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Inhibition of HIV-1 replication in cultured cells with phosphorylated dideoxyuridine derivatives encapsulated in immunoliposomes[1]

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Summary

Among the 2′,3′-dideoxynucleoside 5′-triphosphates containing a physiological base, 2′,3′-dideoxyuridine 5′-triphosphate (ddUTP) has been reported to be among the most powerful inhibitors of human immunodeficiency virus (HIV) reverse transcriptase (RT) in cell-free systems. However, in contrast to other dideoxynucleosides, 2′,3′-dideoxyuridine (ddU) is inactive in treatment of HIV-infected cells in culture, since it is a poor substrate for cellular nucleoside kinases. This problem cannot be overcome by the use of phosphorylated ddU because such compounds are unable to cross cell membranes. To promote entry and thus bypass the limiting steps of intracellular phosphorylation, we have encapsulated mono- and tri-phosphorylated ddU in liposomes coupled to monoclonal antibodies (immunoliposomes). We investigated antiviral effects in two human T cell lines (MT-4, CEM). We observed that ddU nucleotides remain phosphorylated for several weeks after encapsulation in immunoliposomes, and potent antiviral activity is obtained when these drugs are delivered

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into infected cells by cell-specific antibodies ($ED_{50} \leqslant 1$ μM on CEM). In contrast, no inhibition was observed with non-targeted liposomes containing phosphorylated ddU, or with empty liposomes, whether targeted or not.

HIV; Liposome; Antiviral therapy; Dideoxynucleoside; Dideoxyuridine; Endocytosis

Introduction

Since the discovery of human immunodeficiency virus (HIV) as the etiologic agent of acquired immunodeficiency syndrome (AIDS), many approaches designed to inhibit the replication of HIV have been developed. Among antiviral agents, the family of 2′,3′-dideoxynucleosides (ddN) has been extensively investigated. 3′-Azido-2′,3′-dideoxythymidine (AZT) is the most widely used anti-HIV drug. 2′,3′-Dideoxycytidine (ddC) and 2′,3′-dideoxyinosine (ddI) have undergone intensive clinical investigation (Mitsuya et al., 1991) and have recently been approved by the Food and Drug Administration (Antiviral Agents Bulletin (1992) 5, 7). These drugs inhibit reverse transcriptase (RT) after phosphorylation by cellular kinases, and the 5′-triphosphate forms are potent and specific inhibitors of HIV RT by acting as DNA chain terminators (Cooney et al., 1986; Furman et al., 1986; Mitsuya et al., 1990). However, the toxicity of these agents and the development of resistance during therapy limit their long term usefulness (Mitsuya et al., 1991). This has promoted studies of other nucleoside analogs with potential antiviral activity. Among the phosphorylated 2′,3′-dideoxynucleosides containing a physiological base, 2′,3′-dideoxyuridine 5′-triphosphate (ddUTP) has been shown to be an efficient inhibitor of HIV RT activity in cell-free systems (Hao et al., 1988; Hao et al., 1990), but 2′,3′-dideoxyuridine (ddU) was ineffective in blocking HIV-infection in cell culture (Baba et al., 1987; Balzarini et al., 1987; Balzarini et al., 1988; Hao et al., 1990). Investigations into the reason for this lack of effect revealed that ddU is a poor substrate for cellular nucleoside kinases and is consequently not converted into its active form in the cell (Hao et al., 1990). ddUTP cannot be used as such, since phosphorylated nucleosides are unable to cross cell membranes owing to their ionic character and their low lipophilicity (Cohen, 1975). Moreover, phosphorylated nucleosides are susceptible to rapid degradation in tissues by nucleotidases and non-specific phosphatases.

To promote entry of phosphorylated ddU into cells, Sastry et al. (1992) developed a membrane-permeable pivaloyloxymethyl ester 'prodrug' of ddU 5′-monophosphate (ddUMP) that inhibits HIV infection. This neutral lipophilic phosphotriester penetrates cells by passive diffusion and ddUMP is released by cellular esterases. This prodrug was active in the micromolar range in the inhibition of viral protein (p24) synthesis (Sastry et al., 1992).

An alternative approach to the nucleotide cell permeability problem is to use immunoliposomes (liposome coupled proteins, such as monoclonal antibodies

or protein A) as a transport system to deliver phosphorylated molecules directly into cells, a technology which has been extensively studied for other membrane-impermeable molecules (Heath, 1988; Noé et al., 1988). These liposomes permit targeting encapsulated agents to cells expressing the determinant recognized by the coupled proteins (Leserman et al., 1980; Leserman et al., 1981). Studies by several groups have shown that immunoliposomes enter into lymphoid cells by an endocytic pathway, the efficiency of which depends on the physiology of the targeted surface molecule, the type of cell expressing it and on the size of liposomes (Machy et al., 1983; Matthay et al., 1989; Suzuki et al., 1991). Encapsulation of phosphorylated ddU also offers protection from possible degradation because of its isolation from the external medium. As non-phosphorylated ddU is inactive, the liposome-encapsulated product would be expected to be active only if delivered into the cytoplasm in its phosphorylated form.

In this paper we studied the antiviral effects of 2′,3′-dideoxyuridine 5′-mono, and -triphosphate encapsulated in immunoliposomes. Liposome encapsulation does not destabilize the ddU nucleotides, which remain phosphorylated for several weeks at 4°C. Our results reveal that it is possible to efficiently deliver this antiviral drug into HIV-infected cells via immunoliposomes and, that inside the cell, the phosphorylated ddU profoundly inhibits HIV replication.

Materials and Methods

Synthesis of ddU and phosphorylated ddU

ddU was obtained from Synthelabo-Recherche (France). General procedures and instrumentation used have been described previously (Puech et al., 1990).

Antibodies

Antibodies used in this study are B1.23.2 (anti-human HLA-BC) (Rebaï et al., 1983a), 8H8.1 (anti-human CD7) (Haynes, 1986), H40–164.3 (40B) (anti-mouse I-A^k and I-E^k, that cross-reacts with human HLA-DR) (Rebaï et al., 1983b) and H100.5/28 (anti-mouse H2-K^k without cross-reaction on human cells) used as control (Lemke et al., 1979). All are monoclonal mouse $IgG2_a,\kappa$ antibodies purified on Protein A-Sepharose CL-4B columns (Pharmacia).

Cells and virus

CCRF-CEM cells (CEM), a T-lymphoblastoid cell line, was obtained from American Type Culture Collection, Rockville, MD, USA, (Ref. CCL 119). MT-4 cells are human T-cells infected by human T-cell leukemia virus type 1 (HTLV-I), that undergo lytic infection with HIV (Harada et al., 1985).

HIV-1 (BRU) (Barré-Sinoussi et al., 1983), provided by L. Montagnier (Institut Pasteur, Paris, France), was maintained and amplified on CEM cells.

Cells and infected cells were cultivated in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM glutamine, 5×10^{-5} M 2-mercaptoethanol, 2 mM

sodium pyruvate, 2 mM nonessential amino acids and antibiotics at 37°C in a 5% CO_2 atmosphere.

Preparation of liposomes

Liposomes composed of 50 μmol total lipid (64 mol% dipalmitoylphosphatidylcholine (Avanti Polar Lipids, Birmingham, AL, USA), 35 mol% cholesterol (Sigma), and 1 mol% dipalmitoylphosphatidylethanolamine (Sigma) modified with *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) (Pharmacia)) (Leserman et al., 1980), were prepared with the aqueous phase composed of a solution of 10 or 15 mM 5′-mono- or tri-phosphorylated 2′,3′-dideoxyuridine in saline buffered to pH 7.4 with 10 mM Hepes, or with this buffer alone as control. The solution was alternately frozen in liquid nitrogen and thawed by heating to 55°C five times with intermittent vortex mixing. The multilamellar liposomes thus produced were passed through an 'Extruder' (Lipex Biomembranes, Vancouver, Canada), mounted with 0.08 μm polycarbonate filters (Nucleopore, Pleasanton, CA, USA), according to the manufacturer's instructions and published references (Hope et al., 1985), at 55°C. Liposomes were covalently coupled to *Staphylococcus aureus* Protein A (Pharmacia), as described (Leserman et al., 1980). Uncoupled Protein A and unencapsulated products were separated from liposomes on a Sepharose 4B (Pharmacia) column. Liposomes were sterilized by filtration through 0.45 μm Gelman filters. Liposome encapsulation efficiency varies with lipid concentration and liposome size. In this case, it was about 2%, but this number has no real meaning because of this dependence. Liposomes always contained ddUMP or ddUTP at their original concentrations (10 or 15 mM). The final concentration of phosphorylated ddU in the medium was obtained by dilution of the stock preparation. The nucleotide concentration used thus refers to that in the tissue culture wells, not in the liposomes. Liposomes containing buffer were diluted to equivalent phospholipid concentrations, as determined by the method of Stewart (Stewart, 1980).

Stability of ddUMP and ddUTP encapsulated in liposomes

Extraction procedure for HPLC analysis. To 200 μl of buffer containing the liposome-encapsulated agents were added 200 μl of methanol and 200 μl of chloroform. The mixture was vortex-mixed 5 min, then heated at 50°C for 2 min, vortex-mixed again for 5 min and centrifuged 10 min at 4000 RPM. The $MeOH/H_2O$ upper layer (about 200 μl) was transferred to conic inserts of the autosampler. 50 μl were chromatographed immediately. The extraction procedure is at least 90% efficient (data not shown).

HPLC analyses. The Waters-Millipore instrument was equipped with a Model 712 autosampler and a Model 990 diode-array UV detector. The ion-exchanger column (Ultranucleotide 4000–05, 10 μm, 4.6 × 100 mm) and the corresponding guard column (4.6 × 10 mm) were purchased from SFCC/Shandon (Eragny, France). A linear gradient was performed in 10 min from

solvent A (20 mM potassium phosphate buffer, pH 6) to solvent B (20 mM potassium phosphate buffer, 0.2 M potassium chloride, pH 6). The solvents were prepared with distilled water purified on a Milli-Q System (Millipore, Bedford, MA USA) and with 'Proanalysis grade' KH_2PO_4, K_2HPO_4 and KCl (Merck, Darmstadt, Germany). Three-dimensional chromatograms were recorded from 195 to 350 nm. The structure and concentration of products were characterized by comparison (retention times, UV spectra and areas of signals at 263 nm) with authentic samples (injection: 10 μl of 1 mM solutions in water).

Antiviral assay on CEM cells. CEM cells, at a concentration of 5×10^6 cells/ml, were infected with 1000 $TCID_{50}$ of virus and incubated for 30 min at 4°C in 96 well flat bottom microtitre plates, in a total volume of 200 μl. Cells were washed, diluted to 5×10^5 cells/ml and incubated at 37°C in the presence or absence of 20 μg/ml of antibody and various concentrations of liposomes or with ddU and phosphorylated ddU free in solution in 24 tissue plates in 500 μl. At day four, the cell concentration was readjusted to 5×10^5 cells/ml. At day seven, samples were removed to determine RT activity as described previously (Rey et al., 1984) and p24 expression.

Antiviral assay on MT-4 cells. MT-4 cells, concentrated to 3×10^6 cells/ml, were incubated in the presence or absence of 20 μg/ml of antibody and infected with 1000 $TCID_{50}$ of virus for 30 min at 4°C. Cells were washed, diluted to 3×10^5 cells/ml and incubated at 37°C with appropriate concentrations of liposomes or with ddU and phosphorylated ddU free in solution. This modification of the protocol for MT-4 cells relative to that used for CEM was to prevent inhibition of viral proliferation by subsequently added antibodies, as has been reported for this cell line but not CEM (Corbeau et al., 1991). 5 Days following infection, culture samples were removed to determine RT activity as described previously (Rey et al., 1984) and p24 expression. Data presented from RT and p24 determinations represent means normalized to percentage inhibition values +/-S.D., for a minimum of three independent experiments.

Determination of p24 expression. The assay is a twin-site sandwich ELISA. Briefly, p24 antigen is captured from a detergent lysate of virions onto a polyclonal anti p24 antibody (Ref. D7320, Aalto Bioreagents, Dublin, Eire) adsorbed on a solid phase support (MaxiSorp, Nunc, Roskilde, Denmark). Bound p24 is detected with an alkaline phosphatase-conjugated anti-p24 monoclonal antibody (Ref. EH12E1, MRC Reagent Programme) and the AMPAK ELISA amplification system (DAKO, France).

Results

Chemical synthesis

2'3'-Dideoxyuridine 5'-monophosphate (ddUMP) (Sodium salt). 2',3'-Dideoxyuridine (ddU) (0.1 g, 0.47 mmol) was dissolved in trimethylphosphate (4 ml) and phosphorous oxychloride (58 μl, 0.61 mmol) ($POCl_3$) was added at O°C. The mixture was left at 0°C for 3 h, neutralized with 1 M triethylammonium bicarbonate (TEAB) buffer and then successively extracted with toluene (2 × 10 ml) and with ether (2 × 10 ml). The aqueous layer was applied to a column (30 × 2.5 cm) of DEAE Sephadex A25 (HCO_3^- form) and eluted with a linear gradient of aqueous TEAB (from 0 to 0.4 M; total volume: 1 L). Fractions containing ddUMP were pooled and evaporated. Residual triethylammonium bicarbonate was removed by successive lyophilizations in water. The residue was dissolved in ethanol and a 1% solution of sodium iodide in acetone was added. The white precipitate that formed was collected by centrifugation and dried over P_2O_5 in vacuo (0.12 g, 76%); R_fTLC: 0.41 (Isopropanol/H_2O/NH_4OH, 11:7:2 v/v); UV (H_2O) λ_{max} 262 nm (ε, 8900), λ_{min} 232 nm (ε, 2100); ^{1}H-NMR (DMSO-d_6), δ_H 11.3 (br s, 1H, NH-3), 8.05 (d, 1H, H-6; $J_{5,6}$ = 8.1Hz), 5.9 (m, 1H, H-1'), 5.58 (d, 1H, H-5), 4.10 (m, 1H, H-4'), 3.8 and 3.7 (2 m, 1H each, H-5' and H-5''), 2.2 (m, 1H, H-2'), 1.9 (m, 3 H, H-2'', 3', 3''); ^{31}P-NMR (D_2O) δ_p −4.6 (s); mass spectrum (FAB < 0, matrix: glycerol): 335 $(M-H)^-$, 313 $(M-Na)^-$, 291 $(M+H-2\,Na)^-$; HPLC purity at 263 nm: >99%.

2'3'-Dideoxyuridine 5'-triphosphate (ddUTP) (Sodium salt). To a solution of ddU (0.115 g, 0.54 mmol) in trimethylphosphate (4.6 ml) was added $POCl_3$ (66 μl, 0.7 mmol), and the stirring was pursued at 0°C for 3 h. Then a solution of 0.5 M *bis*(tributylammonium)pyrophosphate in DMF (2.7 ml) and tributylamine (0.54 ml, 2.2 mmol) were added. The reaction mixture was stirred at 0°C for 1 h, then neutralized with aqueous 1 M TEAB, and evaporated. The residue was dissolved in water and applied to the top of a column (30 × 2.5 cm) of DEAE Sephadex A25 (HCO_3^- form). The elution was performed with a linear gradient of aqueous TEAB (from 0 to 1 M; total volume: 2 L). The fractions containing ddUTP were collected, concentrated under reduced pressure, and then lyophilized 4 times with water. The residue was dissolved in ethanol and a 1% solution of sodium iodide in acetone was added. The white precipitate that formed was collected by centrifugation and dried over P_2O_5 in vacuo (0.085 g, 32%); R_fTLC: 0.25 (dioxane/H_2O/NH_4OH, 6:4:3, v/v); UV (H_2O) λ_{max} 263 nm (ε, 9100), λ_{min} 232 nm (ε, 2200); ^{1}H-NMR (D_2O), δ_H 8.05 (d, 1H, H-6; $J_{5,6}$ = 8.1 Hz), 6.17 (m, 1H, H-1'), 5.95 (d, 1H, H-5), 4.45 (m, 1H, H-4'), 4.3 and 4.2 (2 m, 1H each, H-5' and H-5''), 2.5 (m, 1H, H-2'), 2.2 (m, 3 H, H-2'', 3', 3''), ^{31}P-NMR (D_2O) δ_p −10.03 (m, 2P, P-α and P-γ), −22.58 (m, 1P, P-β); mass spectrum (FAB < 0, matrix: glycerol): 539 $(M-H)^-$, 517 $(M-Na)^-$, 495 $(M+H-2Na)^-$, 473 $(M+2H-3Na)^-$, 451 $(M+3\,H-4Na)^-$; HPLC purity at 263 nm: >98%.

Stability of encapsulated phosphorylated ddU derivatives in immunoliposomes. Analyses of ddUMP and ddUTP encapsulated in liposomes were performed 2 weeks after encapsulation and storage at 4°C. In the chromatogram (not shown) obtained for encapsulated ddUMP the main signal at 2.5 min corresponds to ddUMP (C = 31 μM). Signals X centered at 1.5 min correspond to unknown products which were found in all Protein A coupled liposome extracts, including empty liposomes. Their UV spectra show the absence of nucleoside derivatives. No other product was detected, showing the stability of the ddUMP in the sample. Analysis of encapsulated ddUTP shows the presence of ddUTP (79%), ddUDP (21%) and ddUMP (traces) with a global concentration of 19 μM. We re-analyzed (Fig. 2) a sample from the same preparation 8 weeks after encapsulation (storage at 4°C) and detected ddUTP (74%, Retention time (R_t) 9.3 min), ddUDP (20%, R_t 6.6 min) and ddUMP (6%, R_t 2.6 min). This confirms a slow decomposition (less than 1%/week) of the tri- and di-phosphorylated compounds, but the total concentration of ddU derivatives remained unchanged.

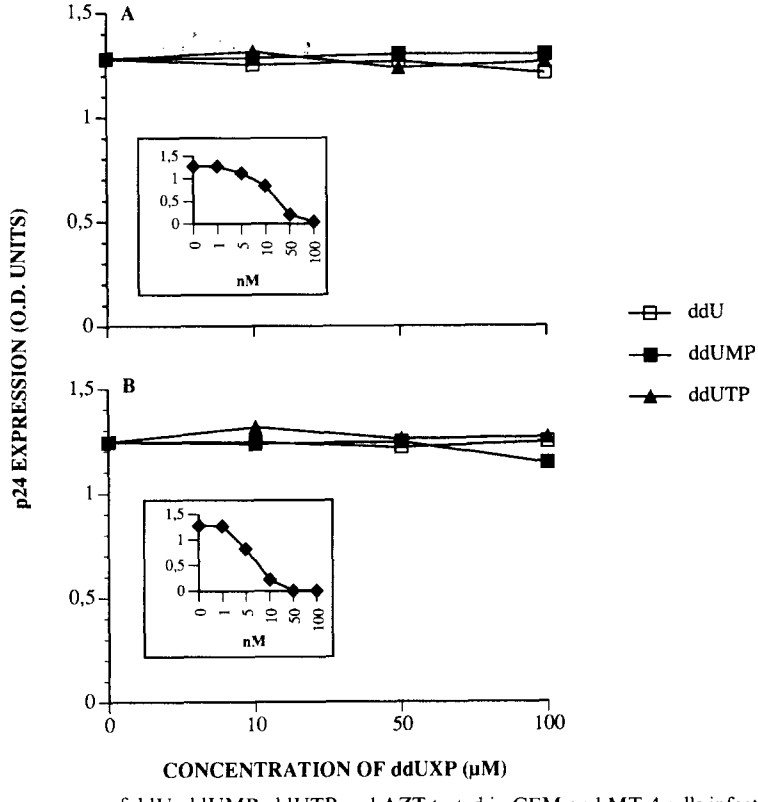

Fig. 1. Dose responses of ddU, ddUMP, ddUTP and AZT tested in CEM and MT-4 cells infected de novo with HIV-1 (BRU) as described in Materials and Methods. A: CEM. B: MT-4. Inserts: effects of AZT on the same cells. Note the difference in the concentrations used for ddU and its derivatives compared to AZT.

Antiviral activity of free phosphorylated and non-phosphorylated ddU. Antiviral activity was evaluated on de novo infected CEM and MT-4 cells. Effects of free ddU, ddUMP and ddUTP were monitored as compared to AZT (Fig. 1). No inhibition of p24 expression was observed for any of the ddU compounds at concentrations as high as 100 μM, while AZT was active in the nanomolar range (see insert in Fig. 1). Comparable results were obtained for RT (data not shown). This confirms existing data (Baba et al., 1987; Balzarini et al., 1987; Balzarini et al., 1988; Hao et al., 1990), which shows that ddU cannot, or can only weakly, inhibit HIV replication in cell culture compared to other ddN containing natural bases, or to AZT. We subsequently investigated the potential of immunoliposomes to introduce phosphorylated ddU into cells.

Antiviral effects of immunoliposomes containing phosphorylated ddU. We have previously reported fixation and internalization of fluorescent, methotrexate-containing liposomes by CEM cells (Suzuki et al., 1991). Liposomes were

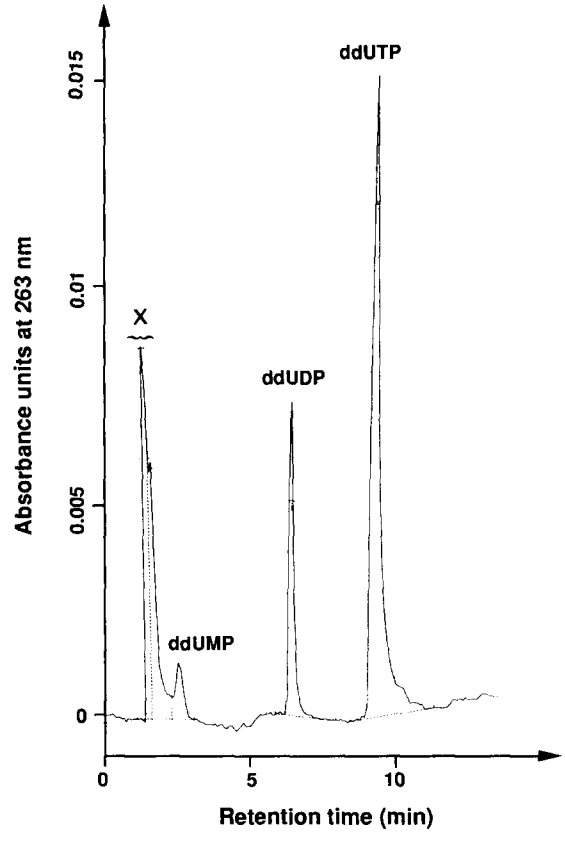

Fig. 2. HPLC analysis of the encapsulated ddUTP sample performed 8 weeks after encapsulation. For the origin of signals x, see the text.

TABLE 1

Antiviral effects of non-targeted immunoliposomes on HIV-infected CEM and MT-4 cells

	% inhibition			
	CEM		MT-4	
	RT	P24	RT	P24
Empty liposomes[a]	12.5 ± 10	2 ± 3	14 ± 18	22 ± 19
ddUMP-liposomes[b]	9 ± 12	4 ± 4	8 ± 16	12 ± 9
ddUTP-liposomes[b]	15 ± 21	14 ± 16	24 ± 13	29 ± 10

Antiviral assays were performed as described in Materials and Methods.
[a]Empty liposomes were adjusted to the same lipid concentration as the other liposomes.
[b]Liposomes were tested at 2 μM phosphorylated ddU.

coupled to protein A, which binds with high affinity to the Fc region of several antibody classes, including the mouse IgG2a antibodies used here. These were targeted to CEM cells by CD7 and HLA class I-specific antibodies. These results showed that CD7 and class I molecules expressed on CEM are good targets for specific delivery of liposomal methotrexate. Using the same experimental approach as with CEM, we observed that HLA class I and class II molecules were good candidates for targeting immunoliposomes on MT-4 cells, but the CD7 molecule was a poor target, presumably because of its low level of expression. The HLA class II molecule is not expressed on CEM (data not shown).

Liposomes containing buffer or any of the ddU compounds at 2 μM final concentration failed to show significant anti-viral activity on CEM or MT-4 cells in the absence of antibody (Table 1), or in the presence of an irrelevant (mouse-specific) antibody (Tables 2 and 3). Liposomes containing ddUMP and ddUTP and targeted by relevant antibodies showed significant antiviral effect, in contrast to liposomes containing buffer targeted by the same antibodies. With CEM cells, anti-HLA class I antibodies seemed more efficient than anti-CD7 antibodies: as can be seen in Table 2, the ddUTP-liposomes targeted to HLA class I molecules inhibited RT activity by 95%, while there was only 67%

TABLE 2

Antiviral activity of targeted immunoliposomes against HIV-infected CEM cells

	% inhibition					
	H2-K^{k} [a]		HLA-BC[a]		CD7[a]	
	RT	P24	RT	P24	RT	P24
Empty liposomes[b]	8.5 ± 12	2 ± 0.5	24 ± 24	13 ± 9	23 ± 16	8 ± 8
ddUMP-liposomes[b]	0 ± 0	16.5 ± 17	84 ± 10	73 ± 15	68 ± 16	68 ± 17
ddUTP-liposomes[b]	7 ± 10	8 ± 9	95 ± 1	76 ± 14	67 ± 17	70 ± 19

Antiviral assays were performed as described in Materials and Methods.
[a]Cell surface molecules targeted by immunoliposomes. H2-K^{k} is not expressed by these cells.
[b]The concentration of compounds is the same as described in Table 1.

TABLE 3
Antiviral activity of targeted immunoliposomes against HIV-infected MT-4 cells

	% inhibition					
	H2-K^{k} [a]		HLA-BC[a]		HLA-DR[a]	
	RT	P24	RT	P24	RT	P24
Empty liposomes[b]	3 ± 6	0 ± 0	2 ± 3	11 ± 4	10 ± 5	9 ± 10
ddUMP-liposomes[b]	21 ± 15	30 ± 14	66 ± 32	86 ± 12	62 ± 21	66 ± 25
ddUTP-liposomes[b]	21 ± 25	25 ± 8	79 ± 13	90 ± 6	70 ± 15	80 ± 21

Antiviral assays were performed as described in Materials and Methods.
[a]Cell surface molecules targeted by immunoliposomes. H2-K^{k} is not expressed by these cells.
[b]The concentration of compounds is the same as described in Table 1.

inhibition when targeted to CD7. A similar effect was seen with MT-4 cells: ddUMP-liposomes targeted to HLA class I molecules inhibited p24 expression by 86%, while the same liposomes targeted to HLA class II molecules only inhibited by 66%. This may correlate with the higher expression of class I molecules on these cells.

We evaluated the toxicity of compounds encapsulated in liposomes with and without antibodies on both infected and uninfected cells and found that all preparations of empty liposomes and those containing ddUMP and ddUTP were devoid of cytotoxicity as measured by trypan blue exclusion and [^{3}H]thymidine incorporation (data not shown).

From the dose response curve (Fig. 3), the 50% antiviral effective dose (ED_{50}) of ddUMP-liposomes and ddUTP-liposomes were evaluated for infected CEM cells. They were both $\leqslant$ 1 μM in the cases where the liposomes were targeted with anti-HLA class I antibodies. These values were slightly higher with anti-CD7 antibodies (about 1.5 μM and 1.8 μM, respectively). As expected from results both from this and earlier work (Leonetti et al., 1990), empty liposomes had absolutely no antiviral activity when used with an irrelevant antibody (H2-K) or targeted with anti-HLA class I and anti-CD7 antibodies.

Discussion

ddU is inactive as an anti-HIV agent, presumably because it is poorly phosphorylated in human cells which have been tested (Balzarini et al., 1988; Hao et al., 1990). ddUTP is an active inhibitor of HIV RT in cell-free systems (Hao et al., 1988); however, nucleosides cannot cross cell membranes in their phosphorylated form (Cohen, 1975). In this study we have used immunoliposomes to deliver phosphorylated ddU into cells. No decomposition of liposomal ddUMP was observed 2 weeks after encapsulation when stored at 4°C. From the HPLC analyses performed at 2 and 8 weeks, encapsulated ddUTP is slowly degraded (less than 1%/week). It is known that ddUDP and

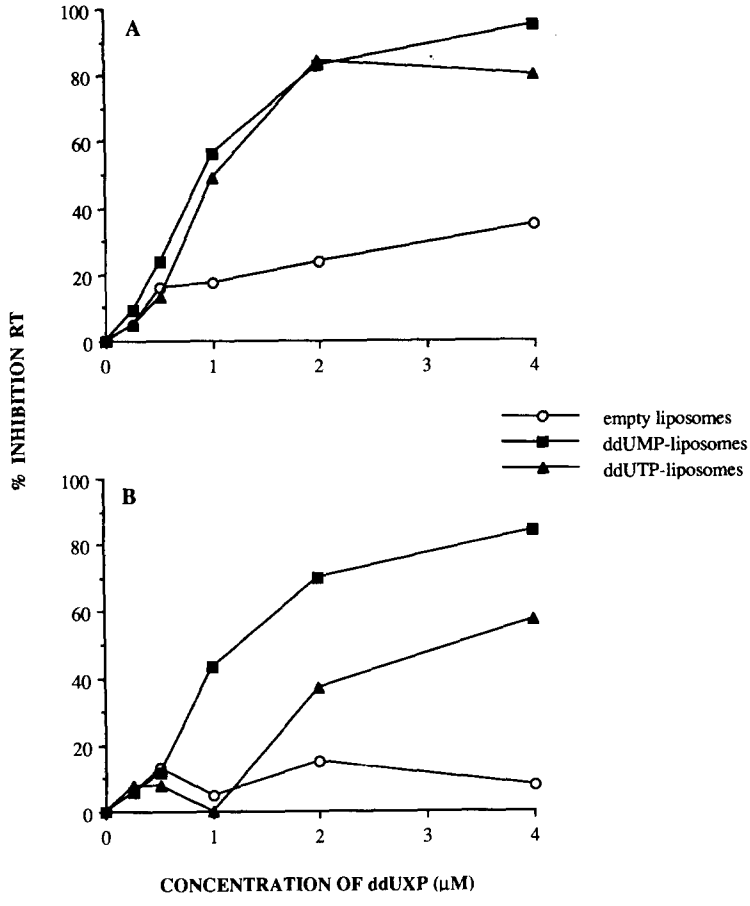

Fig. 3. Dose responses of empty liposomes, ddUMP-liposomes and ddUTP-liposomes in CEM cells infected de novo with HIV-1 (BRU) as described in Materials and Methods. A: Immunoliposomes targeted to HLA class I molecules. B: Immunoliposomes targeted to CD7 molecules.

ddUTP are slowly dephosphorylated in aqueous media, even when stored at 4°C; therefore the observed degradation should not be attributed to instability in liposomes. The rate of degradation cannot explain the 21% decomposition of ddUTP after 2 weeks storage. Probably the main degradation occurs during liposome preparation (heating 30 min at 55°C.). This conclusion is supported by other results (not described here) on the stability of ddUDP encapsulated in liposomes (24% degradation after 2 weeks and 31% after 8 weeks). Nevertheless, the preparation procedure did not affect the stability of ddUMP, as no ddU was detected in our analyses.

ddUMP and ddUTP encapsulated in liposomes show prominent antiviral effects when they are delivered into infected cells. We have also shown here, in agreement with previous publications (Konopka et al., 1990; Kumagai et al., 1991), that liposomes composed of neutral lipids have no effect on HIV

infectivity, in as much as liposomes containing only buffer were inactive whether or not targeted by antibodies. This is in contrast to results reported by the same groups showing that positively or negatively charged non-targeted liposomes altered HIV infectivity.

Our results confirm those obtained by Sastry et al., showing that HIV infection is inhibited by a ddUMP derivative able to enter into infected cells (Sastry et al., 1992). Their results suggested that the initial phosphorylation of ddU by cellular kinases is the limiting step, since their prodrug delivered only the monophosphate form of ddU into cells. This is confirmed directly in our study, since ddUMP blocked HIV infection as effectively as ddUTP, indicating that the second and third phosphorylations are not limiting in the inhibition of HIV infection by these compounds. Moreover, though comparison between different experimental systems is difficult, inhibition by ddUMP within immunoliposomes seems more efficient than that reported for membrane permeable ddUMP, as indicated by an ED_{50} approximately 10-fold lower for MT-4 cells for inhibition of RT. This may be explained by the protection and targeting of phosphorylated ddU as a consequence of its encapsulation within liposomes, in contrast to the membrane permeable prodrug.

Other dideoxynucleosides and other antiviral agents have been encapsulated in targeted or conventional liposomes. For example, AZT encapsulated within liposomes had anti-retroviral activity, while the free drug did not. However most of the non-polar AZT leaked from liposomes (Phillips et al., 1991; Phillips et al., 1992). This diffusion was also observed by another group for ddC (Szebeni et al., 1990), and by us for 'encapsulated' non-phosphorylated ddU, the activity of which, in consequence, could not be evaluated in immunoliposomes (data not shown).

Dideoxycytidine 5′-triphosphate encapsulated in liposomes (Szebeni et al., 1990) or in erythrocytes (Magnani et al., 1992) inhibited viral replication in monocytes and macrophages, which constitute an important reservoir for HIV (Gartner et al., 1986). Since non-phosphorylated forms of AZT, ddC (and of all other major dideoxynucleosides except ddU) are pharmacologically active, the liposome-dependence of their encapsulated nucleotides is difficult to evaluate, because dephosphorylation and leakage of only a small fraction of liposome contents would be sufficient to account for their activity. In principle, the lack of entry of leaked phosphorylated or activity of dephosphorylated ddU would limit the systemic toxicity of this nucleoside. Another feature of delivery of triphosphate forms into cells is the ability to bypass the phosphorylation process, which is slow in most mammalian cells (Waqar et al., 1984). This abrogates variation in effectiveness of the ddN in different cell lines which may reflect variations in the levels of nucleoside cellular kinases. Moreover, this approach may circumvent acquired resistance to nucleoside analogues caused by the loss or depletion of cellular nucleoside kinases.

The pharmacologic effect of liposome-encapsulated reagents was less than that of free AZT, which has the advantage of freely diffusing into cells. The amount of ddU which was actually delivered into the cytoplasm cannot be

determined with precision, because there is no way of knowing how much of the cell associated liposomes' contents actually reach their intracellular site of action. This will depend on the development of techniques capable of measuring intracellular nucleotides and their degradation products. The liposomes used in this study were selected for their stability; other liposome formulations may be more efficient for intracytoplasmic delivery. The delivery of phosphorylated ddU, in addition to published data for RNA (Renneisen et al., 1990) and DNA (Leonetti et al., 1990) antisense reagents, indicates that liposomes may be a versatile delivery system for a wide range of biologically active molecules.

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