Bioactivity of prolactin isoforms: lactation and recovery of menses in nursing women

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To assess whether plasma prolactin (PRL) characteristics relate to lactogenesis and absence or presence of menstrual cycles, we measured bioactive PRL (BIO-PRL) using the Nb2 assay, immunoreactive PRL (IR-PRL) by radioimmunoassay, calculated equations describing the BIO-PRL–IR-PRL relationship and separated charged PRL isoforms (by chromatofocusing) in five amenorrhoeic and five cycling nursing women at 6 months postpartum and in 10 cycling non-nursing women. Plasma samples were drawn before and 30 min after a suckling episode at 0800, 1600 and 2400 h in nursing women and at the same hours in non-nursing women. BIO-PRL and IR-PRL concentrations were highest in amenorrhoeic nursing women, intermediate in cycling nursing women and lowest in cycling non-nursing women. The BIO-PRL–IR-PRL relationship shows that a given amount of IR-PRL corresponds to equivalent amounts of BIO-PRL in cycling nursing and cycling non-nursing women, and to a larger extent in amenorrhoeic nursing women. IR-PRL was present in plasma as several charge isoforms. Bioactive isoforms eluting at pH 6.0–5.1 were found in amenorrhoeic and cycling nursing women, reaching similar concentrations after suckling. Bioactive isoforms eluting at pH 7.0–6.1 were found only in amenorrhoeic nursing women. We speculate that isoforms eluting at pH 6.0–5.1 may play a role in lactation and isoforms eluting at pH 7.0–6.1, in lactational amenorrhoea. Key words: lactation/lactational amenorrhoea/prolactin isoforms

Introduction
In women, breast-feeding is a physiological situation in which hyperprolactinaemia and adequate milk production may coexist with the absence or presence of menstrual cycles. Suckling maintains hyperprolactinaemia and milk production (Howie and McNeilly, 1982; Tucker, 1994). The determinants of the resumption of menstrual cycles during lactation are not well known. Concentrations of immunoreactive follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Glasier et al., 1983), of bioactive LH (Seron-Ferre et al., 1995) or the patterns of pulsatility of immunoreactive LH (Nunley et al., 1991; Tay et al., 1992; Díaz et al., 1995) showed no correlation with the duration of lactational amenorrhoea in fully breast-feeding women. An association between prolactin (PRL) and duration of lactational amenorrhoea has been shown by our group, studying prospectively immunoreactive PRL (IR-PRL) in a group of fully breast-feeding women of similar age, weight, suckling frequency and infant growth rate, some of whom recovered their menses before 6 months postpartum and some who did so after 6 months postpartum. In these women, the duration of amenorrhoea was correlated directly with IR-PRL concentrations. At 3, 6 and 9 months postpartum, women belonging to the long amenorrhoea group had higher IR-PRL than those belonging to the short amenorrhoea group. However, these latter women, when already menstruating at 6 months postpartum, maintained hyperprolactinaemia (Díaz et al., 1989).

In a following study, we found at 3 months postpartum, when all women were in amenorrhoea, that besides the differences in IR-PRL concentrations, there were also differences between the women in post-suckling BIO-PRL concentrations (Campino et al., 1997) and in the equations relating BIO-PRL and IR-PRL concentrations (Campino et al., 1994). For the same amount of IR-PRL, women who were to experience long amenorrhoea had more BIO-PRL than those who were to experience short amenorrhoea. Plasma human PRL is present as multiple isoforms that differ from each other in size (Larrea et al., 1989), charge (Brue et al., 1992; Larrea et al., 1992) and in bioactivity (Markoff and Lee, 1987; Markoff et al., 1988). Although there is scanty information about the types of PRL isoforms present during lactation and their contribution to total plasma PRL bioactivity, a difference in isoform composition could very well explain the discrepancies in IR-PRL and BIO-PRL found in these fully nursing women at 3 months postpartum.

In the present study, we explored whether differences in PRL isoforms may relate to the dissociation between the maintenance of lactation and the presence or absence of menstrual cycles in these nursing women at 6 months postpartum. Our hypothesis was that breast-feeding and the absence or presence of ovarian function are associated with overall plasma BIO-PRL, different plasma BIO-PRL–IR-PRL relationship and with particular bioactive isoforms of PRL. To test the hypothesis we compared these PRL characteristics in amenorrhoeic and cycling nursing women at 6 months postpartum, and in cycling non-nursing women.
Table I. Characteristics of amenorrhoeic and cycling nursing women at 6 months postpartum and of cycling non-nursing women

<table>
<thead>
<tr>
<th>Variable</th>
<th>Nursing (n = 5)</th>
<th>Non-nursing (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>25.4 ± 1.8</td>
<td>24.2 ± 2.8</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>55.6 ± 10.5</td>
<td>67.5 ± 8.8</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>157.0 ± 4.0</td>
<td>157.0 ± 8.0</td>
</tr>
<tr>
<td>Postpartum (days)</td>
<td>189.6 ± 15.4</td>
<td>177.2 ± 6.7</td>
</tr>
<tr>
<td>Months after weaning</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Infant’s weight (g)</td>
<td>3482 ± 271</td>
<td>3472 ± 242</td>
</tr>
<tr>
<td>At birth</td>
<td>7864 ± 124</td>
<td>7918 ± 788</td>
</tr>
<tr>
<td>Breast-feeding status</td>
<td>Exclusive 2</td>
<td>2 NA</td>
</tr>
<tr>
<td>Supplemented</td>
<td>1 2</td>
<td>NA</td>
</tr>
<tr>
<td>Non-dairy food introduced</td>
<td>2 1</td>
<td>NA</td>
</tr>
<tr>
<td>Nursing episode/24 h</td>
<td>10.8 ± 0.8</td>
<td>10.0 ± 2.3</td>
</tr>
<tr>
<td>Suckling contacts/24 h</td>
<td>21.4 ± 1.3</td>
<td>23.2 ± 7.8</td>
</tr>
<tr>
<td>Suckling duration (min)/24 h</td>
<td>137.8 ± 41.1</td>
<td>128.2 ± 37.1</td>
</tr>
</tbody>
</table>

Values are mean ± SD; NA = not applicable.

Materials and methods

Subjects

Nursing women
Ten healthy nursing women at about day 180 postpartum who were fully breast-feeding and whose infants had a normal growth rate participated in the study. Volunteers were recruited at the time of delivery among healthy women who wanted to breast-feed their child for as long as possible. They were 20–30 years old, had a parity 1–3 and a normal pregnancy ending at 38–40 weeks gestation in vaginal delivery of a healthy child of normal weight. They were not using therapeutic drugs or hormonal contraception. The women were instructed not to feed their babies any liquid or solid food or water and to use their breast as the only source of water and nutrients during the first 6 months postpartum, except for the administration of vitamin drops (exclusive breast-feeding). All the women were fully breast-feeding for the first 5 months postpartum. Supplements and non-dairy food were introduced by some women in each group between months 5 and 6 postpartum (Table I). They were followed for 12 months at the clinic recording the exact date of their first and following postpartum menses. Five of the women recovered their cycles between 3 and 6 months postpartum and continued cycling regularly thereafter. Recovery of menstrual cycles was defined as the occurrence of the first postpartum bleeding, consisting of at least 1 day of normal bleeding or 3 consecutive days of spotting, followed by another bleeding within 60 days. In these women, samples for the present study were drawn during the follicular phase of the cycle. The other five women remained in amenorrhoea for more than 6 months (range 8–10 months). Amenorrhoeic and cycling nursing women at 6 months postpartum had a similar breast-feeding pattern (Table I). Subjects were part of the previous studies on IR-PRL (Diaz et al., 1989) and BIO-PRL (Campino et al., 1994, 1997) around day 90 postpartum mentioned above. A detailed description of the selection procedure is found in these reports.

Non-nursing women
Ten fertile cycling women with IR-PRL within normal range (3.7–16.6 μg/l), were selected as controls. They were 23–33 years old. All of them had one or two previous pregnancies and had breastfed their children. The time interval from the end of breast-feeding to the beginning of this study was 2.4–5.4 years. All women had regular menstrual cycles and none had galactorrhoea. They were not using therapeutic drugs or hormonal contraception. Blood samples were taken during the follicular phase of the cycle. Age, weight and height were similar in nursing and non-nursing women.

The experimental procedure was explained to all women and they gave their consent in writing. The study protocol was approved by the Institutional Ethical Committee of the Pontificia Universidad Catolica de Chile.

Blood sampling protocol

Nursing women
The women and their infants were hospitalized for 48 h in the metabolic ward of the Hospital Clínico, Pontificia Universidad Catolica de Chile. Standardized meals were provided at fixed hours. The women remained sitting or lying down for the major part of the day, took care of their infants assisted by a nurse and continued their usual breast-feeding practice while in the unit. The first 24 h was allowed for adaptation to the environment and on the second day a butterfly needle was inserted into an antecubital vein between 0630 and 0715 h. During the 24 h sampling period the time and duration of each nursing episode were recorded. A nursing episode was defined as an event in which the infant suckled one or both breasts, separated by at least 30 min from the preceding and following episodes. Within each episode one or several suckling contacts took place, defined as periods of time during which the baby sucked continuously (Diaz et al., 1989). Between 10–11 nursing episodes occurred in 24 h. Pre-suckling (basal) blood samples were collected at 2 h intervals, starting at 0800 h and ending at the same hour on the following day. In addition, to study the effect of suckling, blood was collected in six of the 11 suckling episodes. Samples were drawn 10 and 30 min after the initiation of the suckling episodes that occurred at approximately 0800, 1200, 1600, 2000, 2400 and 0400 h (Campino et al., 1994). In each woman, the sampling schedule was adjusted to the breast-feeding pattern in order to obtain the basal samples at least 90 min after the end of the preceding nursing episode. Five millilitres of blood were obtained each time in tubes containing heparin. The blood samples were centrifuged and the plasma was stored at −20°C until assayed.

We evaluated IR-PRL and BIO-PRL at 0800, 1600, 2400 h in the plasma before and 30 min after the initiation a suckling episode (mentioned as basal and post-suckling samples respectively in the text). The stability of BIO-PRL and IR-PRL has been demonstrated previously (Campino et al., 1994).

Non-nursing women
The women were hospitalized for 24 h in the metabolic ward of the Hospital Clínico, Pontificia Universidad Catolica de Chile. Standardized meals were provided at fixed hours. A butterfly needle was inserted into an antecubital vein between 10–11 h and blood samples were drawn at 1600 and 2400 h on the same day and 0800 h on the next day. Ten millilitres of blood was obtained each time in tubes containing heparin. The blood samples were centrifuged and the plasma was stored at −20°C until assayed.

Laboratory assays

Plasma IR-PRL was measured by radioimmunoassay using the reagents and methodology of WHO Program for the Provision of Matched Assay Reagents for the RIA of Hormones in Reproductive Physiology Program. BIO-PRL was measured by the Nb2 lymphoma cell assay (Campino et al., 1994). Human growth hormone (hGH) was measured by radioimmunoassay in all the samples, using the reagents of National Hormone and Pituitary Program of the NIDDK (Bethesda, MD, USA) (Campino et al., 1992). The details of these methods were described in a previous study (Campino et al., 1994).
Preparation of samples for chromatofocusing

Plasma PRL isoforms were separated by chromatofocusing. To have an amount of PRL in the eluted fractions within the sensitivity of the radioimmunoassay, we made separated pools of equal amounts of plasma from the basal and post-suckling samples obtained at 0800, 1600 and 2400 h from each group of nursing women. A similar procedure was used to make a plasma pool from non-nursing cycling women. Occasional samples which had hGH concentrations over 2.7 µg/l were not included in the pools to avoid interference in the Nb2 lymphoma cell assay (Tanaka et al., 1980). The pre and post-suckling plasma pools from amenorrhoeic nursing women included 14 basal and 14 post-suckling samples. The pre- and post-suckling plasma pools from the cycling nursing women included 11 basal and 11 post-suckling samples. Non-nursing women plasma pool included 27 samples from the 10 women. In all the groups the missing samples were randomly distributed at all time intervals.

Chromatofocusing

The procedure was performed at 4°C. Plasma (3.5 ml) was applied to a 22×12 cm polybuffer exchanger column (Pharmacia Biotech AB, Uppsala, Sweden), equilibrated previously with 0.025 M ethanolamine, pH 9.9 (Sigma E-9508; Sigma Chemical Co., St Louis, MO, USA). Proteins were eluted according to their isoelectric point using a pH gradient from 10 to 3.7. The gradient was initiated by passing polybuffer 96, (Pharmacia, pH 5.9, diluted 1:10 with water) at 12 ml/h, then aliquots (3 ml) were collected. When the pH of the eluate reached 6.0, polybuffer 74, (Pharmacia, pH 4.0 diluted 1:8 with water) was applied until the pH of the eluate reached 4.0. Finally, 1.0 M NaCl was applied to the column to elute molecules remaining attached to the polybuffer exchanger, and 20 more aliquots were collected. The pH of each aliquot was measured in an Orion Research Digital Ionalyzer/501 pHmeter (sensitivity 0.01). To eliminate polybuffers, the proteins were precipitated by adding 0.5 ml of 1% bovine serum albumin and 2.3 g of powdered ammonium sulphate to each fraction. After thoroughly mixing, the mixture was incubated at 4°C for 2 h and afterwards centrifuged at 1500 g for 3 h. The supernatants were discarded. The remaining precipitates were resuspended in 0.5 ml of buffer 0.01 M PHS (phosphate buffered saline), pH 7.4, and two consecutive aliquots were mixed to maximize the concentration of PRL. In each of the resulting fractions we analysed IR-PRL by RIA. All the fractions from each chromatofocusing were analysed in duplicate in the same assay. An aliquot of the respective plasma pool was included in the IR-PRL assay. Recovery was assessed by dialysing known amounts of 125I-PRL and hPRL standard diluted in known volumes of seven chromatofocusing fractions of different pH that did not contain detectable IR-PRL, obtained from the plasma pool of cycling non-nursing women. IR-PRL and BIO-PRL were lost to similar extents during dialysis. After dialysis, 43.93 ± 0.68% of 125I-PRL, 44.01 ± 1.3% of IR-PRL and 43.97 ± 0.76% of BIO-PRL remained inside the dialysis tube. We considered the recovery of BIO-PRL and IR-PRL after dialysis as 44%.

BIO-PRL and IR-PRL were measured in the dialysate of the fraction containing the peak (highest IR-PRL concentration) of each isoform. Several dilutions of the peak fraction were bioassayed to assure that the BIO-PRL concentration was within the linear range of the standard curve of the Nb2 cell assay. In addition, several fractions, selected at random in which IR-PRL was undetectable, were dialysed and bioassayed. To obtain the mass of IR-PRL and BIO-PRL after dialysis in the peak of each isoform, the results were corrected by the recoveries of the dialysis and of the respective chromatofocusing. Results were expressed as mean ± SE.

Data analysis

The results were analysed using Wilcoxon and Wilcoxon signed rank tests (Zar, 1974) and the likelihood-ratio tests (Mardia et al., 1982). This last test investigates whether the population fits best one or two straight lines. To that effect, it first calculates the best-fit line for all points and the deviation of each point from this line (error). Then, it fits the data to two independent lines and again calculates the error for each point. Finally, it compares the error for each model by a Fisher test (Mardia et al., 1982). If the model of two lines has a lower error than the one-line model, this last one is rejected. Differences were considered significant when P < 0.05.

Table II. Bioactive prolactin (BIO-PRL) and immunoreactive prolactin (IR-PRL) concentrations1 in amenorrhoeic and cycling nursing women at 6 months postpartum and in cycling non-nursing women

<table>
<thead>
<tr>
<th>Groups of women</th>
<th>BIO-PRL</th>
<th>IR-PRL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
</tr>
<tr>
<td>Nursing</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amenorrhoeic</td>
<td>55.5 ± 6.3</td>
<td>74.7 ± 7.0*</td>
</tr>
<tr>
<td>Cycling</td>
<td>13.8 ± 2.5†</td>
<td>14.7 ± 2.8†</td>
</tr>
<tr>
<td>Non-nursing</td>
<td>6.0 ± 0.7</td>
<td>NA</td>
</tr>
</tbody>
</table>

1Mean of day (µg/l) ± SE; pre = pre-suckling; post = post-suckling; *Post versus pre, P < 0.05 Wilcoxon signed ranks test. †Cycling nursing women versus amenorrhoeic nursing women and versus cycling non-nursing women, P < 0.05 Wilcoxon test. NA = not applicable.

Results

BIO-PRL, IR-PRL concentrations and BIO-PRL–IR-PRL relationship

Several differences were found between the three groups of women. Mean daily BIO-PRL and IR-PRL concentrations were highest in amenorrhoeic nursing women, intermediate in cycling nursing women, and lowest in cycling non-nursing women (Table II). Differences between both groups of nursing
women were larger for BIO-PRL than for IR-PRL. The PRL response to suckling was also different: an increase in IR-PRL after suckling was observed in the two groups of nursing women, but was larger in the amenorrhoeic nursing women; however, only these nursing women showed a significant response of BIO-PRL to suckling (Table II, $P < 0.05$, Wilcoxon signed ranks test).

The relationship between plasma BIO-PRL and IR-PRL concentrations (Figure 1) fitted different regression lines in amenorrhoeic and cycling nursing women. Suckling did not change the BIO-PRL–IR-PRL relationship. Considering the pre- and the post-suckling samples, the equations were BIO-PRL = 0.48 IR-PRL + 22.28 ($r = 0.69$) for the amenorrhoeic nursing women and BIO-PRL = 0.30 IR-PRL + 1.27 ($r = 0.78$) for the cycling nursing women ($P < 0.05$, likelihood ratio test).

The BIO-PRL–IR-PRL relationship in amenorrhoeic nursing women was similar to that reported in cycling nursing women at 3 months postpartum (BIO-PRL = 0.56 IR-PRL + 17.7, $r = 0.62$), when those women were in amenorrhoea, 2–3 months prior to recovery their menstrual cycles (Campino et al., 1994).

Cycling non-nursing women, although having lower basal values of BIO-PRL (about half) and of IR-PRL (about one-third, Table II) showed a similar BIO-PRL–IR-PRL relationship than cycling nursing women (BIO-PRL = 0.29 IR-PRL + 2.35, $r = 0.58$).

The equations for the BIO-PRL–IR-PRL relationship suggest that a given amount of IR-PRL corresponds to the same amount of BIO-PRL in cycling nursing and cycling non-nursing women, while it corresponds to a larger amount of BIO-PRL in amenorrhoeic nursing women who are 2–3 months from their first menses.

**PRL isoforms**

Prolactin was present as several IR-PRL isoforms in the plasma pools of the three groups of women studied. BIO-PRL was detected in the peaks of some of the larger isoforms, indicated by arrows in Figure 2. A combination of low initial IR-PRL concentrations and methodological losses during dialysis (see Materials and methods) resulted in concentrations below the limit of the sensitivity of the Nb2 cell assay and radioimmunoassay for many of the other peaks. No bioactivity was found in the chromatofocusing fractions that did not contain IR-PRL isoforms. The overall profiles of IR-PRL isoforms in basal samples were alike in cycling nursing and cycling non-nursing women, showing a larger number of IR-PRL isoforms compared with amenorrhoeic nursing women. Bioactivity was detected in isoforms eluting at a more acidic range of pH in the basal and post-suckling samples in nursing women and in isoforms that were more alkaline in cycling non-nursing women (Table III).

In the basal samples of amenorrhoeic nursing women, IR-PRL was distributed in two clearly separated isoforms in the pH range 7.0–5.1. The amount of BIO-PRL found in the peak of these isoforms is shown in Table III. The ratio BIO/IR of the isoform eluting at pH 6.0–5.1 was 0.3. It was not possible to determine this ratio in the peak of the isoform eluting in pH 7.0–6.1. Suckling increased total IR–PRL (Table II), resulting in a marked increase in the IR-PRL isoform eluting pH 7.0–6.1. The peak of this isoform was slightly more acidic than the isoform found in the basal samples at this pH range; it contained a large amount of BIO-PRL (27.9 ng) and had a BIO/IR ratio of 1.2. A second, smaller IR-PRL isoform, eluting at pH 6.0–5.1 was detected in the down slope of this isoform. The peak of this isoform contained 5.2 ng of BIO-PRL and had a BIO/IR ratio of 0.9, suggesting that it is different from that eluting at pH 7.0–6.1. In addition, this isoform is probably different from that eluting at pH 6.0–5.1 in the basal sample since it is slightly more alkaline and has a higher BIO/IR ratio.

In the basal plasma pool of cycling nursing women, IR-PRL was distributed in several IR-PRL isoforms. Of these, we detected a small amount of bioactivity in the peak of the isoform eluting at pH 6.0–5.1. The BIO/IR ratio at the peak was 0.3. Suckling increased total plasma IR–PRL, resulting in an increase in one isoform eluting at pH 6.0–5.1. BIO-PRL 4 ng were detected in the peak of this isoform and the BIO/IR ratio was 0.9, suggesting that, although eluting in the same pH than the isoform found in the basal samples, it is not the same isoform. A second isoform, in a more alkaline range (pH 8.0–7.1) showed a small amount of bioactivity (Table III). The BIO/IR ratio of this isoform could not be determined.

Thus, in amenorrhoeic and cycling nursing women bioactive IR-PRL isoforms were present in the pH range 6.0–5.1. The similarity in eluting pH and BIO/IR ratios suggests that the same isoform was present in both groups of nursing women in the basal samples. An analogous situation was observed after suckling, in which comparable amounts of an isoform eluting at pH 6.0–5.1 but with a higher BIO/IR ratio than that found in the basal samples was present in both groups of women.

Nursing women differed in that large amounts of IR-PRL and BIO-PRL were found only in the pH range 7.0–6.1 in the basal and post-suckling plasma pools of amenorrhoeic nursing women. In the post-suckling plasma pool, the BIO/IR ratio of this isoform was 1.2, the highest detected in the present study.
Figure 2. Immunoreactive prolactin (IR-PRL) isoform profiles in basal and post-suckling plasma pools of amenorrhoeic nursing women (upper panel), cycling nursing women (central panel) and in basal plasma pool of non-nursing cycling women (lower panel). Please note the different scale in amenorrhoeic nursing women. Bioactive PRL isoforms are indicated with arrows (see text for details). Each coloured interval represents a pH unit. The horizontal broken lines show the limit of sensitivity of the PRL radioimmunoassay.

Cycling non-nursing women, although having the lowest plasma PRL concentrations, also showed several IR-PRL isoforms. We detected bioactivity in two of these isoforms, eluting at pH 10–9.1 and 8.0–7.1. It was not possible to measure BIO/IR ratio in these isoforms. BIO-PRL in an isoform in the pH range 8.0–7.1 was also detected in nursing cycling women after suckling, although we could not assess whether it corresponded to the isoform found in non-nursing women.

Discussion
Our study shows that in women, lactation and the absence or presence of menstrual cycles during breast-feeding are
associated with differences in total plasma BIO-PRL, in the amount of BIO-PRL for a given amount of IR-PRL concentrations (indicated by the equations of the BIO-PRL–IR-PRL relationships) and in the presence of particular bioactive isoforms of PRL. We suggest that these isoforms may play a role in maintaining lactogenesis and lactational amenorrhoea.

Although PRL is necessary for milk production (Tucker, 1994) and suckling maintains hyperprolactinaemia (Howie and McNeilly, 1982), we found a poor correlation between breast-feeding, total BIO-PRL and the amount of BIO-PRL for a given amount of IR-PRL according to the BIO–IR relationship. Cycling nursing women had about twice the concentration of BIO-PRL than cycling non-nursing women, but a similar BIO–IR relationship. Between nursing women there were big differences in BIO-PRL concentrations and in BIO-PRL–IR-PRL relationships, despite having a similar suckling frequency and their babies reaching similar weights. Amenorrhoeic nursing women had about four-fold more BIO-PRL than cycling nursing women in basal samples; differences were augmented after suckling since only these women showed a BIO-PRL response. Successful nursing seems not to require a PRL of high biological activity, since the amount of BIO-PRL for a given amount of IR-PRL was similar in cycling nursing and non-nursing women and less than that found in amenorrhoeic nursing women. In contrast, the same data indicate that amenorrhoea in nursing women is accompanied by a PRL of high biological activity.

The above observations correlated with the presence of combinations of charge isoforms of PRL in the three groups of women. The overall profiles of IR-PRL isoforms in basal samples were alike in cycling nursing and cycling non-nursing women, showing a larger number of IR-PRL isoforms than amenorrhoeic nursing women. Differences between amenorrhoeic and cycling nursing women were accentuated when comparing the profile of IR-PRL obtained after suckling. Most IR-PRL appeared in isoforms eluting at pH 7.0–6.1 and at pH 6.0–5.1 in amenorrhoeic nursing women and at pH 6.0–5.1 in cycling nursing women. To assess the contribution of these isoforms to the BIO-PRL–IR-PRL relationships we measured PRL biological activity in the peak of each IR-PRL isoform. Characterization of the residue determining the charge of the isoform or the molecular weight was not attempted because of the small mass of PRL available. Detectable bioactivity was found only in a few of the IR-PRL isoforms present in each group of women. In nursing women, PRL isoforms had BIO/IR ratios ranging from 0.3 to 1.2.

Higher BIO/IR ratios were present after suckling. Isoforms eluting at pH 6.0–5.1 had a BIO/IR ratio of 0.3 in the basal plasma pools and 0.9 in the post-suckling plasma pool, suggesting the presence of a different isoform in basal and post-suckling samples but common to amenorrhoeic and cycling nursing women. After suckling the amount of BIO-PRL in this isoform was similar in amenorrhoeic and cycling nursing women. In addition, in cycling nursing women a small amount of BIO-PRL appeared after suckling in an alkaline isoform, eluting at pH 8.0–7.1, that was also present in cycling non-nursing women. The increase in BIO/IR ratio observed in isoform eluting at pH 6.0–5.1 may involve an effect of suckling upon processing of PRL in the lactotrophs. One possible explanation could be the secretion of non-glycosylated PRL, more bioactive than glycosylated PRL (Markoff and Lee, 1987; Markoff et al., 1988). Both types of PRL have been found in plasma of nursing women (Markoff and Lee, 1987; Hashim et al., 1990). In amenorrhoeic nursing women, large amounts of BIO-PRL were found in the peak of the isoform eluting at pH 7.0–6.1; this had a BIO/IR ratio of 1.2, the highest detected in the present study. As we could not measure the BIO/IR ratio of this isoform in the basal samples, we do not know whether it was changed by suckling. The presence of large amounts of the isoform eluting at pH 7.0–6.1, and its high BIO/IR ratio after suckling, may help to explain the increase in total plasma BIO-PRL after suckling observed in amenorrhoeic nursing women and the BIO-PRL–IR-PRL relationships in total plasma, indicating more BIO-PRL for a given amount of IR-PRL in these women than in cycling nursing women.

In the present study, the main difference in PRL isoforms between amenorrhoeic and cycling nursing women was the presence of the bioactive PRL isoform eluting at pH 7.0–6.1 only in amenorrhoeic nursing women. The design of this study does not allow us to distinguish whether this difference is due to changes in PRL regulation related to the re-initiation of menstrual cycles and/or the endocrine environment provided by the cycling ovary. However, our data show that decreased PRL biological activity precedes the recovery of menses by 2–3 months. In the amenorrhoeic nursing women, the BIO-PRL–IR-PRL relationships in total plasma showed that PRL biological activity diminished between three (Campino et al., 1994) and 6 months postpartum. In addition, at 6 months postpartum the BIO–IR-PRL relationship of these women became similar to that found at 3 months postpartum in the nursing women that are now cycling (Campino et al., 1994). Whether this diminution of PRL biological activity preceding re-initiation of menstrual cycles in nursing women involves changes in the amount of the PRL isoform eluting at pH 7.0–6.1 remains to be investigated.

The association of particular bioactive PRL isoforms with breast-feeding and with lactational amenorrhoea allows some speculation on a possible role of the isoforms. Since both groups of nursing women shared bioactive isoforms eluting at

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### Table III. BIO-PRL (µg/l) present in the peak fraction of each plasma IR-PRL isoform in amenorrhoeic and cycling nursing women at 6 months postpartum and in cycling non-nursing women

<table>
<thead>
<tr>
<th>Groups of women</th>
<th>pH range</th>
<th>Pre-suckling</th>
<th>Post-suckling</th>
<th>Cycling</th>
<th>Non-nursing cycling</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amenorrhoeic</td>
<td>10–9.1</td>
<td>nd</td>
<td>3.7</td>
<td>27.9</td>
<td>0.6</td>
</tr>
<tr>
<td>Pre-suckling</td>
<td>9.0–8.1</td>
<td>nd</td>
<td>2.6</td>
<td>5.2</td>
<td>0.6</td>
</tr>
<tr>
<td>Post-suckling</td>
<td>8.0–7.1</td>
<td>nd</td>
<td>0.7</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Cycling</td>
<td>7.0–6.1</td>
<td>nd</td>
<td>0.7</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Non-nursing</td>
<td>6.0–5.1</td>
<td>nd</td>
<td>0.7</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td>5.0–4.1</td>
<td>nd</td>
<td>0.7</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

nd = non-detectable BIO-PRL; – = no IR-PRL isoform.
ph 6.0–5.1 and reaching comparable amounts of bioactivity after suckling, these isoforms could act upon the mammary gland to regulate milk production. Such a situation could explain the discrepancy between the similar weight of the infants and the several-fold differences in total plasma BIO-PRL concentration and in BIO-PRL–IR-PRL relationship between nursing women that are amenorrhoeic or cycling. A second speculation relates to the association of lactational amenorrhoea with the presence in plasma of large amounts of BIO-PRL in the isoform eluting at pH 7.0–6.1. The presence of some PRL is necessary for normal ovarian function, as suggested by the observations of deranged cycles in hypo- and hyperprolactinaemic women (Kauppila et al., 1988). Moreover, in pathological hyperprolactinaemia, high biological activity of PRL is required for the presence of amenorrhoea, since menstrual cycles are not altered when low bioactivity isoforms of PRL (so-called big-big PRL) predominate (Whitaker et al., 1984; Jackson et al., 1985; Larrea et al., 1992; Leite et al., 1992). In this regard, the increase in BIO-PRL in the isoform eluting at pH range 7.0–6.1 after suckling, could be part of the unknown mechanisms by which a high suckling frequency contributes to the duration of lactational amenorrhoea (Howie and McNeilly, 1982). Our speculations imply that the target organs (mammary gland and ovary) selectively recognize PRL isoforms. Three types of PRL receptors have been identified: long, short and intermediate. The long form is present in human breast tissue, while long and short forms have been identified in several rat tissues, including mammary gland and ovary (Bole-Feyso et al., 1998). The intermediate form is present in Nb2 lymphoma cell (Kelly et al., 1992). The three forms of the PRL receptors have a common extracellular domain but differ in the length of the intracellular tail and in the signal transduction mechanisms utilized (Bole-Feyso et al., 1998). In the rat, the number of PRL receptors present in different tissues changes under different physiological conditions and the increase or decrease is tissue specific (Nagano and Kelly, 1994). In the human endometrium, treatment with levonorgestrel increases the number of PRL receptors (Critchley et al., 1998), suggesting that PRL receptors are regulated, as has been shown in the rat. In this species, the number of ovarian PRL receptors is elevated during pregnancy and decreases considerably at term, while the mammary gland maintains a higher number of receptors (Nagano and Kelly, 1994; Telleria et al., 1997). In our study, the higher biological activity of isoform pH 7.0–6.1 in the Nb2 lymphoma cell assay suggests that it interacts with higher affinity with the extracellular domain of the PRL receptor than isoform pH 6.0–5.1. Since in-vivo biological actions of PRL require binding of a second molecule of receptor and homodimerization (Bole-Feyso et al., 1998), the combination of high affinity and a large concentration of the isoform pH 7.0–6.1 may result in inhibition by saturation of receptors in the monomer state as proposed by Fuh et al. (1993), impairing homodimerization of the receptor and intracellular signal transduction. This inhibition was not observed in the Nb2 cell assay since PRL isoforms were diluted to fit into the standard curve. If in amenorrhoeic nursing women, the number of PRL receptors was higher in the mammary gland than in the ovary, then the ovarian receptor could be saturated by isoform pH 7.0–6.1 while the mammary gland would still have some receptor available to be stimulated by the lower affinity isoform pH 6.0–5.1.

In summary, our results suggest that the differences in plasma overall BIO-PRL concentration and in the BIO-PRL–IR-PRL relationship in amenorrhoeic and cycling nursing and non-nursing women may be determined by the combination of PRL isoforms present. Further studies are needed to establish the physiological role of these isoforms.

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