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Antisense oligodeoxynucleotide phosphorothioate complementary to Gag mRNA blocks replication of human immunodeficiency virus type 1 in human peripheral blood cells

(AIDS/therapy)

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ABSTRACT **Gene-expression modulator 91 (GEM91) is a 25-nt antisense oligodeoxynucleotide phosphorothioate complementary to the Gag mRNA of human immunodeficiency virus type 1 (HIV-1). Cellular uptake and intracellular distribution of GEM91 within cells suggest that this oligomer is readily available for antisense activity. GEM91 inhibited HIV-1 replication in a dose-dependent and sequence-specific manner. In a comparative study, 2 μM GEM91 was as effective as 5 μM 3′-azido-3′-deoxythymidine in blocking virus replication during the 28-day treatment of an HIV-1-infected T-cell line. GEM91 also completely inhibited (>99%) the growth of three different HIV-1 isolates in primary lymphocytes and prevented the cytopathic effect of the virus in primary CD4$^+$ T cells. Similarly, treatment with GEM91 for 3 weeks of HIV-1/BaL-infected primary macrophages blocked virus replication. Based on GEM91 anti-HIV-activity, safety, and pharmacokinetic profile in animals, a clinical trial was started using this compound as an antisense oligonucleotide drug for the treatment of the acquired immunodeficiency syndrome.**

One possible treatment of human immunodeficiency virus type 1 (HIV-1) infection is the use of sequence-specific antisense oligodeoxynucleotides to inhibit virus replication (1–3). It was previously shown that antisense oligodeoxynucleotide phosphorothioates could inhibit HIV-1 replication in immortalized T-cell lines (4–8). To further develop antisense oligodeoxynucleotides in the direction of effective anti-HIV drugs, we synthesized a 25-mer oligodeoxynucleotide phosphorothioate, gene-expression modulator 91 (GEM91) (9), targeted to the translational initiation site of the Gag mRNA (Fig. 1*A*). GEM91 may inhibit HIV-1 replication through two different mechanisms: it could block the translation of the Gag mRNA and also affect the viral assembly by interruption of the secondary structure of the target RNA required for packaging and dimerization (10, 11).

METHODS

Oligonucleotide Synthesis. Oligodeoxynucleotide phosphorothioates were synthesized, purified, and characterized as previously reported (12).

Short-Term Anti-HIV-1 Assay. An immortalized T-cell line, MOLT-3 (5×10^5 cells per ml), was infected with HIV-1/IIIB (multiplicity of infection, 1) and cultured in the presence of 0.1–4 μM GEM91 or random oligodeoxynucleotides. Four days after infection, HIV-1 replication was monitored by syncytium formation and p24 antigen-capture assay (Coulter), and cell viability was detected by trypan blue exclusion. The percentages of sequence-specific inhibition were calculated from the p24 values after treatment with various concentrations of GEM91 and random oligomers.

Long-Term Antiviral Assay. MOLT-3 cells (4×10^5 per ml) were infected with HIV-1/IIIB (multiplicity of infection, 1) or HIV-1/571. HIV-1/571 is a primary isolate which was isolated by cocultivation of phytohemagglutinin-activated peripheral blood mononuclear cells (PBMCs) from a patient with acquired immunodeficiency syndrome (AIDS) and phytohemagglutinin-activated PBMCs from a normal blood donor; at the time of this study HIV-1/571 had been passaged only twice in activated PBMCs from normal donors. Culture supernatant of HIV-1/571-infected PBMCs was directly used for infection of MOLT-3 cells. After 2 hr of infection, cells were washed and treated with oligodeoxynucleotide phosphorothioates or AZT at concentrations indicated in Fig. 1. Cells were split to 4×10^5 per ml every 3 or 4 days and treated with drugs at the same concentrations. HIV-1 replication was monitored by p24 antigen-capture assay, and cell viability was detected by trypan blue exclusion.

Antiviral Assay on Primary Lymphocytes. PBMCs were isolated from peripheral blood of normal donors by Ficoll/Hypaque density gradient centrifugation, activated with phytohemagglutinin (Difco), and cultured in RPMI 1640 medium supplemented with 15% fetal bovine serum and interleukin 2 (100 units/ml; Boehringer Mannheim). After 3 days PBMCs (10^6 per ml) were infected with HIV-1 (multiplicity of infection, 0.1–0.2). After 2 hr cells were washed and treated with various concentrations of oligodeoxynucleotides. The medium was changed every 3 or 4 days and oligodeoxynucleotides were added at these times. Seven days after infection, cells were split to 10^6 per ml. HIV-1 replication was monitored by p24 antigen-capture assay, and viable cell number was determined as described by Pauwels *et al.* (13).

Flow Cytometry. Fourteen days after infection, aliquots of the cells were analyzed for expression of CD4 and CD8 antigens by cytofluorometry. Briefly, cells were washed and treated with fluorochrome-labeled monoclonal antibodies to CD4 or CD8 (Becton Dickinson) at 2 μg/ml. The cells were washed again and fixed with 2% paraformaldehyde before the analysis.

Antiviral Assay on Primary Macrophages. Primary human monocytes were isolated from peripheral blood of healthy volunteers by counterflow centrifugal elutriation (14). These cells were plated in RPMI 1640 supplemented with 15% fetal bovine serum and granulocyte/macrophage-colony-stimulating factor (50 units/ml) to promote the differentiation of

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Abbreviations: AZT, 3′-azido-3′-deoxythymidine; GEM91, gene-expression modulator 91; HIV, human immunodeficiency virus; PBMC, peripheral blood mononuclear cell.

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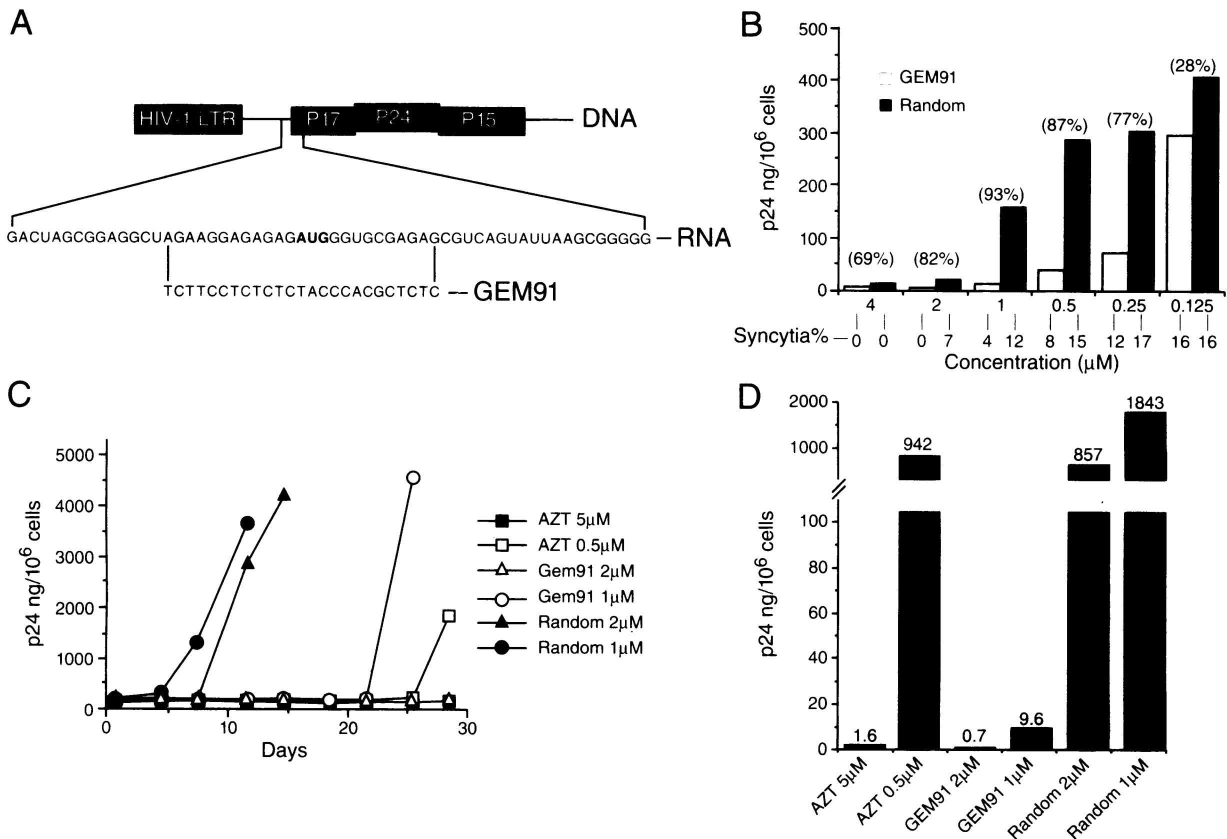

FIG. 1. (*A*) Sequence of GEM91 and the targeted region in the HIV-1 genome (9). LTR, long terminal repeat. (*B*) Short-term antiviral activity of GEM91 compared with the random oligomer preparation. (*C*) Long-term inhibition of HIV-1/IIIB replication by GEM91 and 3′-azido-3′-deoxythymidine (AZT) in MOLT-3 cells. (*D*) Inhibition of HIV-1/571 replication by GEM91 and AZT in MOLT-3 cells 28 days after infection. In the untreated infected cells, p24 production peaked 7 days after infection (13,000 ng per 10^6 cells).

monocytes to macrophages. After 2 weeks in culture, macrophages (2×10^5 per ml) were infected with HIV-1/BaL (reverse transcriptase activity, 30,000 cpm/ml). After intensive washing, cells were treated with 1.2 μM oligodeoxynucleotide phosphorothioate every second day, and the cultures were maintained for 21 days. HIV-1 replication was monitored by p24 antigen-capture assay, and cell viability was detected by trypan blue exclusion. All antiviral assays described above were carried out at least twice and similar results were obtained.

Confocal Microscopy. Cells were incubated with 1 μM fluorescein-conjugated GEM91 (3′-labeled) and submitted to flow cytofluorometry and laser-assisted confocal microscopy to study uptake and intracellular distribution (15, 16), respectively. Intracellular distribution was observed after 20 hr of GEM91 incubation and optical sections of 1 μm were obtained for each sample.

RESULTS AND DISCUSSION

Inhibition of HIV-1 Replication in an Immortalized T-Cell Line. To demonstrate the anti-HIV activity of GEM91, MOLT-3 cells were infected with HIV-1/IIIB (17) and treated with GEM91 and a control oligonucleotide preparation. Since oligodeoxynucleotide phosphorothioates are known to have a sequence-independent inhibitory activity, which is dependent on the length of the oligomers (18–20), we used a 25-mer random oligomer mixture [theoretically containing 4^{25} sequences (21)] as a non-sequence-specific control for GEM91. GEM91 inhibited HIV-1/IIIB replication in a sequence-specific and dose-dependent manner (Fig. 1*B*).

Several anti-HIV-1 drugs, including some oligodeoxynucleotide phosphorothioates, were demonstrated to be effective in short-term infection assays but were not able to suppress virus replication in long-term cultures (20). To evaluate the long-term efficacy of GEM91, infected cells were maintained in the presence of GEM91 or random oligodeoxynucleotide phosphorothioates for 28 days. GEM91 inhibited HIV-1/IIIB (17) replication, and a dose higher than 1 μM was required to maintain the inhibitory effect longer than 20 days (Fig. 1*C*). In the presence of the random oligodeoxynucleotide phosphorothioates a transient suppression of HIV-1 replication was observed, as previously reported (21). We compared the antiviral effect of a reverse transcriptase inhibitor, AZT, with that of GEM91. Treatment with 0.5 μM AZT or 1 μM GEM91 showed similar efficacy: both totally inhibited HIV-1 replication for up to 20 days but failed to prevent virus replication thereafter. In contrast, higher doses of both drugs, 2 μM for GEM91 and 5 μM for AZT, completely blocked HIV-1/IIIB replication for up to 28 days, which was the duration of the experiment.

Because of the sequence variability among different HIV-1 isolates, GEM91 was designed to hybridize to a region of the HIV-1 genome which is known to be highly conserved (22). Therefore, we expected that GEM91 could be an effective inhibitor of replication of an unrelated HIV-1 isolate, especially if it was not adapted to cell culture (primary isolate). MOLT-3 cells were infected with a primary isolate, HIV-1/571, and treated with GEM91 and AZT. Replication of HIV-1/571 was delayed compared with HIV-1/IIIB, since HIV-1/571 was not previously adapted to cell lines. In cultures treated with random oligonucleotides (1 or 2 μM), the virus replicated at high levels. In contrast, treatment of HIV-1/571-infected cells with 2 μM GEM91 blocked the replication for up to 28 days (Fig. 1*D*). GEM91 was less effective at 1 μM than at 2 μM. Treatment with 0.5 μM AZT

Table 1. HIV-1 replication in primary PBMCs treated with oligonucleotide phosphorothioates

HIV-1 isolate	Oligonucleotide phosphorothioate(s)	Concentration, μM	p24, ng per 10^6 cells		
			Day 7	Day 11	Day 14
HIV-1/IIIB	GEM91	1.0	6.4	1.0	3.7
		0.5	11.8	7.3	23.5
		0.25	115.9	19.4	12.8
	Random	1.0	149.3	34.6	29.2
		0.5	111.9	16.9	14.4
		0.25	100.0	20.9	19.2
	None	—	153.0	11.4	12.7
HIV-1/MN	GEM91	1.0	0.06	0.1	0.03
		0.5	6.0	22.6	18.3
		0.25	26.0	69.6	17.3
	Random	1.0	36.5	50.2	22.1
		0.5	169.7	44.6	10.7
		0.25	148.5	47.8	8.0
	None	—	53.7	37.0	12.6
HIV-1/571	GEM91	1.0	0	0.08	0.01
		0.5	1.0	7.0	7.2
		0.25	26.1	18.8	9.9
	Random	1.0	2.7	5.0	18.5
		0.5	23.7	25.7	13.8
		0.25	27.4	23.4	10.4
	None	—	48.5	19.2	8.6

was ineffective on HIV-1/571, although this virus was sensitive to 5 μM AZT. These results indicate that GEM91 can inhibit replication of primary HIV-1 isolates. Also, GEM91 has been known to be effective against AZT-resistant strains (unpublished data), because mutations contributing to the development of AZT resistance are located in the coding sequence of the reverse transcriptase gene, not in the complementary sequence of GEM91 (23).

Antiviral Effect of GEM91 on Primary Lymphocytes. The main targets of HIV-1 infection *in vivo* are $CD4^+$ T lymphocytes and macrophages. Since the uptake and bio-availability of antisense oligodeoxynucleotides may vary between immortalized and primary cells, it was important to demonstrate the efficacy of GEM91 in the primary target cells of HIV-1. Activated PBMCs from normal donors were infected with HIV-1 and cultured in the presence of GEM91 or random oligonucleotide phosphorothioates. To evaluate the antiviral effect of GEM91 on various HIV-1 isolates, we used HIV-1/IIIB (24), HIV-1/MN (25–27), and HIV-1/571 for infection (Table 1). In the untreated control cultures, virus replication peaked 7 days after infection. Treatment of infected cells with 1 μM GEM91 inhibited the replication of all three HIV-1 isolates by >96% compared with 1 μM random oligonucleotides. Treatment with 0.5 μM GEM91 was also effective, although the inhibition was dose-dependent (Table 1). Treatment with 1 μM GEM91 almost completely and sequence-specifically blocked the replication of all three HIV-1 isolates for up to 14 days, at which time the experiment was terminated (Table 1; Fig. 2 *A–C*).

Antiviral Effect of GEM91 on Primary Macrophages. Another major target cell group for HIV-1 infection are the macrophages. Fully differentiated macrophages were infected with HIV-1/BaL (24), a macrophage-tropic isolate, and treated with GEM91 or random oligonucleotides. GEM91 blocked the replication of HIV-1/BaL by >99% compared with the random control (Fig. 2*D*). It is noteworthy

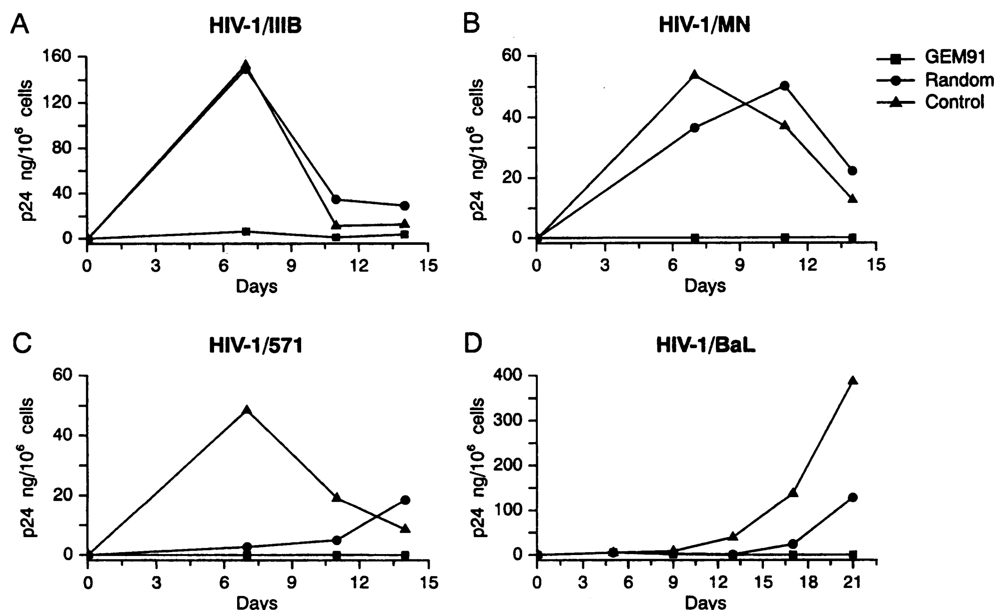

FIG. 2. GEM91 blocks the replication of HIV-1/IIIB (*A*), HIV-1/MN (*B*), HIV-1/571 (*C*), HIV-1/BaL in primary human macrophages (*D*).

Table 2. HIV-1 replication in the $CD4^+$ population of primary PBMCs treated with oligonucleotide phosphorothioates

HIV-1 isolate	Oligonucleotide phosphorothioate(s)	Concentration, μM	$CD4^+$ cells, no. per ml	$CD4^+/CD8^+$ cell ratio	p24, μg per 10^6 $CD4^+$ cells	% inhibition*
HIV-1/IIIB	GEM91	1.0	249,300	48	10	99
		0.5	86,000	16	305	94
		0.25	13,400	2	1169	83
	Random	1.0	29,500	4	1093	
		0.5	3,300	<1	5576	
		0.25	3,500	<1	7029	
	None	0	2,000	<1	8100	
HIV-1/MN	GEM91	1.0	233,000	49	<0.1	>99
		0.5	93,500	15	234	49
		0.25	50,200	7	311	2
	Random	1.0	60,200	10	448	
		0.5	44,200	4	456	
		0.25	32,300	5	319	
	None	0	33,900	7	240	
HIV-1/571	GEM91	1.0	219,200	49	<0.1	>99
		0.5	219,300	34	35	93
		0.25	75,300	10	140	52
	Random	1.0	223,100	38	92	
		0.5	43,400	4	497	
		0.25	49,300	5	292	
	None	0	16,500	3	469	

*Dose-dependent sequence-specific inhibition by GEM91 in $CD4^+$ lymphocytes. Percent inhibition was calculated by dividing the μg of p24 per 10^6 $CD4^+$ cells of cultures treated with GEM91 by the μg of p24 per 10^6 $CD4^+$ cells of cultures treated with the same concentration of random oligomers.

that the random oligomer preparation had a sequence-independent antiviral activity on macrophages, since it delayed but did not effectively inhibit HIV-1 replication. A similar effect was observed on HIV-1 infection of MOLT-3 cells (21).

Effect of GEM91 on CD4 and CD8 T-Cell Subsets. One of the major immunological markers correlated with the progression to AIDS is the decline in $CD4^+$ T lymphocytes. *In vitro*, HIV-1 induces loss of $CD4^+$ T-cells, but not all HIV-1 isolates cause syncytium formation in cultured human cells. HIV-1/IIIB is cytopathic on MOLT-3 cells but induces only limited syncytium formation on activated PBMCs, similar to HIV-1/MN. In contrast, HIV-1/571 is a highly cytopathic primary isolate which can induce formation of syncytia on primary human lymphocytes. To see whether GEM91 treatment can inhibit the cytopathic effect of different HIV-1 isolates, we analyzed the expression of CD4 and CD8 antigens on the surface of the cultured PBMCs 14 days after infection. GEM91 treatment preserved $CD4^+$ T cells from the cytopathic effect induced by all three HIV-1 isolates (Table 2). This protection was dependent on the concentration of the GEM91 and was inversely correlated with the level of virus production. Because the major HIV-1 producers are the $CD4^+$ T cells, the specific effect of GEM91 inhibition was even more evident after calculation of the $CD4^+$ T cells' survival. Treatment with 1 μM GEM91 blocked the production of all three HIV-1 isolates by >99% after 14 days of infection (Table 2). Treatment with 0.5 μM GEM91 was at least 60% more effective than treatment with the same concentration of the control random oligodeoxynucleotides. These results suggest that the blockage of HIV-1 replication parallels the suppression of the cytopathic effects of the virus in primary human lymphocytes.

Cellular Uptake and Intracellular Distribution of GEM91. Human lymphocytes and macrophages were incubated with fluorescein-labeled GEM91 under the experimental conditions used for inhibition of HIV-1 replication. The intracellular content of GEM91 was ascertained by cytofluorometric analysis and the intracellular distribution was visualized by a confocal laser scanning microscope as previously described (15, 16). The cellular uptake of GEM91 in MOLT-3 cells was slow (Fig. 3*A*); oligodeoxynucleotides were mainly localized in cytoplasmic vesicles. This intracellular distribution pattern indicates the involvement of an endocytic process in GEM91 uptake, as previously observed for other oligonucleotide phosphorothioates in MOLT-3 cells (15) and other lymphoma cell lines (16). Therefore, the efficacy of these oligodeoxynucleotides may be hindered by their localization in endosomal vesicles, leading to a lower availability in cellular compartments where antisense activity occurs, such as cytoplasm or nucleus. Macrophages incorporated GEM91 much faster and to a greater extent (Fig. 3*B*) than MOLT-3 cells. Similar to the immortalized T cells, in macrophages the internalized GEM91 was principally found in the endosomal vesicles. The level of cellular uptake of GEM91 into PBMCs (Fig. 3*C*) was similar to that observed with MOLT-3 cells. However, in contrast to MOLT-3 cells, the intracellular distribution of GEM91 in primary human lymphocytes appeared very diffuse, suggesting that most of the incorporated oligodeoxynucleotides were available for antisense activity in the cytoplasm and the nucleus.

Sequence-specific inhibition of the replication of four different HIV-1 isolates, including a primary isolate, suggests that the complementary sequence of GEM91 is an excellent target for antisense inhibition. Although this region is highly conserved in HIV-1 (22), it remains possible that HIV-1 will develop escape mutants after GEM91 treatment *in vivo*. However, we presume that a longer period of time may be required to escape from antisense treatment than from AZT treatment because a single point mutation, which can confer AZT resistance (23), may not be sufficient for viral escape from antisense inhibition. Therefore, GEM91 treatment could remain effective for a longer time than AZT treatment.

We have demonstrated that an antisense oligodeoxynucleotide phosphorothioate complementary to the Gag mRNA (GEM91) can be rapidly taken up by lymphocytes and macrophages and effectively block HIV-1 replication. Pharmacokinetic study of oligodeoxynucleotide phosphorothioates in mice, rats, and monkeys showed that following intravenous administration (28), oligonucleotides have a long plasma half-life, 30–40 hr (based on radioactivity) (29), and are biodistributed to all major organs, including lymph nodes. In

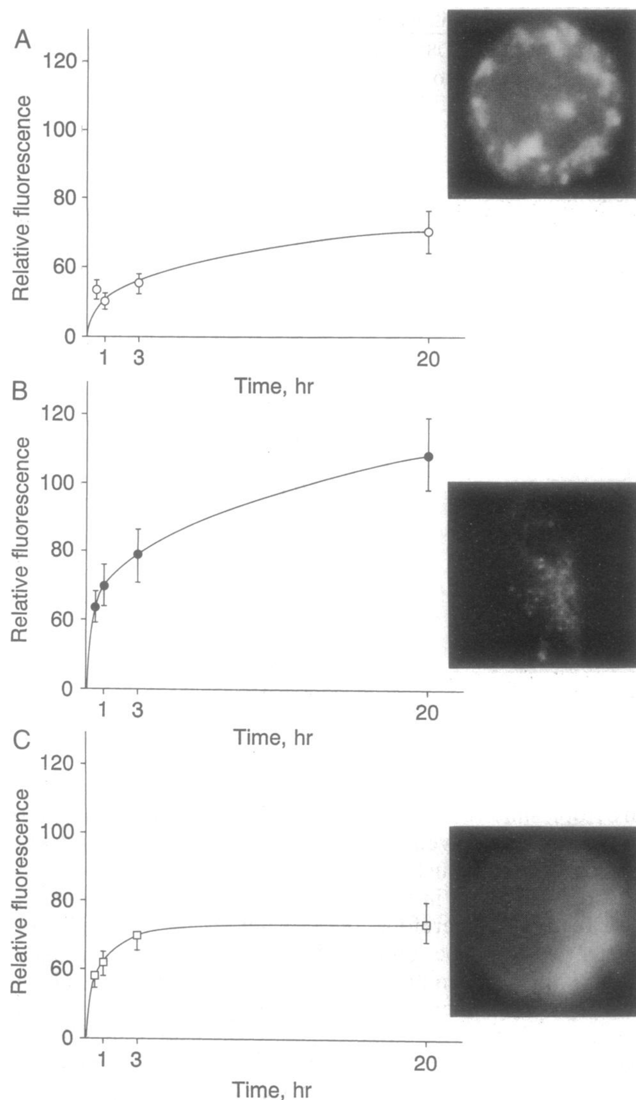

FIG. 3. Intracellular uptake and distribution of GEM91 into MOLT-3 cells (*A*), monocyte-derived macrophages (*B*), and primary human lymphocytes isolated from a normal donor (*C*). (*A* and *C*, ×495; *B*, ×100.)

this regard, we may expect that GEM91 could be taken up by cells and persist in the cytoplasm or in the nucleus, where, in the case of HIV-1 infection, it could function as an effective antisense compound. GEM91 is presently in phase I human clinical study in the United States and France.

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