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A possible cause of the variable detectability of macroprolactin by different immunoassay systems

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Abstract

Background: Macroprolactinaemia is a major cause of hyperprolactinaemia. The detectability of macroprolactin varies widely among different immunoassay systems, but the causes are not fully known. This study aimed to identify the factors influencing the detectability of macroprolactin by immunoassay systems.

Methods: The study included 1544 patients who visited an obstetric and gynaecological hospital. Macroprolactinaemia was screened using the polyethylene glycol (PEG) method and confirmed using gel filtration chromatography and the protein G method. The prolactin (PRL) values determined by enzyme immunoassay (EIA) were compared with those of a chemiluminescence immunoassay system (Centaur) that is known to cross-react the least with macroprolactin.

Results: Macroprolactinaemia was found in 62 of 1544 patients (4.02%) who visited an obstetric and gynaecological hospital. The ratio of EIA-determined total PRL to free PRL in the supernatant after PEG precipitation was significantly elevated in all 62 serum samples with macroprolactin compared to those in 1482 serum samples without macroprolactin. In contrast, the ratio of Centaur-determined total PRL to free PRL was significantly elevated in 32 serum samples (group 1) and was within the normal range in 30 (group 2) of 62 serum samples with macroprolactin. The prevalence of non-IgG-type macroprolactin was significantly higher in group 1 than in group 2. Centaur diagnosed hyperprolactinaemia less frequently than EIA (n=2 vs. 16) in 62 patients with macroprolactinaemia. Those two hyperprolactinaemic patients diagnosed by Centaur had non-IgG-type macroprolactin.

Conclusions: Macroprolactinaemia was present in 4% of patients visiting an obstetric and gynaecological hospital. The nature of macroprolactin (IgG-type or non-IgG-type) may partly explain why macroprolactin detectability varies among different immunoassay systems.

Keywords: hyperprolactinaemia; immunoassay; macroprolactin.

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Introduction

PRL in the serum exists in three forms identified by gel filtration chromatography: monomeric PRL (MW 23 kDa), big PRL (MW 50–60 kDa) and big-big PRL (MW >150 kDa). In normal subjects, the proportions of monomeric, big, and big-big PRL components were reportedly 85.8%±2.3%, 9.1%±0.9%, and 5.1%±1.7%, respectively, and big PRL was shown to be a PRL dimer [1]. Macroprolactinaemia is a condition in which big-big PRL is substantially increased in the serum, often causing hyperprolactinaemia [2–9]. The prevalence of macroprolactinaemia is reportedly 10%–26% in patients with hyperprolactinaemia [2–6] and 3.68% in healthy hospital workers [9]. The aetiologies of macroprolactin are heterogeneous, but macroprolactin is primarily a complex of PRL with immunoglobulin (Ig)-G, particularly with anti-PRL autoantibodies [9–16]. Serum PRL determination is a key laboratory test for the differential diagnosis of obstetric and gynaecological symptoms, such as irregular menses and infertility. Correct diagnosis of the causes of hyperprolactinaemia is essential for the successful treatment of such patients. Overlooking macroprolactinaemia may lead to the misdiagnosis and mistreatment of patients with hyperprolactinaemia.

The detection of macroprolactin depends on the characteristics of the reagent antibodies in immunoassay systems. There are several PRL immunoassays that cross-react with macroprolactin less frequently [17–21]; however, it is unclear what type of macroprolactin they do not recognise and with what type of macroprolactin they still cross-react.

In this study, we examined the prevalence of macroprolactinaemia in 1544 women who visited an obstetric and gynaecological hospital with various symptoms, and analysed the factors that influence the detectability of

macroprolactin by comparing PRL values determined using an enzyme immunoassay (EIA) and the chemiluminescence immunoassay system (Centaur) that is known to be one of the least cross-reactive platforms with macroprolactin.

Materials and methods

Subjects

From 2012 to 2014, 1544 women aged 36.4±7.9 years visited the Hamada Obstetric and Gynaecological Hospital for infertility (n=378), menstrual disorders (n=512), polycystic ovary syndrome (PCOS; n=443), metrorrhagia (n=85), menopausal disorders (n=112), and unknown reasons (n=14). PRL was routinely measured in all patients who visited this hospital for the first time regardless of their symptoms. Blood was drawn in the morning, and the serum was separated and then stored at –30 °C until they were analysed for macroprolactin. The Clinical Research Review Board of Hamada Hospital approved this study.

Polyethylene glycol (PEG) precipitation and PRL assays

Macroprolactin was screened for using the polyethylene glycol (PEG) method as described previously [22]. In brief, each serum sample (50 µL) was mixed vigorously with 50 µL of cold PEG solution (final concentration 12.5% (w/v)) and centrifuged at 9100×*g* for 10 min. Free PRL was measured in the supernatant after PEG precipitation. The PEG-precipitable PRL (%), which might represent the amount of macroprolactin, was calculated as follows: (total PRL – free PRL)/total PRL×100. The proportion of PEG-precipitable PRL in the 1544 serum samples was 31.9±12.1% (mean±SD).

The concentrations of PRL were measured in duplicate using an enzyme immunoassay (EIA) for human PRL as previously described [9]. In brief, serum samples were incubated with polystyrene balls (Precision Plastic Ball Co., Chicago, IL, USA) that were coated with anti-human PRL antiserum (NIDDK-Anti-human PRL IC-5) at 37 °C for 6 h. After washing, the balls were incubated at 4 °C overnight and at 20 °C for 6 h with affinity-purified, anti-human PRL Fab' fragments conjugated to horseradish peroxidase. After washing, the peroxidase activity bound to the balls was assayed in an enzyme reaction with 3-(*p*-hydroxyphenyl) propionic acid (Aldrich Chemical Co., Milwaukee, WI, USA) as a substrate by measuring the fluorescence intensity using a spectrofluorophotometer (FP-6200ST; JASCO Co., Tokyo, Japan), with excitation at 320 nm and emission at 405 nm. Highly purified recombinant human PRL (human PRL-RP-2: conversion factor=1/35 for mIU/L to µg/L, according to the instructions) and anti-human PRL polyclonal antibody (rabbit) were provided by Dr. A. F. Parlow of the Harbour-UCLA Medical Centre, National Hormone and Peptide Program, Torrance, CA, USA (parlow@humc.edu). The limit of detection was 0.36 mIU/L, with a mean+3SD of fluorescence intensity of the blank. The intra- and inter-assay coefficients of variation were 4% and 5%, respectively, based on a serum sample with a total PRL concentration of 178.5 mIU/L. The study population range of PRL was defined as the mean±1.96 SD of ln-transformed PRL values; back-transformed these values were 91.0–1015.0 mIU/L for the total PRL and 63.0–637.0 mIU/L for the free PRL (n=1544).

PRL concentrations were also measured using a chemiluminescence immunoassay system (Chemilumi ACS: Centaur; Siemens Healthcare Diagnostics, Inc., East Walpole, MA, USA). Only total PRL data were available, and the range of PRL using this assay system, determined in the same manner as the range for EIA, was 73.9–713.2 mIU/L. The conversion factor from mIU/L to ng/mL is 1/21.1 [20].

Gel filtration chromatography

Gel filtration chromatography was conducted using a 1×60-cm column of Ultrogel AcA 44 (IBF, La Garenne, France) equilibrated with 0.01 mol/L sodium phosphate buffer (pH 7.0), which contained 0.1 mol/L NaCl, 0.1% (w/v) bovine serum albumin, and 0.01% (w/v) NaN_3. Serum samples (50–500 µL) were applied to the column, and 1-mL fractions were collected for PRL determinations. The column was calibrated with various molecular weight markers (Sigma, St. Louis, MO, USA). Among 1456 serum samples with a PEG-precipitable PRL ratio <50%, 37 serum samples were randomly selected as controls. Small amounts of big PRL (9.4%±2.0%) and big-big PRL (3.7%±4.0%) were observed in 37 control serum samples. Macroprolactinaemia was defined when the ratio of big-big PRL exceeded 15.7% (mean+3 SD in 37 control sera) on gel filtration chromatography.

Measurement of IgG-bound PRL

Protein G Sepharose (GE Healthcare, Uppsala, Sweden), which characteristically binds to IgG, was used to determine the IgG-bound PRL [9]. The ratio of IgG-bound PRL (%) was calculated using the following equation: PRL in the bound fraction/(PRL in the unbound fraction+PRL in the bound fraction)×100. A small amount of PRL (3.7%±2.6%) bound to the column in 37 controls. The serum samples were judged to contain IgG-type-macroprolactin when the ratio of PRL that bound to the column exceeded 11.5% (mean+3 SD in 37 control sera).

Statistical analysis

Values are expressed as the mean±SD. Fisher's exact test was used to compare the prevalence of non-IgG-type macroprolactin. Regression analysis was performed to determine the correlations between two parameters. Several parameters in groups 1 and 2, and the ratios of big-big PRL in patients with macroprolactinaemia and in the controls, were compared using Student's unpaired t-test. $p<0.05$ was considered significant.

Results

Prevalence of macroprolactinaemia

PRL was eluted primarily at the position of 23 kDa with small amounts of big PRL (9.4%±2.0%) and big-big PRL (3.7%±4.0%) in the controls. In macroprolactinaemia,

the ratio of big-PRL (12.8%±10.8%) was not significantly different, but the ratio of big-big PRL (44.4%±23.5%) was significantly higher than that in the controls (Figure 1). There was a significant correlation between the ratios of PEG-precipitable PRL and big-big PRL on gel chromatography (r=0.83) (Figure 2). Macroprolactinaemia is usually suspected when PEG-precipitable PRL exceeds 60% of the total PRL (recovery <40%). Forty-two of 43 serum samples with PEG-precipitable PRL >60% were confirmed to have macroprolactin by gel chromatography. However, 20 of 45 serum samples with PEG-precipitable PRL between 50% and 60% were also confirmed to have macroprolactin by gel chromatography. Therefore, macroprolactinaemia was diagnosed in 62 of 1544 women (4.02%) who visited an obstetric and gynaecological hospital.

Total PRL values by EIA and Centaur

We then compared the total PRL values determined by the EIA and Centaur immunoassay systems in 1544 serum samples including the 62 with macroprolactin and 1482 without macroprolactin. For reference purposes, to examine the influence of macroprolactin on the total PRL concentrations, we used free PRL (post-PEG PRL) concentrations determined by EIA. There was a significant correlation (r=0.980) between the concentrations of EIA-determined total PRL and free PRL in the 1482 serum samples without macroprolactin (Figure 3A). The ratio of EIA-determined total PRL to free PRL was 1.46±0.21, and the 62 serum samples containing macroprolactin all exceeded 1.87 (mean+1.96 SD). There was a significant correlation (r=0.975) between the concentrations of Centaur-determined total PRL and the free PRL in the 1482 serum samples without macroprolactin (Figure 3B). The ratio of Centaur-determined total PRL to free PRL was 1.90±0.36. In contrast to the EIA-determined total PRL, the ratio exceeded 2.6 (mean+1.96 SD) in 32 of the 62 serum samples with macroprolactin, and it was within the normal range in the other 30 serum samples. Patients with ratios >2.6 were designated as group 1, and those with ratios within the normal range were designated as group 2. The average ratios of macroprolactin determined by gel filtration chromatography were similar in both groups (Table 1).

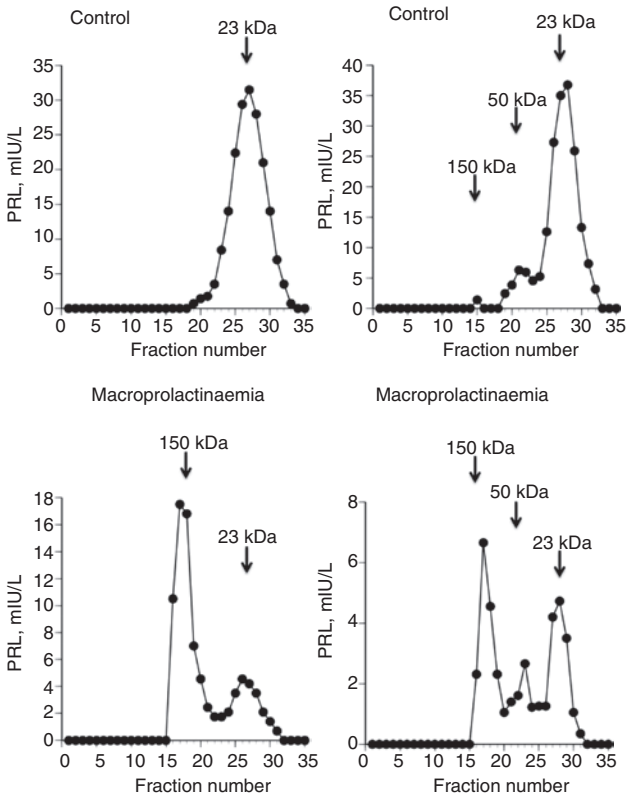

Figure 1: Gel filtration chromatography.
Representative gel filtration profiles of PRL in the controls and patients with macroprolactinaemia. Serum samples (50–500 µL) were applied to an Ultrogel AcA 44 column (1×60 cm) and 1-mL fractions were collected. PRL concentrations in each fraction were measured using EIA.

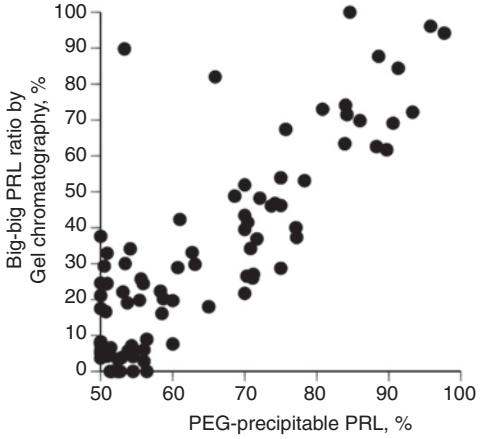

Figure 2: Relationship between the ratios of PEG-precipitable PRL and big-big PRL on gel filtration chromatography.
PEG-precipitable PRL (%) was defined as (total PRL–free PRL)/total PRL×100. Big-big PRL (%) was defined as the ratio of PRL whose molecular weight was >150 kDa in the serum PRL on gel chromatography. Serum samples with a PEG-precipitable PRL ratio >50% (n=88) were examined.

Non-IgG-type macroprolactin

Non-IgG-type macroprolactin was found in 20 of the 62 patients with macroprolactinaemia (32.3%). The prevalence of non-IgG-type macroprolactin was significantly

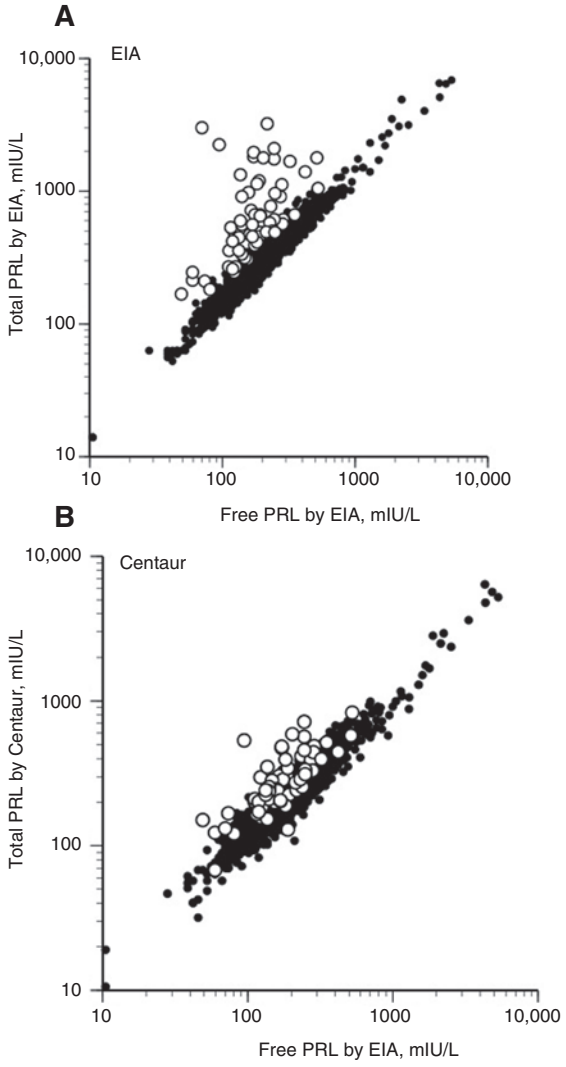

Figure 3: Relationship between PRL values measured using an enzyme immunoassay (EIA) and a chemiluminescence immunoassay (Chemilumi ACS: Centaur).
(A) The total PRL values determined by EIA in the serum samples from 1482 patients without macroprolactin (●) and in the 62 patients with macroprolactin (○) are shown with respect to the free PRL. There was a significant correlation (r=0.980) between the EIA-determined total PRL and free PRL in patients without macroprolactin and the ratio of the EIA-determined total PRL to free PRL was 1.46±0.21. Note: The vertical and horizontal axes employ a logarithmic scale. (B) The total PRL values determined by Centaur in sera from the 1482 patients without macroprolactin (●) and in the 62 patients with macroprolactin (○) are shown with respect to the free PRL. There was a significant correlation (r=0.975) between the Centaur-determined total PRL and free PRL in patients without macroprolactin and the ratio of the EIA-determined total PRL to free PRL was 1.90±0.36. Note: The vertical and horizontal axes employ a logarithmic scale.

higher in group 1 than in group 2 (50.0% vs. 13.3%) (Figure 4). In the 62 patients with macroprolactinaemia, Centaur diagnosed hyperprolactinaemia less frequently than EIA (two patients [3.2%] vs. 16 patients [29.0%]). Two macroprolactinaemic patients with hyperprolactinaemia detected by Centaur belonged to group 1 and had non-IgG-type macroprolactin.

Macroprolactinaemia in hyperprolactinaemia

Of the 1544 women who visited an obstetric and gynaecological hospital, hyperprolactinaemia was diagnosed in 53 by EIA and in 37 by Centaur. The prevalence of macroprolactinaemia in patients with hyperprolactinaemia was 30.2% (n=16) by EIA and 5.4% (n=2) by Centaur.

Discussion

We demonstrated that 1) macroprolactinaemia is present in 4% of women with various obstetric and gynaecological symptoms; 2) the Centaur PRL immunoassay, which reportedly least recognises macroprolactin, reacted with macroprolactin in approximately 50% of those patients with macroprolactinaemia; and 3) the Centaur PRL immunoassay preferentially detected the non-IgG form of macroprolactin relative to the more commonly encountered IgG-PRL complex.

There are no definite criteria for the diagnosis of macroprolactinaemia on gel chromatography; some have adopted a ratio of big-big PRL (MW 150–170 kDa) >8% [3], and others have used 21% [2], according to the normal range of big-big PRL. Big PRL, which has a molecular mass of 50 kDa, is thought to be a covalently bound dimer of PRL and is not included in the component of macroprolactin [1, 6]. In this study, macroprolactinaemia was defined as occurring when the ratio of big-big PRL exceeded 15.7% (mean+3 SD in controls) on gel filtration chromatography. Although the technique of gel filtration chromatography is considered the 'gold standard' for the diagnosis of macroprolactinaemia, it is time and labour intensive. In contrast, the PEG method is a simple and easy technique for the screening of macroprolactinaemia. Macroprolactinaemia is usually suspected when PEG-precipitable PRL exceeds 60% of the total PRL (recovery <40%). In this study, we took the strategy of screening for macroprolactinaemia using the PEG method with a cut-off value of 50% so as not to miss it and to confirm macroprolactinaemia by gel filtration chromatography.

Smith et al. tested to which degree nine immunoassay platforms recognised macroprolactin by using

Table 1: Characteristics of patients with macroprolactinaemia whose macroprolactin was recognised or unrecognised by Centaur immunoassay system.

	Macroprolactinaemia absent	Macroprolactinaeima present	
	Total	Group 1 (Recognised)	Group 2 (Unrecognised)
n	1482	32	30
Age, years	36.4±8.0	37.9±7.9	37.4±7.4
PRL (EIA), mIU/L	91.0–941.5	850.5±731.5	798.0±623.0
PRL (Centaur), mIU/L	73.9–744.8	346.0±173.0[a]	271.8±116.1
Free PRL (EIA), mIU/L	63.0–651.0	168.0±91.0	210.0±98.0
Macroprolactin on GC, %	3.7±4.0 (n=37)	46.2±25.9	42.5±20.9

[a]p=0.023.

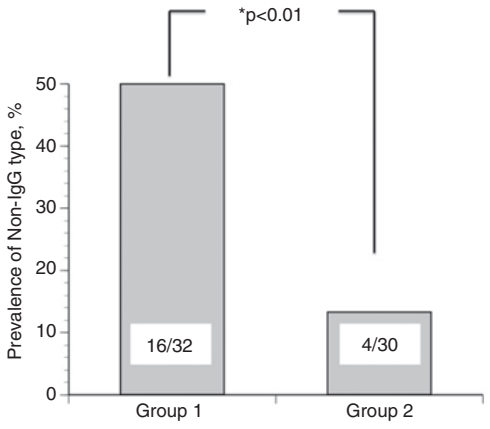

Figure 4: Prevalence of non-IgG-type macroprolactin.
Patients with macroprolactinaemia were classified into two groups: those whose serum macroprolactin was recognised by Centaur (group 1) and those whose serum macroprolactin was not recognised by Centaur (group 2). The figures in the bars indicate the number of serum samples containing non-IgG-type macroprolactin/the number of serum samples in each group.

10 hyperprolactinaemic samples containing macroprolactin that were originally identified by the DELFIA immunoassay system [17]. They found that the serum PRL concentrations of nine immunoassay platforms ranged in difference by 2.3- to 7.8-fold among the serum samples and that Centaur was the least cross-reactive with macroprolactin. The Roche Elecsys prolactin II assay was also less reactive with macroprolactin, similar to Centaur [21]. We examined the relationships of the total PRL concentrations determined by EIA and Centaur with those of the free PRL by EIA in the 1544 serum samples. As expected, the ratio of the EIA-determined total PRL to the free PRL was elevated in all 62 sera containing macroprolactin. Because the total PRL is the sum of the free PRL and macroprolactin, the ratio increases if macroprolactin is present and recognised by the immunoassay system. The ratio of the Centaur-determined total PRL to free PRL was within the normal range in 30 of the 62 serum samples with macroprolactin, suggesting that the Centaur immunoassay system is less reactive with macroprolactin than EIA. Nonetheless, the finding that the ratio of the Centaur-determined total PRL to free PRL was higher than the normal range in 32 of the 62 macroprolactinaemic patients indicates that the Centaur immunoassay system still recognises macroprolactin in approximately half of the patients with macroprolactinaemia. Two macroprolactinaemic patients who were diagnosed with hyperprolactinaemia by Centaur were included in this group. Although the other 30 patients in group 1 did not show hyperprolactinaemia on this occasion, macroprolactin was more or less recognised by the Centaur immunoassay systems, resulting in higher total PRL values than true ones. Because serum PRL levels fluctuate physiologically [23], it is possible that these patients might become hyperprolactinaemic on occasion. Because serum PRL determination is a key laboratory test for the differential diagnosis of obstetric and gynaecological disorders, screening of macroprolactinaemia in patients with hyperprolactinaemia is essential for the successful treatment of such patients.

The reasons why different immunoassay systems may vary in their ability to detect macroprolactin are not fully understood; it may be related to the degree to which anti-PRL autoantibodies and reagent antibodies share epitopes that are presented on the PRL molecule [15]. If the epitopes of PRL for reagent antibodies are already occupied by anti-PRL autoantibodies, the immunoassay systems that employ such reagent antibodies would not be able to recognise macroprolactin. We found that the prevalence of a non-IgG-type macroprolactin was significantly higher in group 1 (recognised by Centaur) than in group 2 (unrecognised by Centaur), suggesting that the Centaur immunoassay system tends to recognise non-IgG-type macroprolactin. This finding raises another

possibility: that the nature of the macroprolactin, i.e. whether it is an IgG-type or non-IgG-type, may be related to the varying ability of different immunoassay systems to detect macroprolactin. Further study may be necessary to elucidate the causes of macroprolactinaemia other than anti-PRL autoantibodies so that new immunoassay systems can be developed that are not subject to macroprolactin interference.

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