

PHYSIOLOGY

A Corpus Luteum is not a Prerequisite for the Expression of Progesterone Induced Blocking Factor by T-Lymphocytes a Week After Implantation

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Purpose: To determine if production of the immunomodulatory protein, progesterone induced blocking factor (PIBF), requires merely progesterone or whether other factors made by the corpus luteum are required.

Methods: The percentage of peripheral lymphocytes expressing PIBF was determined by obtaining a blood sample from women 9–12 days after embryo transfer. The embryos were either fresh ones following hyperstimulation and oocyte retrieval or were frozen-thawed embryos. Preparation for frozen embryo transfer required corpus luteum suppression with exogenous estrogen. The percentage of lymphocytes expressing PIBF was determined by an immunocytochemistry method.

Results: PIBF expression (>1%) was found in 20.5% of COH and 13.3% of frozen embryo transfer cycles. There either was a significant difference or a trend for higher pregnancy rates when PIBF expression was detected.

Conclusions: These data corroborate previous conclusions that PIBF is detected in a minority of women in the

late luteal phase. A corpus luteum is not required for its expression.

KEY WORDS: Corpus luteum; immunomodulatory protein; progesterone.

INTRODUCTION

Progesterone receptor positive natural killer (NK) cells have been demonstrated in the decidua (1). These progesterone receptor positive NK cells will produce a 34 kDa immunomodulatory protein when exposed to progesterone which is named the progesterone induced blocking factor (PIBF) (2).

Progesterone induced blocking factor has been shown to inhibit degranulation of peripheral NK lymphocytes and thus inhibits perforin liberation from NK cells, thus inhibiting NK cell activity (2,3).

When PIBF was measured by an enzyme linked immunosorbent assay (ELISA), there were lower levels in those who aborted versus those who successfully completed the pregnancy (4). Using a cut-off value of 197.5 $\mu\text{g}/\text{mL}$ for PIBF, 52 of 87 women would eventually abort either immediately or up to 12 weeks later if the PIBF levels were less than the cut-off level (4). When PIBF was evaluated using an immunocytochemistry method, between the 9th and 40th week of gestation, the percentage of PIBF expressing lymphocytes in the peripheral blood of 96 healthy pregnant women was 67% versus only 6.5% in 62 women with pathological pregnancies (5).

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Using the immunocytochemistry method, progesterone induced blocking factor has been detected as early as the late luteal phase (6). It has been suggested that detection that early may be associated with conception outcome (6). This study, by evaluating the presence of PIBF expression in cycles of fresh embryo transfer (ET) versus frozen ET (where the corpus luteum had been suppressed by exogenous estrogen), hoped to determine if the corpus luteum plays any role in the facilitation of PIBF expression. Furthermore, the study would try to corroborate or refute the suggestion that detection even that early may be associated with positive conception outcome.

MATERIALS AND METHODS

A prospective study was conducted. Patients undergoing embryo transfer either following oocyte retrieval or the thawing of cryopreserved embryos were enrolled in the study. Oocyte recipients were excluded. Patients had an extra tube of blood obtained at the same time of obtaining a serum β -hCG level and a serum progesterone level about 9–12 days after embryo transfer.

The measurement of PIBF expression was determined by an immunocytochemistry method using a PIBF-specific polyclonal antibody. Mononuclear cells were removed using Isoprep (Robbins Scientific, Sunnyvale, CA) and cold centrifugation, and were adjusted to a concentration of 2×10^6 /mL; 100 μ L aliquots of cell suspension were added to sample chambers and air dried then fixed in cold acetone. The cells were first incubated with protein blocking agent and then incubated overnight with anti-PIBF. The cells were washed in phosphate buffered saline (PBS) (Gibco, Grand Islands, NY) and then covered with anti-rabbit peroxidase. Following a second PBS wash, fresh chromogen solution was added and the cells incubated; the reaction was then stopped with distilled water and the cells counterstained with hematoxylin, and the slides were read under oil immersion (100 \times objective). A positive reaction was indicated by a reddish precipitate at sites of specific cellular antigen localization; 300 cells were counted. The percent of the cells positive was then determined. A test was considered positive if there were at least four lymphocytes of the 300 counted demonstrating the reddish precipitate. This cut-off level of >1% was chosen based on previous unpublished data using this immunocytochemistry technique in which the large majority of nonpregnant women showed

\leq 1% of the lymphocytes demonstrating a positive reaction.

Oocyte retrieval followed stimulation using either the luteal phase leuprolide acetate/gonadotropin protocol (7) or the follicular phase leuprolide acetate/gonadotropin protocol (8). Embryo transfer was performed three days following retrieval. Assisted hatching and intracytoplasmic sperm injection were used as needed. The transfer of thawed cryopreserved embryos was performed in cycles in which patients were given graduated doses of oral estradiol for 2 weeks beginning at 2 mg and ending at 6 mg followed by progesterone vaginal suppositories 200 mg 2 \times /day and IM progesterone (100 mg/day). Down regulation with leuprolide acetate 0.5 to 1 mg daily SC was added if indicated.

The outcome measures studied were PIBF expression, positive pregnancy test, and viable pregnancy (viable fetus detected by ultrasound at the end of the first trimester). Chi-square analysis was used to evaluate the association between outcome of embryo transfer and PIBF expression and was also used to evaluate association between expression of PIBF and presence of the corpus luteum. A *p* value of 0.05 was used to determine significance.

This study was approved by the ethics committee of the Cooper Institute for Reproductive Endocrinology. It was not necessary to submit to the Institutional Review Board of Cooper Hospital because treatment rendered to patients was unaltered whether PIBF was positive or not, and the blood tests for PIBF were obtained at the same time that other hormonal studies were obtained so that no extra venipunctures were necessary. Patients were not charged for these tests, but were made aware that they were taken, and informed consent forms were signed.

RESULTS

One hundred and seven cycles following controlled ovarian hyperstimulation (COH) and in vitro fertilization (IVF)-ET were evaluated. Progesterone induced blocking factor expression was detected in 16 women and 11 (68.8%) had a positive beta human chorionic gonadotropin (hCG) level >100 IU/mL. There were 91 cycles where PIBF expression was not detected and a pregnancy was achieved in 35 (38.5%) cases. Comparing these same values using viable pregnancy (ultrasound demonstrating a viable fetus after first trimester) as the criteria, 7 of 16 (43.8%) demonstrating PIBF expression had

Table I. Association of Progesterone Induced Blocking Factor (PIBF) Expression By Natural Killer Lymphocytes and Pregnancy

	<i>n</i>	Not pregnant	Pregnant	Viable pregnancy
Fresh ET				
PIBF-	91	56	35	31
PIBF+	16	5	11	7
Frozen ET				
PIBF-	78	40	38	29
PIBF+	12	3	9	8

viable pregnancies versus 31 of 91 (34.1%) where PIBF expression was not detected (the chemical pregnancy rate was significantly different between those expressing and not expressing PIBF but the viable pregnancy rates were not). The data are summarized in Table I.

Evaluating these same results in a different way, PIBF expression was detected in 11 of 46 (23.9%) of women with positive β -hCG levels versus 5 of 61 (8.2%) of nonpregnant patients ($p < .05$). Progesterone induced blocking factor was detected in 7 of 38 (18.4%) of viable pregnancies versus 9 of 69 (13.0%) of those without a viable pregnancy ($p = ns$). The data are summarized in Table I.

For frozen ETs, 9 of 12 (75%) patients who demonstrated PIBF expression achieved a pregnancy versus 38 of 78 (48.7%) who did not demonstrate PIBF expression ($p = 0.166$). When evaluating viable pregnancy rates, 8 of 12 (66.7%) who showed PIBF expression achieved a viable pregnancy versus 29 of 78 (37.2%) with negative PIBF expression ($p = 0.106$). Looking at the data in a different manner, 8 of 37 (21.6%) women with viable pregnancies demonstrated PIBF expression versus 4 of 53 (7.5%) who did not have viable pregnancies ($p = 0.106$). The data are summarized in Table I.

DISCUSSION

Evidence shows that 80% of the maternal lymphocytes present in the human decidua during the early weeks of pregnancy are NK-like cells (9). They have the characteristic morphology of large granular lymphocytes and have a distinctive phenotype (CD3-, CD56bright, CD16-) (10-12). Unlike CD56+ NK cells in peripheral blood, decidual CD56+ cells lack type III Fc receptors (CD16) and do not express significant levels of either type 1 FcR (CD64) or type II FcR (CDw32) (13). There are data suggesting that these decidual CD56+ lymphocytes are to a high extent the same population as

decidual lymphocytes that express the gamma/delta T cell receptor (TCR) (14). Most gamma/delta T cells recognize unprocessed foreign antigens without major histocompatibility antigens (14). These cells all express progesterone receptors (15), the 34 kDa protein PIBF (2,14), and very late antigen 1 (VLA-1, CD49a) (16). These data do not necessarily exclude the possibility that a major role is not played by alpha/beta T cells.

Normal lymphocytes in non-pregnant women do not demonstrate progesterone receptors (17). However normal human lymphocytes express progesterone receptors after in vitro allogeneic or mitogenic stimulation (18). Also progesterone receptors were also demonstrated in peripheral lymphocytes of liver transplanted and transfused patients (19). In peripheral blood of pregnant women there is an increased ratio of gamma/delta TCR positive lymphocytes and more than 90% of these cells are activated and express progesterone receptors (15,20). The exact nature of the paternal antigen that stimulates the progesterone receptor is not known but some data suggests that the antigens may be class I or class I-like molecules (21-23). In the presence of progesterone, progesterone receptor positive lymphocytes synthesize the immunomodulatory protein PIBF (2).

Though it is known that PIBF is produced by decidual CD56+ cells and can be detected easily by the end of the first trimester (4,5), only one study found that it could be detected as early as the late luteal phase (6). Though allogenic stimulation has been found to be sufficient to induce progesterone receptors in these decidual NK cells with the capacity for PIBF expression (18-20), the possibility exists that some factor produced by the corpus luteum facilitates PIBF expression in the late luteal phase shortly after exposure to paternal allogeneic stimulus after trophoblast invasion. However, the demonstration that PIBF could be detected in the late luteal phase even in women having frozen ET whose corpora lutea were inhibited by the use of exogenous estrogen, shows that the corpus luteum is not involved in PIBF expression by decidual NK cells. This study also corroborated previous data that PIBF expression can be detected in the late luteal phase. This may not seem so surprising in that there are data that MHC class I mRNAs are synthesized soon after conception and even prior to implantation (24).

The results of this study showed a significantly higher pregnancy rate in those expressing PIBF than those not expressing it, and a trend for higher viable pregnancy rates following fresh ET, and a

trend for higher pregnancy and viable pregnancy rates following frozen ET. These data thus also corroborate a previous study showing a trend for a positive correlation of PIBF expression by peripheral lymphocytes and conception (25). However this association does not necessarily prove that early production of PIBF plays any role in aiding implantation. If it was essential, one could argue that the 38.5% who conceived despite no evident PIBF expression by peripheral lymphocytes may have had PIBF expression by decidual NK cells but there was insufficient production to allow measurable quantities in the peripheral lymphocyte pool.

However other theories could explain successful pregnancy without PIBF demonstration. It is known that normal pregnancy is established partly through a TH2 cytokine dominance and that abortion occurs when there is a TH2-TH1 shift (26). Though the immunological pregnancy protective affect of PIBF may be manifested by controlling cytokine production (27), there are several cells and soluble factors that could be mediators of immunomodulation, e.g., trophoblast cells (28), decidual cells and cells of the lymph nodes draining the uterus (29), factors secreted by cultured placental cells (30,31), and trophoblast cell lines (32-34).

Those patients who either did not conceive or aborted despite PIBF expression could be explained by nonimmunologic factors, e.g., chromosome abnormalities or other immune problems that may not be neutralized by early PIBF expression; for example, some studies suggest that some spontaneous abortions may be related to high preconceptual NK activity (35). On the other hand, it may also be that only patients with a high preconceptual NK activity level need to produce a higher level of PIBF to overcome this problem.

The fact that 38.5% of patients not demonstrating PIBF expression achieved a pregnancy could be explained on another basis. Some previous data found the early murine fetus is protected from the maternal immune system at least during the first half of pregnancy by an intrinsic ability of the early embryo to resist cell mediated lysis (36). This may also apply to the human fetus.

The development in the near future of a more pure PIBF protein should allow for the development of a more sensitive assay for PIBF. Perhaps a more sensitive test will show an even greater dichotomy of women attaining pregnancy showing positive levels and those not achieving pregnancy with negative

levels. Possibly some women may be found whose constant failure to conceive despite ET is associated with failure to demonstrate PIBF expression in the late luteal phase. This would be an interesting group to study whether allogeneic stimulation with paternal lymphocytes may allow PIBF expression and whether the production of this immunomodulatory protein is associated with attaining pregnancies (37).

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