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Thymidine Concentrations in Human Sera: Variations in Patients with Leukaemia and Megaloblastic Anaemia

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Abstract—*Thymidine concentrations have been measured in human sera by radioimmunoassay using an antibody raised in rabbits with a conjugate of* 1-β-D *ribofuranosyl thymine and human serum albumin. The mean level in* 234 *normal blood donors was* 0.36 μM (*range* 0.10–1.16 μM). *In* 154 *patients with leukaemia and lymphoma, the mean serum thymidine level was significantly raised* (*mean* 0.61 μM; *range* <0.1–7.4 μM). *There was no significant difference between the different subgroups of leukaemia or lymphoma but each group was significantly raised compared to normal. In* 63 *patients with untreated megaloblastic anaemia, the mean serum thymidine level was also significantly raised* ($P<0.01$, *mean* 0.47 μM, *range* 0.1–1.64 μM). *The source of thymidine in human serum is not known but it is suggested that release from cells dying in the bone marrow and from red cell maturation in bone marrow with extrusion of nuclei may be important components. The thymidine concentrations in human serum indicate that thymidine* salvage *might be quantitatively a major route of thymidylate synthesis in some tissues. Thymidine modulates the toxicity and therapeutic index of several nucleic acid antimetabolites, and variations in serum thymidine levels in different patients may influence tumour sensitivity and the toxicity of antimetabolite chemotherapy.*

INTRODUCTION

THE METABOLIC steps involved in thymidylate synthesis have been studied extensively and two principal pathways have been defined—the *de novo* pathway, which involves a series of enzyme catalysed reactions starting with carbamyl phosphate and aspartate, and the *salvage* pathway, by which pyrimidine bases and nucleotides derived from the diet or cell breakdown are re-incorporated into nucleic acid. The relative contributions of *de novo* and *salvage* pathways in thymidylate synthesis in mammalian cells have not been extensively studied. Inhibitors of the *de novo* pathway are frequently cytotoxic and this has supported the notion that the *de novo* pathway is of primary importance. On the other hand, the incorporation into deoxyribonucleic acid (DNA) of labelled thymidine has been used to identify cells engaged in DNA synthesis and it has been widely assumed that all cells engaged in DNA synthesis will be labelled.

Studies in animal tumour models have indicated that nucleosides may modify the toxicity of nucleic acid antimetabolites [1–10]. For example, it has been reported that thymidine will modify the toxicity of methotrexate in tumour-bearing animals without impairing its anti-tumour activity [4, 5], while Harrap's group has reported that a combination of hypoxanthine, allopurinol and thymidine can prevent the toxic effects of methotrexate in mice, and can be used as an alternative to folinic acid as a rescue agent [6]. Thymidine has also been shown to increase the toxicity of fluorouracil treatment *in vivo*, but its precise effect on the therapeutic index has not yet been determined [8, 9]. Cytosine arabinoside toxicity in cell culture systems has been shown to be increased by thymidine in the culture medium [10], and there are experimental studies in progress evaluating the combination of cytosine arabinoside and thymidine in animal tumour systems. Thus, it is clear that

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exogenous nucleosides can modify the toxicity and anti-tumour effects of several nucleic acid antimetabolites.

De novo and *salvage* thymidylate synthetic enzymes in human tissues have been measured only infrequently and their relative activities in different tissues have not been reported. The contribution of the *de novo* and *salvage* thymidylate pathways to DNA synthesis must be influenced by the tissue enzyme levels and the concentration in the medium of the appropriate substrates. There have been very few reports of the serum levels of nucleosides or bases [11–13], and this paper reports the measurement of serum thymidine levels in normal blood donors and in patients with leukaemia, and untreated megaloblastic anaemia due to folic acid or vitamin B12 deficiency in which decreased *de novo* thymidylate synthesis is known to occur. Our results indicate that thymidine concentrations in plasma are sufficiently high to suggest that some tissues may utilise the thymidine *salvage* pathway as a major source of thymidylate for DNA synthesis. Since in some patients with acute leukaemia the serum thymidine level was elevated markedly, it is possible that serum nucleoside levels may influence the clinical toxicity and efficacy of nucleic acid antimetabolites.

MATERIALS AND METHODS

Reagent chemicals were obtained from Hopkins and Williams, nucleosides from Sigma Laboratories, and ^{125}I-iododeoxyuridine (spec. act. >2000 Ci/mmole from New England Nuclear, ^{3}H-thymidine (spec. act. 46 Ci/mmole) and ^{14}C-deoxyuridine (30–45 Ci/mmole) were purchased from the Radiochemical Centre, Amersham.

Thymidine was measured in human sera using the competitive binding assay described by Hughes *et al.* [14] The thymidine antiserum was prepared by immunising rabbits with a conjugate of 1-β-D ribofuranosyl thymine and human serum albumin prepared according to the method of Erlanger and Beiser [15]. Rabbits were injected with conjugate twice, 14 days apart, and the serum was monitored by specific binding to labelled iododeoxyuridine.

The specificity of the thymidine antiserum was determined by comparing the displacement of label with different nucleosides and bases. In addition ultrafiltrates of sera to which tracer amounts of ^{3}H-thymidine and ^{14}C-deoxyuridine had been added were run on PEI cellulose plates (Schleicher and Schull 1440PEI/LS254) in water saturated butanol. The PEI cellulose plates were then divided into 1 cm strips and the cellulose scraped off for elution with water. Cold thymidine and deoxyuridine were run as reference markers. The eluates were assayed for thymidine by radioimmunoassay, and the radioactive tracers detected by liquid scintillation counting.

Recovery of thymidine was determined by adding known quantities of the nucleoside to sera prior to ultrafiltration. The coefficient of variation was determined on the same sample in 10 different assays and a single sample repeated several times in the same assay. The effects of serum storage temperature and technique of blood specimen collection for thymidine assay were studied. Thymidine levels were measured in ultrafiltrates of plasma separated immediately from heparinized blood by centrifugation and in serum from blood left to clot at room temperature, or 4°C for varying times before ultrafiltration. The stability of thymidine in ultrafiltrates was studied in specimens stored at −20°C for several months.

Patients

Serum was obtained from 234 normal blood donors, from 154 patients with leukaemia or lymphomas in relapse, and from 63 patients with untreated megaloblastic anaemia, haemoglobin range 4.5–11.0 g/dl. The group with leukaemia included patients with acute myeloid or lymphoblastic leukaemia (untreated or in relapse), patients with chronic granulocytic or chronic lymphocytic leukaemia, patients with Hodgkin's Disease or a non-Hodgkin's Lymphoma, and patients with a miscellaneous group of tumours (hairy cell leukaemia, chronic and subacute myelomonocytic leukaemia, myelosclerosis, polycythaemia vera, myeloma and macroglobulinaemia). Blood was collected at room temperature and allowed to clot. Serum was separated immediately and stored at −20°C until assayed. Prior to assay, sera were ultrafiltered through Amicon filters (Type CF 50A) at 1000 ***g***. This technique excludes more than 97 % of serum protein.

RESULTS

Figure 1 shows the displacement of label which occurred in the presence of deoxyuridine, thymine and 5-methyl uridine. Deoxyuridine gave a similar displacement cur-

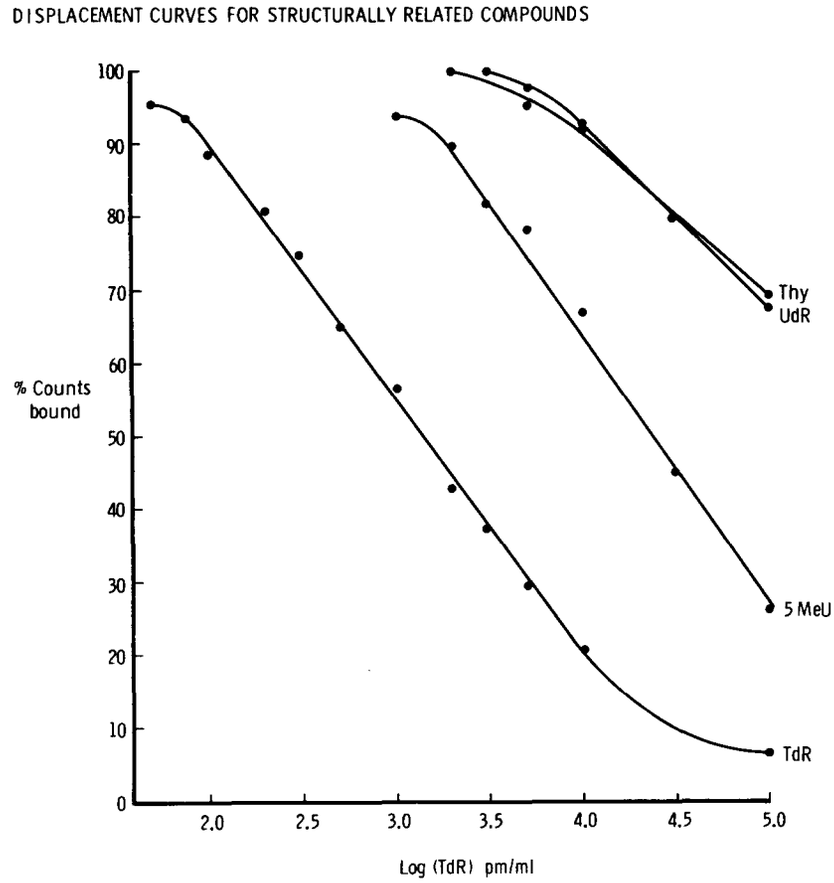

Fig. 1. Displacement curves for thymidine (TdR), 5-methyluridine (5MeU), deoxyuridine (UdR) and thymine (Thy). The abscissa indicates the logarithmic concentration of the displacing compound and the ordinate shows the percentage of the original counts bound. 5MeU shows the greatest degree of cross reactivity with thymidine.

ve to thymidine but at 100 times the concentration. No other naturally occurring compound tested showed the same degree of cross-reactivity. Of the structurally related artificial analogues tested, iododeoxyuridine (not shown) shows a higher affinity for the antiserum even than thymidine. 5-Methyl uridine also shows a high degree of cross-reactivity, although the affinity is less than for thymidine. Figure 2 shows the peaks of labelled ^{3}H-thymidine and ^{14}C-deoxyuridine in chromatographed serum as well as the fractions reacting in the thymidine assay. The peak of ^{3}H-thymidine corresponds well with the peak for thymidine measured by radioimmunoassay. There is no radioimmunoassay reactive material travelling with ^{14}C-deoxyuridine.

Using the optimum antiserum dilution of 1/30 with saline the coefficient of variation for the same sample measured in different assays was 17%, and for a single sample assayed multiple times within the same assay, the coefficient of variation was 3.5%. The lower limit of detection of thymidine was 0.1 μM, and mean recovery of thymidine added to serum prior to ultrafiltration was 97%.

The thymidine level in sera did not change significantly with variation in blood handling. Serum which had been allowed to clot and then stand for up to 24 hr at room temperature or 4°C before ultrafiltration showed no difference in thymidine value from the same sample which had been heparinized or ultrafiltered immediately after clotting. Thymidine was stable in ultrafiltrates of serum stored at −20°C for several months. Table 1 and Fig. 3 show the serum thymidine levels for the three groups of subjects studied. The scatter of thymidine levels within each group is wide but there were significant differences between the mean levels in normal sera, and in sera from patients with leukaemia, lymphoma, and megaloblastic anaemia ($P<0.001$ and <0.01, respectively). The leukaemia and lymphoma sera were from patients with five different varieties of disease. A sixth group comprised miscellaneous malignant haematological diseases, and Table 2 shows the thymidine levels

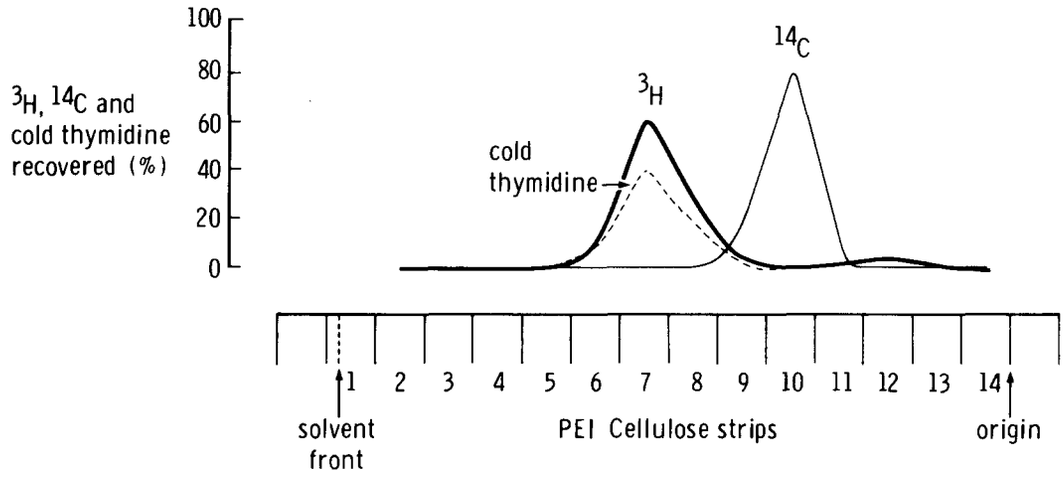

Fig. 2. Peaks of recovered radioactivity measured by liquid scintillation are correlated with the strip numbers and compared with the peak of cold thymidine determined by radioimmunoassay. ^{3}H-Thymidine is located principally in strips 7 and 8, as is the cold thymidine. ^{14}C-Deoxyuridine occurs mainly in strip no. 10.

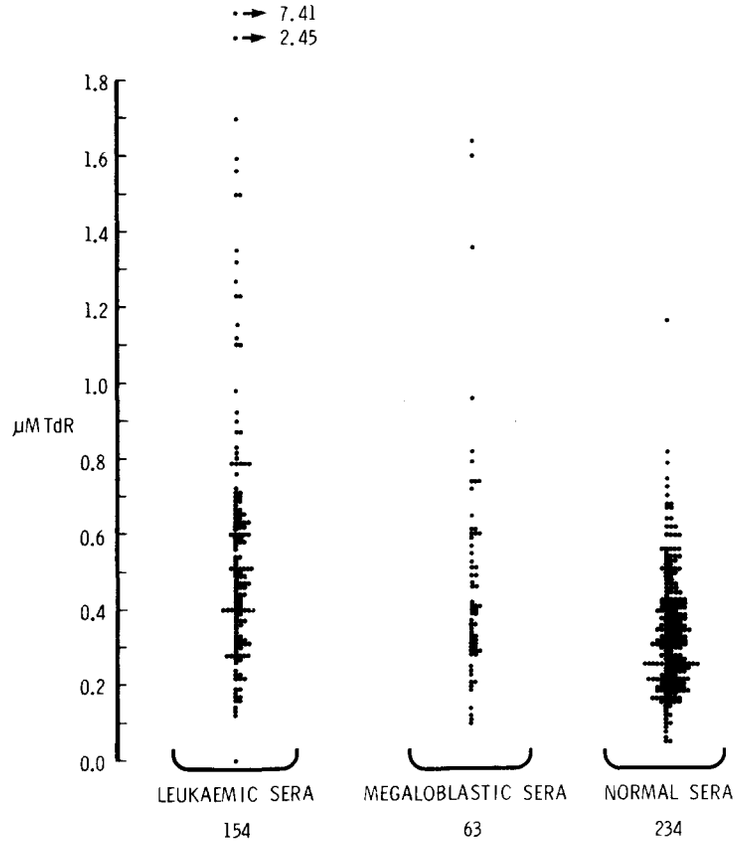

Fig. 3. Scattergram of serum thymidine levels (μM) in three groups of individuals. The leukaemic group has the widest scatter with two very high values. Of 234 normal sera, only one sample has a value greater than 1.0 μM.

in these sera. In each group, thymidine levels are significantly higher than in normals (AML—$P<0.001$ and in other categories $P<0.05$).

In 16 sera from patients with leukaemia, thymidine levels were $>1.0\,\mu$M, and in one patient with lymphosarcoma cell leukaemia, the thymidine level was $7.4\,\mu$M. No correlation was found between the serum thymidine level, the blast cell count, stage of the disease, or recent leukaemic therapy. The possibility that increased thymidine levels

Table 1. *Serum thymidine levels* (μM) *for the three main groups of sera studied—normals, leukaemic and lymphomatous, and megaloblastic*

Group	*n*	Mean TdRμM	S.D.	Range	*P*
Normals	234	0.36	0.16	0.06–1.16	—
Leuks. and Lymphs.	154	0.61	0.65	0–7.4	0.001
Megalo's	63	0.47	0.30	0.1–1.64	0.01

Table 2. *Serum thymidine levels* (μM) *for the six subgroups of the leukaemia and lymphoma patients**

Disease	*n*	Mean TdRμM	S.D.	Range	*P*
AML	69	0.57	0.32	0.0 –1.5	0.001
ALL	11	0.56	0.33	0.31–1.15	0.05
CLL	13	0.68	0.54	0.23–2.45	0.05
CGL	10	0.56	0.25	0.19–1.1	0.05
Lymphomas and Hodgkins	14	0.50	0.24	0.12–1.1	0.05
Miscellaneous	37	0.74	1.159	0.17–7.4	0.05

*Acute myeloblastic leukaemia (AML); Acute lymphoblastic leukaemia (ALL); Chronic lymphoblastic leukaemia (CLL); Chronic granulocytic leukaemia (CGL).

might have been due to impaired liver function with consequent reduction in thymidine phosphorylase activity was excluded by the patients with very high thymidine levels not having particularly deranged liver function.

Serum thymidine levels ranged widely in patients with untreated megaloblastic anaemia, and 3 patients had levels $>1.00\,\mu M$. No correlation was noted between thymidine levels and the cause of the megaloblastic anaemia (folate or vitamin B12 deficiency) or with the degree of anaemia. Although many of the megaloblastic patients had elevated serum LDH levels consistent with intramedullary cell destruction, there was no clear correlation between serum LDH level and the serum thymidine level.

DISCUSSION

The thymidine values obtained from normal and abnormal human sera indicate that thymidine is present in biochemically significant concentrations. The origin of serum thymidine is not known, but it seems probable that maturation of red cells during normal erythropoiesis with extrusion of nuclei and subsequent degradation will be one significant source. Breakdown of other haemopoietic cells in the marrow and elsewhere must also be a major component. The increased serum thymidine levels in megaloblastic anaemia are consistent with the marked dyserythropoiesis and increased intramedullary red cell turnover which characterises this anaemia. The elevated serum thymidine levels in leukaemia may also reflect the increased myeloblast numbers and their shortened survival.

The micromolar concentrations of thymidine in human sera suggest that this might be a significant source for cellular thymidylate *salvage* pathway activity. The degree to which thymidine can be utilised for DNA synthesis depends not only on the Km of thymidine kinase in different tissues, but also on the transport mechanism for thymidine across the cell membrane. Previous reports of the kinetics of thymidine transport in a variety of mammalian cells indicate that this is a facilitated diffusion process showing Michaelis–Menton kinetics with saturation occurring at $2\,\mu M$, and Km of $0.24\,\mu M$–$2.26\,\mu M$ [16–20]. The mean thymidine levels in human sera are therefore of the right order for these kinetics.

The relative dependence of different human tissues on *de novo* or *salvage* thymidylate pathways has not been explored in detail. Cooper *et al.* [21] showed that the *de novo* contribution to the thymidine triphosphate pool in normal granulocytes and chronic leukaemic cells remained constant while the contribution of the thymidylate *salvage* pathway varied with the extracellular thymidine concentration. If the extracellular thymidine concentration was greater than $0.5\,\mu M$, the *salvage* pathway contributed almost 80% of DNA thymidine.

Hopkins *et al.* [22] have reported that thymidine kinase is not rate limiting for thymidine uptake in normal or megaloblastic human bone marrow cells, and together these results suggest that the serum thymidine level may be a critical determinant of the balance between *de novo* and *salvage* thymidylate synthesis in myelopoietic cells.

Our results indicate that the thymidylate *salvage* pathway might be the predominant route of thymidylate synthesis in some tissues. An understanding of the variation in tissue dependence on the *de novo* and *salvage* thymidylate synthetic enzymes would be of great value since this might influence the selective tissue toxicity of inhibitors of both pathways. The biochemical basis of tissue selectivity of various pyrimidine antimetabolites such as fluorouracil and fluorothymidine has not been studied extensively. Similarly, the possibility that resistance to thymidylate synthetase blockade by fluorodeoxyuridylate or indirectly by depletion of reduced folate levels might be due to activity of the thymidylate *salvage* pathways has not been explored. Our earlier results in mice showing that thymidine may protect normal tissues from the toxicity of methotrexate without impairing the antitumour effects of this compound have been confirmed by others and extended to human studies [4, 5, 12, 13]. It is clear that the relative activity of *de novo* and *salvage* thymidylate and purine pathways may be important determinants of pyrimidine antimetabolite drug selectivity.

The recent suggestion that very high concentrations of thymidine might in themselves have some selective cytotoxicity [23] raises the possibility that different tissues may have the ability to tolerate high extracellular concentrations of thymidine. Until now, it has been widely assumed that thymidine toxicity is related to the activity of thymidine kinase, and perhaps the cellular levels of deoxycytidine triphosphate. Recent work from our laboratory [24] has shown that T-cells lack thymidine phosphorylase and are particularly sensitive to thymidine toxicity. Ensminger and Rosowsky [25] have shown that diazauracil, an inhibitor of thymidine catabolism, elevates serum thymidine levels in mice, and it is possible that this antimetabolite may have a role in chemotherapy of thymidine sensitive neoplasms.

The elevated serum thymidine levels in megaloblastic anaemia are unexpected, particularly since it has been reported that thymidine supplementation may be a satisfactory, although temporary, treatment for the anaemia of folate and Vitamin B12 deficiency. Hoffbrand *et al.* [26] reported deoxyribonucleoside triphosphate concentrations in megaloblastic human lymphocytes and bone marrow cells and showed that thymidine triphosphate pools were not lowered. The increased avidity of the cells for thymidine is obviously insufficient to reduce the serum concentration in the face of increased cell breakdown.

Recent evidence in animal tumour models suggest that nucleosides may modulate the toxicity and therapeutic index of nucleic acid antimetabolites. There are very few data of purine and pyrimidine base and nucleoside levels in human plasma, and it is possible that variation in these levels may contribute to the intrinsic sensitivity and resistance of the human tumours to antimetabolites with chemotherapy. Radioimmunoassay clearly provides one solution to the problem of measuring low concentrations of these metabolites.

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