Europ. J. Cancer Vol. 15, pp. 693–702.
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0014–2964/79/0501–0693 $02.00/0

The Synthesis of the Glycoprotein Hormone α Subunit by Human Breast Carcinomas

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Abstract—*The glycoprotein hormone α subunit content of 58 primary breast carcinomas was studied using an antiserum prepared against hCG α subunit. α Subunit was detected in 44% of tumour cytosols and 26% of supernatants from short-term tumour culture using a radioimmunoassay and in 23% of tumours examined by an immunoperoxidase method. In the cytosols examined, tumour derived α subunit appeared to be immunologically identical to hCG α but eluted before hCG α on gel chromatography. Detectable α subunit in supernatants was associated with tumour infiltration of axillary lymph nodes.*

Serum α subunit concentrations were determined in 51 patients with stages I and II breast cancer and in 25 of these post-operatively, in 53 women with stages III and IV disease and in 84 age and menopause matched controls. Abnormal concentrations were detected in 2 out of 12 pre-menopausal and 8 out of 92 post-menopausal patients. There was no significant difference between the serum α subunit concentrations in patients with local disease before or after resection of the primary tumour, in patients with advanced disease or in control subjects. α Subunit is rarely found in high enough concentration in serum to be of value as a tumour marker in breast cancer.

INTRODUCTION

ASSESSMENT of tumour burden remains a major problem in the management of patients with most common malignancies. The measurement of a circulating product of certain tumours such as choriocarcinoma and medullary cell carcinoma of the thyroid, provides a quantitative guide to tumour burden and aids early diagnosis, monitoring of therapy and prediction of recurrence. No single satisfactory marker has so far been described for breast cancer and the simultaneous measurement of human chorionic gonadotrophin (hCG) and other tumour related substances has been advocated for monitoring tumour burden [1, 2]. The glycoprotein hormone hCG consists of an α subunit common to all the glycoprotein hormones and a β subunit which is specific to hCG. α Subunit is normally detectable in serum during pregnancy and in post-menopausal women as a result of secretion by the placenta and pituitary respectively. Ectopic secretion of hCG or its isolated α and β subunit has been associated with a wide variety of tumours [3, 4] and isolated α subunit is synthesised by cell lines derived from lung [5], cervix [6], and breast [7] tumours. In breast cancer elevated serum concentrations of hCG and hCG β have found in 10–49% and in 2–14% of patients respectively [1, 8, 9]. The aim of the present work is to define the proportion of human tumours containing the glycoprotein hormone α subunit and to examine the possible clinical value of serum α subunit as a tumour marker in breast cancer. To our knowledge α subunit has not been assessed previously as a tumour marker in breast cancer.

Accepted 26 October 1978.

MATERIALS AND METHODS

Tumour collection and sample preparation

Tumour tissue weighing 0.5–2 g was obtained within 50 min of resection of histologically proven breast cancer from 57 patients. Bilateral malignant tumours were removed during the same operation from one patient and were processed separately. The nodal status of 49 patients was assessed by histologi-

cal examination of axillary nodes sampled during resection of primary disease.

Tissue for cytosol preparation was stored at −40°C in T.E.D. buffer (10 mM Tris pH 7.4, 1.0 mM EDTA, 0.5 mM dithiothreitol). After thawing slowly the tissue was trimmed of fat, connective tissue and large blood vessels and was weighed. Tumour weighing 0.15–1.5 g was sliced, crushed in liquid nitrogen using a Thermovac Autopulveriser (Thermovac Industries Corp., Copiague, N.Y) and homogenised in 5–10 × w:v T.E.D. buffer using a glass–glass homogeniser (Jencons). The homogenate was centrifuged at 100,000 ***g*** for 60 min to obtain the cytosol, aliquots of which were stored at −40°C until assayed for α subunit. All procedures were carried out at 4°C. Results were expressed as nanograms α subunit per gram of wet weight of tumour. α Subunit was measured in cytosols prepared from apparently normal breast tissue adjacent to three carcinomas and in the cytosol of post mortem specimens of liver, brain and kidney from two patients dying from arteriosclerotic disease.

Supernatants from short-term tissue culture were obtained from 42 tumours. Fresh tumour tissue was washed in phosphate buffered saline, pH 7.4 and the sample trimmed and weighed. After cutting into 1–2 mm cubes the tumour was incubated without shaking for 12–24 hr in 14 ml of Ham's F.12 medium containing 10% foetal bovine serum, 10 mU/ml insulin, 1 μg/ml collagenase (Worthington CLS grade) and 100 U/ml penicillin. The disaggregated cells were removed by centrifugation and an aliquot of the supernatant stored at −40°C until assayed for α subunit. A part of each of the 58 carcinomas was formalin fixed and paraffin embedded.

Serum samples were obtained from 51 women about to undergo resection of stages I or II breast cancer and a further sample was obtained from 25 of these patients 1–3 months post-operatively. Serum was also obtained from 53 women with stages III or IV breast cancer who were about to undergo a change of therapy because of clinical evidence of tumour progression. No patient was receiving chemotherapy at the time of venesection. Control sera were obtained from 61 pre- and peri-menopausal women and 23 post-menopausal women all of whom were well. The serum was separated within 2 hr of venesection and aliquots stored at −40°C until assayed. The menstrual history, past medical history and current medication were recorded for each patient. Pre- and peri-menopausal patients were defined as having a history of menstruation within 6 months and a serum FSH of less than 15 i.u./l. The remainder of patients were classified as post-menopausal. The serum creatinine was measured in all the patients. α Subunit, FSH and creatinine were measured on the same serum samples.

α Subunit assay

α Subunit was measured by a double antibody radioimmunoassay. Anti-serum was raised in rabbits to purified hCG α subunit (CR115 kindly donated by R. E. Canfield) and used at a dilution of 1/150,0000. The serum α subunit preparations were iodinated with ^{125}I by a chlorine gas method [10]. Antibody and antigen were initially incubated for 6 hr at 23°C, then for 18 hr at 6°C after the addition of iodinated subunit and finally for 24 hr at 6°C after the addition of a second antibody. After centrifugation at 2000 ***g*** for 30 min the supernatant was aspirated and the precipitate counted for 100 sec in a Wallac gamma well scintillation counter. All samples were assayed in duplicate. The limit of detectability of the α subunit assay was taken as 10% displacement which is equivalent to 1.5 μg α subunit/l. The within and between assay coefficients of variation are 8 and 9% respectively. Cross reactivity in the α subunit is: assay at 50% displacement is: hCG 5%, hCG β 0.3%, LH 11.6%, FSH 16%, TSH 20%, LH β less than 0.3%, FSH β 5%, (human prolactin, hPl, hGh less than 0.3%).

Gel chromatography was performed on a 100 cm × 2.5 cm diameter G100 Sephadex column. The void volume was determined by dextran blue exclusion and the total volume from the column dimensions. The column was calibrated with albumin, chymotrypsinogen and cytochrome C. The column was equilibrated with 0.01 molar phosphate buffer and run at a flow rate of 12 ml/hr with a sample volume of 2.0 ml and a fraction collection volume of 2.25 ml. Detection of α subunit after chromatography required an initial sample concentration of approximately 15 μg/l or greater.

Using the same anti-α subunit antiserum 52 of the formalin fixed specimens were examined for α subunit by an immunoperoxidase technique [11]. FSH and LH were measured by radioimmunoassay [12]. TSH was measured by double antibody radioimmunoassay using reagents supplied by NIH. The cross reactivity of α subunit in the FSH and TSH assays was less than 1%. The cross activity of

whole hCG in the LH assay is greater than 50%.

RESULTS

Tumour content

α Subunit was detected in 24 of 55 (44%) tumour cytosols, in 11 of 42 (26%) culture supernatants (Fig. 1) and in 12 of 52 (23%) tumours examined by the immunoperoxidase method (Fig. 2). When individual tumours were measured by more than one method there was good overall agreement at high concentrations of α subunit between the assays, suggesting that the differences in the positivity rate were largely due to different sensitivities of the three methods. The methods were compared by χ^2 analysis of three 2 × 2 tables of tumours found to be positive or negative for α subunit. Fifty tumours were measured by the cytosol and immunoperoxidase methods ($\chi^2=9.1$ $P=<0.01$), 40 by cytosol and supernatant ($\chi^2=5.0$ $P<0.05$) and 39 by immunoperoxidase and supernatant ($\chi^2=11.0$ $P=<0.001$). The 6 tumours with the highest α concentrations as derived from cytosol measurement were positive for α subunit by the immunoperoxidase and supernatant methods. However, there was some overlap between methods and cytosols from three tumours with relatively high supernatant concentrations (119 ng/g, 104 ng/g and 77 ng/g) did not contain detectable α subunit. Cytosols prepared from apparently normal breast adjacent to the carcinoma contained no detectable α subunit although two of the three tumour cytosols were positive (149 ng/g, 18.3 ng/g). No α subunit was detected in post mortem specimens of brain, kidney and liver from two patients.

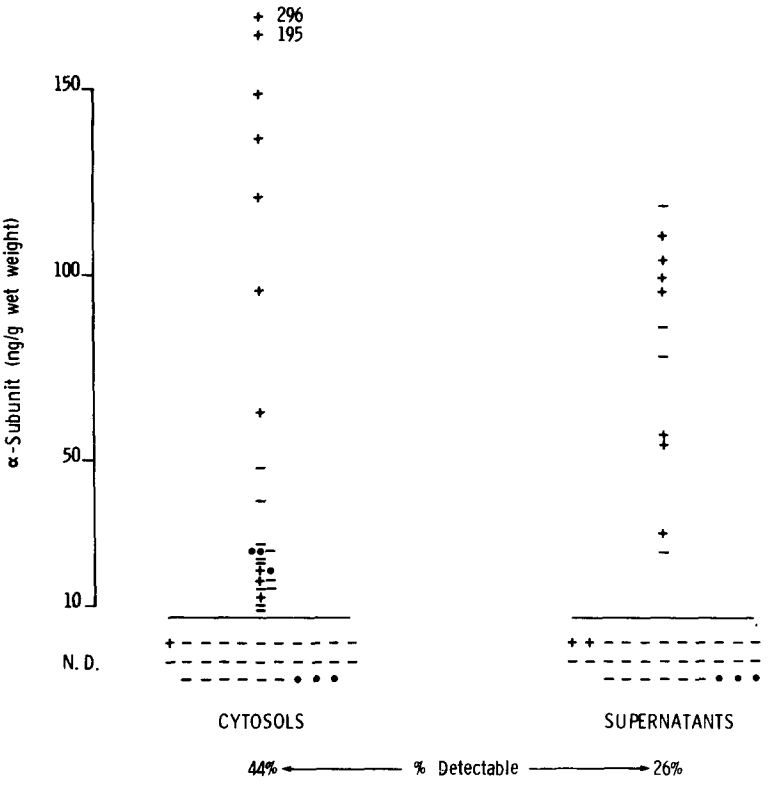

Fig. 1. Tumour concentration of α subunit from measurement of cytosols and culture supernatants. + = immunoperoxidase positive. − = immunoperoxidase negative. • = tumours not examined by the immunoperoxidase technique.

Characterisation

α Subunit measurement of dilutions of two tumour cytosols and one serum sample containing high concentrations of α subunit demonstrated parallelism with the standard curve (Fig. 3).

There was no significant difference detected between these lines using the method of R. Borth [13]. This confirms that the affinities for the antiserum of tumour α subunit and standard hCG α are similar if not identical and implies that in the two cytosols studied tumour derived α subunit and hCG α subunit are immunologically identical. On gel chromatography (Fig. 4) the α subunit of one of these tumour cytosols eluted between ^{125}I-

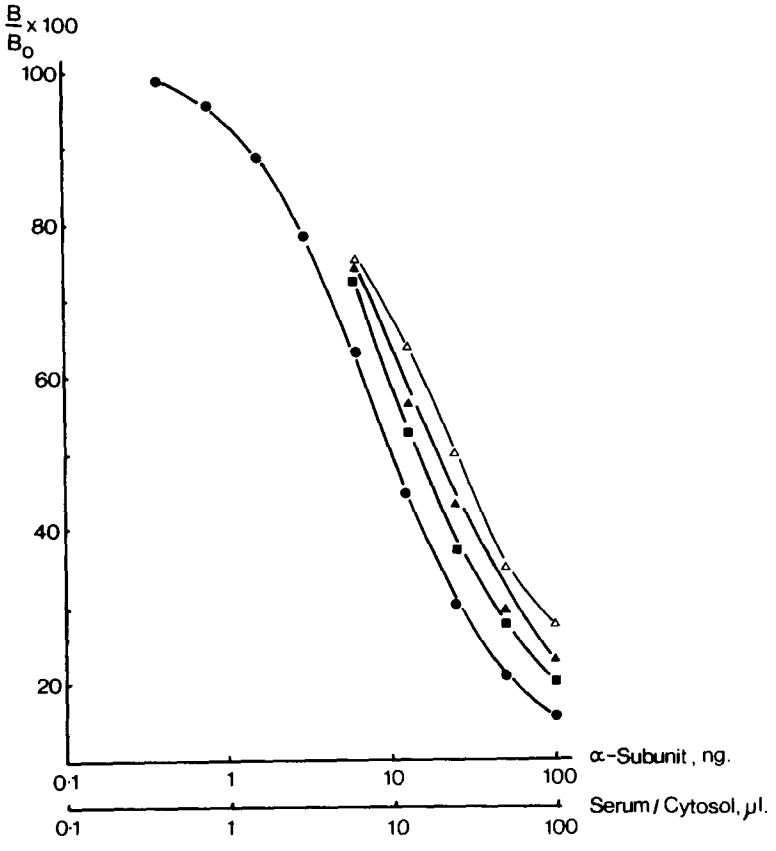

Fig. 3. Serial dilution in the α subunit radioimmunoassay of standard α subunit (●——●), serum (■——■) and cytosols (△——△, ▲——▲), from two patients with primary breast carcinoma.

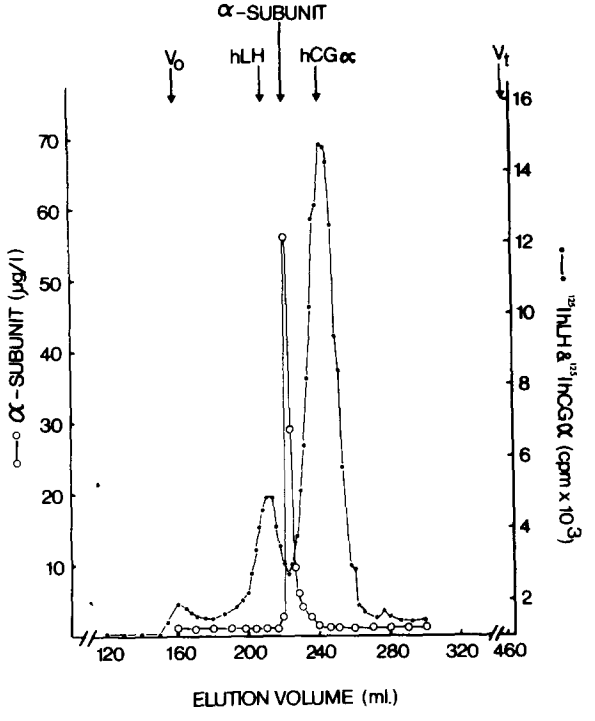

Fig. 4. Chromatography of ^{125}I-LH (●——●), an α subunit positive tumour cytosol (○——○) and ^{125}I-hCG α (●——●).

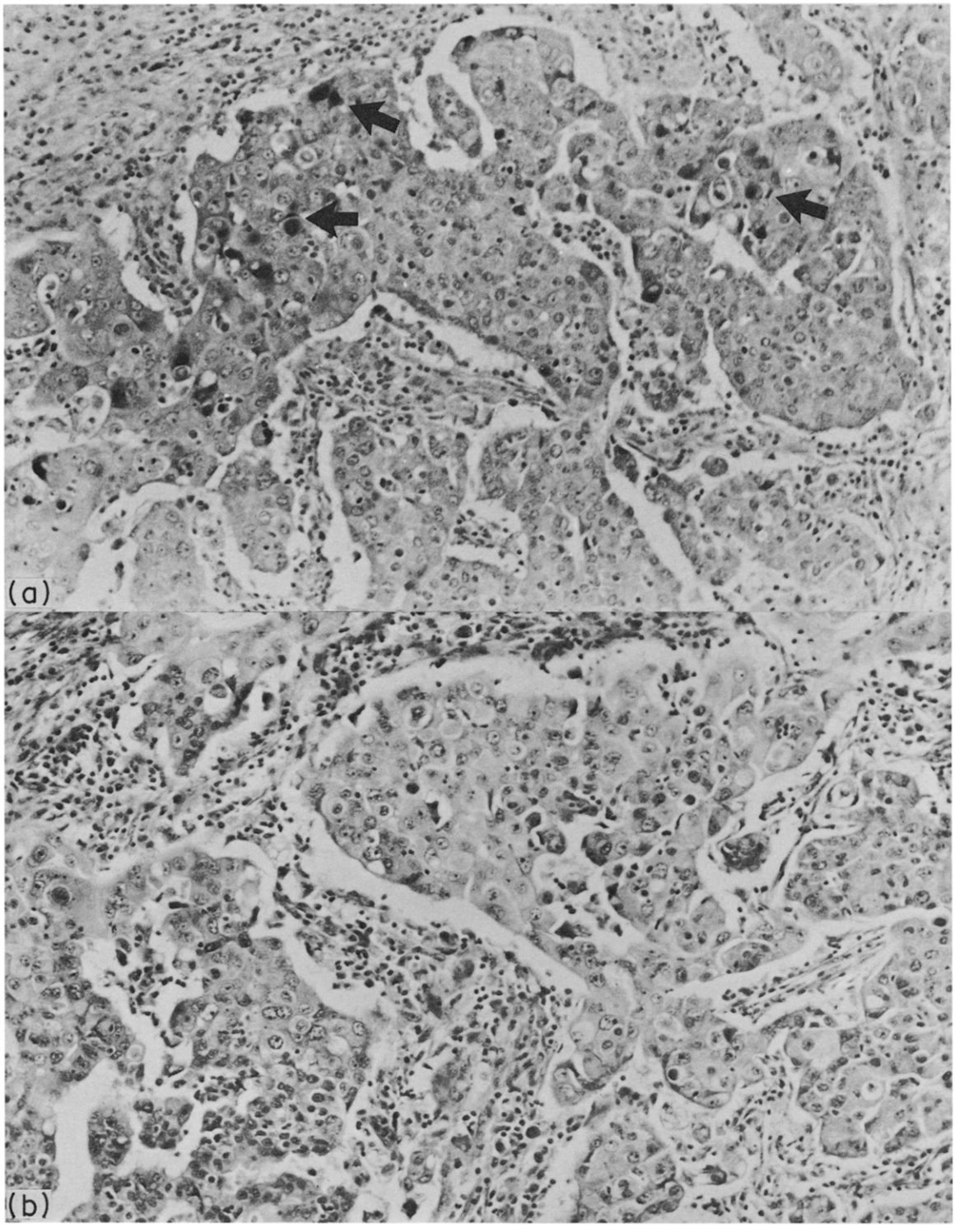

Fig. 2. Sections of the same primary breast carcinoma. Immunoperoxidase method (a). The presence of α subunit is indicated by intracytoplasmic dark staining. H and E stain (b).

LH and ^{125}I-hCG α. Because there is some cross-reactivity in the α subunit assay with whole glycoprotein hormones the cytosol concentrations of TSH, LH and FSH were measured. Immunoreactivity was detected in the LH assay in one of 15 tumour cytosols (concentration = 1.29 ng/g wet weight) in the FSH assay in 12 of 15 tumour cytosols (concentrations = 0.6–6.3 ng/g wet weight, mean 2.0 ng/g) and in the TSH assay in one of 28 tumour cytosols (concentration = 7.0 ng/g wet weight).

Serum (*Fig.* 5)

α Subunit was detected in the sera of 20 out of 61 control pre and peri-menopausal women (range undetectable −4.6 μg/l) and in 21 out of 23 normal post-menopausal women (mean 3.6 μg/l, S.D. = 1.3 μg/l). The upper limits of normal were taken to be 4.6 and 6.3 μg/l for pre- and post-menopausal women respectively. In women with breast cancer the concentrations were elevated in 2 out of 12 pre- and peri-menopausal patients. The four highest values occurred in women with advanced disease and in one of these the serum concentration fell from 42 to 13 μg/l over a period when bony metastases showed partial radiological regression. The highest serum concentration (88 μg/l) occurred in a patient with the highest tumour concentration (296 ng/g) and with an axillary lymph node infiltrated by breast cancer which was also positive (101 ng/g).

No significant difference was detectable in serum α subunit concentrations before (mean = 3.7 μg/l, S.D. = 1.5 μg/l) and after (mean = 3.4 μg/l, S.D. = 1.8 μg/l) resection of local breast cancer in 20 post-menopausal women. There was no significant difference between the serum concentrations of post-menopausal patients with local disease (mean = 3.4 μg/l, S.D. = 1.7 μg/l), of those with advanced disease (mean = 5.4 μg/l, S.D. = 13.1 μg/l) or of control subjects (mean = 3.6 μg/l, S.D. = 1.3 μg/l). In all patients serum creatinine was in the normal range.

DISCUSSION

Using an antiserum raised against hCG α subunit we have found an α subunit like substance in the cytosols of 44% of human breast tumours. Immunological identity between hCG α and the α subunit like substance in breast cancers is suggested by the parallelism on dilution. However, on column chromatography the α subunit of one breast tu-

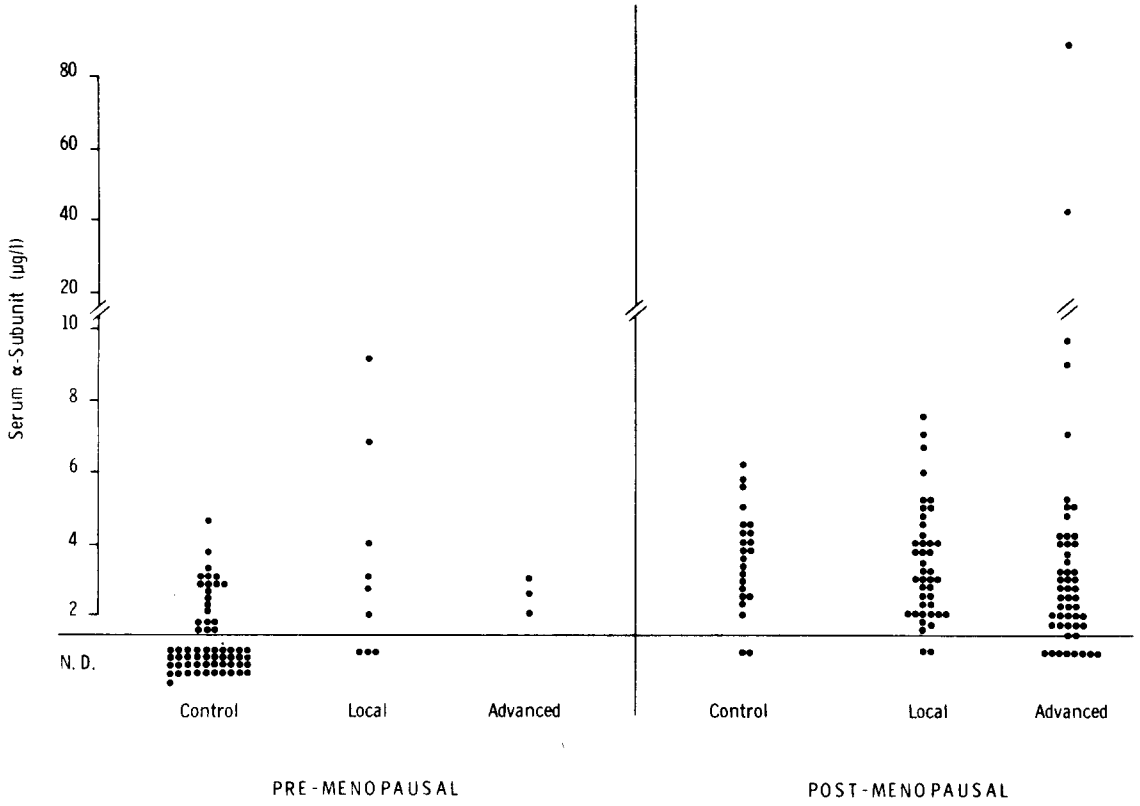

Fig. 5. *Serum concentrations of α subunit according to menopausal status and the stage of breast cancer.*

mour cytosol eluted between hCG α (mol. wt =approx 15,000) and LH (mol. wt=approx 30,000). It is of considerable interest that tumour α subunit derived from HeLa cells [6], gastric carcinoid [3] and a bronchogenic carcinoma cell line [5] were also excluded between the LH and hCG α peaks. These results suggest that tumour derived α subunit is somewhat larger than hCG but the differences do not alter its immunological identity. Further studies are required to find out whether these features are common to all the α subunit positive breast tumours.

Contamination of cytosol with serum α subunit or whole glycoprotein hormones could not account for our cytosol findings for the following reasons. In 11 cases where α subunit was detected in both cytosol and serum, the tumour concentrations were always higher than the serum concentrations suggesting that tumour α subunit was not due to serum contamination. Tumour cytosols and supernatants were diluted 5–15 times weight to volume during preparation which would be expected to reduce the concentration of any α subunit from contaminating serum to below the limit of detection in all but the rare cases with very high serum levels. In one patient who had bilateral tumours resected during the same operation α subunit was found in high concentrations in the carcinoma from the left breast (149 ng/g), in normal concentrations in the serum (3.9 μg/l) but was undetectable in apparently normal breast tissue from the left breast and in the carcinoma from the right breast. TSH, LH and FSH appeared to be detected occasionally in tumour cytosols but only in concentrations 5–20 times below that required to cause significant displacement in the α subunit assay. The presence of low concentrations of LH, FSH and TSH in cytosols may be due to serum contamination of cytosol or an artefact caused by cross reactivity of α subunit in these assays and does not necessarily indicate synthesis by the tumours. We have indirect evidence that whole hcG is not commonly present in high concentrations in tumour cytosols: only one of 30 tumour cytosols contained detectable hCG β [14] although 11 were α subunit positive. As the β and α antisera used have similar cross reactivities for whole hCG, displacement in the α subunit assay alone strongly suggests the presence of isolated α subunit and not whole hCG. Similarly the lack of displacement in the LH assay suggests that significant concentrations of whole hCG do not occur frequently.

Estimation of α subunit in cytosol proved more sensitive than either immunoperoxidase staining or measurement in supernatants. The lower number of positives found by immunoperoxidase may be due to loss or alteration of α subunit during fixation. However, it is possible that minor changes in the antibody concentration used would increase the sensitivity of the method and it has the advantage that it can be used retrospectively on tissue prepared for routine histological examination. Dilution of the tumour in a large volume of culture fluid probably accounts for finding fewer positive supernatants than cytosols. Supernatants from three tumours with negative cytosols contained high concentrations of α subunit (119 ng/g, 104 ng/gm and 77 ng/g) suggesting that at least some tumours are synthesising and releasing α subunit during the period of incubation.

The difference in concentrations of serum α subunit before and after the menopause has been noted previously [15] and indicates the need for adequate age and menopause matched controls in the study of α subunit as a tumour marker. In pre-menopausal women 2 out of 12 patients with breast cancer had elevated serum concentrations. In post-menopausal women 5 out of 50 patients with advanced disease and 3 of 42 patients with local disease had elevated serum concentrations. It is of interest that the four highest values occurred in patients with advanced disease and that partial regression of tumour in one patient was accompanied by a fall in α subunit (42–13 μg/l) suggesting a relationship to tumour burden. Overall however, α subunit concentrations appeared to be largely unaffected by tumour burden and there was no significant difference between control, pre- and post-operative patients with local disease and women with advanced breast cancer. Our results suggest that although serum α subunit concentration occasionally reflects tumour burden in post-menapausal women, markedly elevated values are rare and changes within or close to the normal range are an unreliable guide to tumour burden.

The synthesis of α subunit by 43% of primary breast cancers suggests that it may be a valuable index of the secretory activity of breast tumour cell lines, breast cancers cultures *in vitro* or tumours transplanted into nude mice. Recently there has been considerable interest in sodium butyrate induced stimulation of α subunit release from HeLa cells [16]. We have shown that sodium butyrate has similar effects on the breast tumour

cell line MCF-7 causing increased secretion of α subunit and decreased cellular proliferation [7]. It is possible that with the development of suitable *in vivo* methods of stimulation, serum α subunit could be a valuable index of tumour burden.

hCG and its subunits have immunosuppressive properties *in vitro* and it has been suggested that they might conder a worse prognosis on tumours which synthesise them [17, 18]. If this were the case for α subunit producing tumours we would expect to find more in the poor prognosis node positive patients. Of node positive tumours 9 out of 19 supernatants contained detectable α subunit whereas only 1 out of 20 supernatants from node negative tumours were positive ($\chi^2 = 8.5$, $P = < 0.01$). Although the overall cytosol and immunoperoxidase results showed no significant association between α subunit and nodal status, the five tumours which from cytosol measurement contained the greatest α subunit concentration were all node positive. Patients are being followed up to determine whether α subunit measurement helps to identify those with a poor prognosis.

Acknowledgements—We thank Dr. S. S. Lynch for preparing α subunit antibody, Mrs. S. Griffiths for performing the TSH assays, the West Midlands Regional Health Authority for financial support of D.H.C. through the Sheldon Research Fund and the MRC for financial support. We also thank Professor R. H. Hoffenberg and Professor W. R. Butt for their valuable advice in the preparation of this manuscript.

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