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REPRODUCTIVE PHYSIOLOGY AND DISEASE

Evidence that exposure to progesterone alone is a sufficient stimulus to cause a precipitous rise in the immunomodulatory protein the progesterone induced blocking factor (PIBF)

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Abstract
Purpose To determine if exposure to progesterone alone is sufficient to increase the production of the immunomodulatory protein known as the progesterone induced blocking factor (PIBF). Also to determine what method of progesterone delivery or form of P best stimulates PIBF secretion.
Methods Serum samples from patients with infertility and paid volunteers were evaluated for both PIBF and progesterone at various times during the follicular phase and the luteal phase in both natural cycles and cycles involving embryo transfer after endogenous and exogenous progesterone exposure and after various synthetic progestins. PIBF was measured by a non-commercial research ELISA assay. Comparisons were made of serum PIBF before and after exposure to progesterone, 17-hydroxyprogesterone, and oral contraceptives. PIBF was also measured before and after transfer of embryos.
Results Progesterone alone without exposure to the fetal allogeneic stimulus was able to produce a marked increase in serum PIBF. Neither a synthetic progestin (19-nortestosterone derivative) nor 17-hydroxyprogesterone caused an increase in PIBF. Some PIBF is generally detected even in the follicular phase.
Conclusions A previous concept considered that an allogeneic stimulus, e.g., from the fetal semi-allograft, was necessary to induce de novo progesterone receptors in gamma delta T cells, which, in turn, when exposed to a high concentration of progesterone, would secrete high levels of PIBF. These data show that exposure to an allogeneic stimulus is not needed to cause a marked rise in PIBF, merely progesterone alone is sufficient.

Keywords Immunomodulatory protein · Natural killer cells · Progesterone induced blocking factor · Progesterone · Fetal semi-allograft

Capsule A rise in serum progesterone independent of a conception seems to be the main stimulus for causing a significant rise in the immunomodulatory protein, the progesterone induced blocking factor.

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Introduction

There is an immunomodulatory protein known as the progesterone induced blocking factor (PIBF) that is overexpressed in highly proliferating cells [1]. The parent compound, which measures 90 kDa, resides in the nucleus at a centrosomal position [1]. This protein seems to be unique in that it shows no significant amino acid sequence homology with any known protein [2]. The full length protein consists of 757 amino acid residues and is encoded by PIBF1 cDNA [2]. The 48-kDa N-terminal part of PIBF is biologically active [2]. Exons 2–4 are the part of the molecule responsible for modulating natural killer (NK) cell activity [2]. The parent 90-kDa compound can produce a splice variant that is found in cytoplasm and measures 34–36 kDa [1, 2].

Over 25 years ago, data were presented suggesting that the sensitivity of the immunosuppressive effect of progesterone (P) on NK cell activity was markedly enhanced in the pregnancy state by demonstrating the need to increase the P concentration 100 fold to obtain the same suppressive effect on NK cell activity by non-pregnant vs. pregnant lymphocytes [3, 4]. The use of the P receptor modulator mifepristone abrogated the immune suppression by a factor secreted by gamma/delta TCR+ and/or CD8+ lymphocytes. This suggested that P was needed to interact with a P receptor on these lymphocytes to activate them which then secreted some immunosuppressive factor [5–8]. Thus, the term coined for this immunosuppressive factor was the progesterone induced blocking factor (PIBF), and it has now been purified and synthesized by recombinant DNA technology [2, 9]. The purification of the PIBF protein has allowed the production of a monoclonal antibody against PIBF [10]. Interestingly, the serum protein measures 34 kDa very similar to the intracytoplasmic form [1, 2]. It is not clear if these two proteins are merely similar or identical [2, 9].

The suggestion that the allogeneic stimulus of the fetal-placental unit may be responsible for a hormone independent upregulation of P receptors on gamma/delta T cells was derived from both in vitro and in vivo studies [5, 11]. This concept was further strengthened by the demonstration that lymphocyte immunotherapy can increase P receptors on lymphocytes [12] and can increase PIBF expression [13].

Since, initially, the PIBF protein was not purified, many of the early studies of PIBF used a less sensitive immunocytochemistry technique using polyclonal antibodies to PIBF for its detection [14, 15]. Using the old less sensitive immunocytochemistry technique, the 34-kDa PIBF protein was detected in mid-luteal phase in 20.8 % (14/67) of women receiving controlled ovarian hyperstimulation followed by oocyte retrieval and day 3 embryo transfer (ET) and in 11.4 % (8 of 70) women undergoing day 3 frozen ET [16]. Whereas there was no difference in the pregnancy rates in those having frozen ET who were positive for PIBF (4/8, 50 %) or negative for PIBF (28/62, 45.2 %), there was a significantly lower pregnancy rate in those having in vitro fertilization-embryo transfer (IVF-ET) with positive PIBF (1/14, 7.1 %) vs. those negative for PIBF (23/53, 43.4 %) [16].

Since the leading concept in the very early twenty-first century was that trophoblast invasion was needed to induce P receptors on gamma/delta T cells, the poor pregnancy rates following embryo transfer in those positive for PIBF shortly after ET were attributed to the possibility that controlled ovarian hyperstimulation COH could lead to premature trophoblast invasion with adverse consequences for conception [16, 17]. However, since 50 % of patients having COH and IVF-ET had serum P levels >2 ng/mL on the day of hCG vs. 21 % for those negative for PIBF, an alternate hypothesis is that premature luteinization itself with advancement of the implantation window was responsible for low pregnancy rates. This could also suggest that PIBF may have risen even prior to trophoblast invasion and suggest that P alone without the allogeneic stimulation may induce upregulation of PIBF secretion by gamma/delta T cells [16, 17].

As mentioned, with the purification of the PIBF protein, there has been the creation of monoclonal antibodies to PIBF which could now be used to develop a more sensitive assay, e.g., for an enzyme-linked immunoabsorbent assay (ELISA) for PIBF. The purpose of this study was to corroborate or refute the previously held concept that an allogeneic stimulus by the fetus is important in the sensitization of pregnancy lymphocytes to secrete PIBF in the presence of progesterone, but now perform this evaluation using a much more sensitive research ELISA assay that has been developed.

In addition, using the new ELISA assay, it is hoped that more information can be accrued concerning other aspects of PIBF secretion, e.g., what is the main mechanism of stimulation of this immunomodulatory protein, even if not a prerequisite that the conceptus enhances its secretion? Is some PIBF secreted even in the follicular phase where serum P levels are low? Can exogenous P supplementation and/or controlled ovarian hyperstimulation increase PIBF secretion? A final question of the study was as follows: Are some specific routes of P administration more effective in causing a rise in serum PIBF, i.e., intramuscular (IM) vs. intravaginal, vs. oral? Can PIBF be increased by 17-hydroxyprogesterone or 19-nortestosterone-derived synthetic progestins?

Materials and methods

Study design

Thirty-three subjects had serum PIBF levels obtained on specific days throughout the menstrual cycle in those who were menstruating. There were 23 subjects who were infertility patients in our practice who gave permission to have an extra tube of blood obtained to measure serum PIBF along with nine uncompensated volunteers and one compensated volunteer.

Answers to the following questions were hoped to be attained by this study:

1. With the new more sensitive ELISA assay, can PIBF be detected even in the absence of significant P secretion?
2. Will PIBF increase in the mid-luteal phase in natural cycles following exposure to the endogenous rise of P where unprotected intercourse was precluded? If so, these results would suggest no influence by a conceptus.
3. Does supplemental P, with its subsequent increase in serum P levels, further increase PIBF levels over what

occurs with exposure to endogenous P? Does trophoblast invasion accelerate PIBF production?

4. Will the serum PIBF level be even higher in the late luteal phase vs. mid-luteal phase if one continues the same dosage of exogenous P? In other words, will the difference in P exposure time from mid to late-luteal phase up-regulate PIBF receptors and thus increase the serum PIBF? Is there any correlation with achievement of a pregnancy?
5. If P causes an increase in PIBF, could a 19-nor testosterone derivative synthetic progestin also cause increased secretion of PIBF?
6. Does the type of P supplementation influence the serum PIBF levels (vaginal vs. oral vs. intramuscular)?
7. Does an injection of 17-OHP also increase serum PIBF?

To help answer question 1, 4 ovulatory women in natural cycles, where P levels were <0.6 ng/mL in the follicular phase, had serum PIBF measured. Furthermore, to see if PIBF detected in the follicular phase could be left over by exposure to P in the cycle before, PIBF was measured in 4 post-menopausal women over the age of 55 who were not receiving hormonal replacement. Also, 5 male volunteers had serum PIBF levels obtained.

For question 2, five women with regular menses had 1–3 serum levels of PIBF measured with serum P less than or equal to 1.5 ng/mL, and these were compared to the mid-luteal phase level after an endogenous rise in P in completely natural cycles.

To help answer question 3, three women had PIBF levels obtained 1 h before and 1 h after embryo transfer. To see if controlled ovarian hyperstimulation (COH) has any effect on PIBF, one of these women had COH and IVF-ET, one had COH and FET and had serum PIBF drawn on day 3 of exogenous P. One woman had frozen embryo transfer alone on a graduated estradiol/P regimen, and PIBF was obtained on her 4th day of exogenous P. All 3 of these women also had serum PIBF measured 1 h after embryo transfer. The women having IVF-ET had serum PIBF obtained also 1, 2, and 3 days after embryo transfer, and one woman having frozen ET on a graduated E2/P replacement cycle had a PIBF level obtained 3 days after embryo transfer. The women having COH and IVF-ET used vaginal P supplementation twice daily exclusively (Crinone vaginal gel 8 %—Watson Pharmaceutical, Parsippany, NJ) whereas the one woman having FET on graduated E2 replacement cycle received 100 mg IM P daily plus Crinone vaginal gel twice daily.

To help answer question number 4, 11 women having ET had serum PIBF measured 3 and 11 days after ET. This group consisted of 7 women having COH and IVF-ET, 2 women having fresh ET derived from donor eggs, and 2 women having frozen ET. The 4 women not receiving COH were on a graduated estradiol/progesterone supplementation regimen consisting of 90 mg P vaginal gel (Crinone 8 % vaginal gel —Watson Pharma Inc., Parsippany, NJ 07054, USA) twice daily and 100 mg/day intramuscular progesterone (progesterone injection USP, American Regent Inc, Shirley, NY 11967).

For question number 5, serum PIBF was obtained in a 20-year-old woman after 14 days of an oral contraceptive.

For question 6, one volunteer had a baseline serum PIBF when the serum P was less than 0.6 ng/mL. She then had serum P and PIBF levels obtained during separate courses of progesterone supplementation. The levels were drawn after 7 days of daily Crinone vaginal gel 90 mg/day, after 7 days of micronized P 200 mg/day, and after 4 and 7 days of IM P 100 mg/day. For question 7, the young woman volunteer on oral contraceptives had serum PIBF measured 1 and 3 days after a single injection of 17-hydroxyprogesterone 250 mg I. M. (Makena, Hologic Inc., Sunnyvale, CA). She was taking the entire time an oral contraceptive to block endogenous P secretion. We had already determined that the progestin in this oral contraceptive does not raise PIBF levels (question 6).

The PIBF assay—PIBF assay was performed as follows: A non-commercial enzyme-linked immunoabsorbent assay (ELISA) was used to measure PIBF in serum. Serum specimens were stored at −20 °C. Fifty microliters of recombinant PIBF standard was added to each pre-coated goat anti-rabbit antibody well in duplicate.

The concentrations of the PIBF standard were S0—0, S1—3.2, S2—11.2, S3—40, S4—160, and S5—802 ng/mL. The patient's serum was then added to each well. Next, 50 μl of horse radish peroxidase-conjugated PIBF antigen was added to each well except the zero standard. Next, anti-PIBF IgG antibody was added to each well. The microtiter plate was then incubated in the dark for1 h at 37 °C. After 1 h, the wells were washed with PBS and decanted three times. Next, 50 μl of substrate A (carbamide peroxide) and 50 μl of substrate B (tetramethylbenzidine) were added. The microliter trays were then incubated in the dark at 37 °C for 15 min.

Next, 50 μl of stop solution, whose main component is H_2SO_4, was added. The plates were read within 10 min using a microplate reader at 450 nm. The results were calculated using a 4-parameter logistic curve fit.

It should be noted that this ELISA assay for PIBF is not commercially available. The present assay cannot accurately measure PIBF levels that exceed 800 ng/mL. Nevertheless, the serum PIBF samples are run in duplicate, and the final result is the average of the two samples. Generally, the results of these two samples are similar when using a given batch of anti-PIBF antibody. The reagents for the PIBF assay were obtained from CUSABIO.

Statistical analysis was performed using an up to date statistical program GraphPad Prism 6. Analysis of follicular and luteal phase PIBF levels was done through an unpaired non-parametric *t* test. To analyze the data from patients taking supplemental progesterone vs. endogenous progesterone, vs. the control samples, an ANOVA was used and multiple variables were corrected for by the Tukey method.

Results

Question 1: Can PIBF be detected in the absence of significant P secretion because of the improved sensitivity of the ELISA assay?

To help answer question 1, 13 PIBF specimens were obtained. The serum PIBF levels for the first four (which consisted of ovulatory patients in the follicular phase when serum P was less than or equal to 0.6 ng/mL) were 54, 66, 66, and 79 ng/mL. The next four were post-menopausal women who had random PIBF levels drawn. Their levels were 25.2, 29.9, 34.9, and 41.2 ng/mL. The last five consisted of male volunteers with PIBF levels of 36.6, 43.4, <2, 10.5, and 12.8 ng/mL. Thus, with a more sensitive ELISA, PIBF is present even in the absence of a significant presence of serum P.

Question 2: Will PIBF increase significantly in the mid-luteal phase following exposure to P alone where there can be no influence of a conceptus?

A comparison of serum PIBF in the follicular phase vs. the luteal phase in natural cycles in individual patients is seen in Table 1 in cycles where conception was prevented. There were 11 specimens taken during the patients' follicular phase which yielded a mean of 100.2 ng/mL and a standard deviation of 32.16 ng/mL. They were compared to the mid-luteal phase samples yielding a mean of 443.0 ng/ml and a standard deviation of 167.2 ng/ml. The fourfold increase in serum PIBF levels was significantly higher in the luteal phase compared to the follicular phase using a one way unpaired *T* test ($p = .0005$). Though a relatively small sample size, the significant increase suggests sufficient power to conclude that the influence of a conceptus is not needed to cause a rise in PIBF. Progesterone alone is sufficient.

Question 3A: Does supplemental P therapy in the luteal phase increase serum PIBF more than simply endogenous progesterone?

Table 2 presents serum PIBF levels in women on supplemental vaginal progesterone (attempt to answer question 3). The mean (± SD) PIBF level for such 10 specimens over 4 days where supplemental P was given was 547.3 ± 215.3 ng/mL. The mean serum PIBF level induced in the mid-luteal phase in five specimens without supplemental progesterone (see Table 1) was 443.0 ± 176.2 ng/mL. Comparing the mean mid-luteal phase serum PIBF level of 443.0 ± 176.2 ng/mL to the mean mid-luteal phase levels of 10 women given supplemental P in the luteal phase (547.3 ± 215.3 ng/mL), a significant difference was not attained by ANOVA. The standard error of the difference for this comparison was 89.92 ng/mL. A study with more power will be needed to determine if additional P supplementation increases PIBF over endogenous levels in ovulating women. In planning such a larger study, the design should take into account that the woman's age could possibly influence serum PIBF, so the study groups of endogenous P vs. supplemental P should be age matched.

The mean ± SD for the supplemental P and endogenous P combined was 512.5 ± 203.1 ng/mL. The mean serum

Table 1 Follicular phase levels vs. luteal phase levels of PIBF (ng/mL) in natural cycles without progesterone supplementation

		Serum PIBF (ng/mL)	
Subject	Day	Follicular phase	Mid-luteal phase
1	6	60 (0.9)	
	12	93 (0.2)	
			323 (9.6)
2	3	85 (0.8)	
	8	70 (0.7)	
	15	67 (0.5)	
			370 (14.0)
3	3	170 (0.7)	
	8	94 (0.4)	
	9	103 (0.6)	
			365 (17.1)
4	3	105 (1.5)	
	15	128 (0.6)	
			754 (11.7)
5	10	127 (0.5)	
			403 (13.0)

P levels in parentheses

Table 2 Effect of supplemental P on levels of serum PIBF, and influence of controlled ovarian hyperstimulation and study controls

	Serum PIBF 1° before ET	Serum PIBF 1° after ET	Serum PIBF 1 day after ET	Serum PIBF 2 days after ET	Serum PIBF 3 days after ET
Patient 1 (IVF-ET)	627.8	537.5	618.7	693.6	311.2
Patient 2 (frozen ET)	732.9	703.1			797.3
Patient 3 (frozen ET)	222.4	228.7			

Only patients 1 and 2 received controlled ovarian hyperstimulation

PIBF levels for the follicular controls were 37.6 ± 28.20 ng/mL. The serum PIBF levels for the combined P groups were significantly higher in the luteal phase than the follicular phase controls ($p = 0.0001$, t test). Thus, Table 2 provides additional support for the answer to question 2, i.e., exposure to P significantly causes a rise in serum PIBF (but Table 1 better establishes that a conception is not needed for this precipitous rise in serum PIBF).

Question 3B: Does the conceptus secrete any products while in the uterine cavity or the day of probable implantation to increase serum PIBF levels?

The key conclusion of Table 2 is that the serum PIBF levels were just as high 1 h before embryo transfer as compared to 3 days after embryo transfer, thus showing no influence of the conceptus itself at least in the days in the uterine cavity and possibly the day of implantation. There were only three patients in this study. Thus, with a larger study, one cannot preclude the possibility that perhaps in some women, the presence of an implanted embryo on day 3 could allow a further increase in PIBF. Furthermore, this study did not answer the question as to whether more prolonged exposure to the conceptus following trophoblast invasion could cause a further increase in serum PIBF.

Table 3 provides 11 levels of PIBF obtained after 6 days of P. Seven of these specimens were obtained in a controlled ovarian hyperstimulated (COH) cycle, so the P was derived endogenously from multiple corpora lutea and from P supplementation. Unfortunately, the PIBF assay has not been refined sufficiently to measure levels over 800 ng/mL but 8 of the 11 specimens were over 800 ng/mL. The other 3 were 686, 502, and 390 ng/mL. Thus, only 1 specimen was under the average for natural mid-luteal phase level cycles. These data suggest that raising serum P higher than usually found in the mid-luteal phase of natural cycles increases PIBF and thus provides more data to help answer question 3a, i.e., that if supplemental P or COH itself raises serum P higher, this will lead to higher serum PIBF levels. To be more definitive, a larger prospective study could be designed in which ovulatory women are randomly assigned to a method of P supplementation (e.g., IM) that is known to significantly raise PIBF, and then compare the mean mid-luteal phase PIBF levels in those treated vs. untreated controls. Again, it may be important to restrict the study to one age group, or even more interestingly, determine if mid-luteal PIBF levels are lower in an older aged group in natural cycles not supplemented with P, but if adding IM P increase PIBF levels in the older group comparable to the younger group.

Question 4: Does extending the duration of exogenous P supplementation increase the serum PIBF levels?

Table 3 shows that extending P supplementation to 11 days does not raise PIBF over 6 days of supplementation since only three specimens were >800 ng/mL. This finding helps answer question 4 and suggests that prolonged use of P does not raise serum PIBF levels after a certain point.

Question 5: Do 19-nortestosterone derivative synthetic progestins cause a rise in serum PIBF?

A woman on continuous oral contraceptives only showed a serum PIBF of 34.5 ng/mL. Thus, the answer to question 5 is that at least some progestins that are 19-nortestosterone derivatives do not stimulate a rise in serum PIBF. This does not preclude the possibility that some other progestins could stimulate a rise in serum PIBF.

Table 3 Correlation of serum PIBF level on days 3 and 11 after embryo transfer with serum P at those times and data on achievement of pregnancy

	Serum PIBF (ng/nL) 3 days after ET (serum P)	Serum PIBF 11 days after ET (serum P)	Pregnancy achieved (yes or no)
Patient 1—IVF-ET	>800 (>160)	401 (14.3)	Yes
Patient 2—IVF-ET	686 (49.2)	292 (15.0)	No
Patient 3—IVF-ET	390 (26.5)	762 (58.4)	Yes
Patient 4—IVF-ET	>800 (>160)	375 (54.1)	Yes
Patient 5—IVF-ET	>800 (97.3)	>800 (159.3)	Yes
Patient 6—IVF-ET	>800 (>160)	166 (0.7)	No
Patient 7—IVF-ET	>800 (145.9)	549 (8.8)	No
Patient 8—donor oocyte	>800 (>160)	>800 (136)	Yes
Patient 9—donor oocyte	502 (57.7)	565 (60.4)	Yes
Patient 10—frozen ET	>800 (83.2)	>800 (128.2)	Yes
Patient 11—frozen ET	>800 (91.6)	729 (90.4)	Yes

Question 6: What is the relative effect of vaginal vs. oral vs. IM P on causing a rise in serum PIBF?

Question 7: Is 17-OHP effective in raising serum PIBF?

The data in Table 4 shows that both intramuscular P and oral micronized P raise serum PIBF far greater than vaginal P. Vaginal progesterone does, however, raise the PIBF level. Very interestingly, the rise in PIBF following ingestion of oral micronized P is comparable, in fact, to IM P. These provide answers to question 6. However, 17-OHP failed to increase serum PIBF levels at all (answer to question 7). By comparing PIBF levels in this one individual who was blocked by an oral contraceptive from making endogenous P (with a progestin not found to raise the serum PIBF), it provided the advantage of excluding the confounding effect of possible variation in individual responses to secrete PIBF (by using the same volunteer for all parts of this study), and of course such study keeps the age effect controlled. However, only one patient was evaluated. Thus, the possibility exists that if a larger series was evaluated, a different response could occur in different individuals.

Discussion

Since the fetus is a semi-allograft, it is obvious that certain immune factors need to be inhibited for the fetus to survive. This has to be a selective process rather than a general event so that the mother will not suffer consequences of general immune suppression. The two main effector cells that need to be locally suppressed are cytotoxic T cells and natural killer (NK) cells. Circulating PIBF influenced by P secretion may be an important factor in cellular immune suppression. There are also some recent data that suggest that progesterone may act in an extranuclear (epigenetic or non-genomic) manner to suppress T cell rejection of the fetal semi-allograft [18–20]. Other cells, e.g., TH 17 cells, T reg cells, T helper cells, and dendritic cells, may also play a role.

The progesterone induced blocking factor (PIBF) seems to be involved mostly in suppressing NK cell activity [7, 8]. A priori, one would think that the more allogeneic the stimulus, the more difficult it may be to suppress a maternal immune response against the fetal semi-allograft. However, there was a study published suggesting that there was a survival advantage of the fetus if it was more immunologically distinct from the mother [21]. This was based upon the demonstration that whereas 18 % of couples with recurrent miscarriage showed two similar human leukocyte antigen (HLA) DQ alpha locus alleles, this sharing was only found in only 3 % of fertile couples [21]. This aforementioned study by Ober et al., concerning recurrent miscarriage, was not confirmed by two other studies [22, 23].

Though these latter two studies cast doubt on sharing of HLA DQ alpha 2 antigens as a cause of recurrent miscarriage, one interpretation of the first study by Ober et al. is that sharing of HLA DQ alpha could lead to such early losses that it would manifest more as reduced fecundity rather than higher miscarriage rates [21]. One subsequent study, however, failed to find any association of maternal/paternal sharing of DQ alpha type II HLA antigens to be associated with pregnancy rates or miscarriage rates following IVF-ET [24].

As previously mentioned, some data had suggested that the allogeneic stimulus of the fetal semi-allograft was important in upregulating P receptors in gamma/delta T cells [5, 11, 12]. The data presented in this report clearly show that an allogeneic stimulus is not a prerequisite for a significant rise in serum PIBF. It would appear that exposure to P without an allogeneic stimulus is associated with a very dramatic rise in serum PIBF. The possibility exists that studying a larger group of women could find some women where the conceptus aids in PIBF response to P, though these limited data did not suggest that this was likely.

This study does confirm previous data that had used a far less sensitive immunocytochemistry technique that found that PIBF increases in the serum soon after implantation [14]. However, its presence is not dependent on conception, but merely rises to P exposure. Using the previous less sensitive

Table 4 Serum PIBF (ng/mL) in 1 woman on oral contraceptives after various routes of P and 17-OHP

	Baseline level 1 month on oral contraceptives	Day				
		1	3	4	5	7
IM P	22.3			473		730
Vaginal P (Crinone) 90 mg	22.3					73.2
Oral micronized P 200 mg/day	22.3				618	
17-OHP 250 mg	22.3	22.7	11.7			

17-OHP given as a single injection. P given daily

immunocytochemistry technique, it was found that higher circulating PIBF was positively correlated with pregnancy [15]. Now that it is established that PIBF significantly rises following P exposure, it will be important to evaluate if failure to generate sufficient PIBF levels either during the luteal phase or during the first trimester could be associated with either conception failure or miscarriage.

Theoretically, the body would not waste the significant production of a protein, e.g., PIBF, for no reason. However, the role of generating serum PIBF in allowing a conceptus to progress to a live delivery is not clear. Hopefully, this study will precipitate interest in other scientists to perform studies to determine what role does serum PIBF play in human reproduction.

Progesterone has been found to not only increase the serum PIBF but to also increase the level of a PIBF intracellular splice variant (that is similar in size to circulating PIBF) in rapidly growing cells, e.g., the cells of the fetal placental unit (or cancer cells) [1, 25, 26]. The progesterone receptor modulator abortifacient mifepristone has been found to decrease intracytoplasmic PIBF production of the 34–36-kDa intracytoplasmic splice variant [25]. Yet, mifepristone does not decrease serum PIBF levels [27]. This finding makes the role of serum PIBF even more enigmatic.

It is not known if serum PIBF levels reflect intracytoplasmic PIBF levels in the absence of exposure to a progesterone receptor modulator. If such a correlation is found, and if intracytoplasmic levels of the 34–36-kDa splice variant are found to be the more important factor for the fetal semi-allograft to escape immune surveillance, then knowing which P vehicle stimulates the most serum PIBF could be important in choosing the right type of P supplementation. Of course, even to the present time, there still exists debate about the importance of supplementing extra progesterone to improve the chances of a live delivery [26, 28, 29].

We repeated measuring serum PIBF on the samples used in Tables 1 and 2 on serum samples of an extra tube that was kept frozen. Table 5 lists the original values in the left column and the results using the new antibody kit on the right column. Commercial availability could also stimulate the interest of a lot more clinicians involved in human reproduction. Perhaps a great deal of important clinical information could be generated in a much shorter period of time with not only a more accurate PIBF assay but also using a larger sample size. Antibody variability and small sample size preclude the calculation of a meaningful inter- and intra-variability calculation. However, to provide the reader some idea of the effect of a new batch of anti-PIBF in serum PIBF values, we repeated the PIBF assay on an extra frozen sample using a new antibody kit on the samples used for Tables 1 and 2. The results are seen in Table 5.

The importance of using luteal phase P supplementation to improve fecundity is still an enigmatic question. Despite clinical evidence of its efficacy, there does not appear to be at this present time, except at extremely low serum levels of P, a good method to determine who requires supplementation and who does not [26]. Equally important, if certain women are found to need progesterone, how can one determine if the amount given has corrected the problem? Another important question is what form of progesterone to use to correct the problem? For example, if vaginal P is more efficacious in producing the proper endometrial luteal structure than oral P because the latter is metabolized through first pass through the liver, is there some role for combined vaginal and oral P to improve PIBF levels since oral P is much superior to vaginal P in inducing serum PIBF? Since intramuscular P seems to cause proper endometrial architecture and induces high PIBF levels, should physicians go back to IM P use and only switch to vaginal combined with oral based on side effects?

Our goals are to try to refine the PIBF assay more then try to help answer the above question in a sufficiently powered study to see if one can determine certain discriminatory serum PIBF levels below which there is a lower chance of a live delivery. Unfortunately, the possibility exists that the intracytoplasmic rather than serum level of PIBF is the main factor in preventing immune rejection of the fetus measuring intracytoplasmic PIBF would not be practical. Thus, one could still fail to find that serum PIBF correlates with

Table 5 Original vs. repeat serum PIBF (ng/mL) levels using a different batch of anti-PIBF antibody

	Original sample	Repeat value new antibody
1	60.9	52.5
2	93	77
3	323	302
4	70	82
5	67	49
6	170	95
7	94	90
8	103	86
9	105	71
10	127	110
11	628	642
12	537	492
13	619	580
14	694	710
15	311	385
16	733	729
17	703	685
18	797	755
19	222	255
20	229	199

pregnancy outcome, yet the effect of P on intracytoplasmic PIBF could be its more important action.

Refinement of a more accurate PIBF assay with less variability between batches of antibody products may be crucial to answer the question as to whether there is a need to have an increase in P to generate the same amount of PIBF in women of advanced reproductive age as in younger women.

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Compliance with ethical standards

Statement of human rights The study has been approved by a Western IRB (protocol number 20121249, CIR 110). The studies have been performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

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