radiosensitizer in malignant brain tumors. *Cancer Res* 1982, **42**, 2474–2479.
13. Gouyette A, Apchin A, Foka M, Richard JM. Pharmacokinetics of intra-arterial and intravenous cisplatin in head and neck cancer patients. *Eur J Cancer Clin Oncol* 1986, **22**, 257–263.
14. Hecquet B, Vennin P, Fournier C, Poissonnier B. Evaluation of the pharmacological benefit and determination of the influencing factors of intraarterial cis-diamminedichloroplatinum administration in patients with uterine cervical cancer. *Cancer Res* 1987, **47**, 6134–6137.
15. Hecquet B, Leroy A, Lefebvre JL, Peyrat JP, Adenis L. Uptake of platinum compounds in human tumors. *In vitro* study. *Bull Cancer* 1986, **73**, 535–541.
16. Litterst CL, Gram TE, Dedrick RL, Leroy AF, Guarino AM. Distribution and disposition of platinum following intravenous administration of cis-diamminedichloroplatinum (II) to dogs. *Cancer Res* 1976, **36**, 2340–2344.
17. Hirano A, Matsui T. Vascular structures in brain tumors. *Hum Pathol* 1975, **6**, 611–616.
18. Los G, Mutsaers PHA, Lenglet WJM, Baldew GS, McVie JG. Platinum distribution in intraperitoneal tumors after intraperitoneal cisplatin treatment. *Cancer Chemother Pharmacol* 1990, **25**, 389–394.
19. Andrews PA, Velury S, Mann SC, Howell S. Cis-diamminedichloroplatinum (II) accumulation in sensitive and resistant human ovarian carcinoma cells. *Cancer Res* 1988, **48**, 68–73.
20. Milano G, Troger V, Courdi A, *et al*. Pharmacokinetics of cisplatin given at a daily low dose as a radiosensitiser. *Cancer Chemother Pharmacol*, 1990, **27**, 55–59.
21. Bielack SS, Erttmann R, Looft G, *et al*. Platinum disposition after intraarterial and intravenous infusion of cisplatin for osteosarcoma. *Cancer Chemother Pharmacol* 1989, **24**, 376–380.

Eur J Cancer, Vol. 27, No. 3, pp. 259–263, 1991.
Printed in Great Britain

0277–5379/91 $3.00 + 0.00

Albumin-bound and Non-protein-bound Oestradiol and Testosterone in Postmenopausal Breast Disease

Sarah Pearce, Mitchell Dowsett and J. Alan McKinna

Several studies have recently reported the percentage of non-protein-bound (NPB) oestradiol (E2) to be higher in patients with breast cancer than in normal controls. Using postmenopausal volunteers, we have examined the fractional binding of E2 and testosterone (T), as well as total E2 and T, sex-hormone binding globulin (SHBG), luteinising hormone (LH) and follicle stimulating hormone (FSH), in normal women, those at risk of developing breast cancer and women with breast cancer at first diagnosis and first recurrence. No significant differences were observed in either the concentration or in the percentage of NPB E2 or T, or in any of the other hormones measured. The validity of our observations were confirmed by expected relationships between E2, T, SHBG and body mass.
Eur J Cancer, Vol. 27, No. 3, pp. 259–263, 1991.

INTRODUCTION

A LARGE AMOUNT of epidemiological and experimental evidence implicates oestrogens in the aetiology of breast cancer. However, despite many varied studies the precise role of these hormones remains unknown. Total blood oestradiol (E2) levels have frequently been measured in breast cancer patients, but this may be inappropriate for assessing biological activity as oestradiol circulates extensively bound to sex-hormone binding globulin (SHBG) and albumin, leaving only a small percentage (<2%) of the steroid in the non-protein-bound (NPB) form [1]. Recently there has been much active debate as to the biological availability of these three fractions to the tissue.

In 1981, Siiteri compared the percentage NPB E2 in breast cancer patients with that of controls and found that the level was higher in the cancer patients [2]. Since then five studies [3–7] have all reported a higher percentage of NPB E2 in breast cancer patients than in controls. Two of these studies [5, 6] also showed an increase in the albumin-bound E2 fraction in the breast cancer patients. Two other studies [8, 9], found no significant difference between breast cancer patients and controls. Moore and his colleagues also reported a lower proportion of E2 bound to SHBG in the blood of women who went on to develop breast cancer than in those who did not [10]. Based on this evidence coupled with the observation that Japanese women (who have a lower incidence of breast cancer than western women) also have a higher proportion of E2 bound to SHBG [10], they proposed that this parameter may be a marker for breast cancer risk.

For a given change in SHBG binding capacity, the change in percentage NPB testosterone (T) is greater than that in the percentage NPB E2 [1]. Any difference observed in percentage of NPB E2 would therefore be expected to manifest itself in a greater change in percent NPB T. This parameter would therefore be expected to be a more sensitive marker of breast cancer risk than NPB E2. In addition if this effect were reflected in an increased concentration of NPB T, it may be important in increasing the availability of T to the aromatase enzyme, which is responsible for converting androgens into oestrogens.

In this study we have measured total, albumin-bound and

Correspondence to S. Pearce, Welsh School of Pharmacy, University of Wales Cardiff College, PO Box 13, Cardiff CF1 3XF, U.K.
S. Pearce was previously at, and M. Dowsett is at the Department of Biochemical Endocrinology, and J.A. McKinna is at the Surgical Breast Unit, Royal Marsden Hospital, Fulham Road, London SW6 6JJ, U.K.
Revised 15 Nov. 1990; accepted 10 Dec. 1990.

Table 1. Factor comparison of the 8 menopausal groups: mean (S.E.)

	Control	Control + FH	Diffuse	Diffuse + FH	Benign	Benign + FH	First diagnosis	First recurrence
No.	55	37	34	25	33	18	60	23
Age (years)	58.1 (6.1)	57.4 (5.4)	58.4 (5.4)	57.8 (4.8)	58.6 (7.9)	55.4 (5.2)	63.8 (9.0)	65.0 (7.9)
QI	25.1 (3.5)	24.9 (3.4)	24.1 (3.2)	24.1 (2.6)	26.8 (4.7)	25.7 (3.8)	25.0 (3.4)	29.7 (12.9)
Years past menopause	9.4 (7.2)	9.6 (7.3)	10.3 (7.3)	8.3 (5.4)	10.6 (6.9)	6.9 (5.2)	15.2 (10.8)	13.5 (7.1)
Age at birth of first child	27.5 (4.0)	27.5 (5.3)	28.3 (4.9)	25.4 (4.5)	24.8 (3.8)	25.1 (5.1)	25.6 (4.0)	27.4 (3.9)

FH = family history, QI = Quetelet's index.

NPB E2 and T as well as SHBG, luteinising hormone (LH) and follicle stimulating hormone (FSH) in groups of postmenopausal women: normals with and without a family history of breast cancer; women with diffuse breast disease, with and without a family history of breast cancer; localised benign breast disease patients, with and without a family history of breast cancer; breast cancer patients at first diagnosis and at first recurrence of the disease.

SUBJECTS AND METHODS

Subjects

Clotted blood samples were obtained from the Early Diagnostic Unit and the Breast Clinic of the Royal Marsden Hospital, London. The study was approved by the local ethics committee and volunteers signed a consent form. The following data was recorded at the time the sample was taken: height, weight, age, menopausal status, parity, dietary habits, concurrent medical complaints, medication and family history. All volunteers who had undergone menopause less than two years previously were excluded, as were any women on hormone replacement therapy or who had received any other endocrine treatment within the previous three months. After blood collection, serum samples were stored at −20°C in 1 ml aliquots until analysed.

The volunteers were divided into eight groups for analysis, based on clinical examination: (1) normal; (2) normal with a family history; (3) diffuse benign; (4) diffuse benign with a family history; (5) localised benign; (6) localised benign with a family history; (7) first diagnosis breast cancer prior to surgery; and (8) first recurrence breast cancer prior to therapy. For the purposes of this study family history was defined as at least one first degree relative with breast cancer.

Methods

SHBG binding capacity was measured using the two tier column technique of Iqbal and Johnson [11]. Total E2 was measured by radioimmunoassay using a highly specific antiserum [12]. The sensitivity of the assay was 2.8 pmol/l. Total testosterone was also measured by radioimmunoassay (STRIA, St Thomas' Hospital, London). The sensitivity of this assay was 0.14 nmol/l.

The percentage of NPB steroid was measured by centrifugal-ultrafiltration dialysis (CUFD) [13] with our previously published modifications [14]. The percentage of albumin-bound steroid was measured by ammonium sulphate precipitation [15] also using our previously reported modifications [14].

The percentage of steroid bound to SHBG was calculated by subtraction of the percentage of NPB and albumin-bound steroid. The concentration of the NPB, albumin-bound and SHBG-bound steroid was calculated by multiplication of the measured total steroid concentration and the percentage of the steroid in each fraction. LH and FSH were measured by radioimmunoassay (Chelsea RIA kit). The sensitivity of the assays were 0.7 and 0.3 IU/l respectively.

Statistics

Statistics were performed using SPSS-PC. Pearson correlations, linear regression, analysis of variance, range and Student–Newman–Keuls tests and analysis of covariance were used as appropriate.

RESULTS

Subjects

Initially controls and patients were matched for age, weight and height. Quetelet's index (QI; wt[kg]/ht[m^2]) was used as a measure of body mass. As can be seen from Table 1, first diagnosis and first recurrence breast cancer patients were significantly older than the other groups. The first recurrence group also had a significantly higher QI value than all but the localised benign groups. There were also some differences between the cancer patients and the other groups in the number of years past the menopause, but this is probably a reflection of the age difference in these groups. Throughout the sample population, we observed a significant inverse relationship between QI and SHBG (see Table 2) and a significant correlation between QI and total E2 levels and total T levels. We also observed a correlation between FSH and total E2 levels and QI.

Table 2. Correlations and linear regressions between variables

	r	P	r	P
QI and SHBG	−0.3075	<0.001	0.23469	<0.026
QI and total T	0.1938	<0.01	0.05309	<0.5853
QI and total E2	0.1865	<0.01	0.2009	<0.0371
FSH and E2	0.2609	<0.001	−0.34563	<0.0008
QI and FSH	−0.2113	<0.01	−0.14596	<0.1627
SHBG and %NPB T	−0.3648	<0.001	0.25663	<0.0177
SHBG and %AB T	−0.4765	<0.001	−0.18951	<0.077
SHBG and %NP B E2	−0.2144	<0.01	0.08171	<0.4712
SHBG and %AB E2	−0.3692	<0.001	−0.02304	<0.8293
SHBG and NPB T	−0.2726	<0.001	−0.2357	<0.0319
SHBG and NPB E2	−0.2438	<0.01	−0.26744	<0.0179
SHBG and AB T	−0.3338	<0.001	−0.20483	<0.057
SHBG and AB E2	−0.262	<0.001	−0.23354	<0.0285

For the regression analyses the first parameter is the determinant.

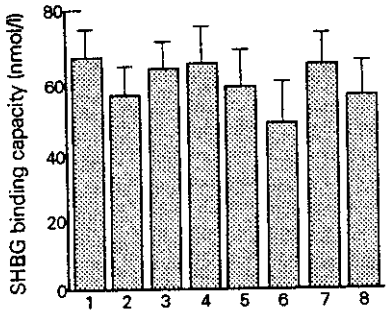

Fig. 1. SHBG binding capacity in (1) normal; (2) normal with family history; (3) diffuse benign; (4) diffuse benign with a family history; (5) localised benign; (6) localised benign with a family history; (7) 1st diagnosis breast cancer; (8) 1st recurrence breast cancer. Bars = mean (S.D.).

The fractional binding of both E2 and T also correlated with SHBG binding capacity.

Storage time

The majority of the samples were collected over a two year period and we investigated whether the length of time a sample had been stored had any influence on any of the endocrine parameters measured. No significant effect of length of storage was observed.

Hormone levels

The hormone levels for each of the groups studied are compared in Figs 1–3. There were no significant differences between any of the groups in the levels of LH, FSH, SHBG (Fig. 1), total oestradiol (Fig. 2a) or total testosterone (Fig. 3a). There were, however, a number of significant differences in some of the subfractions of oestradiol and testosterone.

In patients with diffuse breast disease there was a significantly higher percentage of albumin-bound oestradiol (Fig. 2c) than in patients at first diagnosis of breast cancer. There were, however, no significant differences between any of the groups of normal subjects in this parameter. No significant differences were found between the groups in the percentage (Fig. 2b) or concentration of NPB oestradiol (Fig. 2d), or in the concentration of albumin-bound oestradiol (Fig. 2e).

A significant difference was observed in the percentage of albumin-bound T between the control group and those with diffuse or localised benign breast disease and a family history of breast cancer (Fig. 3c). A significant difference was also observed between patients at first diagnosis of breast cancer and all the increased risk groups (benign, diffuse, and control, diffuse and benign with a family history), but not with the control group. The first diagnosis patients had a lower percentage of albumin-bound T than the others.

Women newly diagnosed as having breast cancer had a significantly lower percentage of NPB T than diffuse patients and control women with a family history of breast cancer (Fig. 3b). Despite all the differences in percentage of albumin-bound T between the groups, the concentration of albumin-bound T (Fig. 3e), was significantly different only between the first diagnosis breast cancer patients and those diagnosed as having localised benign breast disease and a family history of breast cancer. No significant difference was observed in the serum concentration of NPB T (Fig. 3d).

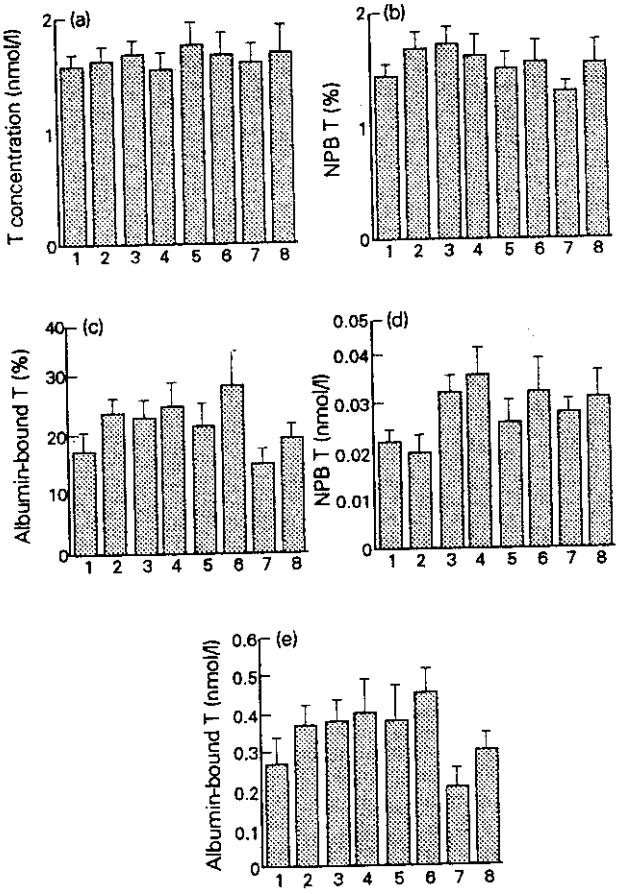

Fig. 2. Fractional binding of oestradiol; (a) total E2; (b) % NPB E2; (c) % albumin-bound E2; (d) concentration of NPB E2; (e) concentration of albumin-bound E2. Groups 1–8 are as for Fig. 1. Bars = mean (S.D.).

Low fat diet

No significant difference was observed in any of the parameters when the women who categorised themselves as being on a low fat diet were compared to those on a "normal" diet.

DISCUSSION

Since the original observation by Siiteri that women with breast cancer had a higher percentage of NPB E2 than normal controls [2], a number of studies have examined this further. We have confined the current study to the investigation of postmenopausal women. Five studies [3–7] all supported the finding of a higher percentage of NPB E2 in patients with breast cancer in this group. Two other studies however [8, 9] could find no difference between the cancer patients and normal controls. Even in the four studies which did show a difference there is a marked discordance in the quantification of the percent NPB E2 and in the relationship of the other binding fractions with breast cancer incidence. Moore *et al* [3] reported a difference in both the proportion and concentration of non-protein-bound E2 between the two groups while Reed *et al.* [4] only observed the difference in the proportion unbound. Bruning *et al.* [8] reported no difference in percentage NPB E2 but with higher total E2 levels found a higher calculated concentration of NPB E2. Differences also exist in the percentage of protein-bound E2 which were measured in the studies of Reed *et al.* [4] and Langley *et al.* [5]. There are thus a series of discordant findings

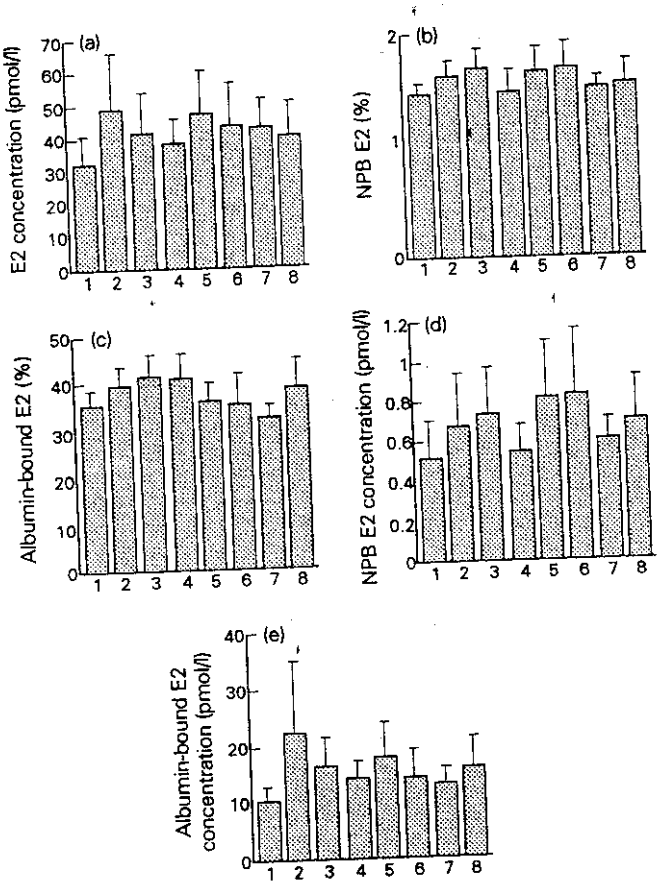

Fig. 3. Fractional binding of testosterone (a) total T; (b) % NPB T; (c) % albumin-bound T; (d) concentration of NPB T; (e) concentration of albumin-bound T. Groups 1–8 are as for Fig. 1. Bars = means (S.D.).

in these studies many of which are probably due to the differences in techniques employed.

In this study we have examined plasma levels of E2 and T and the differential binding of them in not only breast cancer patients and normal controls but also those women thought to be at risk of developing breast cancer, in an attempt to discover whether these parameters have any bearing on breast cancer, the disease and its development. Measurements of E2 have been reported but the fractional binding of T, which because of its binding relationship with SHBG would be expected to show greater changes than that observed with E2, has not been reported previously. The concentration of both non-protein and protein-bound E2 and T were also calculated in this study. No significant differences were found in either the concentration or in the percentage of NPB E2 or T between any of the groups and the controls.

The results in this study support the widely reported observations that body mass index (Quetelet's index) shows a significant inverse correlation with SHBG [16] and a positive correlation with total E2 levels. There was also a significant relation between SHBG binding capacity and the percentage albumin-bound T and E2. These findings are what would be expected from the literature concerning the relation between E2, T, SHBG and body mass. We also observed significant relations between the fractional binding of the steroids and SHBG, which could be predicted from their reported associations. The confirmation of these relations in our data support the validity of our observations.

Moore *et al.* [10] suggested that women who went on to develop breast cancer had lower SHBG binding capacities than normal women. We have been unable to lend support to this as we have observed no difference in SHBG levels between the groups. Although Langley *et al.* [5] found that length of storage at −20°C increased the dissociation rate of steroids from SHBG, we found no evidence of this. Storage at −20°C had no effect on any of the parameters measured. The samples were all stored in 1 ml aliquots and were not thawed and refrozen.

In the data presented here we have found no evidence that there is an association between E2, its differential binding in breast cancer patients or those considered to be at risk of developing breast cancer compared to controls. We also found no reason to support the theory that SHBG may prove to be a good marker for women at risk of breast cancer. The discrepancies between our finding and those of other workers may be partly explained by our careful collection of samples and use of well-validated techniques: potentially confounding variables have not been controlled to the same degree in other studies which are all of similar or smaller size. The size of the effect was so small that power function analysis failed to produce an optimum group size. It is notable also that more recent work of Siiteri's group in a much larger study indicated no significant differences in plasma levels of oestrogens, the differential binding of E2 or SHBG [9].

1. Anderson DC. Sex Hormone binding globulin. *Clin Endocrinol* 1974, **3**, 69–96.
2. Siiteri PK. Extraglandular oestrogen formation and serum binding of oestradiol: relationship to cancer. *J Endocrinol* 1981, **89**, 119.
3. Moore JW, Clark GMG, Bullbrook RD, *et al.* Serum concentrations of total and non-protein-bound oestradiol in patients with breast cancer and normal controls. *Int J Cancer* 1982, **29**, 17–21.
4. Reed MJ, Cheung RW, Noel CT, Dudley HAF, James VHT. Plasma levels of estrone, estrone sulfate and estradiol, and the percentage of unbound estradiol in postmenopausal women with and without breast disease. *Cancer Res* 1983, **43**, 3940–3943.
5. Langley MS, Hammond GL, Bardsley A, Sellwood RA, Anderson DC. Serum steroid binding proteins and the bioavailability of oestradiol in relation to breast disease. *J Natl Cancer Inst* 1985, **75**, 823–829.
6. Ota DM, Jones LA, Jackson GL, Jackson PM, Kemp K, Bauman D. Obesity, non-protein-bound estradiol levels and distribution of estradiol in the sera of breast cancer patients. *Cancer* 1986, **57**, 558–562.
7. Ingram DM, Nottage EM, Willcox DL, Roberts A. Oestrogen binding and risk factors for breast cancer. *Br J Cancer* 1990, **61**, 303–307.
8. Bruning PF, Bonfrér JMG, Hart AAM. Non-protein bound oestradiol, sex hormone binding globulin, breast cancer and breast cancer risk. *Br J Cancer* 1985, **51**, 479–484.
9. Ernster VL, Wrensch MR, Petrakis NL, *et al.* Benign and malignant breast disease: initial study results of serum and breast fluid analyses of endogenous estrogens. *J Natl Cancer Inst* 1987, **79**, 949–960.
10. Moore JW, Clark GMG, Hoare SA, *et al.* Binding of oestradiol to blood proteins and aetiology of breast cancer. *Int J Cancer* 1986, **38**, 625–30.
11. Iqbal MJ, Johnson MW. Study of steroid binding by a novel "two tier" column employing Cibachrome Blue F3 G-A-Sepharose 4B. Sex-hormone-binding-globulin. *J Steroid Biochem* 1977, **8**, 977–983.
12. Dowsett M, Goss P, Powles TJ, *et al.* Use of the aromatase inhibitor 4-hydroxyandrostenedione in postmenopausal breast cancer: optimization of therapeutic dose and route. *Cancer Res* 1987, **47**, 1957–1961.
13. Hammond GL, Nisker JA, Jones LA, Siiteri PK. Estimation of the percentage of free steroid in undiluted serum by centrifugal ultrafiltration dialysis. *J Biol Chem* 1980, **255**, 5023–5026.

14. Pearce S, Dowsett M, Jeffcoate SL. Three methods compared for estimating the fraction of testosterone and estradiol not bound to sex-hormone-binding globulin. *Clin Chem* 1989, 35, 632–635
15. O'Connor S, Baker HWG, Dulmanis A, Hudson B. The measurement of sex steroid binding globulin by differential ammonium sulphate precipitation. *J Steroid Biochem* 1973, 4, 331–339.
16. Moore JW, Key TJA, Bulbrook RD, *et al.* Sex hormone binding globulin concentrations in a population of normal women who had never used exogenous hormones. *Br J Cancer* 1987, 56, 661–666.

Acknowledgements—This work was funded by the Breast Cancer Research Trust.

Eur J Cancer, Vol. 27, No. 3, pp. 263–267, 1991.
Printed in Great Britain

0277–5379/91 $3.00 + 0.00

Thymidylate Synthase from Untreated Human Colorectal Cancer and Colonic Mucosa: Enzyme Activity and Inhibition by 5-fluoro-2′-deoxy-uridine-5′-monophosphate

Godefridus J. Peters, Cees J. van Groeningen, Emile J. Laurensse and Herbert M. Pinedo

Inhibition of thymidylate synthase (TS) by the 5-fluorouracil (5-FU) metabolite FdUMP is considered to be the main mechanism of action of 5-FU. TS from colorectal tumours and normal colon mucosa from 10 untreated patients was studied. There was a large variation in the activity of tumour TS both at 1 and 10 μmol/l of its substrate dUMP; in normal mucosa this variation was less. Inhibition by 10 nmol/l FdUMP in tumours varied from 80 to 90% at 1 μmol/l dUMP; in normal mucosa, inhibition varied from 10 to 80%. The number of FdUMP binding sites ranged from 0.1 to 1 in tumours but such binding sites were not detectable in normal mucosa. The ratio between TS activity and FdUMP binding sites varied considerably in tumours but not in normal mucosa. The deviations from normal kinetics may represent a mutant TS form. Alterations in TS may partly account for differences in response to 5-FU.
Eur J Cancer, Vol. 27, No. 3, pp. 263–267, 1991.

INTRODUCTION

THYMIDYLATE SYNTHASE (TS) is a key enzyme in the *de novo* synthesis of thymidine nucleotides, for which deoxyuridine 5′-monophosphate (dUMP) is the substrate and 5,10-methylenetetrahydrofolate (CH_2-THF) the methyl donor. The K_m for the substrate is about 1–5 μmol/l [1–4], while the K_m for CH_2-THF varies between 10 and 50 μmol/l. Inhibition of TS by the metabolite 5-fluoro-2′-deoxy-UMP (FdUMP) is one of the main mechanisms of action of 5-fluorouracil (5-FU). 5-FU is one of the few drugs useful in colorectal cancer [4]. Leucovorin, a precursor for CH_2-THF, potentiates the effect of 5-FU in patients [5], in mice [6–8] and *in vitro* [8]. Inhibition of TS by FdUMP is probably of crucial importance in the action of 5-FU in patients [8–10].

TS is inhibited by the formation of a covalent ternary complex between the enzyme, FdUMP and CH_2-THF [2–4]. This complex is rapidly formed and the rate of dissociation may determine the efficacy of 5-FU. TS can also be inhibited by an unstable binary complex between FdUMP and TS [4, 11, 12]. In cell and tissue extracts FdUMP is a potent competitive inhibitor of TS (K_i about 1 nmol/l). Retention of inhibition is mainly determined by the stabilisation of the ternary complex by CH_2-THF or one of its polyglutamates [13, 14]. In addition the concentrations of FdUMP and dUMP will influence the extent of inhibition. *In vitro*, resistance to 5-FU or 5-fluoro-2′-deoxyuridine (FUdR) has been related to altered kinetics of TS for dUMP and FdUMP binding [2, 4, 15], disturbed folate pools [14] and the level of enzyme before treatment [15]. Gene amplification of TS has been demonstrated for FUdR-resistant cell lines [16]. Evidence for gene amplication has also been obtained in a patient with colon cancer who developed resistance against 5-FU [17], while in breast cancer patients binding of FdUMP and the effect of CH_2-THF decreased during development of resistance [9].

These aberrations in kinetic properties may affect the extent and duration of inhibition of TS by FdUMP, perhaps even precluding synergism between LV and 5-FU. For instance, one form of TS in a cell line was resistant to inhibition by FdUMP [18]. Therefore we have measured the activity of TS, inhibition by FdUMP and the binding of FdUMP to TS in biopsy specimens of colorectal tumours from previously untreated patients. To establish whether gastrointestinal toxicity may be attributed to enzyme inhibition, we also studied adjacent normal mucosa. Part of the data have been reported in preliminary form [19].

Correspondence to G.J. Peters.
The authors are at the Department of Oncology, Free University Hospital, PO Box 7057, 1007 MB Amsterdam, The Netherlands. E. J. Laurensse is currently at Eurocetas, Amsterdam; H. M. Pinedo is also at the Netherlands Cancer Institute, Amsterdam, The Netherlands.
Revised 8 Nov. 1990; accepted 15 Nov. 1990.