ENHANCED INHIBITION OF VIRUS DNA SYNTHESIS IN HEPATOCYTES BY TRIFLUOROTHYMIDINE COUPLED TO ASIALOFETUIN

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Received 23 April 1979

1. Introduction

The use of some drugs would be greatly improved by concentrating them in those cells where their pharmacological action is required. This might be obtained by conjugating the drug to a macromolecular vector which is selectively taken up by target cells. If the bond between the drug and the carrier is broken down in lysosomes, the drug should be released free and concentrated into the cells where it was transported by the carrier [1,2]. When inhibitors of DNA synthesis are employed as antiviral agents, their concentration in infected cells would allow a more efficient inhibition of virus replication accompanied by a lower toxicity for tissues containing proliferating cells, such as bone marrow and gut. In previous experiments two inhibitors of DNA synthesis, cytosine arabinoside and 5-fluorodeoxyuridine, were concentrated in liver Kupffer cells of mice by conjugation to albumin. The conjugates inhibited the replication of *Ectromelia* virus in these cells, whereas free drugs were ineffective [3].

It was shown that some glycoproteins, such as fetuin, after removal of sialic acid are selectively taken up by hepatocytes where they are digested in lysosomes [4–6]. In the experiments reported here trifluorothymidine (F_3T) was coupled to asialofetuin (AF) in order to concentrate the drug into hepatocytes, as a possible approach to the treatment of hepatitis caused by deoxyriboviruses.

2. Materials and methods

2.1. *F_3T–AF conjugate*

Fetuin was desialylated by neuraminidase according to [7]. The conjugate of F_3T with AF (F_3T–AF) was obtained by coupling the hydroxysuccinimidester of trifluorothymidine glutarate to AF. To a solution of F_3T (500 mg, 1.69 mmol) in 20 ml pyridine, glutaric anhydride (245 mg, 2.15 mmol) was gradually added (2 h) with stirring at 80°C. After keeping at the same temperature overnight, the solvent was evaporated under vacuum; the residue was dissolved in 1.5% aqueous acetic acid and chromatographed on a Sephadex LH20 column (200 × 3 cm) with the same solvent. Fractions 73–86 (each fraction 20 ml) contained F_3T (250 mg); fractions 87–104 contained trifluorothymidine glutarate and were lyophilized (180 mg). Trifluorothymidine glutarate most probably was a binary mixture of 5′-glutarate (main product) and 3′,5′-diglutarate which migrated in high-voltage paper electrophoresis (pH 6.5) faster than the monoester. The ultraviolet spectrum of the glutarates was identical with that of trifluorothymidine (max. 260 nm). The mixture was dissolved in dimethylformamide (5 ml); then *N*-hydroxysuccinimide (79 mg, 0.69 mmol) and dicyclohexylcarbodiimide (142 mg, 0.69 mmol) were gradually added at 4°C. After 72 h at the same temperature the precipitate was filtered and the solution was added over 4 h to a solution of 200 mg AF in 20 ml water at pH 6–7

and 4°C. After stirring overnight at 4°C, the solution was dialyzed thoroughly at the same temperature against water and lyophilized. The residue was dissolved in 10 ml of a 0.05% ammonium bicarbonate solution, chromatographed through a Sephadex G-75 column (100 X 2 cm) and eluted with the same solvent. Fractions 9–24 (each fraction 5 ml) were lyophilized yielding 105 mg F_3T–AF. The molar ratio of F_3T : AF in the conjugate was calculated spectrophotometrically and was found to be 8 (50 μg F_3T in 1 mg F_3T–AF).

2.2. *Blood clearance of labeled AF*

AF was labeled (1.7 X 10^6 dpm/mg) with [^{14}C]-formaldehyde according to [8]. Mice were injected i.v. with [^{14}C]AF at 2 μg/g body wt. Each compound to be tested for competition was administered i.v. at 60 μg/g body wt. At the indicated times mice were bled from the retro-orbital plexus and the radioactivity of plasma was determined.

2.3. *DNA synthesis in liver and bone marrow of* Ectromelia *virus-infected mice*

In each experiment 15 Swiss female mice (28–31 g) were injected i.v. with *Ectromelia* virus at the multiplicity of 2.0 X 10^5 p.f.u./g body wt corresponding to 300-times the LD_{50}. *Ectromelia* virus (Hampstead mouse strain) was grown in L-929 cells and purified according to [9,10]. After 44 h infection mice were divided in 3 groups, each of 5 animals. Animals of groups 1 and 2 were injected i.v. with free F_3T or with F_3T–AF, both administered in saline in 10 μl/g body wt. Animals of group 3 were injected with saline only and used as controls. After 1 h, all mice were injected i.p. with deoxy-[5-^{3}H]cytidine (spec. act. 20 Ci/mmol) at 60 μCi / mouse. After 1 h deoxy-[5-^{3}H]cytidine administration, mice were killed and liver and bone marrow from femurs were rapidly removed. DNA was extracted as in [11], its radioactivity was counted and its concentration was measured according to [12].

3. Results and discussion

Conjugation with F_3T did not change the capacity of AF to interact with the specific receptors on the surface of hepatocytes. Indeed, the clearance of ^{14}C-labelled AF from the blood of mice was competitively inhibited at the same extent by F_3T–AF or by an equal amount of non-conjugated AF. Non-desialylated fetuin and heat-denatured albumin, which do not compete with AF for the surface receptors of hepatocytes [4] did not affect the plasma clearance of labelled AF (fig.1).

Hepatitis caused by *Ectromelia* virus in mice was chosen as experimental model of hepatocyte infection by deoxyriboviruses. *Ectromelia* virus is a poxvirus which, when injected intravenously into mice, is ingested mainly by Kupffer cells in the liver where it carries out the first cycle of replication. After 10–12 h inoculation it starts to infect hepatocytes; thereafter the infected foci spread to surrounding

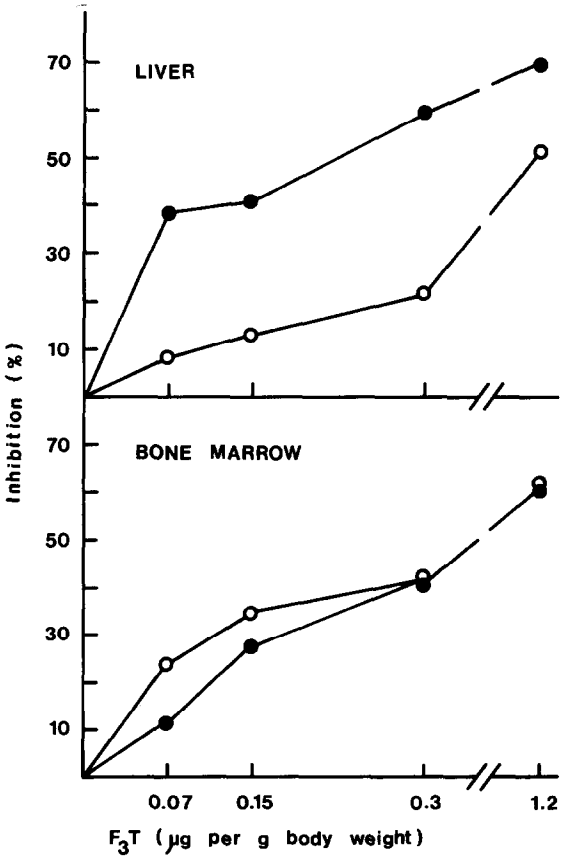

Fig.1. Blood clearance of [^{14}C]AF alone (●) or in the presence of cold, non-conjugated AF (○), F_3T–AF (△), non-desialylated fetuin (▲) and heat-denatured rabbit serum albumin (□). Details are given in section 2.2. Each point represents the mean value of results from 2 animals.

parenchymal cells, ultimately causing liver necrosis [13,14] (and our unpublished electron microscope observations). We observed that DNA synthesis, which is negligible both in normal liver and during the phase of *Ectromelia* virus replication in Kupffer cells, increases several times after 14–50 h virus inoculation, when the infection spreads to hepatocytes. Since poxviruses inhibit cell DNA synthesis during infection [15] this increased DNA synthesis in hepatocytes must be ascribed to virus DNA replication. Under our experimental conditions liver necrosis was observed beginning after 65–70 h virus inoculation.

F_3T and F_3T–AF were injected in infected mice during the phase of active virus replication in hepatocytes and their effect was determined on virus DNA synthesis in liver and on cell DNA synthesis in bone marrow. As shown in fig.2, free F_3T and F_3T coupled to AF inhibited DNA synthesis in liver and in bone marrow. F_3T coupled to AF caused, at the 3 lower doses, an inhibition of DNA synthesis in liver >3-times higher than that produced by the free drug. On the contrary, the percentage of inhibition in bone marrow was similar when F_3T was administered either coupled to AF or as a free drug. These results indicate that, after injection of the conjugate, F_3T was concentrated in active form into hepatocytes.

Three possibilities can be considered to explain the inhibition of DNA synthesis caused by the conjugate in bone marrow.

(1) Bone marrow cells take up the conjugate. This possibility is unlikely because the experiments of competition (fig.1) indicated that conjugated AF behaved like native AF which is taken up almost exclusively (>90%) by the liver [16].
(2) The bond linking F_3T to glutarate in the conjugate is split in the blood.
(3) Part of F_3T released from the conjugate into hepatocytes escapes from these cells into the blood and reaches bone marrow cells as a free drug.

To resolve these points we determined the effect of the conjugate on bone marrow DNA synthesis in mice with hepatic necrosis caused by *Ectromelia* virus or by α-amanitin [17]. In these animals the uptake of AF by the liver is hindered, as demonstrated by a delayed plasma clearance (our unpublished data). Table 1 shows that DNA synthesis in bone marrow was not inhibited by F_3T–AF in mice with hepatic necrosis, whereas free F_3T produced a 50% inhibition as compared to normal controls. These results rule out the first two possibilities and consequently make it very likely that the inhibition of DNA synthesis in bone marrow of normal and infected mice is due to free F_3T escaped from hepatocytes after these cells have taken up the conjugate. This conclusion suggests that the ratio of inhibition of DNA synthesis in liver to that in bone marrow could be further increased by conjugating to AF a drug with a lower ability than F_3T to cross the external membrane of cells.

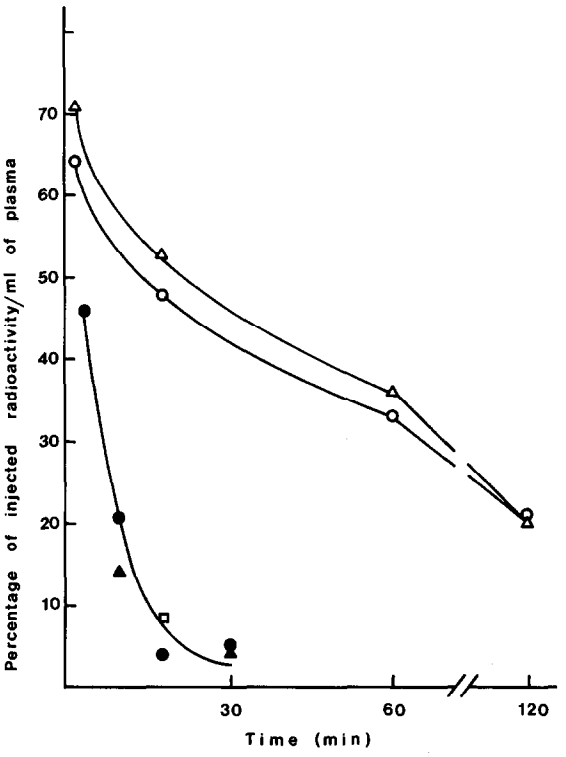

Fig.2. Inhibition of DNA synthesis by free F_3T (○) or F_3T coupled to AF (●) in liver and bone marrow of *Ectromelia* virus infected mice. Experimental details are given in section 2.3. For each dose of free and conjugated F_3T 2 or 3 experiments were performed. Results (dpm/mg DNA) were statistically evaluated by means of Student's *t* test. The difference between deoxy-[5-^{3}H]cytidine incorporation into liver DNA in mice treated with free F_3T or with F_3T–AF was statistically significant for F_3T doses of 0.07, 0.15 and 0.3 μg/g body wt ($P<0.001$, 0.05 and 0.001 for the 3 doses, respectively). The difference was not statistically significant ($P>0.05$) for the F_3T dose of 1.2 μg/g body wt. The difference was not statistically significant for all the F_3T doses in bone marrow.

Table 1
Effect of F_3T–AF and F_3T on deoxy-[5-^{3}H]cytidine incorporation in bone marrow of normal mice and of mice with liver necrosis

Expt		Compounds	dpm/mg DNA		
1	Normal mice (5)[a]	–	261 436 ± 76 182		
	Normal mice (5)	F_3T–AF	153 228 ± 18 056	(41)[b]	$P<0.02$
2	Normal mice (5)	–	369 721 ± 56 586		
	Normal mice (5)	F_3T–AF	203 175 ± 39 134	(45)	$P<0.001$
3	Normal mice (8)	–	290 387 ± 56 530		
	Mice with hepatic necrosis by *Ectromelia* virus (7)	F_3T–AF	301 983 ± 62 928		
4	Normal mice (5)	–	201 680 ± 31 242		
	Mice with hepatic necrosis by α-amanitin (7)	F_3T–AF	203 700 ± 66 110		
5	Normal mice (5)	–	264 623 ± 57 830		
	Mice with hepatic necrosis by α-amanitin (5)	F_3T–AF	246 412 ± 72 616	(7)	NS[c]
6	Normal mice (5)	–	263 849 ± 28 816		
	Mice with hepatic necrosis by α-amanitin (5)	F_3T	127 846 ± 33 734	(51)	$P<0.001$

[a] Number of mice in each group is shown in parentheses
[b] Number in parentheses is the percentage of inhibition
[c] Nonsignificant

In expt 1–5, F_3T–AF was injected i.v. at 6 μg/g body wt corresponding to 0.3 μg F_3T. In expt 6 free F_3T was injected at the dose of 0.3 μg/g body wt. After 1 h animals received an i.p. injection of 60 μCi deoxy-[5-^{3}H]cytidine and were then processed as in section 2.3. In expt 3, F_3T–AF was administered to mice which 80 h earlier had received an i.v. injection of *Ectromelia* virus (4.0×10^5 p.f.u./g body wt corresponding to 600-times the LD_{50}). In expt 4–6, F_3T–AF or F_3T were injected to mice which 40 h earlier had been injected i.p. with α-amanitin (1.5 μg/g body wt). In the groups of *Ectromelia* virus or α-amanitin treated animals, bone marrow was removed only from those mice showing a macroscopic liver necrosis which was subsequently confirmed by histologic examination

Nucleosides with inhibitory activity on DNA synthesis are commonly employed in antiviral chemotherapy. One of them, adenine arabinoside (ara-A), has been recently used in the treatment of human hepatitis type B. Evidence was given that hepatitis B virus replication was inhibited [18,19] but gastrointestinal symptoms and a slight depression of bone marrow functions appeared in treated patients [18]. These side effects which limit the use of ara-A in hepatitis B infection could be avoided or reduced by conjugating the drug to AF, thus causing its selective concentration in hepatocytes.

Acknowledgements

This work was supported in part by Consiglio Nazionale delle Ricerche - Progetto Finalizzato Virus, grants no. 77-00244.84 and 78.00339.84. The excellent technical assistance of Mrs Wilma Rossi-Pusinanti is gratefully acknowledged.

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