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Modified nucleosides in human serum

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ABSTRACT

Methylated purines and pyrimidines derived from the degradation of transfer ribonucleic acid have been shown to be excreted in abnormal amounts in the urine of patients witch cancer. Recent technology developed by Gehrke and Kuo has allowed the separation and quantification of modified nucleosides in serum using reversed-phase high-performance liquid chromatography with diode-array measurement. Serum levels of ten modified nucleosides were measured in 37 normal healthy adults to establish normal values and to correlate activity with age and sex. In addition, serum levels of patients with several malignancies were measured to determine activity in these diseases. Levels of modified nucleosides in normal individuals were consistently reproducible and showed no significant variation among males *versus* females or with age. Patients with malignant diseases showed consistent elevations and these were highest in patients with more advanced disease. The evidence of no significant differences in the mean levels of modified nucleosides in serum with age or sex in normal adults and elevations in patients with malignancies demonstrate the potential value of modified nucleosides as cancer biomarkers.

INTRODUCTION

Methylated purines and pyrimidines and other modified nucleosides, derived predominantly from transfer ribonucleic acid (tRNA), have been shown to be excreted in abnormal amounts in the urine of patients with cancer [1–9]. By contrast, urinary excretion of modified nucleosides by normal adults is relatively low [10]. These excretory products are predominantly minor components of tRNA which originate from the degradation of macromolecules [11]. Evidence indicates that methylation occurs only after synthesis of the intact molecule. Since no kinases have been found that will re-incorporate the monomer units into tRNA, the modified bases and nucleosides are excreted following metabolic degradation of tRNA molecules [12]. Additional studies show that pseudouridine is not catabolized but excreted in urine as the intact molecule [13,14].

Efforts have been made to use methylated nucleosides as biochemical markers for neoplastic diseases. Elevated concentrations in urine have been suggested as possible markers for leukemia [4,9], lymphoma [15,16], mesothelioma [6,17], cancers of the lung [18–23], ovary [24,25], breast [26,27], liver [28], nasopharynx [4], gastrointestinal tract [29,30], and Hodgkin's disease. In addition, urinary levels of modified nucleosides have been suggested as useful for monitoring progress of disease and response to therapy [31,32]. Gehrke *et al.* [33] have shown in longitudinal

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studies that m^2G, t^6A, m^1A, PCNR (see Table I for abbreviations) in serum of osteosarcoma patients serve as prognostic indicators in response to chemotherapy and operative intervention. This work represents the first documented report of modified nucleosides as markers in human osteosarcoma. Furthermore, abnormal levels of these compounds have been detected in hamsters with adenovirus-12-induced tumors [34], rats with thymic lymphoma [35], mice with mammary carcinoma, and laboratory animals subjected to irradiation [36].

Although modified nucleosides and bases have been studied extensively in urine, profiles in serum have not, mainly due to the lack of adequately sensitive chromatographic methods. Krstulović *et al.* [26] and Hartwick *et al.* [37] noted changes in serum of patients with cancer; however, elution interferences did not allow identification or quantitation of the majority of the compounds. Schlimme *et al.* [38] and Boos *et al.* [39] gave developed direct clean-up and analysis of ribonucleosides in physiological fluids using on-line sample processing, and analyzed ribonucleosides in body fluids and correlated their possible role as pathobiochemical markers [40]. The development of a rapid method for analysis of urinary nucleosides by Gehrke *et al.* [41] utilizing reversed-phase high-performance liquid chromatography (HPLC) following concentration by a boronate gel tremendously improved the accuracy of analysis. Further modifications of this method were developed by Gehrke and Kuo and allowed the chromatographic separation and quantitation of serum nucleosides [42–47].

This paper focuses on the modified nucleosides in human serum. Nearly all of the earlier work until 1989 was done on urine as quantitative chromatographic clean-up and measurement methods for serum were not available for application. In addition, the concentration of modified nucleosides is about two orders of magnitude lower than in urine, thus making quantitative analysis much more difficult. With improvement in the chromatographic and clean-up methods that allow identification and quantitation of modified nucleosides in serum, it was felt that serum analysis would offer distinct advantages compared to urine, such as direct measurements of the concentration of the nucleosides per unit volume using a concentration factor without relationship to creatinine and other metabolites. Also, serum nucleosides may be subject to fewer structural alterations than urinary nucleosides. Additionally, the ease of collection and physician preferences for serum values for other analyses favor serum.

The major thrust of this research was to broaden the use of our clean-up and chromatographic methods [45,47] for modified nucleosides in urine to serum, and to establish the values in serum for normal individuals and as a function of age and sex. In this study the concentrations of ten modified serum nucleosides were determined in the serum of 37 normal subjects (twenty males, seventeen females) utilizing reversed-phase HPLC [41–45,47,48]. In addition, serum from patients with several malignant diseases were analyzed and the results compared with normal controls.

TABLE I

NOMENCLATURE OF ROBINUCLEOSIDES

For additional information on the nomenclature of modified nucleosides see tabulation in Part A of ref. 47, pp. 9–22.

Name	Abbreviation
1-Ribosylpyridine-4-one-3-carboxamide	PCNR
1-Methyladenosine	m^1A
1-Methylinosine	m^1I
1-Methylguanosine	m^1G
N^4-Acetylcytidine	ac^4C
N^2,N^2-Dimethylguanosine	m_2^2G
N^6-Threoninocarbonyladenosine	t^6A
N^6-Methyladenosine	m^6A
Xanthosine	X
Pseudouridine	Pseu

EXPERIMENTAL

Our previous publications have described in detail the instrumentation, reagents, nucleoside standard compounds, chromatographic columns, HPLC buffers and phenylboronate affinity column clean-up for nucleoside isolations [42–

45,47]. Included in these references are comprehensive discussions of instrument operation, preparation of chromatographically pure water for buffer and sample preparation, purity of reagents, preparation of the phenylboronate columns and their use for isolation of nucleosides from urine and serum, and the use of ultrafiltration membranes to prepare serum samples for the phenylboronate isolation of nucleosides. The reader is referred to refs. 42–47.

Selection of normal subjects

Donors were selected from normal volunteers with no manifestation of disease. The serum was obtained from twenty men and seventeen women who ranged in age from 25 to 70 years (mean, 38.4 years). All were in good health and none was taking any medication at the time of the study. Additionally, serum samples were obtained from selected patients with a variety of malignant diseases and evaluated.

Blood collection and handling

Serum samples were collected by subcubital venipuncture into vacutainer tubes. After clot formation, the tubes were centrifuged at 2000 rpm at room temperature for 5–10 min, the serum was then transferred to polyethylene sample vials and stored at −20°C. Five serum samples were evaluated prior to freezing and again after thawing to evaluate the effects of freezing on analysis.

Statistical considerations

Initially descriptive analyses for the values of each of the ten nucleosides were carried out. These analyses revealed that the set of measurements were rather skewed, indicating non-normality of the data and suggesting that non-parametric analyses might be appropriate. Further, in view of the fact that there were some missing values for four of the nucleosides, it was not possible to do multivariate tests when comparing the responses by sex or age group. Instead it was necessary to do simple univariate group comparisons for each of the ten nucleosides. The test procedure used was the Wilcoxon Rank Sum test. Since ten tests were done for the sex group comparisons and for the age group comparisons, it was necessary to consider a lower level of significance or, equivalently, to use a modified p value. A commonly used approach to this problem is to use a Bonferroni modification [48]. This approach was used in this analysis.

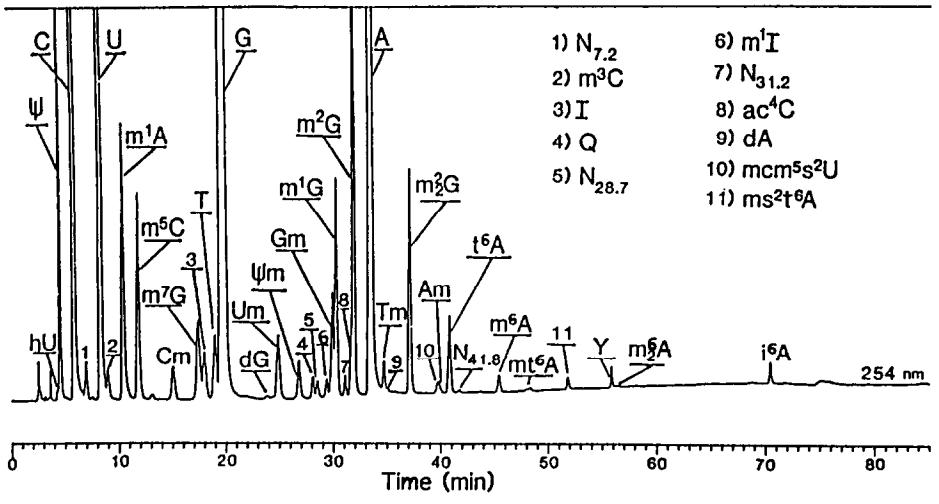

Fig. 1. HPLC of nucleosides in unfractionated calf liver tRNA. Column, Supelcosil LC-18S, 25 cm × 4.6 mm I.D. Elution buffers: A, 2.5% methanol in 0.010 M $NH_4H_2PO_4$ (pH 5.3); B, 20.0% methanol in 0.010 M $NH_4H_2PO_4$ (pH 5.1); and C, 35% acetonitrile in 0.010 M $NH_4H_2PO_4$ (pH 4.9). Temperature, 26°C. Flow-rate, 1.0 ml/min. See Ch. 1, Part C of ref. 47.

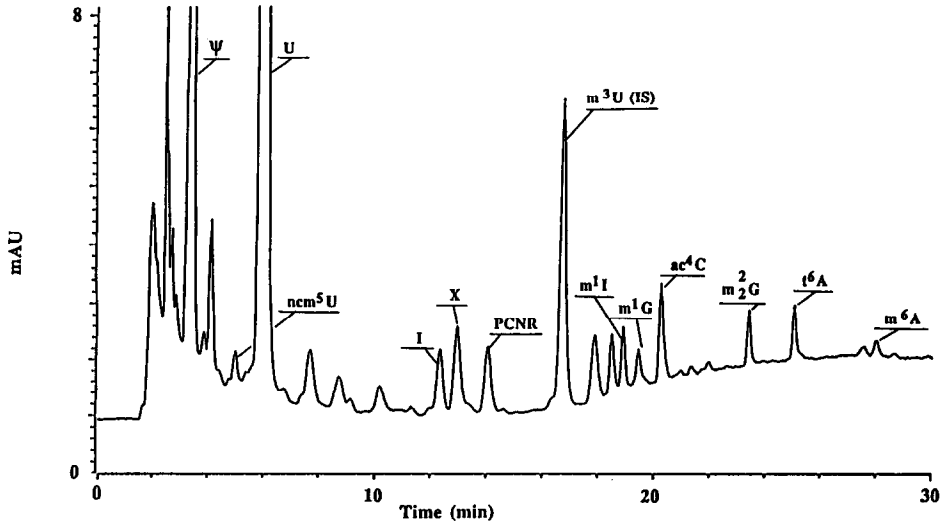

Fig. 2. HPLC of nucleosides in normal human serum. Column, Supelcosil LC-18S, 15 cm × 4.6 mm I.D. All other chromatographic conditions were the same as for Fig. 1. See Ch. 1, Part C, pp. 49–50 of ref. 47.

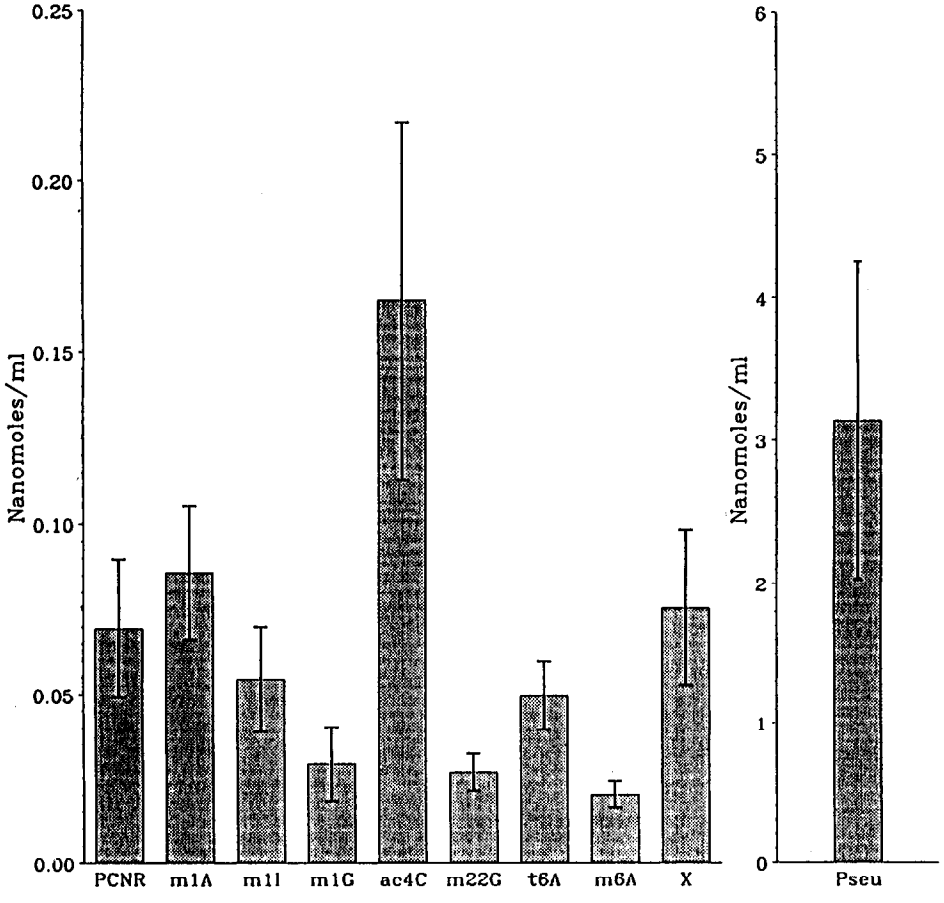

Fig. 3. Mean (twenty men and seventeen women, age 25–70 years) nucleoside levels from normal human serum. Top bar indicates range of standard deviation.

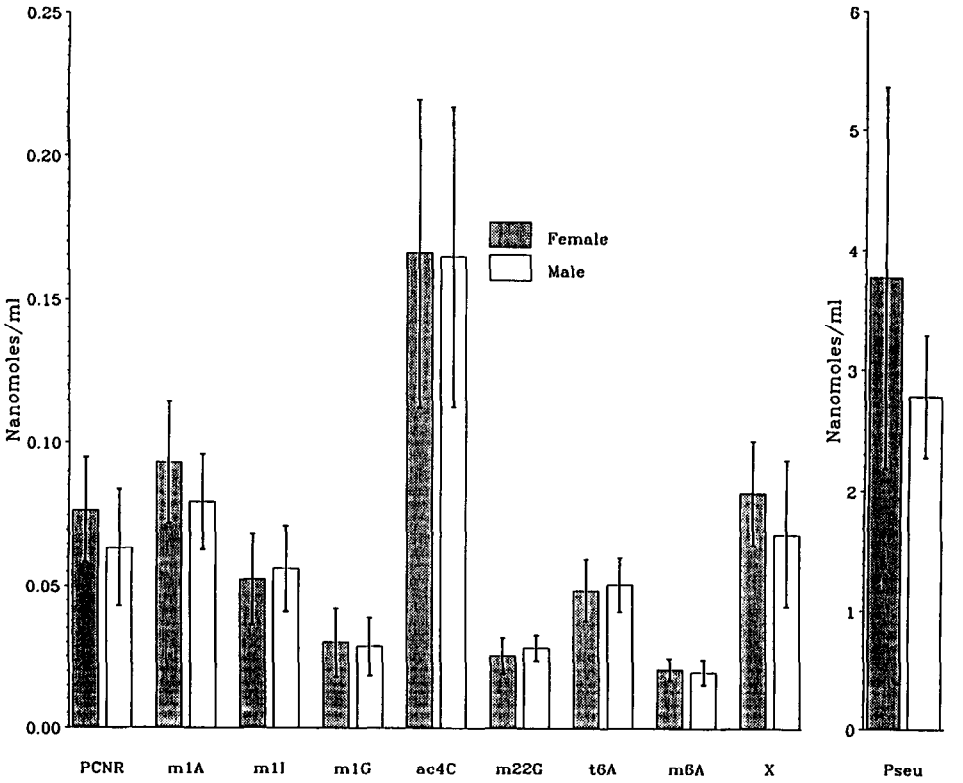

Fig. 4. Graph of comparison of mean nucleoside values in normal human serum of males to females (twenty males and seventeen females).

RESULTS

Representative chromatograms used for identification and quantification of nucleosides are given in Figs. 1 and 2. Fig. 1 is a standard chromatogram showing the separation and resolution of reference nucleosides using the chromatographic conditions described. Fig. 2 is a chromatogram of a normal human serum and is representative of the HPLC method used in this report. Repeat runs demonstrate the accuracy and precision of the technique. Serum from a total of 37 volunteers with no evidence of systemic disease were analyzed for ten nucleosides. The mean age was 38.4 and the range was 25–70. The graphs in Fig. 3 represent the mean levels measured in all samples. The bars indicate the standard deviation observed for each nucleoside measured. Graphs of the levels observed in the serum samples of the twenty males and seventeen females tested are given in Fig. 4. No significant difference was observed between the values obtained from males and females. The unmodified p values all exceeded 0.04 so that, in view of the fact that there were ten nucleosides tested by sex group, the unmodified p values would exceed 0.40.

In order to get age groups of approximately equal size, the subjects were divided into those above 35 years (eighteen subjects) and those 25–35 years (seventeen subjects). There were no significant differences found in the mean nucleoside values with respect to the age groups (Fig. 5). In this case the smallest modified p value was 0.067. In view of the fact that the results of the analysis of age effect could possibly vary with a different choice of age grouping (*e.g.* above 40 *vs.* 40 or below rather than above 35 *vs.* 35 or below) we also looked at Spearman correlations of age with nucleoside value for each of the ten nucleosides. These correlations would then not depend on the choice of age grouping. The smallest modified p

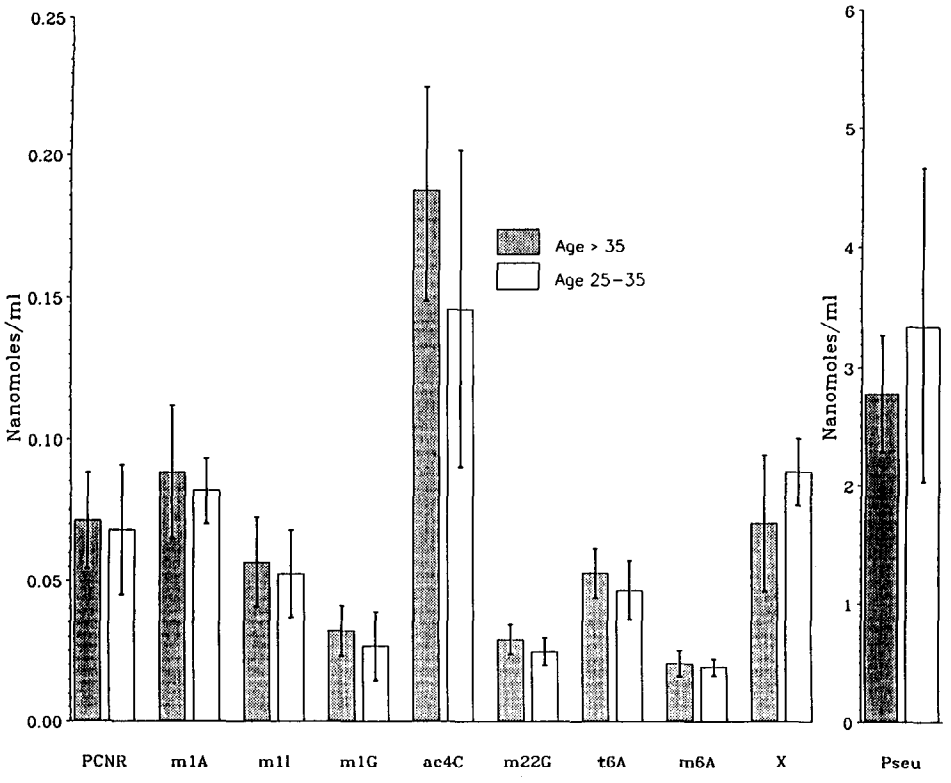

Fig. 5. Bar graph of comparison of mean nucleoside values in normal human serum of individuals above age 35 (range 39–70) to those under 35 (range 25–35): eighteen individuals with age >35, seventeen in age group 25–35 years.

value in this case was 0.029 for nucleoside ac^4C. The next smallest modified p value was 0.16. We do not feel that this mild relationship between age and the ac^4C value has any real significance. Hence we conclude that there is no meaningful difference in the nucleoside values with respect to age.

To show changes in the serum nucleoside values observed with malignant diseases, chromatograms of serum obtained from patients with cancer are shown. The chromatogram obtained from a 47-year-old female with acute myelomonocytic leukemia showed elevation of several nucleosides as evidenced by an increase in the size of the peaks. The bar graph in Fig. 6 represents a comparison of nucleoside levels in this patient to the average normal values. Elevated levels of all nucleosides were demonstrated. The bar graph in Fig. 7 compares levels observed for a 55-year-old male with large cell carcinoma of the lung to normal controls. Significant elevations of most nucleosides were found. These chromatograms and bar graphs were typical of the values obtained in patients with malignant diseases.

Effect of storage conditions

No significant difference was observed in values obtained from serum samples analyzed immediately after collection and those frozen for one, three or six months. Therefore, whole serum may be frozen up to six months without any substantial changes in nucleoside levels.

DISCUSSION

The urinary levels of modified nucleosides derived predominantly from tRNA, have been proposed as useful biochemical markers of malignant diseases [1–9]. An evaluation of carcinoembryonic antigen (CEA), tissue polypeptide anti-

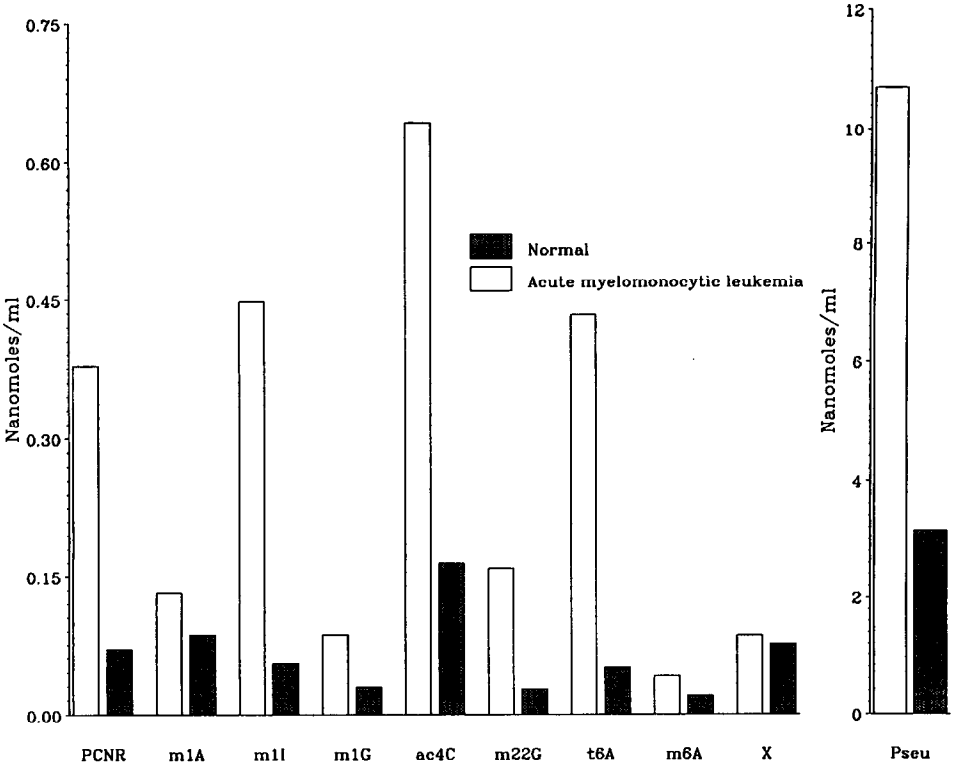

Fig. 6. Bar graph of nucleoside levels in serum from patient with acute myelomonocytic leukemia compared to mean normal levels of 37 individuals.

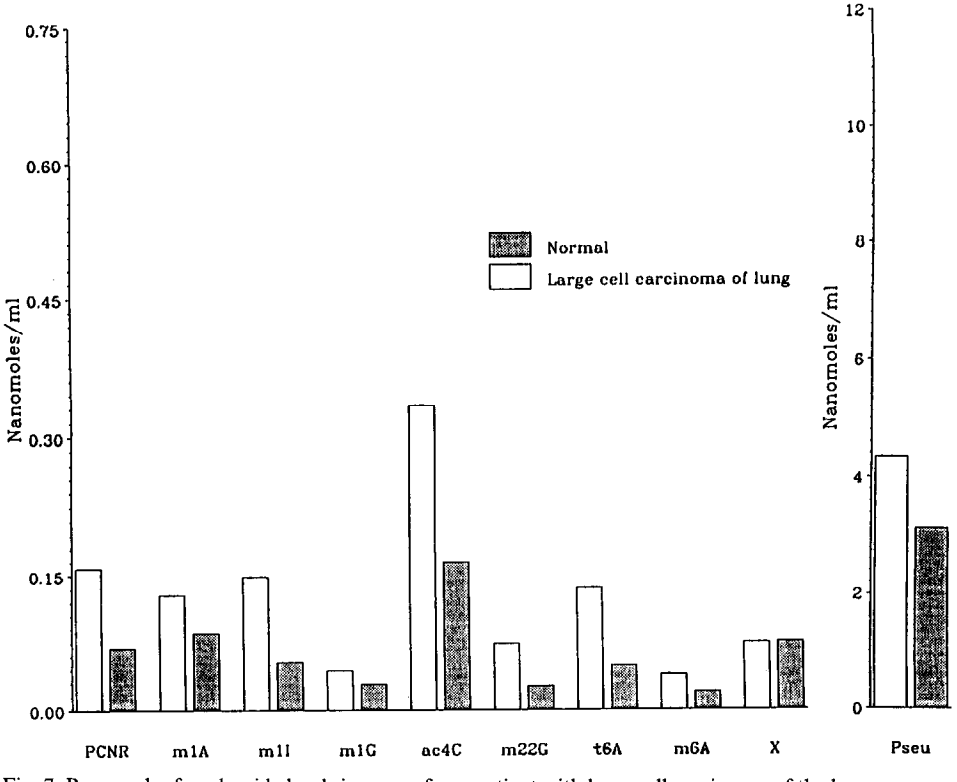

Fig. 7. Bar graph of nucleoside levels in serum from patient with large cell carcinoma of the lung compared to mean normal level of 37 individuals.

gen (TPA), and placental alkaline phosphatase (PLAP) in serum and pseudouridine in urine were analysed in 37 patients with colorectal cancer. The incidence of all four markers increased with advancing stages of disease [8]. The urinary excretion of β-aminoisobutyrate (β-AIB) and pseudouridine were investigated in 26 patients with acute myeloid leukemia (AML) and chronic myeloid leukemia (CML). The excretion of β-AIB correlated to the leukocyte count in CML, while that of pseudouridine correlated positively with the blast count in AML [49]. An elevation of pseudouridine was observed in patients with malignant lymphomas, however, no correlation between the level of excretion and the clinical stage was found [50]. Significantly higher concentrations of 1-methylinosine, N^2,N^2-dimethylguanosine, 1-methylguanosine, and pseudouridine were found in the urine of patients with acute lymphoblastic leukemia at initial diagnosis or in relapse when compared to the concentrations found in normal controls and patients in remission [51]. The urinary excretion of seven nucleosides was measured in patients with chronic myelogenous leukemia and were highest in patients whose disease was in the blastic phase, with the most significant differences noted in the levels of 1-methylinosine, pseudouridine, and N^2,N^2-dimethylguanosine [9].

In a study of nucleosides in patients with small cell carcinoma, the urinary concentrations of pseudouridine, 1-methyladenosine, 1-methylinosine, N^2-methylguanosine, and N^2,N^2-dimethylguanosine correlated to the stage of disease, with elevated values in 40% of patients with limited disease and 81% in patients with extensive disease. Additionally, elevated urinary excretion of one or more nucleoside levels has been demonstrated in patients with breast carcinoma [52], hepatocellular carcinoma [7], and myelomatosis [53].

Studies of urinary nucleosides in animals revealed similar findings. Tumor-bearing mice were found to excrete increased amounts of nucleic acid catabolites, compared to normal controls. Furthermore, the excretion rate of pseudouridine and other nucleic acid catabolites increased prior to tumor diagnosis by other methods. Untreated control mice showed no alteration in the excretion value of any modified nucleoside determined [54]. Levels of hypoxanthine and pseudouridine increased in mice with transplanted mesotheliomas and decreased following growth cessation by chemotherapy [55].

There are several potential advantages of analyzing serum over urine in nucleoside quantitation [41,42,47]. These include direct comparison of data in terms of concentration rather than normalizing on the basis of another molecule such as usually required in urine studies. On the other hand, urine is a much less complex matrix and has much higher levels of most nucleosides of about 100 times.

The development of ultrafiltration and centrifugation methods that separate and remove proteins and other substances has allowed the chromatographic identification and quantitation of nucleosides in serum. Using this technology it is now possible to identify and quantitate more than 65 modified nucleosides [45–47,56]. Precision analysis provides reproducible values and superior resolution.

Prior to the evaluation of this method in malignant diseases it was necessary to establish normal serum nucleoside levels. Evaluation of serum obtained from 37 normal adult donors provided standard serum values for ten nucleosides. These preliminary studies indicate that normal profiles are consistent and reproducible within a narrow range. No significant differences were noted in observed values with regard to sex or age. Elevated levels of some nucleosides were demonstrated in patients with a variety of malignant diseases. It is not clear if individual patterns of serum nucleoside elevations occur in specific cancers or if the levels correlate with extent of tumor burden, other staging and prognostic indicators, or response to changes associated with therapy. The narrow ranges for serum levels of modified nucleosides obtained in normal controls and the degree of elevation observed in patients with malignant diseases suggest the potential value of modified nucleosides as biochemical markers of cancer.

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