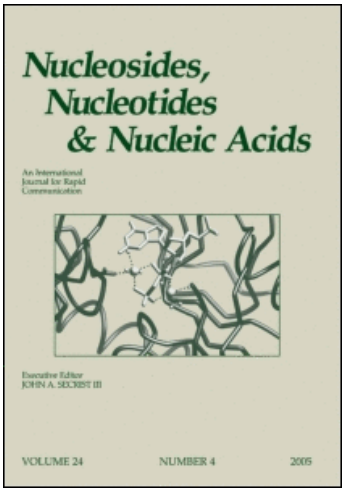

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THE PRODUCTION AND EVALUATION OF ANTIBODIES FOR ENZYME IMMUNOASSAY OF AZTTP

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ABSTRACT

We describe the development of the first enzyme immunoassay for quantifying AZTTP that does not use of radioactive labeling. Anti-AZTTP antibodies were raised in rabbits by immunizing with an AZTTP-kelhoyle limpet hemocyanin (KLH) conjugate. Competitive immunoassays indicated a nanomolar sensitivity to AZTTP. One of the antisera produced was specific for AZTTP.

INTRODUCTION

Nucleosides analogs such AZT, ddC, ddI, d4T, 3TC, are currently used in treatment of AIDS. ddN are converted, successively, into the mono-(ddNMP), di-(ddNDP) and tri-phosphorylated nucleotides (ddNTPs) by the appropriate cellular kinases (1). The active species that inhibit the HIV reverse transcriptase are ddNTPs. The pharmacokinetics of ddNs shows large inter individual differences (2). In addition, plasma concentrations of ddNs are not related to intracellular concentrations of the corresponding 5′-triphosphates (2–5). It is therefore important to define the extent of ddN metabolism and the intracellular concentration of the different metabolites. Different techniques have been described for the quantification of ddN (6,7) and of their metabolites (8–11) but none of them can be used ideally for routine

monitoring of intracellular AZTTP in patients and to define optimum drug dosages. With respect to AZT, for example, the RIA procedure used so far enables only the determination of the total concentration of AZT metabolites (12) except when it is used in combination with HPLC or SPE (13–16) (Solid Phase Extraction). HPLC assay allows the determination of each of these species, but its sensitivity is marginal as compared to RIA (17,18). The sensitivity of HPLC assay can be improved by coupling with a RIA test (19–21), but this method as well as SPE/RIA requires prior separation of AZT metabolites, treatment with phosphatases and quantification of the resultant AZT with a RIA. HPLC with tandem spectrometry constitutes an interesting method for the determination of intracellular level of AZTTP with a limit of detection of 4 fmol/ml (22) but this procedure remains time consuming and costly for routine monitoring. An alternative and attractive method is an immunoassay based on competitive enzyme linked immunoassay (ELISA). This paper describes a specific AZTTP ELISA.

RESULTS AND DISCUSSION

Anti-AZTTP antibodies were produced in rabbits using a keyhole limpet hemocyanin (KLH)-AZTTP conjugate as immunogen **2**. Conjugation of AZTTP to KLH can be achieved either *via* the terminal 5′-phosphate or *via* the base of the nucleotide (23). We previously reported that 5′-O-hemisuccinate ddN can be used to raise anti-ddN antibodies (24). So, we chose to use the immunogen **2** prepared by coupling the phosphate γ moiety of AZTTP to amine groups of KLH. AZT was triphosphorylated in a "one pot" reaction following a protocol developed for phosphorylation of purine nucleoside (25). Briefly, AZT was dissolved in $PO(OEt)_3$ and treated at $-10^{\circ}C$ with 3 eq of $POCl_3$ resulting in the formation of an activated dichlorophosphate which was transformed to AZTTP using an excess of $(Bu_3NH)_2H_2P_2O_7$. The triphosphate 1 was isolated by anion exchange chromatography on DEAE-Sephadex A-25 column with a linear gradient of TEA buffer and purified by HPLC as triethylammonium salt with a 30% yield (Scheme 1) (26).

Immunogen **2** was prepared by coupling AZTTP to KLH through activation of the nucleotide by EDC. This conjugate was isolated after dialysis against a 0.01 M Tris-HCl buffer. Tracer **3** was prepared by coupling AZTTP to acetylcholinesterase (AchE) using the conventional carbodiimide method (27) and isolated by size exclusion chromatography on Biogel A-1.5 M column with EIA buffer.

The conjugate **2** was emulsified in Freund's adjuvant and was injected subcutaneously in three rabbits. Blood was harvested at regular time intervals for monitoring AZTTP antibodies development.

Conventional competitive immunoassays using unpurified rabbit antisera as first antibodies and AZTTP-AChE conjugate as tracer were performed under the conditions described for other haptens (28). Briefly, the different antisera were incubated after performing serial dilutions in EIA buffer with AZTP-AChE conjugate in 96 well immunoplate coated with mouse monoclonal antirabbit IgG antibodies.

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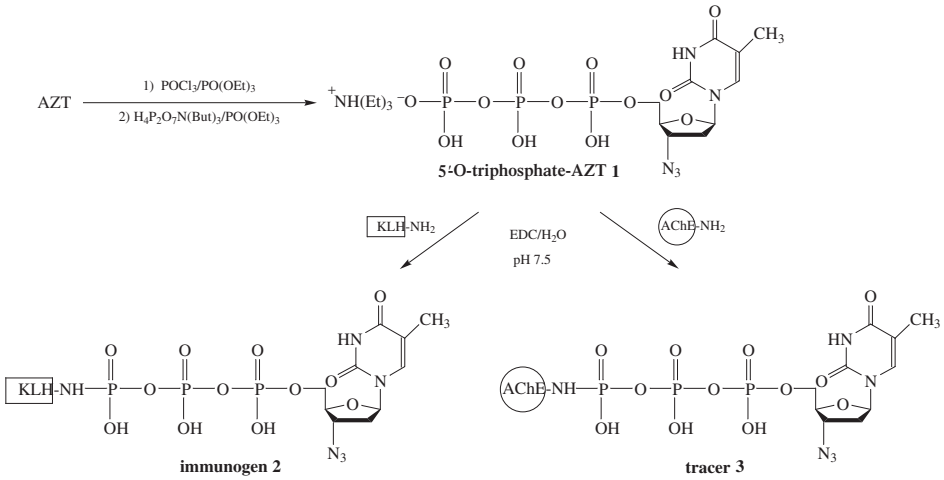

Scheme 1. Synthesis of immunogen and tracer of AZTTP.

After the final booster, anti-AZTTP antibodies were detectable at $1/10^4$ dilution, thus demonstrating the immunogenic potency of AZTTP-KLH immunogen.

Sensitivity and the specificity of the immunoassay were evaluated at the dilution $2/10^4$. The sensitivity of the assay, which is defined by the dose of AZTTP which reduces the binding of AZTTP-AChE conjugate tracer by 50%, was evaluated in presence of serial dilutions of AZTTP in range of 0.2 nM-2 μM. Figure 1 shows a typical standard curve for AZTTP with the best bleeding at $1/10^4$ dilution. The sensitivity was 0.8 nM and the limit of detection (LOD) was around 0.2 nM. The sensitivity of this assay has to be improved to determine intracellular AZTTP concentrations in HIV-infected patients which are very low (0.01–0.6 nM) (16).

The specificity of AZTTP antibodies was analyzed by testing the capacity of AZT, AZTMP and AZTDP to inhibit the binding of the AZTTP-AChE conjugate tracer. Results are presented in Table 1. Two antibodies (AS1 and AS3) recognized AZTTP with a high affinity but they did not discriminate the different phosphorylated forms of AZT. In contrast, AS2 clearly discriminated AZTTP from AZT, AZTMP and AZTDP. The cross-reactivity of antibodies with dTTP and d4TP must be assessed.

Table 1. Study of Specificity of Anti-AZTTP Antibodies

	AZTTP	AZT		AZTMP		AZTDP	
Antisérum	50 % inhibition nM.	50% inhibition nM.	% cross reaction	50% inhibition nM.	% cross reaction	50% inhibition nM.	% cross reaction
AS1	3.7	94	3.9	16.3	22.7	6.7	55
AS2	40	2397	1.6	663	6	913	4.4
AS3	2	356	0.5	1.9	105	1.3	154

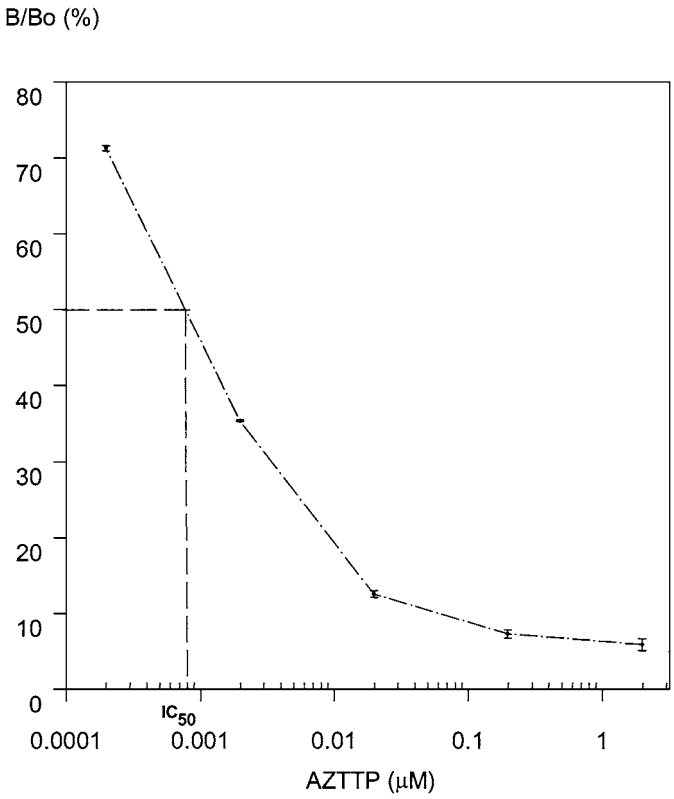

Figure 1. Calibration curve of immunoassay of AZTTP.

In conclusion, this study shows that antibodies specially directed against AZTTP can be raised in rabbits in spite of the poor chemical and *in vivo* stability of the pyrophosphate linkage. An AZTTP-AChE conjugate can also be prepared and allowed the development of a specific ELISA that does not require the use of labelled AZTTP. These tools will be used to assay intracellular AZTTP levels in peripheral blood monocytes of AIDS patients.

EXPERIMENTAL SECTION

AZT was donated by Glaxo Wellcome (France), phosphorous oxychloride, tributylamine, 3-chloroperbenzoic acid, EDC (1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide) and tributylammonium pyrophosphate were purchased from Sigma-Aldrich (Saint-Quentin, France). All other reagents were of analytical grade.

Anion-exchange resin used for separation of AZTTP was SEPHADEX-DEAE A-25 ion-exchange resin (40–120 micron) purchased from Aldrich. The HPLC system for purification or control of purity of AZTTP consists on WATERS apparatus

including 600 pump, 996 photodiode array detector and Millennium chromatographic manager. HPLC purification of AZTTP was achieved on analytical /semi-preparative TSK-GEL DEAE-5PW column with a linear gradient of triethylammonium buffer (TEAB) [0.1–1 M].

Keyhole Limpet Hemocyanin (KLH) was purchased from Pierce. Acetylcholinesterase (AChE) was purified from electric eels (*Electrophorus electricus*) by affinity chromatography as previously reported (29). Ellman's reagent was a solution of 7.5 10^{-4} M acetylthiocholine iodide (enzyme substrate) and 5 10^{-4} M 5,5′-dithiobis (2-nitrobenzoic acid) (chromogen) in 0.1 M phosphate buffer (pH = 7.4).

EIA buffer (pH = 7.4) : $[K_2HPO_4] = 0.08$ M; $[KH_2PO_4] = 0.02$ M; [NaCl] = 0.15 M; [BSA] = 145 μM; $[NaN_3] = 1.5$ mM.

Synthesis of AZT-TP (5′-O-Triphosphate-AZT)

To a solution of AZT (100 mg; 0.37 mmol), in 2 ml of triethylphosphate cooled to −10°C was added freshly distilled phosphorous oxychloride (125 μl; 3.6 eq). The mixture was stirred for 2 h at −10°C under a nitrogen atmosphere. Then tributylamine (1.1 ml; 4.9 mmol) and a anhydrous dimethylformamide solution of anhydrous tributylammonium pyrophosphate (0.5 M; 2.5 ml) were added. The reaction was quenched after 5 min by addition of aqueous solution of NaOH (0.1 M) under vigorous stirring for 20 min at 0°C. The mixture was evaporated under vacuum at room temperature (bath temperature should be kept below 30°C) and lyophilised. The residue was kept in methanol/ethyl ether (1:10, v/v) and cooled at 4°C. The white precipitate was evaporated at room temperature, remained in 5 ml of distilled water and loaded onto DEAE-SEPHADEX A-25 column that was equilibrated with 0.01 M TEAB. The column was eluted with a linear gradient of TEAB (pH = 7.6), [0.1 M–0.3 M; 250 ml each followed by 0.3–0.8 M; 250 ml each]. The appropriate fractions were collected and lyophilised. The 5′-O-triphosphate-AZT (AZT-TP) was purified on analytical/semi-preparative ion-exchange HPLC with a linear gradient of TEAB described in the following table.

Time (min)	%H_2O	%TEAB (0.3 M)	%TEAB (1 M)
0	100	0	0
15	50	50	0
25	0	100	0
40	0	0	100

The 5′-O-triphosphate-AZT was converted into its sodium salt using sodium iodide in acetone. The yield of 5′-O-triphosphate-AZT was around 30%. ^{1}H NMR (D_2O, 200 MHz) δ 7.8 (s, 1 H, H_6), 6.1 (t, 1 H, $H_{1'}$, $J^3_{H1'\text{-}H2'} = 6.8$ Hz), 4.4 (m, 1 H, $H_{3'}$), 4.3 (m,1H, $H_{4'}$), 4.1–4.2 (d, 2H, $H_{5'}$), 2.6 (m, 2H, $H_{2'}$), 1.9 (s, 3H, CH_3); ^{31}P NMR (D_2O, 81 MHz) δ −8.6 (d, 1P, P_γ,$J^2_{P\gamma\text{-}P\beta} = 20.1$ Hz), −11.0 (d, 1 P, P_α,

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$J^2_{P\beta\text{-}P\alpha} = 19.1$ Hz), −21.8 (dd, 1P, P_β, $J^2_{P\beta\text{-}P\gamma} = 20.1$ Hz, $J^2_{P\beta\text{-}P\alpha} = 19.1$ Hz); MS (ESI^-) (m/z) 506 [M-H], 527.9 [M-2H + Na^+]; t_R (HPLC) : 26 min.

KLH-5′-O-Triphosphate-AZT

To an aqueous solution (0.5 ml) of 1 mg (1.6 μmol) of triethylammonium salt of 5′-O-triphosphate-AZT and 325 μg (1.1 eq) of EDC, 1 mg of KLH are added at 0°C. The pH is adjusted at 7.5 by dropwise addition of Tris-HCl buffer (1M). After an overnight incubation at room temperature in the dark, the mixture was dialyzed four times against Tris-HCl buffer (0.01 M) at 4°C. KHL-5′-O-triphosphate-AZT was lyophilised and kept frozen at −20°C until use.

Rabbits were immunized by intradermal injection. Antisera were supplemented with 0.01% sodium azide and stored at +4°C.

AChE-5′-O-Triphosphate-AZT

One milligramme (1.6 μmol) of triethylammonium salt of 5′-O-triphosphate-AZT, 325 μg (1.1 eq) of EDC and 50 μg (156 pmol) of acetylcholinesterase were dissolved in 0.5 ml distilled water. 250 μl of Tris-HCl buffer (1M; pH = 7.4) were added. After an overnight incubation at room temperature in the dark, the enzymatic tracer was purified by size exclusion chromatography on a Biogel A1.5M and stored at −80°C until use. The acetylcholinestérase activity of the tracer was determined after addition of Ellman's reagent.

Competitive Immunoassay

These assays were performed in 96-well microtiter plates coated with mouse monoclonal anti-rabbit IgG antibodies as specific secondary antibodies. The total reaction volume was 150 μl. Each component (tracer, AZTTP standard or sample and rabbit antiserum) being added in a 50 μl volume. AZTTP-AChE conjugate was used at a 2U Ell/ml.

All components of the immunoreaction were dissolved in EIA buffer. All concentrations refer to the concentrations of the reagent in the initial volume before mixing with the other reagents. The sensitivity of the assay was characterized by the dose of AZTTP inducing a 50% lowering of the binding observed in the absence of competitor (B/Bo = 50%).

After an 18 h incubation at +4°C, the plates were washed with 10 mM phosphate buffer pH 7.4 containing 0.05% Tween 20. The enzyme activity was measured by addition of 200 μl of Ellman's reagent.

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