

Development of a Microplate Assay for Serum Chromogranin A (CgA): Establishment of Normal Reference Values and Detection of Elevated CgA in Malignant Diseases

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Chromogranin A (CgA), a marker for neuroendocrine cells, is associated with poor prognosis when detected by immunohistochemical technique in prostate tumors.

We have developed an ELISA on microplates for serum CgA and established the normal reference range. We also attempted to find out whether elevated serum CgA levels could be found in patients with various malignant diseases. Because of non-Gaussian distribution, both medians and 97.5 percentiles of serum CgA levels for men and women of four different age groups were determined. For women, the median and 97.5 percentiles are 20.7 and 63.9 ng/mL for ages

20 to 50, and 32 and 93.8 for 50 to 80 years of age, respectively; for men, they are 27.9 and 78.4 ng/mL for ages 18 to 40 and 41.6 and 92 for 40 to 80 years old, respectively. Elevated serum concentrations of CgA were detectable in patients with prostate cancer not undergoing hormonal treatment, and in patients with various malignant diseases including nonendocrine carcinomas. Most elevated serum CgA levels were associated with sera containing highly elevated serum tumor markers. Drugs targeting neuroendocrine cells should be administered for cancer patients with elevated serum CgA levels. *J. Clin. Lab. Anal.* 13:312–319, 1999. © 1999 Wiley-Liss, Inc.

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INTRODUCTION

Chromogranin A (CgA) is an acidic glycoprotein with a molecular weight of 68 kDa, and an isoelectric point in the range of 4.57 to 4.68. CgA is also present in a variety of polypeptide-secreting endocrine cells that possess secretory granules, such as neuroendocrine cells (1,2). Although serum CgA is a well-known marker for the neuroendocrine cell, due to the lack of commercial assay kits in the U.S., serum CgA rarely has been used for the diagnosis and management of patients with neuroendocrine tumors such as neuroblastoma, pheochromocytoma, small cell lung carcinoma, and carcinoid-like tumors (3,4).

Neuroendocrine cells, in general, have not been found in nonendocrine carcinomas. Neither has serum CgA been detected in carcinomas, except in those tumors whose normal counterparts contain neuroendocrine cells, such as the prostate. In the prostate and urethra, neuroendocrine cells are known to be present in relatively large numbers. These neuroendocrine cells in the prostate are intraepithelial regulatory cells with hybrid neuroendocrine and epithelial characteristic (5). Detection of neuroendocrine differentiation in the pro-

static tumor tissue, based on immunohistochemical methods, was always associated with poor prognosis (6,7). In fact, the CgA score was found to be significantly related to the Gleason score, the volume of the tumor, and the pathologic stage (8,9).

Neuroendocrine differentiation also appears to have an impact on prostate cancer patients undergoing hormonal treatment. Berner showed (10) that prostate cancer patients became resistant to hormonal therapy when there was increasing neuroendocrine differentiation in their tumors. However, in their study, only random specimens were used for serum CgA measurement. When we monitored CgA retrospectively in serial serum specimens from patients known to have resistance to hormonal therapy, we found that in almost one-third of all prostate cancer patients the level of serum CgA was elevated. Also, this elevation was detected at an earlier stage during hormonal treatment (11). Elevation of serum CgA in those patients was usually detected several months prior to the rise of serum PSA. Conceivably, the early modification

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of therapy to target neuroendocrine instead of epithelial tumor cells may improve the chance of survival for those patients (12). The importance of establishing a serum CgA assay is also supported by the study performed by Angelsen et al. (13) which showed that CgA is a more useful serum marker than neuron specific enolase in predicting the extent of neuroendocrine differentiation in prostate tumors.

Using our in-house-developed serum CgA assay we found that serum CgA was elevated not only in prostate cancer patients showing hormonal resistance but also in patients not receiving such treatment. In the latter, the elevation of serum CgA occurred at a much later stage of disease progression—usually so advanced that the serum PSA was also elevated. In our continued investigation we also found both normal and elevated levels of serum CgA in various malignant diseases including nonendocrine carcinomas. This finding was unexpected since neuroendocrine differentiation has never before been reported in these carcinomas. The important clinical implications of this finding warrant additional studies.

MATERIALS AND METHODS

Both the polyclonal rabbit antihuman CgA antibody and the HRP-conjugated monoclonal mouse antihuman CgA antibody were purchased from Dako (Carpinteria, CA). These antibodies are made against the C-terminal 20 kDa fragment of a CgA molecule. Immulon 4 Removawell® strips were from Dynatech Laboratories (Chantilly, VA). SK-N-AS cell line, a human metastatic neuroblastoma cell line was obtained from ATCC (Rockville, MD). The Dako CgA enzyme immunoassay kit and the CIS (CIS Bio International, Cedex, France) radioimmunoassay CgA kit were all generous gifts. TMB peroxidase substrate and K-blue substrate-TMB were from Neogen Corp. (Lexington, KY). Chromatofocusing was performed on Pharmacia (Piscataway, NJ) FPLC automated system using Mono P 5/5 prepacked column as described previously (14).

Specimens

All serum specimens used to establish normal reference values were obtained from normal individuals visiting Chang Gung Memorial Hospital in Taipei, Taiwan, for their annual health check-up. These were adult males and females between age 20 to 80 years old. There were only a few under 20 or over 80 years old. Based on the results of a chemistry profile, these serum specimens did not have any detectable renal and liver abnormalities. Serum specimens from patients with prostate cancer and various carcinomas were sent to clinical laboratories for determining the tumor marker level.

In-House Serum CgA Assay

Antibody coated microplate was prepared by adding to each well 100 μ L of polyclonal anti-CgA antibodies (5 μ g/mL) in

0.1 M carbonate buffer, pH 9.6 and allowing incubation overnight at 4 °C. Blocking solution 150 μ L (PBS containing 1% BSA, 0.05% casein, 0.05% Tween 20 and 2% sucrose) was added at r.t. After 6 h, incubation wells were emptied and washed once with PBS containing 0.05 % Tween 20. The plate was left in the refrigerator in sealed plastic bags during the study.

To assay for CgA 50 μ L HRP-conjugated monoclonal anti-CgA antibody (1:100 dilution) and 50 μ L of sample (or calibrator) were added to each antibody-coated well. After 3 h incubation at r.t. with gentle agitation, wells were washed 3 times with 200 μ L per well of washing solution. For color development, 100 μ L TMB was added to each well. After 12 min, the reaction was stopped with the addition of 150 μ L of 1 N H₂SO₄ and the absorbency was read at 450 nm.

RESULTS

Assay Development

Antibody coating

Various assay parameters were studied in order to determine the optimal conditions for a microplate-based enzyme immunoassay following the sandwich format for serum CgA. The results are depicted in Figure 1.

As shown in Figure 1A we found that coating 100 μ L of 5 μ g/mL polyclonal rabbit anti-CgA antibodies provided the highest sensitivity for CgA detection. Although a coating of 3 to 4 μ g/mL of anti-CgA antibodies could prove sufficient, applying 5 μ g/mL of antibodies would reduce the possibility of hook effects and extend the life of the coated antibody during storage.

Number of incubations

For sandwich-type enzyme immunoassays, one can perform either one or two incubations. We found that an assay using only one incubation, in which 50 μ L serum and 50 μ L detecting antibody were mixed at the same time in the antibody-coated microwell, produced a much higher sensitivity (Fig. 1B).

Incubation time

In general, the longer the incubation time the more sensitive the assay. However, selection of incubation times longer than four hours makes it difficult to complete an assay within one working day. Based on our study (Fig. 1C) we selected four hours as the length of incubation time for our assay.

Detecting antibody

The HRP-conjugated anti-CgA monoclonal antibody we purchased was originally designed for use in immunohistochemical staining. Therefore, the antibody was at a much more diluted form than the antibodies we customarily use for

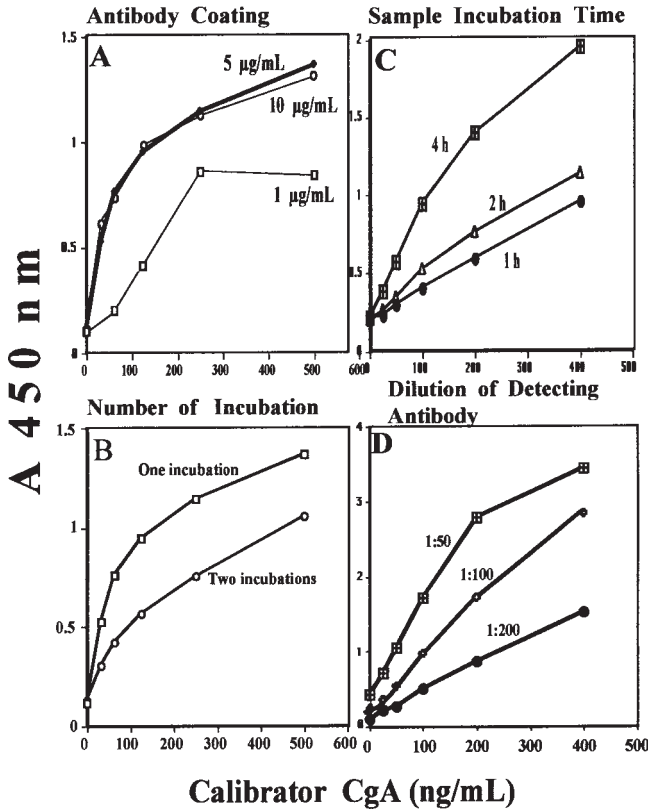


Fig. 1. Assay parameters optimized for the development of a sandwich type enzyme immunoassay for serum CgA. 1A, Determination of an optimal concentration of polyclonal anti-CgA antibodies for coating; 1B, Selection between one and two incubations for the assay. In one incubation, the detecting antibody and the sample were added to the antibody-coated well at the same time; 1C, Determination of the optimal length of incubation time; 1D, Determination of the optimal dilution for the detecting HRP-conjugated anti-CgA monoclonal antibody.

enzyme immunoassays. Because the antibody was HRP-conjugated and ready for immunohistochemical staining, it was well suited for detecting antibody as long as it had a sufficient affinity for the analyte. As shown in Figure 1D we found that dilution of the HRP-conjugated monoclonal antibody 100-fold was most suitable for our assay. At this dilution the assay appeared to be linear up to 400 ng/mL without producing a high background.

Calibration curve

A typical calibration curve for our CgA assay is shown in Figure 2A. The calibration curve covers the CgA concentration up to 500 ng/mL in a linear fashion. Using a serially diluted calibrator of known CgA concentration we found the sensitivity of our assay to be about 5 ng/mL. Dilution of calibrator below 5 ng/mL produced identical absorbance similar to the background absorbance.

Serum CgA and Calibrator

We used CgA contained in the cell culture medium of SK-N-AS cells, a human metastatic neuroblastoma cell line, as the calibrator for our assay without further purification. In order to make certain that the cell line-derived CgA had the same affinity as the serum CgA for the assay antibody, two dose response curves, one using serum CgA and the other using cell-derived CgA, were determined. If there were a difference in affinity between CgA from the two different sources, the slopes would have been different. As shown in Figure 2B, both our cell-derived calibrator and serum CgA exhibited almost identical slopes suggesting that both had the same affinity for the assay antibody. In other words, using CgA from the cell medium of SK-N-AS cells allows us to produce ac-

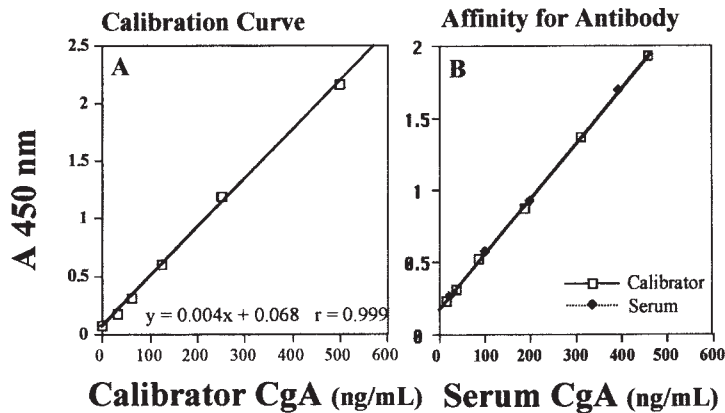


Fig. 2. Typical calibration curve for CgA immunoassay and comparison between calibrator and serum CgA of their affinity (or slope) for assay antibody. 2A, A typical calibration curve of our in-house assay using CgA isolated from SK-N-AS cells. The concentration of our calibrator was calibrated against CIS RIA kit; 2B, Comparison of the slope of two dose response

curves. One is based on the concentration of serum CgA and the other is on the concentration of in-house calibrator. The perfect match of two curves indicates that our in-house calibrator has the same affinity for the assay antibodies as the CgA in the patients' sera. Values of the serum CgA were assigned by dilution.

curate results for our serum CgA assays. CgA from the cell line was also characterized on chromatofocusing. The pI (isoelectric point) of cell line-derived CgA (pI = 5.1) proves that CgA is an acidic protein even though it is slightly different from that of serum CgA (pI = 4.7) (Fig. 3).

Assay precision, sensitivity, and recovery

Using pooled sera from patients with renal insufficiency, we performed the within-day (intra-assay) precision studies at three levels of CgA: CV = 6.25% (20.5 ± 1.3 , N = 12); 6.01% (44.3 ± 2.7 , N = 12); 5.95% (105 ± 6.24 , N = 20). We used controls at two different levels in the routine CgA assays at different days to estimate for the day-to-day precision, CV = 9.8 (110 ± 10.7 , N = 12); CV = 10.2 (42 ± 4.3 , N = 12).

We determined the assay sensitivity by diluting the calibrator of known CgA value continuously until no change or decrease in absorbance at 450 nm could be detected. We found that that assay sensitivity is 5 ng/mL since we could not detect any further decrease of absorbance below that level.

Serum without any detectable CgA was spiked with aliquot of serum containing highly elevated CgA. The recovery was found to be 98 and 102% for serum levels of 35 and 120 ng/mL, respectively.

Normal reference value

As shown in Figure 4 we measured the serum CgA in both men and women of different age groups. Apparently women

at age between 20 to 50 have very similar distributions of their serum concentration of CgA. After they reach the age of 50, serum CgA levels increase. However, distribution of serum CgA is very similar among women between the ages of 50 and 80. On the other hand, the distribution of serum CgA appears to be similar among men 18–40 and 50–80 years old. Because of non-Gaussian distribution of serum CgA we decided to estimate the median instead of mean for serum CgA in various age groups of normal men and women. However, based on the results of the 13 age groups in Figure 4, we decided that four age groups would suffice. The results are shown in Table 1. Apparently, serum CgA levels increase with old age, and men appear to have slightly higher levels. These normal reference values should help the diagnosis and prognosis of patients with various malignant diseases.

Although the normal reference values for serum CgA were based on normal Asian men and women, these values don't seem to be much different from those reported in the literature which were based on a Caucasian population. CIS RIA kit lists a normal mean of \pm SD as 36 ± 18 ng/mL (N = 50 sera) while Kadman et al. reported normal serum CgA as 43 ± 17 ng/mL (15).

CgA in Prostate Cancer

Instead of finding an early rise of serum CgA in those patients with developed hormonal resistance (11), elevated serum CgA was detected in prostate cancer patients without receiving hormonal treatment at a much advanced stage of cancer progression. In other words, elevated serum CgA usually appeared at the time when serum PSA had also become highly elevated such as higher than 100 ng/mL (Fig. 5). More than 50% of specimens containing greater than 100 ng/mL of serum PSA showed elevated serum CgA. On the other hand, only 9% of specimens containing less than 100 ng/mL of PSA had elevated serum CgA. The study was carried out using the Dako EIA kit and their suggested upper normal level was plotted as a dotted horizontal line.

CgA in Various Carcinomas

In nonendocrine carcinomas neuroendocrine differentiation has rarely been observed or reported. Detection of neuroendocrine differentiation in carcinomas is not expected without finding neuroendocrine cells in their normal counterpart (16). There was also some controversy in a few publications concerning neuroendocrine differentiation in detected carcinomas using the immunohistochemical technique (16). Therefore, we made an attempt to study these nonendocrine carcinomas plus other malignant diseases using our sensitive, quantitative serum CgA assay. We were wondering whether it was possible to detect elevated serum CgA as an ectopic tumor marker in advanced stage of carcinomas. In this preliminary study we have measured serum CgA mainly in serum from patients with various malignant diseases containing highly elevated tumor markers.

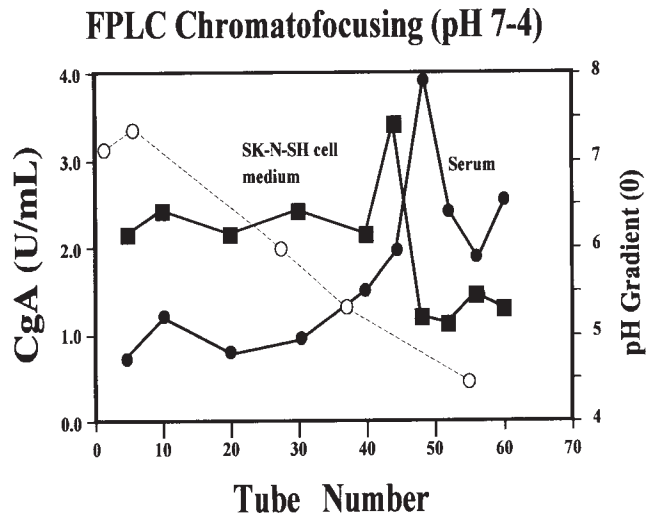


Fig. 3. Elution profile of CgA immunoreactivity by chromatofocusing. Both the cell medium of SK-N-SH cell line (0.2 mL) and serum (0.2 mL) containing highly elevated CgA were subjected to chromatofocusing. Elution buffer was selected to provide a pH gradient of pH 7 to 4. The CgA immunoreactivity elution profile indicated that, although still acidic, the isoelectric point of the CgA molecule of the cell line is slightly higher than the CgA appeared in the serum. A Dako kit was used to determine the CgA concentration in the eluate.

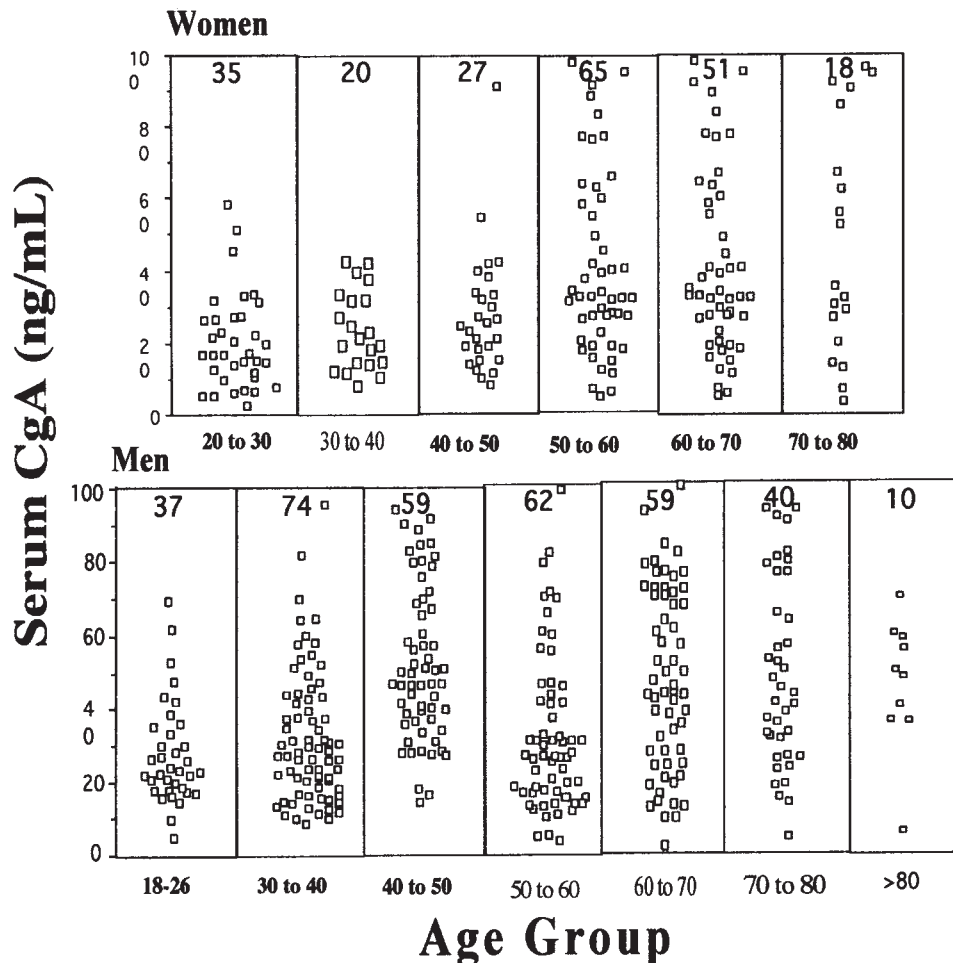


Fig. 4. Concentration of serum CgA determined with in-house assay for normal men and women in different age groups. Numbers at top of each section indicate the number of specimens for each age group.

As shown in Figure 6 the frequency of detecting serum CgA elevation is much higher than we expected. The horizontal dashed lines in the figure correspond to about 90 ng/mL of CgA. Dots above this line relate to elevated serum CgA (assuming most cancer patients are at an older age). Our findings certainly warrant further detailed study.

Effect of Renal Insufficiency and Abnormal Liver Enzymes

Elevated serum CgA was found in patients with renal insufficiency (Fig. 7) and abnormal liver enzymes (Fig. 8). In

TABLE 1. Median and 97.5 percentile of serum CgA for different age groups of women and men

Age group (years of age)	Median (ng/mL)	97.5 percentile (ng/mL)	# of Specimen
Women 20 to 50	20.7	63.9	104
Women 50 to 80	32	93.8	137
Men 18 to 40	27.9	78.4	110
Men 40 to 80	41.6	92	234

patients with renal insufficiency or elevated serum creatinine, the serum CgA concentration could be anywhere from 10- to 100-fold higher than those values detected in normal persons (Fig. 7). The extent of serum CgA elevation found in sera with abnormal liver enzymes, however, was much less than that detected in renal patients. Practically no elevation of serum CgA was detected in sera containing normal liver enzymes.

DISCUSSION

CgA has been known to be a sensitive marker for both neuroblastoma and pheochromocytoma (4). In fact, serum CgA is also useful for the diagnosis and management of patients with small cell lung carcinoma and carcinoid tumors. However, few laboratories offered the serum CgA test and serum CgA has been seldom ordered by clinicians, perhaps having to do with the unavailability of a commercial CgA kit in the United States.

One of the shortcomings of using HPLC for catecholamine quantification has to do with the interference of drugs. For

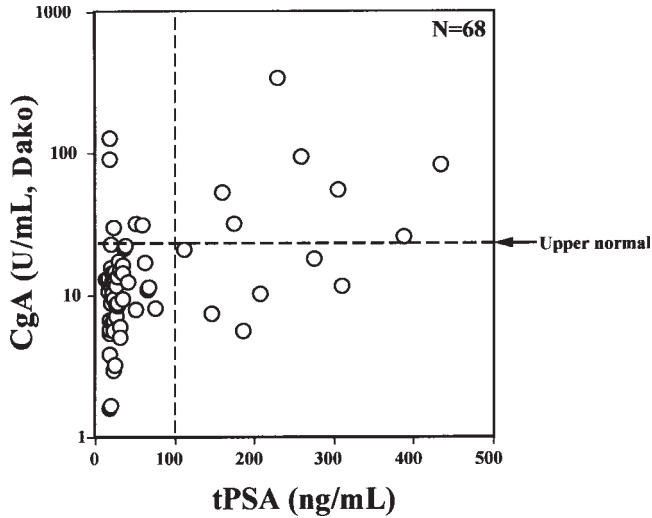


Fig. 5. Levels of serum CgA found in sera from prostate cancer patients containing various concentrations of PSA. All serum specimens were from our clinical laboratory with known PSA values determined by the Hybritech Tandem E immunoassay. The horizontal dotted line is the upper normal limit (18 U/mL) assigned by the Dako assay. The vertical dotted line is the approximate upper normal cutoff for serum CgA levels. An apparently higher percentage of the elevated serum CgA levels was found in specimens containing highly elevated serum PSA levels. The CgA concentration was determined using an CgA enzyme immunoassay kit from Dako.

example, antihypertensive drugs such as labetalol and captopril contained in urine specimens can produce chromatographic peaks interfering or covering the HPLC peaks of epinephrine and norepinephrine, making it impossible to quantify these catecholamines (17). In fact, many metabolites of catecholamine also tend to interfere. In situations when the measurement of catecholamines is used for the diagnosis of pheochromocytoma, measuring serum CgA can become useful; because the CgA test is an immunoassay, it is not subjected to drug interference. Recently, we found that the measurement of serum CgA could also be used to predict whether prostate cancer patients receiving hormonal therapy will develop resistance (11). In one-third of those patients, a slightly elevated serum concentration of CgA will appear several months before the rise of serum PSA levels, serving as an early signal of resistance to hormonal treatment. Since failure in hormonal treatment accounts for the death of a significant number of patients with prostate cancer, the early detection of elevated serum CgA, would allow for early modifications of treatment before it was too late. Drugs used for the treatment of epithelial tumors differ from those recommended for tumors derived from neuroendocrine cell (12). Our findings suggest that prostate cancer patients undergoing hormonal treatment should be routinely monitored by serum CgA, pending further study.

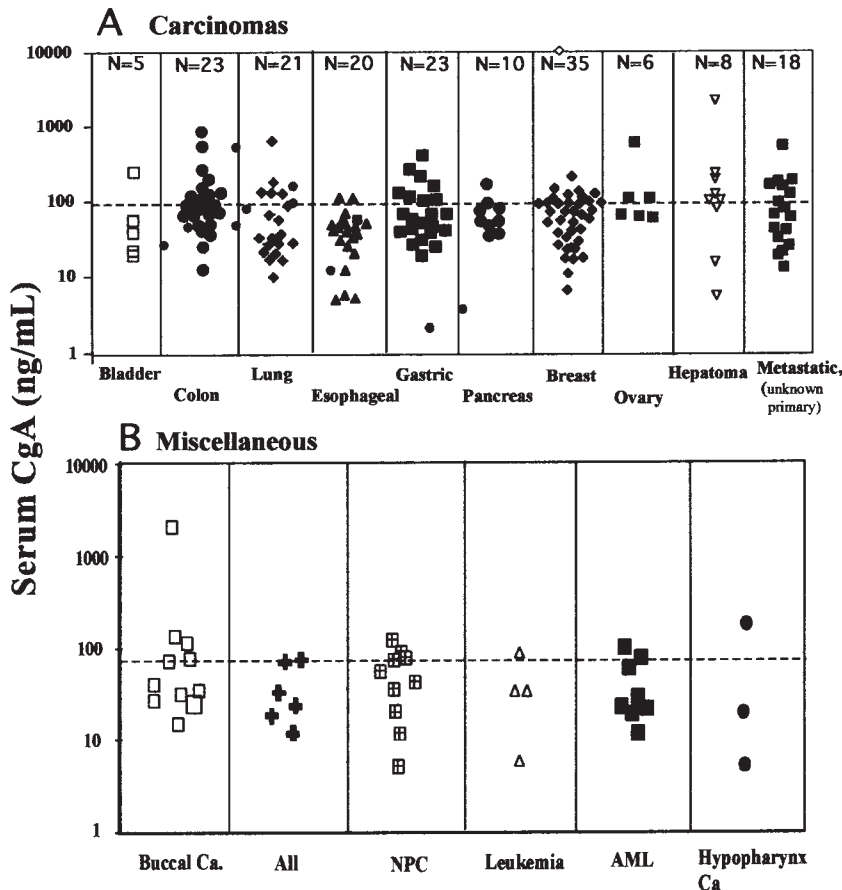


Fig. 6. Detection of elevated concentrations of serum CgA in various malignant diseases including carcinomas. Horizontal dotted lines indicate upper normal cutoffs for serum CgA levels in the older group of men and women.

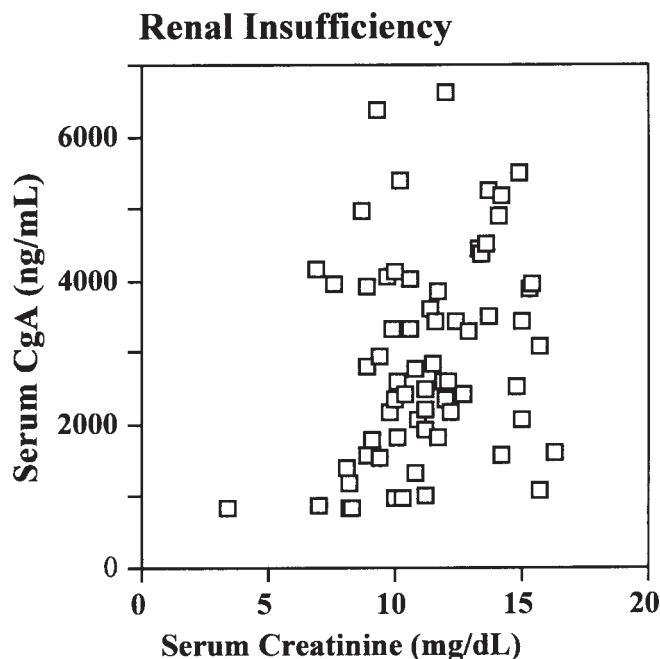


Fig. 7. Detection of highly elevated levels of serum CgA in patients with renal insufficiency. No correlation between the concentration of serum CgA and that of serum creatinine was found.

We felt that it was important to determine the normal reference values of serum CgA for different age groups of women and men because it appears that the normal reference value is age dependent. These normal values should benefit the diagnosis of various neuroendocrine tumors, such as pheochromocytoma, small cell lung carcinoma, and carcinoid tumors, and should also assist the monitoring of carcinoma patients during treatment. The results shown in Figure 4 and Table 1 indicate that separation into four groups is sufficient. Apparently the serum CgA levels are higher in men and at an older age. The lower reference values of serum CgA established for men and women at a younger age provide higher sensitivity for cancer detection, while higher reference values for older people give higher specificity for cancer diagnosis.

To our knowledge detection of CgA in carcinoma tissues other than prostate tissue has rarely been reported regardless of whether the immunoassay or the immunohistochemical technique was used. Our detection of significantly elevated serum CgA in several nonendocrine carcinomas was unexpected. Further studies are warranted. The expression of carcinoembryonic proteins (fetal tumor antigen) and the detection of serum ectopic tumor markers in patients with advanced malignant diseases, such as elevated serum AFP in colon carcinoma and primary gallbladder carcinoma, are well known (18,19). Whether the finding of elevated serum CgA is due to a similar mechanism remains unclear. Neuroendocrine cells are known to have wide-ranging regulatory functions, including the regulation of growth and differentiation,

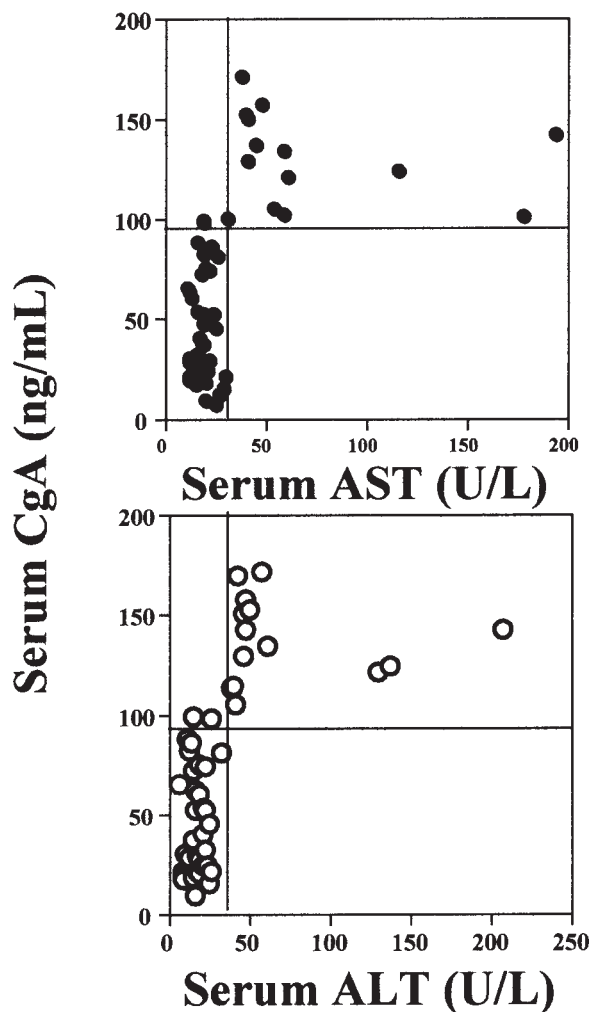


Fig. 8. Elevated levels of CgA detected in serum specimens containing abnormal concentrations of serum AST and ALT liver enzymes.

and exocrine secretory processes (20). The known growth factor activity of the neuroendocrine cell products, an increase in proliferation in cells surrounding neuroendocrine cells, and a lack of androgen receptor expression in neuroendocrine cells suggest mechanisms by which they may be of prognostic significance (5,6).

Detection of elevated serum CgA at advanced stages in carcinomas also has important clinical implications. Carcinomas are the most frequently found cancer. Detection of elevated serum CgA indicates that there is neuroendocrine differentiation and a proliferation of neuroendocrine cells at advanced stages for most carcinomas. Given this fact, treatments should target not only epithelial cells, as most treatments are presently designed to, but also neuroendocrine cells. This is especially the case if the products (neuropeptides such as CgA) of neuroendocrine cells are responsible for promoting the proliferation of epithelial tumor cell growth. It is possible that during the treatment of these types of patients, the

inhibition of neuroendocrine cell growth probably is more critical than the inhibition of epithelial cell growth.

Mezger et al. (21) and O'Connor et al. (3,22) reported before that elevation of serum CgA levels could be found in patients with renal and hepatic failure. Our results not only confirmed their study but also indicated that specimens with serum creatinine > 7 mg/dL may contain close to normal serum CgA levels. We suggest that specimens with elevated serum CgA levels should have their serum creatinine and liver enzymes checked as well. It is important to make certain that the elevated serum CgA detected is not a result of abnormal renal or liver function.

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