity as already suggested by others (25, 38). In experiments where the phosphorothioate oligodeoxynucleotides were added after infection and then maintained until the end of the culture, we observed significant inhibition of virus replication with slight variation among different experiments. At concentrations of both 20 and 10 μM, phosphorothioate oligodeoxynucleotides 2 and 3 inhibited viral rep-

TABLE 1. Inhibition on HIV-1_{Ba-L} replication in human primary macrophages from five different donors induced by the Vpr antisense phosphorothioate oligodeoxynucleotides 2 and 3

Donor	% Inhibition[a]			
	Oligodeoxynucleotide 2		Oligodeoxynucleotide 3	
	10 μM	20 μM	10 μM	20 μM
1	100	100	100	100
2	64	84	76	86
3	ND[b]	ND	53	69
4	44	73	41	70
5	25	51	58	61
Average values	60	77	65	77

[a] Percentage of inhibition of infection yield compared to cultures treated with the relevant phosphorothioate oligodeoxynucleotide control (number 4) measured at day 14 to 15 (also see Fig. 2).
[b] ND, not done.

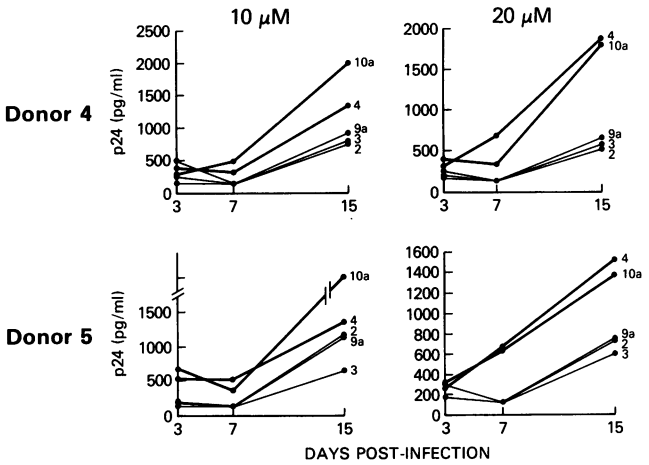

FIG. 3. Kinetics of HIV-1_{Ba-L} infection in human macrophages obtained from two donors in the presence of 10 and 20 μM antisense phosphorothioate oligodeoxynucleotides 2, 3, and 9a and their respective control phosphorothioate oligodeoxynucleotides 4 and 10a. Infection and culture methods were the same as described for Fig. 2.

lication almost completely in macrophage from donor 1 (Fig. 2 and Table 1), whereas phosphorothioate oligodeoxynucleotide 4 did not. A somewhat lesser inhibition, particularly at the lower phosphorothioate oligodeoxynucleotide concentrations, was observed when donors 2 to 5 were used (Fig. 2 and Table 1). In all cases the presence of phosphorothioate oligodeoxynucleotides in the culture interfered to a various extent with the amount of viral p24 protein produced in the culture medium (compare the respective culture without phosphorothioate oligodeoxynucleotide with the control phosphorothioate oligodeoxynucleotide 4) indicating a non-sequence-specific effect of the oligonucleotides on viral infectivity (for a review, see reference 39). The variation observed in the efficacy of the phosphorothioate oligodeoxynucleotides in different experiments appeared to be related to the different donors of peripheral blood monocytes (Fig. 2), as often observed with primary macrophage cultures. The antisense phosphorothioate oligodeoxynucleotide 9, targeted at the initiation codon of the Vpr protein (Fig. 1), also inhibited viral replication approximately 60 to 80% at day 10 at both 10 and 20 μM, but this inhibitory effect was not maintained at 10 μM at day 15 (data not shown).

However, when the size of the oligodeoxynucleotide 9 was increased to 27 bases (oligodeoxynucleotide 9a) the antiviral effect was observed also at 10 μM (Fig. 3) indicating the importance of size in the antiviral activity of this phosphorothioate oligodeoxynucleotide.

Interestingly, although phosphorothioate oligodeoxynucleotides 9 and 9a sequences overlap with the C terminus of the *vif* gene, which is required for viral infectivity in vitro (9, 20, 29, 34, 36, 37, 40) and in vivo (our unpublished results), it did not inhibit viral replication in macrophages to a higher degree than phosphorothioate oligodeoxynucleotides 2 or 3 (Fig. 3), which map exclusively within the *vpr* gene. It is possible that the interaction of phosphorothioate oligodeoxynucleotides 9 and 9a with the 3′ of the Vif mRNA is not sufficient to induce a decrease in the Vif protein synthesis.

To assess whether the decreased viral expression observed in the cultures containing the control oligodeoxynucleotides was to be attributed to the non-sequence-specific effect of the phosphorothioate oligodeoxynucleotides (39) rather than to direct cellular toxicity, the viability of the cultured macrophages in the presence and absence of HIV-1_{Ba-L} and the various phosphorothioate oligodeoxynucleotides was tested. As summarized in Tables 2 and 3, no significant differences were noticed by the trypan blue exclusion method in the macrophage cultures of donor 4 at different times in the experiment. The same results were observed with donor 5 (data not shown). Thus, the non-sequence-specific effects of phosphorothioate oligodeoxynucleotide are more likely to induce a decrease in viral production, probably through a direct effect on reverse transcriptase or gp120 binding to the CD4 receptor, as has been previously demonstrated (25, 38).

Antisense phosphorothioate oligodeoxynucleotides directed against the regulatory *tat* and *rev* genes have been tested and shown to interfere with HIV replication in both acute and chronic infection in vitro (14, 27, 28). However, targeting other genes which might be involved in the late

TABLE 2. Cell viability in uninfected and HIV-1_{Ba-L}-infected macrophage cultures treated with 10 μM phosphorothioate oligodeoxynucleotide

Phosphorothioate oligodeoxynucleotide no. and HIV-1 status[a]	Cell viability[b] on day:		
	7	15	21
2			
−	98.8	97.5	99.8
+	100.2	96.4	96.4
3			
−	99.4	98.1	95.8
+	99.8	96.8	97.8
4			
−	100.3	97.3	98.7
+	98.9	96.7	98.0
9a			
−	99.0	96.8	97.4
+	100.4	96.8	97.1
10a			
−	98.8	97.3	97.4
+	99.3	98.2	96.8

[a] −, negative; +, positive.
[b] Cell viability: percent of the untreated control cultures, as assessed by the trypan blue exclusion method.

TABLE 3. Cell viability in uninfected and HIV-1_{Ba-L}-infected macrophage cultures treated with 20 μM phosphorothioate oligodeoxynucleotide

Phosphorothioate oligodeoxynucleotide no. and HIV-1 status[a]	Cell viability[b] on day:		
	7	15	21
2			
−	99.0	98.2	98.4
+	98.3	98.6	97.9
3			
−	97.7	97.8	96.6
+	98.5	97.5	98.3
4			
−	95.2	95.6	97.6
+	98.5	98.2	98.1
9a			
−	97.3	97.6	97.1
+	99.6	98.4	98.3
10a			
−	96.4	97.1	97.1
+	98.6	98.4	98.1

[a] −, negative; +, positive.
[b] Cell viability: percent of the untreated control cultures, as assessed by the Trypan blue exclusion method.

steps of viral replication may increase the chance of controlling the spread of viral infection in vitro and at the same time minimize viral escape due to genetic mutations. Tat and Rev are viral proteins expressed early in the replicative cycle, and the efficient use of the *vif* and *vpr* spliced mRNAs is dependent on *rev* function (11), suggesting that the expression of Vif and Vpr proteins could be important in the late phases of viral replication. However, since the Vpr protein is virion associated and has transactivating properties (5), it is not inconceivable that its presence might be required early in infection. The Vpr protein might be necessary for transactivation of unintegrated viral DNA, thereby initiating the viral cycle. The presence of the Vpr protein might be particularly important in human macrophages where most, if not all, viral DNA is in episomal form (22). This interpretation, although not supported by experimental data at the present time, might explain the requirement of *vpr* for HIV expression in macrophages but not in T cells, as we have previously shown for HIV-2 (17). To this regard recent work on HIV-1 Vpr mutants also confirms the contribution of the Vpr protein to the efficient expression of HIV-1 in human macrophages (44).

In the case of HIV-1_{Ba-L} we were unable to study the inhibition by anti-*vpr* phosphorothioate oligodeoxynucleotides in primary T cells because this isolate is unable to grow in such cells. However, none of the anti-*vpr* phosphorothioate oligodeoxynucleotides tested substantially inhibits productive viral infection of a continuous T-cell line, PM1 (24), which is susceptible to HIV-1_{Ba-L} (data not shown). These results confirm that *vpr* may be more important for replication of HIV-1 in macrophages than in T cells.

Infection of macrophages is believed to represent an important pathogenic step for AIDS development, as suggested by the finding that the isolation of macrophage-tropic viruses is more frequent during the long asymptomatic period (35). Additionally, we found that abrogation of the *vpr* function in an infectious molecular clone of HIV-2 rendered the virus incapable of establishing persistent infection in rhesus macaques (unpublished results). Thus, the development of specific antiviral inhibitors which interfere with HIV replication in macrophages might be beneficial for the prevention of AIDS development in infected individuals. Furthermore, antiviral therapy based on a combination of antisense oligodeoxynucleotides targeted to different viral genes might help to minimize the problem of viral escape due to genetic variations.

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