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HUMAN RED BLOOD CELLS AS BIOREACTORS FOR THE RELEASE OF 2',3'-DIDEOXYCYTIDINE, AN INHIBITOR OF HIV INFECTIVITY*

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2',3'-Dideoxycytidine (ddCyd) is one of the most potent antiviral nucleosides for killing the human immunodeficiency virus (HIV). ddCyd is currently used in the treatment of severe HIV infections but due to its rapid clearance it must be administered to patients every 4 h reaching concentrations that are toxic. We have synthesized 2',3'-dideoxycytidine-5'-phosphate (ddCMP) as a prodrug, encapsulated it in human erythrocytes and found that it is dephosphorylated by endogenous pyrimidine nucleotidases and subsequently released by the cells as ddCyd. Encapsulated ddCMP does not affect erythrocyte metabolism and was not deaminated by cytidine deaminase. The dephosphorylation reaction has an apparent Km of 6mM, an optimum pH of 6.8 and is not inhibited by ATP or 2,3-bisphosphoglycerate. The efflux of ddCyd from the erythrocyte is a linear function of ddCyd concentration and relatively insensitive to nucleoside transporter inhibitors suggesting that ddCyd permeates the erythrocyte membrane predominantly by nonfacilitated diffusion. Thus, ddCMP-loaded erythrocytes might be used as endogenous bioreactors for ddCyd delivery in the treatment of HIV infection.

2',3'-Dideoxycytidine inhibits retroviral DNA synthesis and mRNA expression in T cell infected by the human immunodeficiency virus (HIV) (1-3). Recently this dideoxynucleoside analogue was selected for clinical development because mole for mole it is about ten times more potent than AZT for killing HIV and is activated by a different phosphorylation pathway (4). Unfortunately the average half-life of ddCyd was found to be only 1.2 h in humans so that to achieve an antiretroviral effect patients received 30 to 90 ug/Kg of ddCyd every 4h. This dose was found to cause toxic effects, in particular neuropathy (4).

* This paper is dedicated to the memory of Prof. Alberto Sols who passed away on August 10, 1989.

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We reasoned that if ddCyd could be encapsulated into a suitable carrier capable of a slow release the toxic effects of ddCyd could probably be reduced, also ensuring long-lasting plasma ddCyd concentrations. Two kinds of carriers for drugs are suitable towards this end: namely, liposomes and erythrocytes (5,6). We believe, as shown below, that erythrocytes should have the main advantage over liposomes in encapsulating prodrugs to be converted into the active drug by resident erythrocyte enzymes. In fact, we first encapsulated ddCyd in human erythrocytes by a procedure of hypotonic hemolysis and resealing, but the entrapped drug was found to escape from the cells and equilibrium between plasma and erythrocytes was reached in 30 min (not shown). Subsequently we decided to synthesize a prodrug hoping to reduce the efflux rate of ddCyd from erythrocytes. This strategy, as shown in this paper, was successful and ddCMP-loaded erythrocytes seem to provide a new ddCyd delivery system in the treatment of HIV infection.

MATERIALS AND METHODS

2',3'-Dideoxycytidine-5'-phosphate was prepared by coupling 2-cyanoethyl phosphate (Fluka) to the 5'-alcoholic group of ddCyd using dicyclohexylcarbodiimide (Fluka) as condensing agent. Removal of the cyanoethyl group from the resulting phosphodiester was then obtained by very mild alkaline hydrolysis (7). The purification of ddCMP was obtained by Dowex 50 H^+ chromatography and it was concentrated in vacuum. The product was found to be more than 98% ddCMP by several methods including NMR spectroscopy, reversed-phase HPLC and UV spectroscopy. The yield of the procedure was about 30%. In some preparations 2',3'-dideoxycytidine-5,6-[^{3}H] (Moravek, Ca) was used to prepare [^{3}H] ddCMP. ddCyd and ddCMP were determined by reverse phase HPLC chromatography.A 5 µm Supelcosil LC 18 column (250 x 4.6 mm i.d., Supelco, Bellefonte, PA) protected with a guard column Pelliguard LC 18 (20 x 4.6 mm i.d.) pellicular packing material, 40 µm particles, were used. The mobile phase consisted of two eluants: 0.1 M KH_2PO_4 solution, pH 6.0 (buffer A), and a 0.1 M KH_2PO_4 solution, pH 6.0, containing 10% (v/v) of CH_3OH (buffer B). The chromatographic conditions were: 1 min 100% of buffer A, 4 min up to 10% of buffer B, 4 min up to 40% of buffer B, 8 min up to 100% of buffer B and hold for 6 min. The gradient was then returned to 100% of buffer A in 1 min and the initial conditions restored in 5 min. The flow rate was 1.3 ml/min and the detection was at 272 nm. Quantitative measurements were carried out by injection of standard solutions of known concentration. The retention time for ddCMP was 6 min and that of ddCyd 16.6 min. The simultaneous detection of 2',3'-dideoxyuridine (a deamination product of ddCyd), if present, is possible since its retention time is 19 min.

Human red blood cells were loaded with ddCMP by a procedure of hypotonic dialysis and isotonic resealing essentially as reported in Ref. 9 with few modifications to adapt the procedure to small scale experiments.

RESULTS

2',3'-Dideoxycytidine-5'-phosphate synthesis

There are no current methods available to prepare 2',3'-dideoxycytidine-5'-phosphate so we have developed a procedure that takes advantage of the cyanoethyl phosphate method to synthesize phosphate esters (7). Since only one alcoholic group is present on ddCyd in the 5'-position no protection of other reacting groups is needed. Some phosphorylation occurs also on the amino group of the pyrimidine ring but the phosphoamide bond is very labile and broke down under conditions used for the removal of the cyanoethyl group. The method provides pure ddCMP in three days (the reaction is left in a stoppered flask for 2 days at room temperature) with 30% yield.

ddCMP dephosphorylation in human red cell lysates

Human erythrocyte lysates were found to catalyze the dephosphorylation of ddCMP yielding stoichiometric amounts of ddCyd (Fig. 1). The reaction was

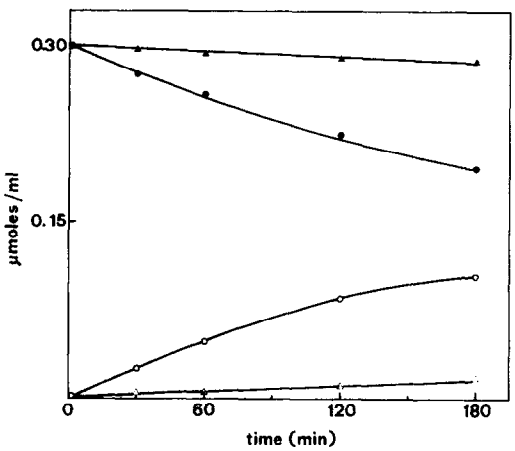

Fig. 1. Human red cell hemolysate catalyzes the dephosphorylation of 2',3'-dideoxycytidine-5'-phosphate. Human red blood cells were freed of white cells and platelets by chromatography on columns of α-cellulose, microcristalline cellulose (14), washed twice with a physiological saline solution (PBS), and lysed by addition of 2 vol of distilled water containing 3 mM 2-mercaptoethanol and 0.5 mM EDTA. After 30 min on ice the cell lysate was centrifuged at 4°C and 12,000 g for 60 min to remove the cell membranes. The membrane-free hemolysate was than dialyzed overnight against 200 vol of 0.9% (w/v) NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 0.02 mM EDTA, pH 7.5 incubated at a concentration of 110 mg/ml of hemoglobin, at 37°C in the presence of 0.3 mM ddCMP. As a control 0.3 mM ddCMP was incubated in the dialysis buffer under identical conditions, without the addition of hemolysate (triangles). At time 0, 30, 60, 120 and 180 min 0.25 ml aliquots were removed and extracted with perchloric acid as in Ref. 15. Neutralized extracts were then used for HPLC analyses of ddCyd (open symbols) and ddCMP (filled symbols).

TABLE 1. Some properties of human erythrocyte ddCMP nucleotidase

K_m	6 mM
V_{MAX}	0.075 μmoles/min/g hemoglobin
pH optimum	6.8
Pb^{2+} inhibition[1]	$3 \cdot 10^{-5}$ M
% Inhibition by[2] dCMP	54
CMP	37
dUMP	0
UMP	0
dTMP	0

[1] Concentration that inhibits by 50% the dephosphorylation rate of 0.3 mM ddCMP at pH 7.0.

[2] Percentage inhibition of dephosphorylation of 0.3 mM ddCMP by 0.3 mM of the listed nucleotides.

proportional to the amount of hemolysate and formation of 2',3'-dideoxyuridine or 2'3'-dideoxyuridine-5'-phosphate was not observed. Apparent Km values and some other properties of the ddCMP dephosphorylation are reported in Table 1. These and other data (lead inhibition, relative activity of other pyrimidine ribo- and deoxyribonucleotides, cytosolic localization) suggest that the dephosphorylation of ddCMP in hemolysates is probably catalyzed by a pyrimidine deoxyribonucleotidase (8).

dd CMP-loading in human erythrocytes and ddCyd release

ddCMP was loaded in human erythrocytes by a procedure of hypotonic dialysis and isotonic resealing (9). This procedure allows encapsulation of 4 μmoles/ml packed erythrocytes of ddCMP but should be adapted to increase or reduce this amount of drug simply adding more or less ddCMP during the dialysis step. The percentage of encapsulation was 25% and the recovery of cells 80%. Several metabolic properties of the ddCMP-loaded erythrocytes are reported in Table 2. The results obtained seem to exclude any significant perturbation of the biochemical properties of ddCMP-loaded erythrocytes. When these erythrocytes were incubated in a physiological saline solution the appearance of ddCyd in the medium was observed together with a decrease of intracellular ddCMP (Fig. 2). In order to characterize also the efflux of ddCyd from the erythrocytes we loaded red blood cells (RBC) with different

TABLE 2. Metabolic properties of ddCMP-loaded erythrocytes and controls

	Unloaded erythrocytes	ddCMP-loaded erythrocytes
Lactate production (nmoles/h/ml RBC)	2,380	2,040
Hexose monophosphate shunt (nmoles CO_2/h/ml RBC)	98	82
ATP (μmoles/ml RBC)	1.62	1.52
GSH (μmoles/ml RBC)	0.91	1.06

The values are obtained after 1 h of incubation at 37°C and are means of 3 experiments that agreed within 10%.

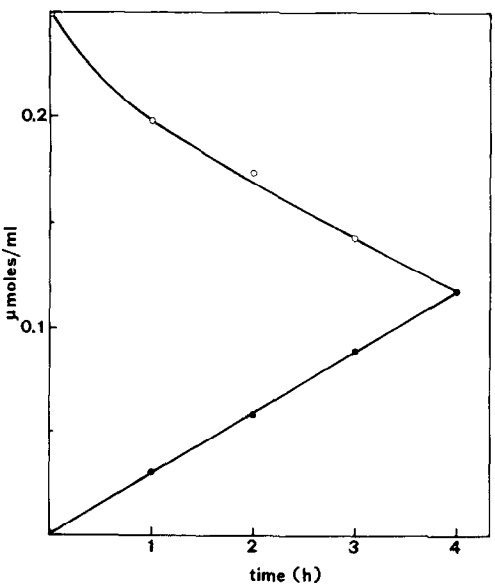

Fig. 2. 2',3'-dideoxycytidine release from 2',3'-dideoxycytidine-5'-phosphate loaded-erythrocytes. Human red blood cells were loaded with ddCMP by a procedure of hypotonic dialysis and isotonic resealing (9) to a final concentration of 3.2 mM. These cells were then resuspended in a physiological saline solution containing 5 mM glucose and incubated at 37°C at an 8% hematocrit. At time intervals as indicated ddCyd released in the medium was measured as described in the legend of Fig. 1. Simultaneously ddCMP was determined on perchloric acid extracts of the whole red cell suspension but after treatment with periodate and methylamine (16) to avoid interferences in the HPLC analyses, ddCMP and ATP (present in the red cell extracts) having similar retention times (6 min and 5.5 min respectively). The results obtained were also confirmed by the use of $[^3H]$-ddCMP prepared from 2',3'-dideoxycytidine-5,6-$[^3H]$ (6 Ci/μmole). In this case the peak of ddCMP eluted by HPLC was collected and counted in a Packard 1500 liquid scintillation spectrometer. Both methods provided values that agreed within 5%. (●), ddCyd released in the medium; (○), ddCMP in the red cell suspension.

concentrations of ddCyd (1 to 5 mM) and measured the nucleoside release in the upper aqueous layer of cell free medium after centrifugation on 1-bromododecane (10). The efflux of ddCyd was found to be a linear function of ddCyd concentration, and relatively insensitive to temperature and to nucleoside transport inhibitors such as Dipyridamole and S-(4-Nitrobenzyl)-6-thioinosine. These data suggest that ddCyd permeates the human erythrocyte membrane predominantly by nonfacilitated diffusion. Further details will be reported elsewhere.

DISCUSSION

The procedure we describe in this paper to synthesize and encapsulate a prodrug within erythrocytes and the fact that human red blood cells have an enzymatic activity able to convert this prodrug into the active one, suggest a potential strategy for ddCyd administration in the treatment of HIV infections. The main advantages of this strategy could be the fact that the prodrug is confined within the erythrocytes so that its toxicity is strongly reduced, and that its delivery is slow enough to ensure a long-lasting plasma concentration. This point appears to be of particular interest since by actual treatment regime patients receive, during 1 h infusion, a new dose of drug every 4 h, 6 times a day and for several months the drug being virustatic and not viruxcide (4).

Attempts to encapsulate cytosine arabinoside monophosphate in erythrocytes have failed due to the presence of cytosine deaminase (11, 12) in human red cells but ddCMP does not seem to be recognized by this enzyme. On the other hand encapsulation of 5'-fluoro-2'-deoxyuridine-5'-monophosphate to obtain the controlled release of the antineoplastic drug 5'-fluoro-2'-deoxyuridine was successful (13) and suggested the strategy we have followed to obtain the results reported in this paper.

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