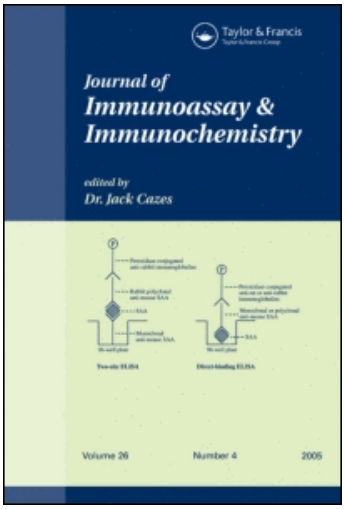

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Hiroyuki Yokoi[a]; Takao Saitoh[a]; Yukiko Nakazawa[a]; Hideto Ohno[a]
[a] Research & Development Center, Yuka Medias Co., Ltd., Ibaraki, Japan

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AN IMMUNORADIOMETRIC ASSAY FOR THYMOSIN α 1

Hiroyuki Yokoi, Takao Saitoh, Yukiko Nakazawa and Hideto Ohno
Yuka Medias Co., Ltd. Research & Development Center
8-5-1 Chuo Ami, Inashiki Ibaraki 300-03, Japan

ABSTRACT

An Immunoradiometric assay for thymosin α 1(1-28) was developed using a monoclonal and a polyclonal antiserum which were raised against synthetic thymosin α 1(1-28) and thymosin α 1(16-28), respectively. A monoclonal antibody, specific for the (acetylated ser1) 1-5 sequence of thymosin α 1, was immobilized on polystyrene beads for the solid phase, and a polyclonal antiserum specific for the 16-28 sequence was employed. This method relies on the formation of an immune complex consisting of a ^{125}I-labelled anti-rabbit IgG goat antibody, polyclonal antiserum, thymosin α 1, and the solid phase monoclonal antibody. Radioactivity on the solid phase is directly proportional to the amount of thymosin α 1 present in the specimen. The minimal detection limit of this assay system was approximately 1.9 pg/ml. The mean values of thymosin α 1 in plasma of healthy subject, ranging in age from 0 to 3 years was approximately five fold higher than that of higher ages. HPLC analysis of plasma of a healthy subjects revealed a single immunoreactive form which eluted with the same retention time as that of synthetic thymosin α 1. This assay will be extremely useful for the measurement of thymosin α 1 in biologic fluids and tissues.

(KEY WORDS: Immunoradiometric assay, Thymosin α 1)

INTRODUCTION

Thymosin α 1 is a small acidic peptide consisting of 28 amino acid residues with a molecular weight of 3108 (1,2). Thymosin α 1, purified from calf thymus tissue, has an acetylated amino terminus. Its role as a thymic hormone was postulated on the basis of its *in vitro* and *in vivo* immunomodulatory activity and its presence in the plasma as defined by radioimmunoassay or enzyme linked immunosorbent assay (3-8). The levels of thymosin α 1 diminish with age in human tissues with the sharpest decrease in the first 10 years of life (3,8).

The immunoradiometric assay(IRMA) has been successfully used for the measurement of several circulating polypeptide hormones (9,10). The theoretical advantage of this approach includes both enhanced sensitivity and specificity compared to the conventional radioimmunoassay. The present report described the development of a sensitive and reliable IRMA for the measurement of plasma thymosin α 1. This assay system depends upon the formation of an immune complex of ^{125}I-labelled anti-rabbit IgG goat antibody, polyclonal antiserum specific for the carboxyl-terminal of thymosin α 1, thymosin α 1, and the solid phase monoclonal antibody specific for the amino-terminal of thymosin α 1. Using this assay system, plasma thymosin α 1 levels in healthy subjects, ranging in age from 0 to 90 years were measured.

MATERIALS AND METHODS

Materials

Polystyrene beads were obtained from Immunochemical Co.(Hiroshima, Japan). Anti-rabbit IgG goat antibody was obtained from DAKO Co.(Carpinteria, CA, U.S.A.), Sep-

Pak C18 cartridges were obtained from Waters Associates(New Bedford, MA, U.S.A.). HPLC columns, TSK-gel ODS 120-T were obtained from Tosho Co.(Tokyo, Japan). Protein A-Sepharose CL-4B, activated CH-Sepharose and Superose 12 column were obtained from Pharmacia Fine Chemicals(Uppala, Sweden). All reagents were of analytical grade.

Peptide synthesis

Thymosin α 1(1-28), (Tyr^{29})-thymosin α 1(1-28), thymosin α 1(1-15), thymosin α 1(16-28), (Cys^{15})-thymosin α 1(16-28) and desacetyl-thymosin α 1(1-28) were synthesized by solid-phase technology using a peptide synthesizer(Applied Biosystems Inc, type 510, CA, U.S.A.). After cleaving from the resin, each crude peptide was purified by HPLC on reverse-phase octadecyl-silica. Peptide concentrations and amino acid compositions were determined by hydrolysis in 6 N HCl for 24 hours at 110 ℃ followed by amino acid analysis on the Hitachi L-8500 analyzer(Tokyo, Japan). The purified peptides gave the expected amino acid ratios and were homogenous by HPLC chromatography.

Production of monoclonal antibody

Thymosin α 1(1-28)-BSA conjugate in Freund's adjuvant was injected subcutaneously into BALB/C female mice. Subsequent intraperitoneal injections of the conjugate with Freund's complete adjuvant were done at 2 week intervals. Intravenous immunizations were given to mice with the highest anti-thymosin α 1 titer and 3 days later spleens were taken for the cell fusion. Spleen cells were fused to P3U1 mouse myeloma cells according

to standard techniques (11). All wells containing hybrids were screened for antibody to thymosin α1. The screening was performed by RIA(see MATERIALS AND METHODS). Positive cells were cloned twice in a limiting dilution system and the cloned cells were inoculated into peritoneal cavities of pristane treated BALB/C mice. Monoclonal antibody(MTH33G2) from ascites fluid was used in this study.

Production of polyclonal antibody

(Cys^{15})-thymosin α1(16-28)-bovine thyroglobulin conjugate(2.0 mg thymosin α1 / rabbit, dissolved in 1.0 ml of saline) was emulsified with Freund's complete adjuvant and the emulsion was injected intracutaneously at multiple sites on 5 New Zealand White rabbits. Immunization was repeated every 2 weeks using 1.0 mg thymosin α1. Rabbits were bled 14 days after the 10th immunization. Immunizations were repeated until a sufficient titer was detected by RIA (see MATERIALS AND METHODS). After 4 months, production of antibody to thymosin α1 was detected in all of the immunized rabbits. Antiserum(MCR0577) was used in the present study. The antiserum was purified by affinity chromatography using thymosin α1(1-28) coupled to activated CH-Sepharose according to the manufacturer's instructions. Purified polyclonal antibody was used in this study.

Characterization of monoclonal and polyclonal antibody

Monoclonal and polyclonal antibodies used in this assay were characterized by RIA using synthetic thymosin α1(1-28), thymosin α1(1-15), thymosin α1(16-28) and

desacetyl-thymosin α 1(1-28). The RIA procedure used was as follows: to each incubation tube, Dulbecco's PBS(0.1 ml), antiserum or ascites supernatant(0.1 ml), synthetic thymosin α 1(1-28) or thymosin α 1 fragments(0.1 ml) and ^{125}I-(Tyr^{29})-thymosin α 1(1-28)(0.05 ml)(10,000 cpm) were mixed. The mixture was incubated for 20 hours at 4℃. Diluted goat anti-rabbit γ -globulin serum(0.1 ml), 20% polyethylene glycol(PEG 6000)(0.2 ml) and diluted normal rabbit serum(0.1 ml) were added. In the case of monoclonal antibody, diluted goat anti-mouse γ -globulin serum(0.1 ml) and diluted mouse serum(0.1 ml) were used. After incubation for 30 min at 4℃, the mixture was centrifuged at 3,000 rpm for 20 min at 4℃. The supernatant was aspirated, and the radioactivity of the precipitate was counted in a gamma-counter.

Preparation of monoclonal antibody immobilized on polystyrene beads

Monoclonal antibody MTH33G2 in ascites fluid was purified on a protein A-Sepharose CL-4B and then immobilized on polystyrene beads. The monoclonal antibody(750 μg) dissolved in 150 ml of potassium phosphate buffer, pH 7.2(0.05 M) was mixed with 500 polystyrene beads(6.35 mm diameter). After 20 hours at room temperature, the buffer was aspirated and the beads were washed with potassium phosphate buffer, pH 7.2(0.05 M). The beads were dried and stored at 4℃.

Iodination of anti-rabbit IgG goat antibody

Prior to iodination, anti-rabbit IgG goat antibody was purified by a Superose 12 column(10x300 mm). The column was eluted with potassium phosphate buffer, pH 7.5(0.1

M). The purified antibody was iodinated by the Chloramine-T method (11) and the resulting labelled antibody was applied to a Superose 12 column(10x300 mm), and eluted with Tris-HCl buffer, pH8.5(0.05 M).

Collection and extraction of plasma

Blood samples were collected into ice-chilled tubes containing EDTA(4 mmol/L). Samples were centrifuged immediately and plasma was stored at -40℃. Plasma samples were obtained from 137 healthy subjects without evidence of endocrine disease, ranging from 0 to 90 years old.

Plasma(1.0 ml) of healthy subjects was diluted 2-fold with HCl(0.1 M), and applied to Sep-Pak C18 cartridges which were then washed with 4%(v/v) acetic acid(10 ml), followed by elution with a mixture of H_2O and acetonitrile(75:25 v/v, 2.5 ml). The organic solvent was evaporated under a stream of nitrogen and the remaining aqueous solution was lyophilized. Lyophilized samples were reconstituted for assay with potassium phosphate buffer pH 7.2(0.1 M), containing 1.0 mg/ml BSA, 1.0 mg/ml Tween20 and 0.5 mg/ml sodium azide.

Immunoradiometric assay of thymosin α 1

IRMA was designed to produce an immune complex of ^{125}I-labelled anti-rabbit IgG goat antibody, polyclonal antiserum, thymosin α 1, and the solid phase monoclonal antibody. The standard diluent buffer was potassium phosphate buffer, pH 7.2(0.1 M) containing 1.0 mg/ml BSA, 1.0 mg/ml Tween 20 and 0.5mg/ml sodium azide. For dilution

of ^{125}I-labelled anti-rabbit IgG goat antibody, a potassium phosphate buffer, pH 7.2(0.1 M) containing 20 mg/ml BSA, 0.5 mg/ml bovine γ -globulin, 1.0 mg/ml Tween 20 and 0.5 mg/ml sodium azide, was used.

The assay was carried out in polystyrene tubes(12x75 mm). In each incubation tube, the above described potassium phosphate buffer(0.05 ml), synthetic thymosin α 1(1-28) or extracted plasma sample(0.2 ml), the antiserum diluted 1/2500 in standard diluent buffer(0.05 ml) and the solid phase monoclonal antibody(1 bead) were mixed. After incubation for 20 hours at 4℃, the solid phase was washed 3 times with distilled H_2O(2.0 ml) and ^{125}I-labelled anti-rabbit IgG goat antibody(0.2 ml)(approximately 200,000 cpm) was added. The mixture was then incubated for 20 hours at 4℃. The solid phase was washed 3 times with distilled H_2O(2.0 ml). Radioactivity of the solid phase was counted in a gamma-counter.

RESULT

Synthesis of thymosin α 1 and its fragments

Synthesis of thymosin α 1 and its fragments were of high purity as judged by various analytical criteria.

Production and characterization of antibodies and IRMA

With the use of synthetic thymosin α 1, we produced monoclonal and polyclonal antibodies to thymosin α 1 which were indispensable for the development of the IRMA. In the present study, a conjugate of synthetic thymosin α 1(1-28) with BSA was used as

immunogen for producing monoclonal antibody, and a conjugate of synthetic (Cys^{15})-thymosin α 1(16-28) with porcine thyroglobulin was used as immunogen for producing polyclonal antibody. The cross-reactivities of both the monoclonal antibody(MTH33G2) and polyclonal antibody(MCR0577) were shown in Fig. 1. The monoclonal antibody was shown to be N-terminal specific, since a synthetic fragment thymosin α 1(1-15), cross-reacted identically to that thymosin α 1(1-28).

The monoclonal antibody failed to detect both thymosin α 1(16-28) and deacetyl-thymosin α 1(1-28). It was found that the monoclonal antibody recognize mainly the amino terminal including residues acetylated Ser1 of thymosin α 1. The absence of this residue would account for the lack of cross-reactivity of thymosin α 1 in the RIA. On the other hand, polyclonal antibody was found to recognize C-terminal sequence, since a synthetic fragment thymosin α 1(16-28), when compared with that of thymosin α 1(1-28). These data indicate that the antibodies are specific for different regions of thymosin α 1(1-28) and they are useful for the development of IRMA.

The standard curve for thymosin α 1 is shown in Fig. 2. A useful operation range was between 2.5 and 200 pg/ml. The detection limit of the assay, estimated from the interpolated response at 2 SDs above the zero standard, was 1.9 pg/ml. The present IRMA for the measurement of thymosin α 1 in plasma is specific, as indicated by the data in Fig. 2. The assay failed to detect any tested fragments of thymosin α 1(1-15), thymosin α 1(16-28) and deacetyl-thymosin α 1(1-28).

Table 1 shows an analytical recovery of thymosin α 1 from plasma samples. When physiological amounts of thymosin α 1 were added to plasma of healthy subjects and

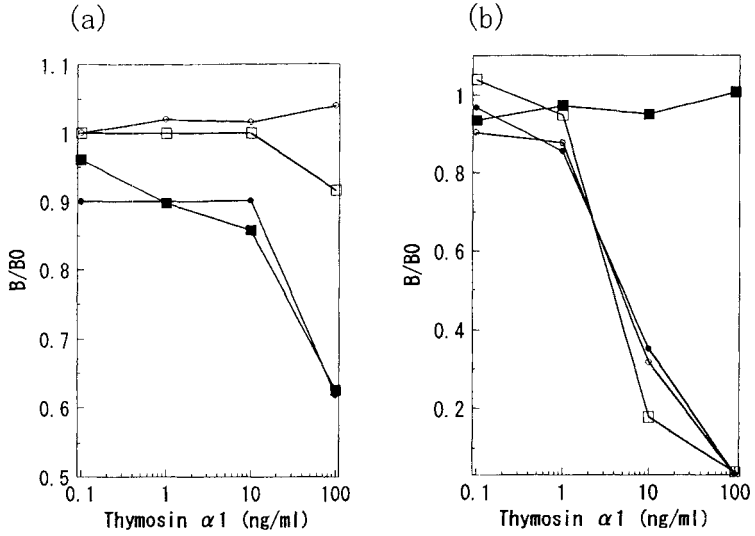

FIGURE 1 Standard curves for the thymosin α 1 RIA with use of monoclonal antibody(MTH33G2) (a) and polyclonal antibody(MCR0577) (b).
RIA was performed as described in the Materials and Methods section using thymosin α1 (1-28) as the peptide standard (●), desacetyl-thymosin α1 (1-28) (○), thymosin α1 (1-15) (■) and thymosin α1 (16-28) (□).

carried through the standard extraction and assay procedure, the recovery was virtually complete regardless of the amount of thymosin α 1 added. In addition, the intra- and interassay coefficients of variance (Table 2) were in an acceptance range, with the intraassay coefficient of variance below 2.9 % and the interassay coefficient of variance below 3.7 %.

Fig. 3 shows reverse phase HPLC separation of plasma of healthy subjects. Each fraction was assayed for thymosin α 1. A single immunoreactive form coeluted with synthetic thymosin α 1(1-28). The coincidence of the elution position of an immunoreactive material

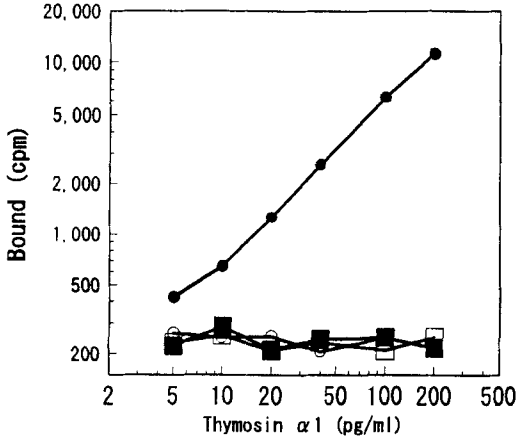

FIGURE 2 Standard curves for the thymosin α 1 IRMA. IRMA using thymosin α 1(1-28)(●), desacetyl-thymosin α 1(1-28) (○), thymosin α 1(1-15) (■) and thymosin α 1(16-28) (□).

TABLE 1

Analytical recovery of thymosin α 1(1-28)

Sample	Added (pg/ml)	Expected (pg/ml)	Measured (pg/ml)	Recovery (%)
1	-	-	2.1	-
2	10	12.1	11.9	98.3
3	50	52.1	53.7	103.1

TABLE 2

Precision of measurments of plasma thymosin α 1(1-28)

Sample	intra-assay variation			inter-assay variation		
	n	Mean±SD	%CV	n	Mean±SD	%CV
A	3	14.3±0.4	2.9	5	14.6±0.5	3.5
B	3	29.7±0.7	2.3	5	29.4±0.8	3.2
C	3	79.9±2.3	2.8	5	80.8±2.0	3.7

with that of synthetic thymosin α 1(1-28) suggests the presence of thymosin α 1 (1-28) in plasma.

The levels of thymosin α 1 in plasma of healthy subjects, ranging in age from 0 to 90 years old, are shown in Fig. 4. The thymosin α 1 level decreased with age. The concentration of thymosin α 1 in blood was highest in utero(23.3 ± 9.7 pg/ml)(n=3) when compared to the levels of healthy individuals after parturition. The average levels in sera of individuals over 4 years of age was 2.7 ± 0.8 pg/ml.

DISCUSSION

Several RIAs have been reported for measuring thymosin α 1 in human species. None of established RIAs has the sensitivity and specificity required to measure only intact thymosin α 1(1-28). It is well known that the theoretical advantage of IRMA includes both enhanced sensitivity and specificity compared to RIA. The present IRMA procedure was found to be more sensitive than the RIA method, since as little as 2 pg of thymosin α 1 could be detected in the IRMA but RIA using a unique antiserum had reported sensitivities

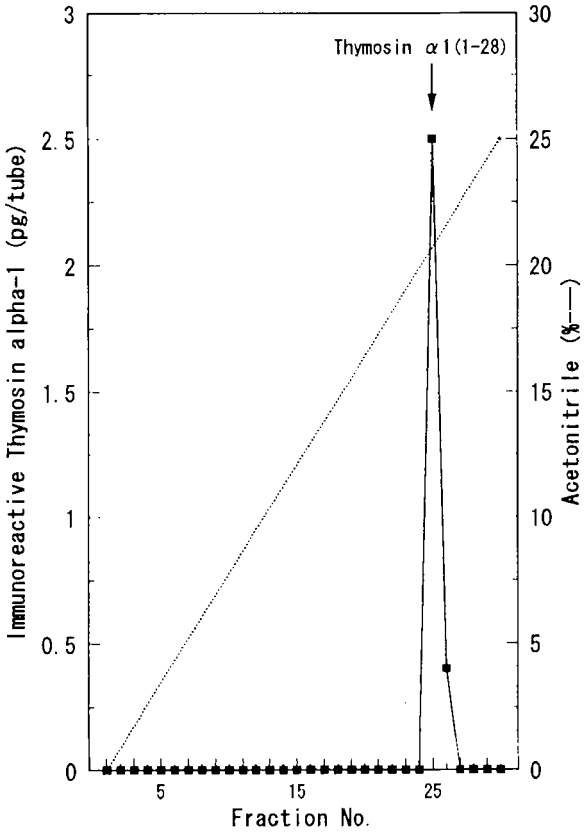

FIGURE 3 Immunoreactive thymosin α 1 in human plasma.

Plasma(1.0ml) of normal subjects was extracted with Sep-Pak C18(MATERIALS AND METHODS) for HPLC analysis. The HPLC column(TSK-gel ODS 120T;4.6mm x 250mm) eluted at a flow rate of 1.0ml/min with solvent B/solvent A (0/100→100/0, v/v, 30min) : solvent A = H_2O : acetonitrile : 10% aqueous TFA = 100 : 0 : 1 (v/v/v) and solvent B = H_2O : acetonitrile : 10% aqueous TFA = 50 : 50 : 1(v/v/v). Fractions(1.0 min intervals) were collected and lyohilized for IRMA. The arrows show the elution positions of the authentic thymosin α 1(1-28).

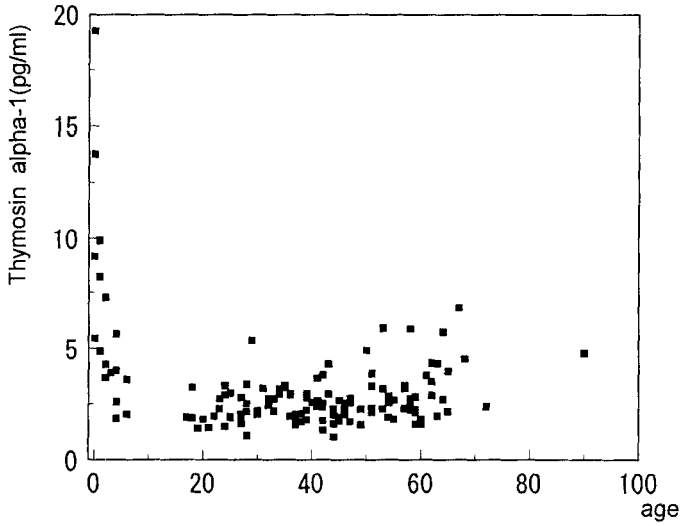

FIGURE 4 Thymosin α 1 levels in plasma of normal subjects, ranging in age from 0 to 90 years old.

of 200 pg/ml (7) and 40 pg/ml with incubation periods of 48-72 hours (12). The sensitivities of our monoclonal and polyclonal antibodies determined by the RIA were 1000 pg and 100 pg/ml, respectively. It is of interest that neither of monoclonal and polyclonal antibodies employed in IRMA were of sufficiently high avidity for a sensitive RIA. However, the choice of optimal antibody combinations allows the establishment of very high sensitive IRMA.

Since we first reported IRMA for thymosin α 1, a number of studies have demonstrated normal and abnormal levels of thymosin α 1 in plasma. Lower levels are often associated with immunodeficiency diseases. Our assay should be sufficiently sensitive for such patient samples since thymosin α 1 concentration of all healthy subjects tested was above the

sensitivity of the IRMA. The lower IRMA values in human blood compared to previous reports by using RIAs may represent limited difference in the specificity between IRMA and RIA.

Thymosin α 1 and its cross-reacting materials have been detected in human peripheral blood and in rat thymus by using RIAs and combining HPLC and RIA analysis demonstrated that the major immunoreactive form in normal serum was thymosin α 1. The level of thymosin α 1 diminish with age in human tissues with the sharpest decrease for prothymosin α in thymus in the first 10 years of life (8). Consistent with previous reports, this study confirms that the major immunoreactive form in human blood is thymosin α 1(1-28) and its concentration is highest in utero, decrease sharply after birth and then remain constant during the life. Naruse et al. reported that the levels of immunoreactive thymosin α 1 in thymoma tissue extracts were relatively higher than those on patients with normal thymuses (13). However, no previous study has described thymosin α 1 levels in plasma of patients with thymoma. Although we are now accumulating additional specimens of patients with thymus related diseases, our preliminary analysis indicate that plasma thymosin α 1 values of patients with thymoma will be >10 pg/ml. Elevated blood levels may correlate well with the function of the thymus. The thymosin α 1 IRMA will allow us to pursue detailed studies of the blood levels in both health and pathologic states.

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