Radioimmunoassay for Human Thyroxine-Binding Prealbumin*

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ABSTRACT

A radioimmunoassay (RIA) for human thyroxine-binding prealbumin (PA) is described. It employs highly purified PA, anti-human PA serum at 1:30,000 final dilution, normal bovine serum as a carrier, and polyethylene-glycol to precipitate the immune complexes. This assay is extremely sensitive (limit of detection less than 0.2 μg per dL or less than 3.6 × 10⁻¹⁵ moles per tube), accurate (recovery = 98.7 ± 9 percent, mean ± S.D.) and reproducible (intra- and inter-assay coefficients of variation = 3.6 to 6.3 percent and 7.2 to 9.5 percent, respectively). There was a highly significant correlation when the RIA was compared with radical immunodiffusion or with PA maximal binding capacity for thyroxine (r = 0.944 and r = 0.724, respectively, p < 0.001). Concentration of PA in sera from normal subjects (age range = 20 to 88 years) averaged 27.7 ± 0.5 mg per dL (mean ± S.E.M.), with significantly higher values in males than in females in all age groups with the exception of the older subjects (20 to 50 years: males = 26.5 to 37 mg per dL; females = 23.1 to 33.8 mg per dL). Levels of PA progressively declined after the fifth decade of life. Pregnancy, hyperthyroidism, chronic liver diseases, cystic fibrosis, cancer and

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other non-thyroidal illnesses were associated with decreased levels of serum PA. Untreated hypothyroidism and chronic renal diseases showed widely scattered values of PA. Inherited thyroxine-binding globulin (TBG) abnormalities and bisalbuminemia had no apparent effect on concentrations of serum PA.

Introduction

Studies conducted during the past two decades have shown prealbumin (PA) to be a stable liver-synthesized protein composed of four 127 residue subunits, each with a molecular weight of 14,000. The protein possesses two thyroid hormone binding sites and four independent binding sites for retinol-binding protein (RBP), the specific carrier for vitamin A. Therefore, it is also known as thyroxine-binding prealbumin (TBPA) or transthyretin.

Recent studies have demonstrated:

1. The presence of a deoxyribonucleic acid (DNA) binding site on its outer surface, from which it has been inferred an ancestral connection between nuclear receptors and PA;

2. Sequence homologies with gastrointestinal prohormones, which suggest not only the existence of a super-family of related proteins but also possible evolutionary links with gastrointestinal peptides;

3. The presence of PA in normal human islet alpha cells of the pancreas and markedly elevated levels of serum PA in patients with islet cell carcinomas;

4. A thymic hormone-like activity;

5. The localization of PA ribonucleic acid (mRNA) in specific regions of the brain as well as, although to a lesser extent, in heart, skeletal muscle, stomach, and spleen of the rat, strongly suggesting the de novo synthesis of PA in extra hepatic tissues;

6. The structure of human PA gene and its localization to chromosome 18;

7. A close relationship with hereditary amyloidosis, since the extracellular amyloid deposits consist of an abnormal PA molecule with a methionine for valine substitution at position 30. The association of PA with another inherited disorder (alpha-1-antitrypsin deficiency) is also probable.

Believing that PA might play currently unknown roles in the economy of the body, we are interested in elucidating aspects of metabolism of PA in humans that so far are uninvestigated. To this end, the first step was the development of a sensitive radioimmunoassay (RIA) for human PA. To date, measurement of human PA concentration has been carried out by (1) determination of maximal binding capacity of PA for thyroxine (TBPAcap), (2) radial immunodiffusion, (3) radial diffusion on acetate strips sprayed with diluted antiserum, (4) densitometry after polyacrylamide, or (5) starch gel electrophoresis. Measurement of TBPAcap is subject to experimental artifacts, owing to pH and buffer effects. All of the other techniques are cumbersome, time-consuming, and unsuitable to detect extremely low plasma concentrations of PA. Furthermore, it is not possible with these methods to assay many samples in the same run.

In the present study, a radioimmunoassay for human PA is validated and the data obtained in healthy individuals and patients compared with those reported by others employing different methods.

Materials and Methods

Buffer

Unless otherwise stated, the buffer was 0.05 M barbital buffer, pH 8.6, containing one percent bovine serum albu-
min (BSA) and 1:100 normal bovine serum (NBS).

ANTIGEN

Highly (98 percent) purified and RBP-free human PA was obtained.* The lyophilized material was reconstituted in 0.06 M Tris-HCl buffer pH 8.6, stored in aliquots at −20°C, and thawed immediately before use.

ANTIBODY

Two antisera raised in rabbits were tested. The first† had a titer of 0.28 g per L; the second one‡ had a titer of 0.175 g per L. In the routine assay, the latter serum was used at a final dilution of 1:30,000, which gave a 20 percent binding in the absence of unlabeled PA.

IODINATION OF PA

Prealbumin was labeled using the lactoperoxidase method.37 Following two min incubation at 4°C and 60 min incubation at −20°C, the reaction was stopped by dilution with barbital buffer. Iodide was separated from iodinated protein by a Sephadex G-25 gel-filtration column (0.5 × 10 cm) using barbital buffer for elution and the protein peak used as labeled antigen in the RIA. The 125I-PA electrophoretic mobility, as analyzed by polyacrylamide gel electrophoresis, was found to be the same as that of unlabeled PA (data not shown). The efficiency of PA iodination was approximately 20 percent. Specific activity ranged 10 to 14 μCi per μg protein. Storage at −20°C was chosen since deiodination occurred more quickly at 4°C.

RIA PROCEDURE

Standard or sample (100 μl), diluted antiserum (100 μl) and labeled PA (100 μl; about 15,000 cpm) were incubated in duplicate with 100 μl normal bovine serum as carrier. After overnight incubation (18 to 24 hours) at room temperature, one ml of an 8 percent polyethylene glycol (PEG) 6,000 solution was added to separate bound from free antigen. Tubes were centrifuged at 2,000 × g for 20 min at 4°C and radioactivity was counted in the pellet. Serum dilutions (1:10,000 to 1:50,000) were selected so that the percent B/B₀ ratios fell in the central portion of the standard curve. There was no difference between serum dilution with assay buffer or deionized water. As reference serum, human serum* with a certified mean value of 24.5 mg per dL of PA (radial immunodiffusion) was used.

COLLECTION OF SERUM SAMPLES

The study group is illustrated in table I. Details on patients with inherited thyroxine-binding globulin (TBG) abnormalities, bisalbuminemia, and cystic fibrosis have been given elsewhere.2,3,4,5,12 Chronic liver diseases were proven by biopsy in each patient. Etiology of renal diseases was unclear in most of the patients. Many patients were on hemodialysis and samples for assay of PA were obtained prior to dialysis. Patients with cancer included 32 in-patients and 64 out-patients. The primary tumor sites included pituitary, breast, esophagus, stomach, colon-rectum, liver, pancreas, thyroid, lung, and pleura. Also examined

* Lot #101169, Behringwerke, Mahrburg/Lahn, West Germany.
† Lot #103015A, Behringwerke.
‡ Lot #108F, Dako Industries, Denmark.
* Lot #047099Q, Behringwerke.
TABLE I

Characteristics of the Study Group

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. of Subjects</th>
<th>Age (yr) Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Males</td>
<td>Females</td>
</tr>
<tr>
<td>Normals</td>
<td>35</td>
<td>50</td>
</tr>
<tr>
<td>Pregnancy*</td>
<td>-</td>
<td>31</td>
</tr>
<tr>
<td>Inherited TBGf abnormalities</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>Hyperthyroidism§</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>Hypothyroidism§</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>Chronic liver diseases:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Active hepatitis</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>2. Cirrhosis</td>
<td>12</td>
<td>18</td>
</tr>
<tr>
<td>Chronic renal diseases</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>Cystic fibrosis</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Cancer</td>
<td>26</td>
<td>70</td>
</tr>
<tr>
<td>Acromegaly</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Other nonthyroidal illnesses§</td>
<td>14</td>
<td>19</td>
</tr>
</tbody>
</table>

*Weeks of pregnancy ranged from 21 to 40.
†Thyroxine-binding globulin.
‡Toxic diffuse goiter (n = 8) and toxic nodular goiter (n = 6).
§Hashimoto's thyroiditis (n = 4), idiopathic myxedema (n = 5) and endemic cretinism (n = 1).
¶Ulcerative colitis (n = 7), myocardial infarction (n = 5), completed stroke (n = 12) and fever (n = 7).

were non-Hodgkin's lymphomas (stage III to IV), chronic myeloid leukemia, and polycythemia. In order to compare different methods to a measure of PA, concentrations of PA in a number of healthy individuals and patients were also determined by radial immunodiffusion using Behringwerke plates or by TBPAcap14 using 0.1 M Tris-HCl, pH 8.6.

Results

PERFORMANCES OF THE PA RADIOIMMUNOASSAY

In figure 1, is shown a typical standard curve, with the central portion between 0.39 and 1.56 µg per dL (390 to 1,560 pg per tube) of unlabeled PA and a sensitivity of less than 0.2 µg per dL (<3.6 × 10−15 moles per tube). Use of the Behringwerke antiserum resulted in a slight loss of sensitivity. Accuracy was tested by the parallelism and recovery tests. The excellent parallelism \[ Y = 0.44 + (0.49 \times); r = 0.9996 \] indicates that no interference was present under the described conditions. The parallelism between the standard curve and that of one serum sample suggests immunological identity of standard and endogenous PA (figure 1). The mean recovery was 98.7 ± 3.9 percent (mean ± S.D.).

Some cross-reactivity was observed with human serum albumin (HSA) at 2.5 and 5.0 g per dL (table II). This interference was negligible, since an extensive dilution (1:10,000 or more) of the unknown serum was used because of the high assay sensitivity. Such cross-reactivity depended upon the presence in the tracer of minimal amounts (about two percent) of 125I-HSA. In fact, when excess anti-HSA serum was used in the assay instead of the anti-PA serum, 2.5 to 3.9 percent of the total radioactivity was precipitated.

The reference serum and a serum containing 6 mg per dL of PA were routinely

![Figure 1. Standard curve of human prealbumin (circles) and its parallelism with the displacing curve obtained by diluting one serum sample (asterisks).](image)
TABLE II
Crossreactivity of Different Substances in Prealbumin-Radioimmunoassay

<table>
<thead>
<tr>
<th>Substance</th>
<th>µg/Tube</th>
<th>B/B₀</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human serum albumin</td>
<td>0.01 to 15</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>2.500</td>
<td>68</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.000</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>4 to 500</td>
<td>103</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2,500</td>
<td>102</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5,000</td>
<td>107</td>
<td></td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>15</td>
<td>107</td>
<td></td>
</tr>
<tr>
<td>Human TBG*</td>
<td>6</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Human thyroglobulin</td>
<td>0.005 to 5</td>
<td>105</td>
<td></td>
</tr>
<tr>
<td>Tissue polypeptide antigen</td>
<td>0.000003 to 0.03 units</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Thyroxine</td>
<td>0.00004</td>
<td>99</td>
<td></td>
</tr>
<tr>
<td>Triiodothyronine</td>
<td>0.00002</td>
<td>101</td>
<td></td>
</tr>
<tr>
<td>NBS†</td>
<td>(1:11)</td>
<td>105</td>
<td></td>
</tr>
<tr>
<td>NRS‡</td>
<td>(1:1)</td>
<td>104</td>
<td></td>
</tr>
<tr>
<td>Human prealbumin</td>
<td>0.000012</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

*Thyroxine-binding globulin
†Normal bovine serum
‡Normal rabbit serum

assayed in 24 different sets of assays to evaluate precision. The inter-assay coefficient of variation (C.V.) was 7.2 percent for normal levels of PA and 9.5 percent for low levels of PA. The corresponding intra-assay C.V. (16 replicates) were 3.6 and 6.3 percent. A good correlation between RIA and radial immunodiffusion (r = 0.944, p < 0.001) and between RIA and TBPA<sub>cap</sub> (r = 0.724, p < 0.001) was obtained.

Clinical Validation of the Assay (Figure 2)

NORMAL SUBJECTS AND THYROID DISEASES

Mean concentrations of serum PA in 85 healthy subjects aged 20 to 85 years were 27.7 ± 0.5 mg per dL (mean ± S.E.M.; range = 16 to 37). In table III are illustrated the small variations in levels of PA which were observed during adulthood. Levels of PA progressively decreased after the fifth decade of life and were 30 percent lower in those 70 years or older as compared to those aged 30 to 50 years. There were slight to marked differences between males and females aged 20 to 60 years (p = 0.10 to p < 0.02; analysis of variance) as well as between pregnant and nonpregnant women aged 20 to 40 years (20.8 ± 0.6 vs 29.3 ± 0.7 mg per dL, p < 0.001).

Levels of serum PA in two females with bisalbuminemia (21.8 and 26.1 mg per dL) and in nine subjects with TBC abnormalities (26.7 ± 1.3 mg per dL) fell within the range assessed in age and sex-matched controls (table III). Mean values of PA in untreated hyperthyroidism were 14.8 ± 0.7 mg per dL, which is significantly lower (p < 0.001) than in euthyroid controls. Values measured in the hypothyroid patients (24.4 ± 1.5 mg per dL) were not different.

NONTHYROIDAL ILLNESSES

As in hypothyroid subjects, levels of serum PA ranged widely (10 to 30 mg per dL; 22.6 ± 6.0, mean ± S.E.M.) in patients with chronic renal insufficiency. Markedly subnormal values of PA (<15 mg per dL) were detected in the two patients with proteinuria (nephrotic syndrome). Concentrations of PA in seven patients with chronic active hepatitis overlapped those found in 30 patients with liver cirrhosis (12.4 ± 0.7 and 10.4 ± 0.7 mg per dL, respectively). In those with liver cirrhosis, the lowest values were observed in the more seriously ill patients with hepatic encephalopathy, ascites, bleeding varices, and signs of disseminated intravascular coagulation. Circulating PA in infants and children with cystic fibrosis averaged 16.7 ± 1.4 mg per dL, a value significantly lower (p < 0.01) than that measured in 26 age and sex-matched controls. Mean serum PA in the 96 cancer patients was 16.8 ± 0.6 mg per
dL (p < 0.001 vs normal controls), with levels in the 32 in-patients significantly lower than in the 64 out-patients (14.0 ± 0.9 vs 18.2 ± 0.8 mg per dL, p < 0.001). Among the former, the presence of one or more metastases was associated with a remarkably lower circulating PA in comparison to the metastases-free group (10.3 ± 0.8 vs 15.8 ± 0.7 mg per dL, p < 0.01). Moreover, in the outpatient group, 19 individuals on chemotherapy had levels of serum PA significantly higher than those found in 45 patients sampled during the first visit or long after withdrawal of therapy (23.8 ± 1.3 vs 15.8 ± 0.7 mg per dL, p < 0.001). Four patients with well-differentiated pancreatic carcinoma and two patients with hepatoma had markedly reduced (below 16 mg per dL) levels of PA, while a supranormal concentration of PA (39.5 mg per dL) was detected in one out of three acromegalic patients. A fall in blood PA was observed in patients with other non-thyroidal illnesses, such as stroke (14.2 ± 2.8 mg per dL), ulcerative colitis (16.0 ± 1.9 mg per dL), fever (14.1 ± 1.0 mg per dL), and myocardial infarction (17.7 ± 2.1 mg per dL).

**Discussion**

This paper describes an accurate, precise, and remarkably specific RIA for human PA. Radioimmunoassay is the most sensitive method so far available to
detect minimal amounts of a given substance in any biological fluid. As expected, sensitivity of our PA-RIA was very high (<0.2 μg per dL or 1.1 to 3.5 mg per dL of other techniques). This will enable the present authors to undertake studies aimed at elucidating possible functions of human PA other than those of a simple carrier protein.

In agreement with previous results obtained by measuring TBPA\textsubscript{cap}, sex and age effects on circulating PA were observed using RIA. It progressively decreased from adulthood to the elderly; in males it was higher than in females, especially during pregnancy. Immunoassayable PA remains superimposable in both sexes from birth to nine years; sex discrepancies become evident only after the onset of puberty. These changes, therefore, can be attributed to the opposite effects on concentration (and synthesis) of serum PA exerted by androgens (increase) and estrogens (decrease). The mean values and the range of healthy subjects are consistent with those previously reported using other techniques.

Investigation of subjects with inherited TBG abnormalities is noteworthy since low TBPA\textsubscript{cap} values were found in subjects with TBG excess, while high TBPA\textsubscript{cap} values were associated with TBG deficiency. Our data do not confirm these differences. Concentrations of serum PA were normal in either variety of TBG abnormality as well as in inherited bisalbuminemia, which supports the view of an independent synthesis of PA with respect to the other major thyroid hormone binding proteins (TBG and HSA).

As to changes of PA in thyroid diseases, different studies have shown a reduction of PA in thyrotoxic patients, but normal or slightly elevated TBPA\textsubscript{cap} values are shown in hypothyroid patients. Our results confirm that immunoassayable concentrations of serum PA are decreased in hyperthyroidism and normal, with some scattering, in hypothyroidism.

The clinical usefulness of the determination of PA, however, is seen in other pathological conditions. A wide spectrum of stressful conditions, such as traumas, burns, surgery, malnutrition, inflammatory and neoplastic processes, are associated with a drop of PA in blood. Under these and any other circumstances characterized by debilitation coexisting or not with fever, plasma con-
centrations of a group of proteins named acute phase reactants (APR) (C-reactive protein, alpha-1-acid glycoprotein, ceruloplasmin, etc.) become abnormally high. This preferential synthesis of APR occurs at the expense of PA and other proteins of definite physiological significance (HSA, transferrin). Such a shift in the protein synthesis apparently has the significance of an adaptative response to the stress. In fact, a drop in the level of PA will produce an increase in free thyroid hormone and RBP availability to the tissues. It is likely that uncomplexed thyroid hormones and RBP may be necessary for the appropriate cell response to stress.

As stressed in the material following, there is clear-cut evidence concerning the value of PA as a reliable indicator of the clinical condition of severely ill patients. Prealbumin is the first plasma protein to decline in response to protein deprivation, allowing the detection of subtle forms of malnutrition. Different studies have demonstrated that PA is the most sensitive indicator of hepatic malfunction, when measured against more than 20 other serum proteins. Other studies found that persistently depressed levels of PA in patients with ulcerative colitis indicate the need for surgical treatment. Moreover, PA has been claimed as a useful index of cancer burden and a sensitive test for predicting impending death in patients with cancer of the colon. Our data are in agreement with these concepts. In particular, the patients with extremely severe derangement of liver function (hepatic encephalopathy) exhibited the lowest levels of serum PA observed in our study group—a fact not surprising given that the liver is the site of synthesis of PA. Instead, the unequivocally low concentrations of PA coexisting with normal or supranormal TBG levels (48 μg per ml in one patient) may appear unexpectedly in patients with hepatomas, since both of the thyroid hormone binding proteins are synthesized in the liver. Increased levels of serum TBG in such a malignancy have been previously reported, however, parallel measurement of both TBG and PA in such patients is something of which the present authors are unaware. The pathogenesis and the biological significance, if any, of this discrepancy probably deserves further attention.

In contrast, our results on cystic fibrosis and well-differentiated pancreatic adenocarcinomas, taken together with the finding of excess of serum PA in patients with islet cell carcinomas, indicate that pancreatic secretion of PA into circulation is associated with a neoplastic lesion at the islet level and not with a lesion (even malignant) of the exocrine portion of the pancreas. It is likely that the existence of a physiological islet cell secretion of the protein may account for the so far unreported cases of the deficiency of PA in the severe liver dysfunction.

The discrimination operated by radioimmunoassayable PA between patients with and without metastases as well as between treated and untreated cancer patients demonstrates that PA reflects well the severity of the illness. Determination of PA in serum is, therefore, recommended to monitor the clinical recovery (or the deterioration in the general conditions) of cancer patients. The supranormal levels of PA in one acromegalic patient confirms the unique observation of frequently enhanced TBPA values in GH-secreting pituitary tumors, probably as a consequence of an augmented androgen secretion.

The observation of reduced levels of PA in patients with stroke seems of particular interest because admission concentrations of PA in the five patients who died were 50 percent lower than in the seven who survived (9.0 ± 2.0 vs. 18.4 ± 1.8 mg per dL). No information was so
far available on changes of PA in cerebrovascular accidents. Should these differences be confirmed, PA might also be of value in this illness. Renal failure per se, instead, appears not to affect appreciably the metabolism of PA unless proteinuria is present.

In conclusion, the development of a RIA for human PA has been reported. Although it was not our aim to set up a RIA for the routine determination of circulating PA, this method was applied to the measurement of serum PA in physiological and pathological conditions to validate the method. The results obtained were generally in agreement with those reported using other methods. Our study shows that radioimmunoassayable PA is sex and age-dependent in healthy persons, definitely reduced in thyroid overactivity, widely variable in thyroid failure, and normal in the inherited abnormalities of TBG and HSA. Most importantly, this study stresses how serum PA can be a useful prognostic index in nonthyroid illnesses, such as chronic liver diseases, cancer, and stroke.

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References


