0021-972X/06/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 91(3):1048–1055

doi: 10.1210/jc.2005-1831

Human Macroprolactin Displays Low Biological Activity via Its Homologous Receptor in a New Sensitive Bioassay

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Context: Macroprolactinemia is a frequent finding in hyperprolactinemic individuals, usually without clinical impact. Data on biological activity of macroprolactin (bbPRL) are controversial and mostly based on a heterologous rat Nb2 cell bioassay. Biological activity of bbPRL observed *in vitro* but not *in vivo* may be due to its high molecular weight, preventing its passage through capillary barrier. Alternatively, bbPRL bioactivity may differ depending on the prolactin (PRL) receptor (PRLR) species specificity.

Objective: The objective of the study was to characterize the bioactivity of bbPRL in a homologous bioassay: Ba/F-3 cells stably expressing the human PRLR.

Design/Setting/Patients: Chromatography-purified bbPRL from macroprolactinemic individuals (group I, n = 18) and monomeric PRL from hyperprolactinemic patients without macroprolactinemia (group II, n = 5) were tested in Nb2 and Ba/F-LLP bioassays. Both groups were followed up at the neuroendocrinology outpatients' clinic.

Main Outcome Measure: Biological activity of bbPRL presented in the two bioassays was measured.

Results: In group I, no patient had hypogonadism. Mean ratio bioactivity to immunoactivity of bbPRL in the Nb2 assay was 0.69. There was no dose-response in 15 of the 18 samples tested in Ba/F-LLP assay. In group II, three patients had galactorrhea and all five had hypogonadism. Mean ratio bioactivity to immunoactivity of monomeric PRL samples was 1.35 in Nb2 and 0.91 in Ba/F-LLP assay.

Conclusion: Whereas both bioassays achieve similar results with respect to monomeric PRL activity, our results indicate that the activity displayed by bbPRL toward the rat receptor may be inappropriate because it is not observed in the human PRLR-mediated assay, consistent with the apparent absence of bioactivity *in vivo*. **(*J Clin Endocrinol Metab* 91: 1048–1055, 2006)**

PROLACTIN (PRL) IS a polypeptide hormone that can undergo various forms of posttranslational processing, including molecular aggregation. PRL isoforms with different molecular weights can be classified as monomeric PRL (mPRL) , with a molecular mass of 23 kDa, big PRL (bPRL), with 45–60 kDa, and macroprolactin or big big PRL (bbPRL), with more than 100 kDa (1). In the majority of serum from normal and hyperprolactinemic individuals, the main PRL isoform is mPRL, corresponding to more than 80% of the total PRL. Macroprolactinemia is usually defined when bbPRL is the predominant circulating PRL isoform (2).

The usual clinical presentation of hyperprolactinemia includes menstrual disturbances, hypogonadism, galactorrhea, infertility, and decreased libido. Causes of hyperprolactinemia can be divided into physiological, drug-induced, pathological, macroprolactinemia, and idiopathic ones. Although macroprolactinemia can be found in any of the above-cited situations, it more frequently occurs in asymptomatic subjects.

The origin of macroprolactinemia is still poorly understood. Some authors (3, 4) described the occurrence of a PRL autoantibody, and it is possible that such an antibody causes hyperprolactinemia because bound PRL escapes from clearance/degradation in kidney and target organs, which might affect its autoregulatory mechanisms. PRL autoantibodies were found in idiopathic hyperprolactinemia and at a lower frequency, in hyperprolactinemia from other causes, including prolactinomas (4). Other authors described bbPRL in the absence of PRL autoantibody as a polymer of mPRL bound by disulfide bridges, noncovalent partially glycosylated aggregates of mPRL (5), or PRL linked with IgG by disulfide bridges (6).

The gold standard for diagnosing macroprolactinemia is gel-filtration chromatography, but because this method is laborious and expensive, polyethylene glycol (PEG) serum

First Published Online December 29, 2005

Abbreviations: BA/IA, Bioactivity to immunoactivity ratio; bbPRL, big big PRL; bPRL, big PRL; h, human; mPRL, monomeric PRL; Nb, Noble strain; PEG, polyethylene glycol; PRL, prolactin; PRLR, PRL receptor; rPRL, recombinant PRL; wPRL, Third International Standard of human PRL WHO 84/500 PRL.

JCEM is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.

precipitation has been used as a screening method for large series studies (7–9).

The prevalence of hyperprolactinemia varies from 0.4% in healthy adults (10) to 70% in women with amenorrhea and galactorrhea (11). In a nonselected population, the prevalence of macroprolactinemia was 0.15% (12), and it varied between 15.4 and 46% in hyperprolactinemic patients (7–9, 12–17). However, in large series studies, the majority of macroprolactinemic subjects were asymptomatic or poorly symptomatic considering their serum PRL levels (18).

Although these data emphasize the low biological activity of macroprolactinemia *in vivo*, studies on bbPRL biological activity *in vitro* are still controversial. Initially, PRL activity has been studied in radioreceptors (19) and, more recently, in Nb2 cells, a pre-T cell clone derived from a transplantable lymphoma of a rat of the Noble (Nb) strain (20). Based on Nb2 cell proliferation induced by lactogenic hormones, such as human PRL, GH, and placental lactogen, the Nb2 assay was also used to evaluate the bioactivity of bbPRL. Some authors showed that bbPRL is less active than mPRL (2, 3, 21, 22), whereas others showed a similar activity (23–30). Some of them (3) postulated that although bbPRL has the ability to exert PRL-like activity *in vitro*, it would be inert *in vivo* because its high molecular weight could prevent its crossing through the capillary blood barrier.

Prolactin receptor (PRLR) is a single membrane-bound protein that belongs to class 1 of the cytokine receptor super family. Numerous PRLR isoforms have been described, varying in length of their cytoplasmic domain. In rats, three PRLR isoforms have been identified: short, intermediate, and long (31). Interestingly, an intermediate PRLR has been described only in Nb2 cells, in which it represents the main PRLR isoform. In contrast to the isoforms described in many species, which occur by alternative splicing of the primary transcript (31), the Nb2 intermediate PRLR results from a 594-bp deletion in the PRLR gene, leading to 198-amino acid deletion in mature protein. The impact of this deletion on the biological properties of the PRLR are still unknown. The main signaling pathways described for the long PRLR are preserved in the Nb2 isoform, and its affinity for PRL is three to four times higher, compared with the long isoform (32). Although a variety of PRLR isoforms have also been described in humans, none of them is homologous to the Nb2 receptor (33). Accordingly, we have previously shown, using reporter gene transfection assays, that there is a clear species specificity in the biological response observed with lactogens (34). Therefore, using a bioassay involving a mutated, intermediate-sized nonhuman receptor (Nb2) may not be suitable to characterize the actual bioactivity of human bbPRL, and we decided to analyze bbPRL activity using a recently developed bioassay (Ba/F-LP, for low PRL) involving the homologous (human) PRLR (35). Because we suspected the bioactivity of bbPRL to be rather low, we first developed an improved version of the Ba/F-LP exhibiting sensitivity similar to that of Nb2 cells. Our results provide evidence that the biological activity of bbPRL is considerably lower toward the homologous receptor than in the rat Nb2 assay, which better correlates with the absence of symptoms observed in the majority of patients suffering from macroprolactinemia.

Subjects and Methods

Subjects

Postpubertal individuals older than 18 yr and hyperprolactinemic (serum PRL > 30 μg/liter) were included in the study and divided into two groups. Women using oral or parenteral contraceptives, submitted to hysterectomy or postmenopausal, were excluded. Group I consisted of macroprolactinemic subjects, defined by bbPRL levels more than 50% of total serum PRL, and group II consisted of five patients with prolactinoma and without macroprolactinemia, considered as a positive control to the bioassays. The subjects were selected from the outpatient clinic of the Neuroendocrine Unit, Hospital das Clinicas, University of Sao Paulo Medical School. All individuals studied signed the informed consent, approved by the local Research Ethics Committee.

To compare clinical findings to bbPRL bioactivity by bioassays, a clinical score based on a numeric variable was designed. For men, score was 0 if serum testosterone level was normal and III if its levels were lower than normal range. For women, score was 0 if there was ovulatory cycles, I for short luteal phase, II for oligomenorrhea, and III for amenorrhea. For both genders, the score was 0 in the absence of galactorrhea, I for mild galactorrhea at expression, II for important galactorrhea at expression, and III for spontaneous galactorrhea.

Imaging

Subjects were submitted to a magnetic resonance imaging of the sellar region (Sigma LX GE, Milwaukee, WI), 1.5T, and gradient of 23 mT/m. The slices were axial, coronal, and sagittal in T1, pre- and postgadolinium, and T2.

Biochemical and gonadal hormone assessment

Serum concentrations of free T_4, TSH, creatinine, aspartate aminotransferase, and alanine aminotransferase were measured to exclude hypothyroidism, renal failure, and hepatic insufficiency as cause of hyperprolactinemia. LH, FSH, estradiol, and progesterone in the luteal phase of the menstrual cycle were determined in all female patients in at least two nonconsecutive cycles. LH, FSH, and at least two measurements of total testosterone in distinct days were determined in all male patients.

PRL assay

Serum PRL was quantitatively determined in at least two different instances, between 0800 and 1100 h, by a immunofluorimetric assay (Wallac AutoDELFIA. PerkinElmer Life Sciences. Boston, MA). The normal references values ranged from 2 to 10 μg/liter for men and 2 to 15 μg/liter for women. Intra- and interassay coefficients of variation were 1.05 and 2.60%, respectively. The same method was used for all PRL measurements to avoid diverse results in different assays because of the presence of bbPRL (36).

Macroprolactinemia assessment

PRL assay was requested elsewhere based on different reasons, as depicted in Table 1. Because hyperprolactinemic individuals presented mild or no symptoms in group I, macroprolactinemia was investigated, and confirmation was performed by gel-filtration chromatography using a column of 1.6×30 cm Superdex 200 (Pharmacia, Uppsala, Sweden), eluted by FPLC with 20 mM Na_2HPO_4/NaH_2PO_4, 15 mM NaCl, and 10 mg/liter of gentamicin (pH 7.5). The column was calibrated with blue dextran and different protein standards with known molecular weight; 0.5 ml serum was used in the column eluted at a speed of 1 ml/min. Aliquots of 1.5 ml were collected, and measurement of PRL and GH was performed in each aliquot (Wallac AutoDELFIA). The area under the curve expressed as percentage of mPRL, bPRL, and bbPRL were calculated for each serum sample.

The aliquots from gel-filtration chromatography were maintained at −80 C, and in the day of the bioassay, they were diluted with a specific assay medium in three different concentrations.

PRL autoantibody determinations

PRL autoantibodies were identified with the method described by Hattori *et al.* (37) to be correlated with clinical and laboratory findings,

TABLE 1. Clinical and laboratorial features of cases from groups I and II

Patient	Sex	Age (yr)	Reason for serum PRL assessment	Clinical score	PRL (μg/liter)	mPRL (μg/liter)	bbPRL (μg/liter)	BA/IA (Nb2)	BA/IA (Ba/F LLP)
Group I									
1[a]	F	52	Irregular menses since 18 yr	I	155.0	35.80	95.98	0.47	
2	F	42	Hirsutism	0	57.8	4.96	48.98	0.38	
3	F	27	Casual	I	87.2	6.69	72.39	0.39	
4	F	42	Galactorrhea for 6 yr	I	51.2	0.26	38.15	0.74	
5	M	33	Casual	0	30.8	1.93	27.12	0.79	
6	F	45	Previous irregular menses	0	56.1	4.09	47.21	1.26	
7	F	28	Investigation of Cushing syndrome	0	34.6	1.77	29.83	0.26	
8	F	28	Hair loss	0	110.0	5.54	101.47	0.57	
9	F	41	Galactorrhea	0	166.0	0	159.18	0.70	
10	F	37	Skin lesions	0	58.2	3.85	41.13	0.56	
11	F	23	Previous irregular menses	I	47.7	3.07	27.81	0.11	
12	F	25	Acne	0	30.7	1.75	21.31	0.35	
13	F	41	Casual	0	107.9	9.11	80.05	0.37	0.59
14	F	37	Previous galactorrhea	0	124.0	2.16	113.65	0.79	0.73
15	F	27	Headache	0	43.8	1.02	25.54	0.32	
16	M	54	Erectile dysfunction	0	37.5	1.59	35.14	1.37	0.33
17[a]	F	27	Previous galactorrhea	0	40.3	4.81	31.68	2.80	
18	F	43	Headache	0	82.5	5.4	67.3	0.3	
Group II									
19	F	37	Amenorrhea and galactorrhea	IV	117.0	50.6		1.13	0.82
20	F	37	Amenorrhea and galactorrhea	IV	107.0	34.6		1.87	0.97
21	M	30	Hypogonadism and galactorrhea	IV	108.0	93.9		1.28	1.24
22	M	33	Hypogonadism	III	53.1	39.3		1.66	0.85
23	F	30	Amenorrhea	III	129.0	88.46		0.81	0.68

M, Male; F, female.

[a] Individuals on dopaminergic agonist; BA/IA was determined on chromatography purified bbPRL and mPRL.

mainly with bbPRL bioactivity *in vitro*. Summing up, 100 μl serum and 50 μl [^{125}I] PRL (15,000 cpm/50 μl phosphate buffer) were incubated for 1 h at 37 C, 150 μl of 25% PEG were added, and the reaction volume was vortexed and centrifuged at 4500 rpm for 30 min. The pellet was washed with 12.5% PEG and the radioactivity measured with a γ-counter. Serum samples were considered as containing anti-PRL if the radioactivity exceeded mean +2 SD in 39 serum samples used as negative controls (21 samples with normal PRL levels and 18 samples with hyperprolactinemia due to mPRL).

Bioassays

Chromatography-purified bbPRL from group I and mPRL from group II, at least in triplicates for each isoform concentration, in three different concentrations were tested in both bioassays.

Nb2 bioassay

Cells were routinely maintained as suspension cultures in RPMI 1640 medium supplemented with 10% horse serum, 10% fetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin (Invitrogen, Carlsbad, CA), and 0.1 mM β-mercaptoethanol. The Nb2 bioassay was performed as described by Tanaka *et al.* (20), with slight modifications. The cells were incubated at 37 C and 5% CO_2. Cell number was assessed 72 h after plating by MTS [3(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt] (Promega Corp., Madison, WI) assay. MTS dye at 2 mg/ml in PBS was mixed at a 20:1 ratio (vol/vol) with phenazine methosulfate (Sigma, St. Louis, MO), 0.92 mg/ml in PBS. Twenty microliters of the mixture were then added to each well, and 2 h after incubation at 37 C before reading the absorbance at 490 nm in a microplate reader (model MR4000; Dynatech, Chantilly, VA). The Third International Standard of human PRL World Health Organization (WHO) 84/500, referred as wPRL in this study, with a bioactivity of 53 mIU per 2.5 μg was used as reference preparation. The wPRL was kindly provided by the National Institute for Biological Standards and Control (South Mimms, UK).

Ba/F-LLP bioassay

Ba/F-3 cells are murine pro-B cells dependent on IL-3 for growth. They were previously transfected with a plasmid encoding the long isoform of the human (h) PRLR, and PRLR-expressing cells were selected using G418 antibiotic resistance (35). Substitution of recombinant hPRL for IL-3 further ensured selecting cells PRL dependent for their growth. After a few passages under these conditions, a stable cell population named LP (low PRL) was obtained by adding 10 μg/liter PRL in routine culture medium; accordingly, these cells exhibit maximal proliferation at a PRL concentration of 10 μg/liter (35). After a few passages in culture medium containing only 1 μg/liter PRL, a more sensitive population was selected, namely Ba/F-LLP (low low PRL). Cells were routinely maintained in suspension in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum; 2 mM glutamine; 50 U/ml penicillin; 50 μg/ml streptomycin; 700 μg/ml geneticin, and 1 μg/liter recombinant PRL (rPRL) (35). rPRL was produced from bacteria and purified as described earlier (34). Before proliferation assays, cells were starved for 6 h in 1% fetal calf serum RPMI 1640 medium with additives and then distributed in a flat-bottom 96-well plate at a density of 5×10^4 cells/well in a final volume of 200 μl. After 72 h at 37 C and 5% CO_2, the number of viable cells was assessed using the MTS assay. A standard curve with rPRL was constructed in each assay. We calculated the bioactivity to immunoactivity (BA/IA) ratio for bbPRL for each case, based on the standard curve. For those exhibiting a dose-dependent response, we repeated the assay under the same conditions in the presence of 1:4,000 diluted hPRL antibody reagent (rabbit antihuman pituitary prolactin antiserum, NIDDK-anti-hPRL-3, AFP-C11580) to confirm the specificity of the response (efficiency of antibody activity ≤ 1:10,000).

Ratio BA/IA

The ratio BA/IA was used as a parameter to compare samples potency. Bioactivity was measured by biological response (cellular proliferation) stimulated by PRL. It was calculated as standard PRL concentration required to achieve the same biological response of a sample (mPRL or bbPRL). Immunoactivity was measured as a concentration of PRL in a sample by an immunofluorimetric method (autoDELFIA; Wallac, Turku, Finland).

Data analysis

Data were presented as mean ± SD unless otherwise stated. Student's *t* test, paired *t* test, ANOVA with repeated measures, and Bonferroni test

were applied for BA/IA, slopes, and serum PRL levels for groups I and II. When extreme departure from normality was observed, Kruskal-Wallis test was applied (38). The level of significant difference was set at $P < 0.05$.

Results

Clinical data

In group I (cases 1–18), with macroprolactinemic individuals, there were 18 individuals (16 females), median age 37 yr (27–52 yr old) and in group II (cases 19–23), with hyperprolactinemia without bbPRL, there were five patients (three females), with median age 33 yr (30–37 yr old). Clinical score was I for cases 1, 3, 4, and 11 because of mild galactorrhea and 0 for the remaining 14 cases (Table 1). Serum testosterone levels were normal in men, and all women presented ovulatory cycles. In group II, both men presented hypogonadism (score III) and one presented galactorrhea (total score IV); all three women presented galactorrhea and amenorrhea (score IV) (Table 1).

PRL profile

In group I, total serum PRL concentrations and bbPRL were 73.4 ± 42.6 and 59.8 ± 38 μg/liter, respectively. All patients but one (case 1) had normal serum levels of mPRL (Table 1); therefore, any symptom related to hyperprolactinemia might be due to bbPRL, which represented 58.3–95.9% of the total PRL. Figure 1 compares the chromatographic profiles of a macroprolactinemic case (case 9) with a symptomatic hyperprolactinemic patient (case 21) with a predominance of mPRL isoform from group II. In group II, total PRL serum concentrations and mPRL were 102.8 ± 29 and 61.4 ± 28 μg/liter, respectively. mPRL represents from 32.3 to 87% of total PRL, the remaining being bPRL.

Because human GH has bioactivity in Nb2 and Ba/F-LLP assay and GH could coelute with mPRL or bbPRL, depending on the isoforms present in serum samples, GH concentrations in chromatography fractions were measured. GH amount was almost negligible; therefore, it did not interfere with PRL samples bioactivity.

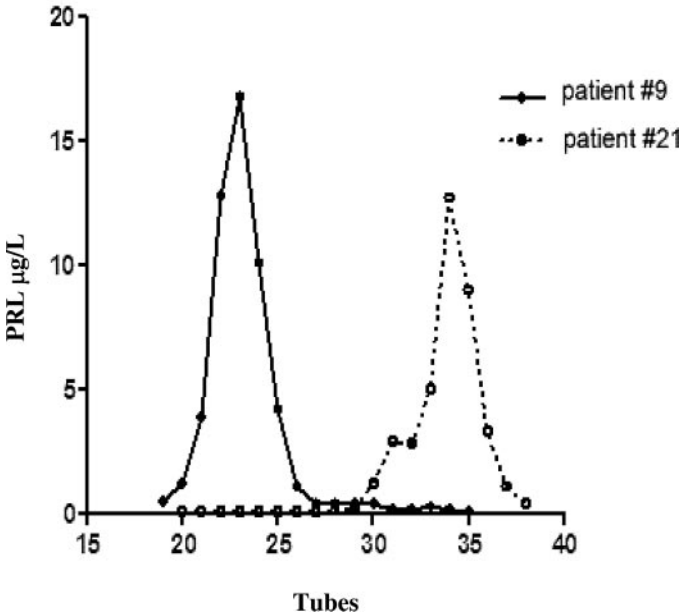

FIG. 1. Typical chromatographic profiles of a macroprolactinemic case (case 9), showing the predominance of bbPRL (tubes 20–25), and of a symptomatic hyperprolactinemic patient (case 21), mainly depicting the mPRL isoform (tubes 33–36).

PRL autoantibody assessment

Using the method described by Hattori *et al.* (37), PRL autoantibody was present in seven macroprolactinemic patients (2, 3, 9, 13, 15, 16, 18) (38%). [^{125}I]PRL bound was dose-dependently displaced by unlabeled PRL (NIDDK-hPRL-I-8: 800–1,600 μg/liter) in this system. No anti-PRL determination was performed in group II.

Imaging

In group I, magnetic resonance imaging of the sellar region disclosed a partial empty sella in case 1, and cases 3, 4, 8, 9, and 11 presented slight homogeneous pituitary enlargement. In group II, cases 19 and 22 had microadenomas and cases 20, 21, and 23 had macroadenomas.

Bioassays (Ba/F-LLP and Nb2)

After the cultures sat for a few weeks in medium containing low PRL concentration (1 μg/liter), a subpopulation from Ba/F-LP cells was grown and called Ba/F-LLP. Dose-response assays were performed using Ba/F-LLP cells, and maximal cell growth was dropped from 10 μg/liter for Ba/F-LP cells (35) to approximately 1 μg/liter of rPRL for Ba/F-LLP cells (Fig. 2B), indicating that the assay sensitivity could be increased only by modifying the PRL concentration in routine culture medium. We routinely observed that maximal response occurred in the 0.5–1 μg/liter range, which probably reflects the number of cells at the starting assay day, the proliferation rate in individual experiments, and the self-antagonism (35).

In Ba/F-LLP assay, the intraassay variability is 12.5%; the interassay variability is 16%; the mean slope of the dose-response curve is 2.27. The minimal detectable dose, which corresponds to the PRL concentration resulting in a response 2 SD away from the zero dose response, is 0.023 μg/liter, similar to the reference Nb2 assay (20). The ED_{50} is 0.134 μg/liter (Fig. 2A). These data presented were obtained from an assay performed with rPRL in eight replicates and are representative of eight experiments.

Regarding Ba/F-LLP specificity, lactogenic hormones [mouse GH, human GH, ovine PRL rPRL (WHO 97/714), and wPRL] were tested, and all of them presented similar bioactivity, except mouse GH (data not shown).

Interestingly, rPRL appeared to be 2- to 2.5 times more active than wPRL (Fig. 2B), probably in part because the former presents only nonglycosylated monomeric isoform. Our chromatographic analysis showed that wPRL presented 90% of mPRL. In Nb2 assay, despite their parallelism, rPRL appeared to be 20% more active than wPRL.

Because wPRL was used as a standard in Nb2 assay and rPRL as a reference in Ba/F-LLP assay, ratio BA/IA obtained from the latter assay was normalized against wPRL.

Macroprolactin (bbPRL) purified from all group I indi-

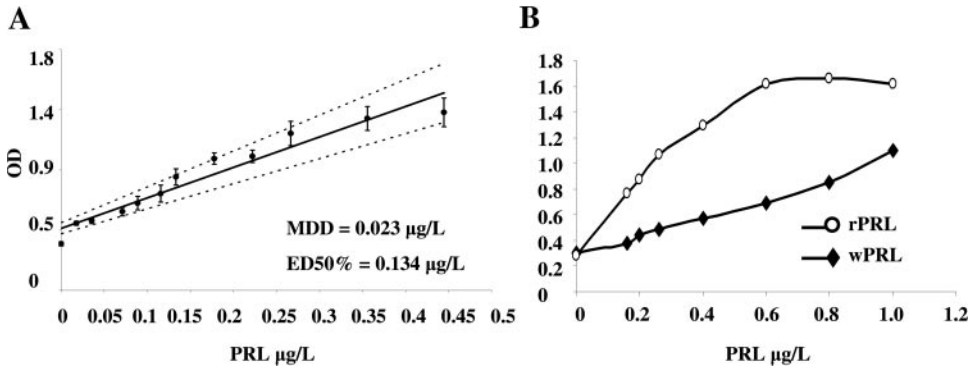

FIG. 2. Characterization of the Ba/F-LLP bioassay. A, Minimal detectable dose (MDD) and ED_{50} of the Ba/F-LLP proliferation assay. B, Comparison of wPRL (standard PRL from WHO) and rPRL (standard rPRL) bioactivity, showing the higher activity of rPRL, compared with wPRL.

viduals exhibited measurable activity in the Nb2 bioassay, with BA/IA ratio ranging from 0.11 to 2.8 (Table 1). In sharp contrast, 15 of the 18 cases with macroprolactinemia failed to induce any dose response in the Ba/F-LLP assay, thus preventing any BA/IA ratio to be calculated. Therefore, for these 15 samples, we used the slope of the curve obtained by PRL concentration *vs.* OD, in Ba/F-LLP assay, to compare bioactivity samples. Their slopes were always low and comparing them with rPRL slope, the values were below the 95% confidence interval; therefore, we considered no dose response for the range of sample concentration studied.

Remarkable exceptions were bbPRL from cases 13, 14, and 16, for which BA/IA ratios were 0.59, 0.73, and 0.33, respectively. Figure 3 illustrates typical dose-response curves obtained using Nb2 (A) or Ba/F-LLP (B) assays for bbPRL purified from case 2 (representative of the majority of group I samples) and case 14. As expected, the specificity of the response for these three cases was demonstrated by the abolition of biological response in the presence of hPRL antibody reagent (Fig. 3B).

Mean sample slopes from group I, containing bbPRL, and from group II, containing mPRL, were evaluated in Ba/F-LLP and Nb2 assays. Statistical analyses were performed among these four subgroup using Bonferroni test, and the results are represented in Fig. 4. The only statistically significant difference was related to the mean slope obtained in the Ba/F-LLP assay for group I patients, which was lower, compared with that obtained in the Nb2 assay for the same group ($P = 0.000$), and with that obtained from Ba/F-LLP assay for group II ($P = 0.02$). Interestingly enough, the same parameters did not show significant difference between the two groups with respect to the Nb2 assay ($P = 1.0$), further indicating that Ba/F LLP assay distinguishes samples when Nb2 assay does not. In group II, although the BA/IA ratio was higher in Nb2 than Ba/F-LLP assays (1.35 ± 0.42 and 0.91 ± 0.21), whereas mean slopes went the opposite (1.38 ± 0.51 in Ba/F LLP *vs.* 1.09 ± 0.27 in Nb2), these differences did not reach statistical significance ($P = 0.442$). These data also reinforced that the Ba/F LLP assay is as reliable as the reference Nb2 bioassay to display the bioactivity of mPRL.

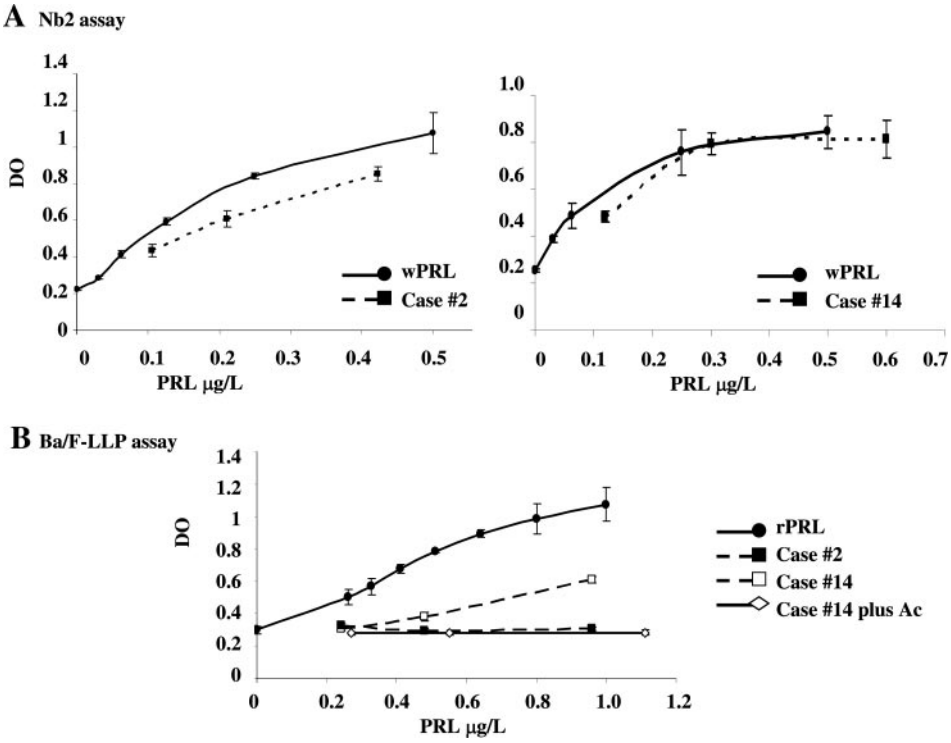

FIG. 3. Biological activity of bbPRL in Nb2 and Ba/F-LLP assays: two representative cases from group I. Various dilutions of chromatography-purified bbPRL activity were compared with a reference dose-response curve obtained with standard PRL. A, Nb2 assay: both cases (like the 17 others) induce dose-response proliferation. B, Ba/F-LLP assay: whereas case 2 did not induce any response (like 14 other samples), case 14 (like cases 13 and 16) showed a dose response. Specificity of this response was confirmed by addition of PRL antibody reagent (Ac).

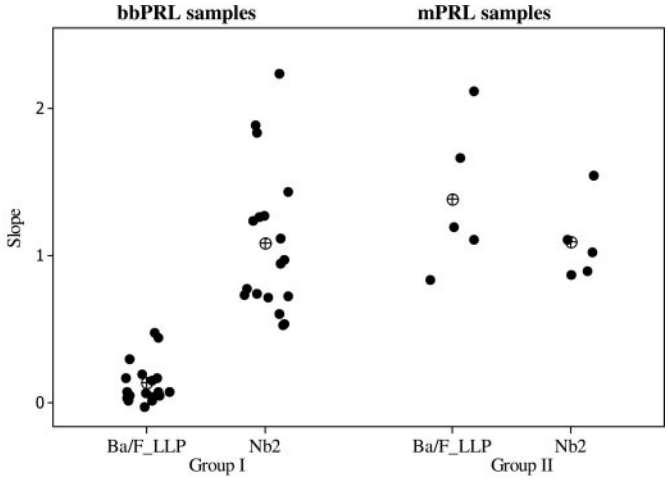

FIG. 4. Slopes for each sample were calculated based on dose-response curve (PRL concentration *vs.* OD), which was built using three different sample concentrations, in triplicates. Sample slopes from group I, containing bbPRL, and group II, containing mPRL, were evaluated in Ba/F-LLP and Nb2 assays. Statistical analyses were performed among these four subgroups. The figure depicts individual slopes values for each sample in each assay (●) as well as mean slope (⊕) for samples from groups I and II, in both Ba/F-LLP and Nb2 assays. This figure highlights that the mean slope obtained in Ba/F-LLP assay for group I patients was significantly lower, compared with that obtained in the Nb2 assay for the same group, and with that obtained in the Ba/F-LLP assay for group II.

Finally, in group I, there was no statistical difference between mean slopes in subgroups containing or not PRL autoantibody, and this was true for both bioassays ($P = 0.784$ for Nb2, 0.507 for Ba/F LLP), excluding autoantibodies as a parameter to be considered for explaining the differences between assays.

Discussion

In this study, we show that biological activity of bbPRL purified from serum of individuals with macroprolactinemia is lower in a human PRLR-mediated assay, compared with the commonly used heterologous Nb2 assay. These results are consistent with the usual absence of clinical manifestations in subjects with macroprolactinemia. In fact, in 10 of our 18 cases, serum PRL assessment was performed routinely or due to clinical manifestations unrelated to hyperprolactinemia (Table 1), reflecting the indiscriminate request for hormone tests that may result in diagnosis pitfalls (39). Regarding the remaining eight subjects, complaints at the time of the initial PRL assessment could be related to hyperprolactinemia. During our clinical investigation, however, only four women presented with mild galactorrhea (Table 1), and galactorrhea is not always related to hyperprolactinemia. Kleinberg *et al.* (40) studied patients with galactorrhea and in 86% of women without menstrual irregularities, PRL levels were normal.

There was no case of hypogonadism at the time of investigation. Case 16, who had normal serum testosterone levels, had erectile dysfunction, probably related to psychosocial issues that improved with psychotherapy. Cases 1 and 17 had irregular menses, which normalized after dopamine agonist administration, suggesting previous pathological hyperprolactinemia. Serum PRL levels in these patients were high before starting treatment, and ovulatory cycles were restored despite no complete resolution of hyperprolactinemia on bromocriptine.

PRL autoantibodies were found in 38% of our macroprolactinemic individuals. De Schepper *et al.* (30) identified PRL autoantibodies in 92% of macroprolactinemic subjects. They did not find differences between subjects with or without PRL-IgG complexes, regarding clinical findings and PRL activity in Nb2 assay. On the other hand, Mounier *et al.* (41), using the same method we did, did not find PRL autoantibodies in five macroprolactinemic individuals. As suggested in the literature (18), macroprolactinemia is a heterogeneous entity, a fact that could explain differences in clinical presentation and different behaviors in biological assays. Interestingly, two of the three samples (cases 13 and 16) in group I that showed bbPRL activity in the homologous assay contain PRL autoantibodies. Nevertheless, the presence or absence of PRL autoantibodies did not influence our clinical and laboratorial findings.

Regarding comparison of clinical and *in vitro* findings, it is noteworthy that bbPRL always had some activity in Nb2 cells, in a dose-dependent manner, despite normal gonadal functions in all cases. In Ba/F-LLP cells, however, there was dose-dependent activity in only three asymptomatic patients. The bioactivity of these patients' bbPRL vanished when hPRL antibody reagent was added, confirming the specificity of PRL action in Ba/F-LLP assay. However, no obvious reason was found for such results. Samples of symptomatic hyperprolactinemic patients with prolactinomas were tested to assess the bioactivity of mPRL in Ba/F-LLP assay. The BA/IA ratio was close to 1 for all these patients, arguing for the reliability of our new Ba/F-LLP assay.

The parameter of species specificity of bioassays for lactogen has already been addressed in previous studies. Gertler and Djiane (42) showed that the ruminant placental lactogen could activate the human GH receptor but not the homologous GH receptor. Moreover, Goffin *et al.* (43) and Bernichtein *et al.* (35) also showed that the apparent activity of PRL variants, either agonists or antagonists, could markedly differ depending on the assay used (43, 44). These studies pointed out assay sensitivity and species specificity as two major parameters directing the apparent biological activity of PRL analogs in various *in vitro* bioassays. Because the two assays used in this study (Ba/F-LLP and Nb2) exhibit similar sensitivity and both involve lactogen-induced proliferation of lymphoid cells, it is likely that the dramatic difference between bbPRL activity in these assays mainly results from the species specificity of ligand-receptor interaction. The molecular bases of this species specificity are currently unknown because rat and human receptors are highly conserved. A BLAST 2 Sequences comparison (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html) indicated 71% homology between Nb2 and human long PRLR, uniformly spread over the extracellular, transmembrane and cytoplasmatic domains. Although the binding determinants of hPRL have been extensively studied (43), this is not true for the receptor, and it is difficult to predict why bbPRL is less active toward the human

than the rat receptor. In addition, as already mentioned, bbPRL is a heterologous entity; therefore, it is likely that its mechanism of interaction with the PRLR differs from what has been described for mPRL.

In conclusion, our study strongly suggests that, in the current state of the art, using a homologous receptor-mediated bioassay is probably more suitable to characterize the actual activity of bbPRL. Data obtained using the Ba/F-LLP assay correlate well with the assumption that bbPRL activity is very low *in vivo*. Further studies with a larger number of macroprolactinemic subjects, asymptomatic or not, will be useful to confirm our data. The use of Ba/F-LLP assay, as a promising diagnostic tool, could help to further understand the pathophysiological importance of macroprolactinemia.

Acknowledgments

We acknowledge the collaboration of biologists from Fleury Laboratory (Leda Hitomi Obara, Maria Teresa Camargo, Teresinha Tachibana, and Sonia Nishida); researchers from Instituto de Pesquisas Energeticas e Nucleares-Comissão Nacional de Energia Nuclear (Susana da Rocha Heller, Cibele Nunes Pieroni, and Paolo Bartolini); and fellows from Institut National de la Santé et de la Recherche Médicale U584 (Jean-Baptiste Jomain and Sophie Bernichtein).

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This work was supported by Grant 02/00761-7 and 02/02688-5 from Fundação de Amparo à Pesquisa do Estado de São Paulo and BEX 2381/02-8 from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior.

The authors have no conflict of interest.

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