A radioimmunoassay for deoxythymidine triphosphate

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The intracellular concentrations of deoxyribonucleoside triphosphates are important indicators of metabolic perturbations in antimetabolite-treated cells. However, their measurement in small cell numbers has often proved to be difficult owing to the simultaneous presence of 100–1000-fold concentrations of the corresponding ribonucleoside triphosphates. Excellent resolution is possible with h.p.l.c. but uv. detection often lacks the sensitivity required. The DNA polymersase assay is insensitive to deoxyribonucleoside triphosphates and cannot discriminate between dUTP and dTTP [1]. This is important when antifolates and thymidylate synthetase inhibitors are being investigated as significantly increased dUTP concentrations have been observed [2]. A feasible alternative for the measurement of low concentrations of deoxyribonucleoside triphosphates is radioimmunoassay (RIA). RIA procedures for the measurement of dCTP [3] and dUTP [4] have been described. The production of an antiserum to dTTP and the development of an RIA procedure for the measurement of dTTP in small numbers of cells (approx. 10⁵) is described here.

Standard nucleosides and nucleotides were obtained from Pharmacia and Sigma Chemical Co. dTTP tetrasodium salt (methyl-¹H) was purchased from New England Nuclear (50–80 Ci/mmol).

dTTP was conjugated to ovalbumin using a carbodi-imide condensation reaction [5] and the immunogen (10–14 mol of dTTP/mol of protein) was used to immunize four sheep and four rabbits. A positive immune response was obtained in all animals, but only one sheep, HP/S/1524, produced an antiserum suitable for assay development. Conventional RIA techniques were employed for affinity chromatographic separation but were used. The antiserum (affinity constant, K, 3.90 x 10⁶ l/mol) could be used at an initial dilution of 1/330 and the standard curve range was 28–1409 pg dTTP added (0.05–2.57 pmol). Fifty percent inhibition of binding occurred with the addition of 180 pg of dTTP. The antiserum cross-reacted with dTDP by 86%, dUTP and dUDP by 7.8% and UTP by 0.3%. Cross-reactivity with dT, dTMP, dUMP, dCTP and dATP was less than 0.1%.

Although the cross-reaction with dUTP was relatively low, attempts were made to affinity purify the antiserum to ensure that any interference from the relatively high concentrations of dUTP in thymidylate synthetase inhibited cells could be minimized. The IgG from the crude antiserum was prepared with saturated ammonium sulphate and passed through an affinity column of activated CH-Sepharose 4B to which a thyroglobulin-dUTP conjugate had been attached. The titre of the purified antiserum was 1/48 demonstrating that antibodies which recognized both dTTP and dUTP had been removed. The procedure had not significantly reduced the cross-reactivity with dUTP (4.7%) and it was concluded that there was not a distinct population of antibodies which recognized dUTP. However, using samples containing different relative amounts of dTTP and dUTP, it was shown that the avidity of the antibodies for dTTP was sufficient to overcome competition from dUTP. Using the affinity-purified antiserum (K, 1.72 x 10⁶ l/mol), the standard curve was slightly less sensitive (66–2800 pg added) and 50% inhibition of binding occurred with 480 pg of dTTP.

The final assay procedure for the measurement of dTTP in cells involved extraction with ice-cold perchloric acid; sodium periodate treatment [6] to remove any interference from UTP, and chromatography on QAE Sephadex using a stepwise elution with 0.3 m- and 0.7 m-potassium phosphate to eliminate interference from dTMP and dTDP. The RIA using purified antiserum was carried out on the fractions from the columns or aliquots of each fraction. The recovery of standard dTTP applied to the QAE Sephadex columns was 84.6% (n = 3, s.d. = 13.7). The reproducibility of the assay was between 4% and 15% of coefficient of variance using the linear portion of the standard curve.

The procedures described above were used in preliminary experiments to quantify dTTP levels in human lung carcinoma A549 cells treated with antimetabolites. Control levels in untreated cells were 177.8 ± 23 pmol/10⁶ cells. In A549 cells exposed to 20, 60 and 200 nm-methotrexate for 24 h, dTTP concentrations decreased to 95.9%, 30.2% and 15% of control values, respectively. The specific thymidylate synthetase inhibitor CB3771 [N-[4-N-(2-amino-4-hydroxy-6-quinazolynyl)methyl[prop - 2-pyridin]- i-butyric acid] caused a 100-fold decrease in dTTP levels after exposure to 30 μM concentrations for 24 h.

Although two pre-assay steps are involved in this assay, i.e. sodium periodate treatment and chromatography, the RIA procedure is sensitive and reproducible and can be performed on the small numbers of cells available from experiments in vitro or clinical biopsies. The QAE Sephadex step is unable to separate dTTP from dUTP and providing that specific antisera are available, both dTTP and dUTP can be measured in aliquots of the same fractions. The availability of such procedures will facilitate the investigation of mechanisms of resistance to antimetabolites and their modes of action.

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Abbreviation used: RIA, radioimmunoassay.