

# Technical Note: A Radioimmunoassay for Porcine Intrauterine Folate Binding Protein<sup>1</sup>

J. L. Vallet<sup>2</sup>, R. K. Christenson, and H. G. Klemcke

USDA, ARS, Roman L. Hruska U.S. Meat Animal Research Center, Clay Center, NE 68933

**ABSTRACT:** A RIA was developed for porcine intrauterine folate binding protein (FBP). Displacement of [<sup>125</sup>I]FBP caused by increasing dilutions of uterine flushings collected from either d-15 pregnant or nonpregnant gilts or media from culture of endometrial tissue from d-15 pregnant or nonpregnant gilts was parallel to the displacement caused by the standard curve. Addition of known amounts of purified allantoic fluid FBP to dilutions of either intrauterine flushings or endometrial culture medium were measured accurately with the RIA. To test specificity, 2-mL samples of uterine flushings collected from d-15 pregnant and nonpregnant gilts were preincubated with 10  $\mu$ Ci of [<sup>3</sup>H]folic acid and then chromatographed using Sephadex G-100 (Sigma Chemical Co., St. Louis, MO). The fractions were subsequently assayed for radioactivity by liquid scintillation spectrophotometry and for FBP by RIA. The [<sup>3</sup>H]folic acid and FBP peaks coincided, indicating that the RIA is specific for FBP. Uterine flushings were collected on d

10, 11, 12, 13, 14, and 15 of the cycle or pregnancy from 1) White crossbred, 2) progesterone-treated White crossbred (200 mg of progesterone at 48 and 72 h after estrus), and 3) Meishan gilts and assayed for FBP. Total FBP increased 140-fold from d 10 to 15, and the pattern of change across day did not differ between pregnant and nonpregnant gilts. Progesterone treatment increased intrauterine FBP content on d 10 and 11. No difference in FBP concentrations was detected between White crossbred and Meishan gilts. These results indicate that the RIA for FBP is valid, allowing measurement of this protein in uterine flushings and endometrial culture medium. The onset of FBP secretion by the uterus between d 10 and 15 of the cycle or pregnancy is influenced by the timing of onset of progesterone influence in a manner similar to the endometrial proteins uteroferrin and retinol binding protein. In contrast to these endometrial proteins, FBP concentrations are similar in Meishan and White crossbred gilts.

Key Words: Endometrium, Pregnancy, Vitamins, Progesterone

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## Introduction

Folic acid is a vitamin that participates in biochemical pathways involving methyl transfer reactions, including methionine, purine ring, and thymidine synthesis (Shane and Stokstad, 1985). Tissues undergoing rapid cell division, such as developing swine conceptuses, would have a high demand for folic acid to maintain DNA synthesis (Babior, 1990; Blount et al., 1997). Recently, a folate binding protein (FBP)

was isolated from porcine allantoic fluid and used to generate antiserum (Vallet et al., 1998a). Immunoblotting using this antiserum indicated that a FBP is present in uterine flushings from early pregnant and nonpregnant gilts, and it was hypothesized that this protein is involved in transport of folate to developing swine conceptuses. In a previous study (Vallet et al., 1998b), progesterone treatment accelerated uteroferrin and retinol binding protein secretion from the uterus. Also, the uterus of Meishan gilts secreted less uteroferrin and retinol binding protein than the uterus of White crossbred gilts. It was of interest to determine whether FBP was also influenced by these treatments. The objective of the following experiments was 1) to develop and validate a RIA to measure concentrations of FBP in uterine flushings and endometrial culture medium and 2) to use the RIA to determine conceptus, progesterone, and breed effects on concentrations of FBP within the intrauterine environment.

<sup>1</sup>Mention of names is necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the same by USDA implies no approval of the product to the exclusion of others that may also be suitable.

<sup>2</sup>To whom correspondence should be addressed: P. O. Box 166, State Spur 18D (phone: 402-762-4187; fax: 402-762-4382; E-mail: vallet@email.marc.usda.gov).

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## Materials and Methods

Uterine flushings and endometrial culture medium collected in a previous experiment (Vallet et al., 1998b) were used to develop and validate the RIA for FBP and to examine conceptus, progesterone, and breed effects on FBP concentrations. Briefly, uterine flushings were collected from nonpregnant and pregnant 1) White crossbred, 2) progesterone-treated White crossbred (200 mg of progesterone per day given 48 and 72 h after estrus), and 3) Meishan gilts on d 10, 11, 12, 13, 14, and 15. Endometrium from these gilts was also collected and cultured in Minimal Essential Medium containing one-tenth the normal amount of leucine along with 50  $\mu$ Ci of [ $^3$ H]leucine using conditions described previously (Vallet and Christenson, 1993). Culture medium was used for assay validation only.

The FBP used in standards was purified from d-60 allantoic fluid as described previously (Vallet et al., 1998a). Iodination of FBP was performed using chloramine T as described previously for retinol binding protein (Vallet, 1994). For RIA, standards (.125, .25, .5, 1, 2, or 4 ng in 100  $\mu$ L of 50 mM Tris, 5% Tween 20, pH 8.2 assay buffer) or samples (100  $\mu$ L of the appropriate dilution in assay buffer) were combined with 100  $\mu$ L of .5% bovine gamma globulin in assay buffer, 50,000 cpm of [ $^{125}$ I]FBP in assay buffer, 100  $\mu$ L of 1% BSA in assay buffer, and 100  $\mu$ L of a 1:100,000 dilution of FBP antiserum (Vallet et al., 1998a) in assay buffer. Assay tubes were incubated for 16 to 18 h at 4°C, and then antibody-bound [ $^{125}$ I]FBP was separated from unbound protein by precipitation with polyethylene glycol as previously described (Vallet, 1994). Radioactivity in assay tubes was then quantified using a gamma counter (1 min counting time).

To validate the RIA, serial dilutions ranging from 1:100 to 1:12,800 of uterine flushings from pregnant and nonpregnant gilts and serial dilutions ranging from 1:10 to 1:160 of medium from culture of endometrium from pregnant and nonpregnant gilts were assayed, and the displacement of [ $^{125}$ I]FBP in the presence of 100  $\mu$ L of these dilutions was compared with the displacement resulting from the standard curve. To determine accuracy, .25, .5, 1, and 2 ng of purified FBP were added to 1:1,600 dilutions of uterine flushings from d-15 pregnant and nonpregnant gilts and 1:32 dilutions of medium from culture of endometrium from d-15 pregnant and nonpregnant gilts, and these samples were then assayed. No related proteins were available with which to demonstrate specificity of the antisera using cross-reaction analysis. Therefore, to demonstrate assay specificity, 2-mL samples of uterine flushings obtained from pregnant and nonpregnant gilts on d 15 were stripped of endogenous folate and then incubated with [ $^3$ H]folic acid as described previously (Vallet et al., 1998a). Samples were chromatographed using G-100 Sepha-

dex. Aliquots of each fraction obtained from chromatography were subjected to scintillation counting to quantify [ $^3$ H]folate for FBP and were also assayed with the RIA. For further validation, uterine flushing samples in which the amount of folate binding had been determined using ligand binding procedures (Vallet et al., 1998a) were also assayed with the RIA and the results were compared.

To determine the effect of the presence of the conceptus, early progesterone treatment, and breed on concentrations of FBP in the intrauterine environment, the uterine flush samples described above were assayed using the FBP RIA. Intra- and interassay CV for these assays were 13.3 and 7.1%, respectively. Limit of detection was 1.25 ng/mL, which is the lowest standard in the standard curve.

## Statistical Analysis

Parallelism of sample dilutions with the standard curve was analyzed using homogeneity of regression analysis (Snedecor and Cochran, 1967) using SAS software (SAS Institute Inc., Cary, NC). Accuracy of the RIA was determined using regression analysis. Total content of FBP in uterine flushings was calculated by multiplying the concentration of FBP in uterine flushings by the flush volume. The influence of the conceptus, progesterone treatment, and breed on intrauterine FBP content was determined with homogeneity of regression using a model that included the effect of status (pregnant or nonpregnant), treatment/breed (White crossbred untreated, White crossbred progesterone-treated, or Meishan untreated), treatment/breed  $\times$  status, the linear and quadratic effects of hours after estrus, the interactions of status with the linear and quadratic effects of hours after estrus, the interactions of treatment/breed with the linear and quadratic effects of hours after estrus, and the interaction of treatment/breed  $\times$  status with the linear and quadratic effects of hours after estrus.

## Results

Displacement from dilutions of uterine flushings and endometrial culture medium from d-15 pregnant and nonpregnant gilts was parallel to the standard curve (Figure 1). Regression analysis of the amount of FBP added vs the amount of FBP measured in the assay indicated that the assay accurately measured the added FBP (slopes of the regression lines were .99, 1.10, .93, and .91 for uterine flushings from pregnant and nonpregnant gilts and media from culture of endometrium from pregnant and nonpregnant gilts, respectively). Chromatography of uterine flushings from pregnant and nonpregnant gilts and subsequent assay of the fractions for FBP and for [ $^3$ H]folic acid indicated that peak immunoreactive FBP values coincided with the radioactive peak in

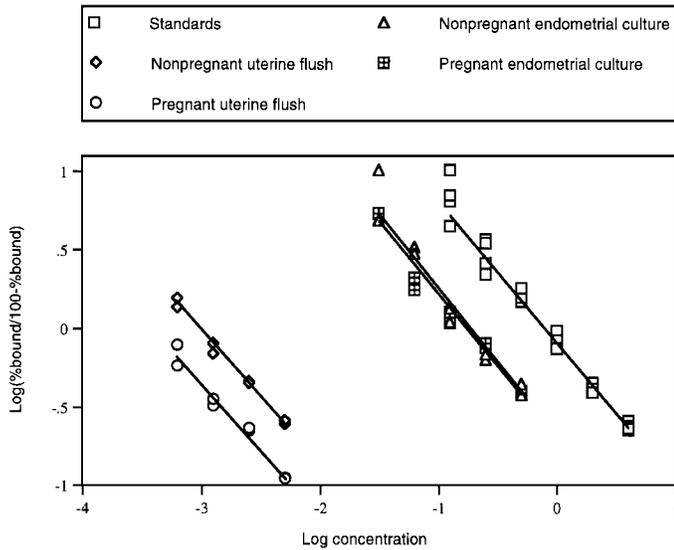


Figure 1. Displacement of [ $^{125}$ I]folate binding protein resulting from decreasing dilutions of uterine flushings and endometrial culture media from d-15 pregnant and nonpregnant gilts are compared to the displacement caused by the standard curve. Slopes of the lines did not differ.

folate binding (Figure 2), indicating that the RIA was specific for FBP. Folate binding (Vallet et al., 1998a) and FBP, measured by RIA, in samples of uterine flushings from pregnant and nonpregnant gilts were correlated ( $r = .87$ ;  $P < .01$ ). Taken together, these results indicate that the RIA accurately measured the amount of FBP in uterine flushings and culture medium samples.

The amount of FBP in uterine flushings increased ( $P < .01$ ) from 240 to 360 h (d 10 to d 15) after estrus. Regression analysis indicated that the pattern of change over time ( $P < .01$ ) and the overall mean ( $P < .01$ ) intrauterine FBP concentrations differed between untreated White crossbred gilts and progesterone-treated White crossbred gilts (Figure 3). Examination of the data indicated that this was due to earlier onset of FBP secretion in progesterone-treated gilts than in untreated gilts. There was no detectable difference between breeds or between pregnant and nonpregnant gilts in either the pattern of change with time or the overall mean FBP concentrations.

## Discussion

This seems to be the first report of a validated RIA for porcine intrauterine folate binding protein. Results confirm the dramatic increase in the amount of FBP measured by ligand binding in uterine flushings during the period of maternal recognition of pregnancy reported previously (Vallet et al., 1998a). No difference in the amount of FBP in uterine flushings was found between pregnant and nonpregnant gilts, con-

firmed previous results and suggesting that FBP is not influenced by the presence of the conceptus. Progesterone treatment significantly advanced the onset of FBP secretion into the uterine lumen, indicating that FBP, like retinol binding protein and uteroferrin (Vallet et al., 1998b), may be controlled, at least in part, by the number of days the uterus is under the influence of progesterone. Unlike uteroferrin and retinol binding protein, content of FBP in uterine flushings did not differ between Meishan and White crossbred gilts.

Several studies indicate that administering exogenous folic acid to gestating gilts improves reproduc-

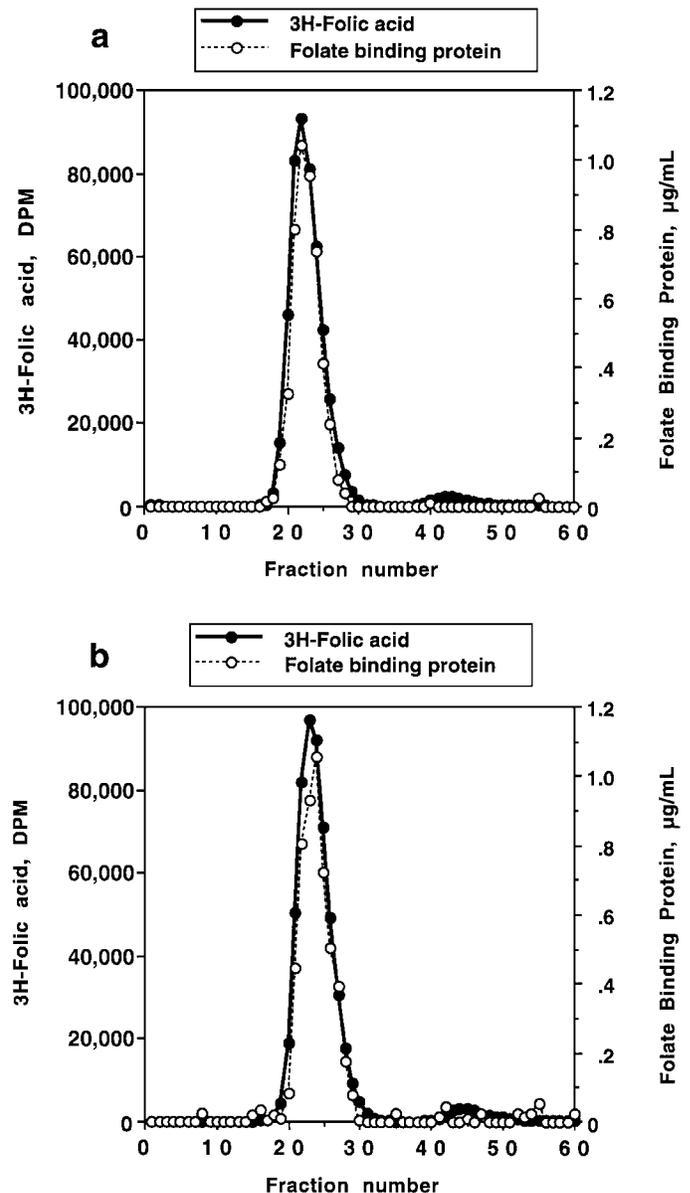


Figure 2. Radioactivity and folate binding protein concentrations in fractions from G-100 Sephadex chromatography of d-15 pregnant (a) and nonpregnant (b) uterine flushing samples preloaded with [ $^3$ H]folic acid are illustrated.

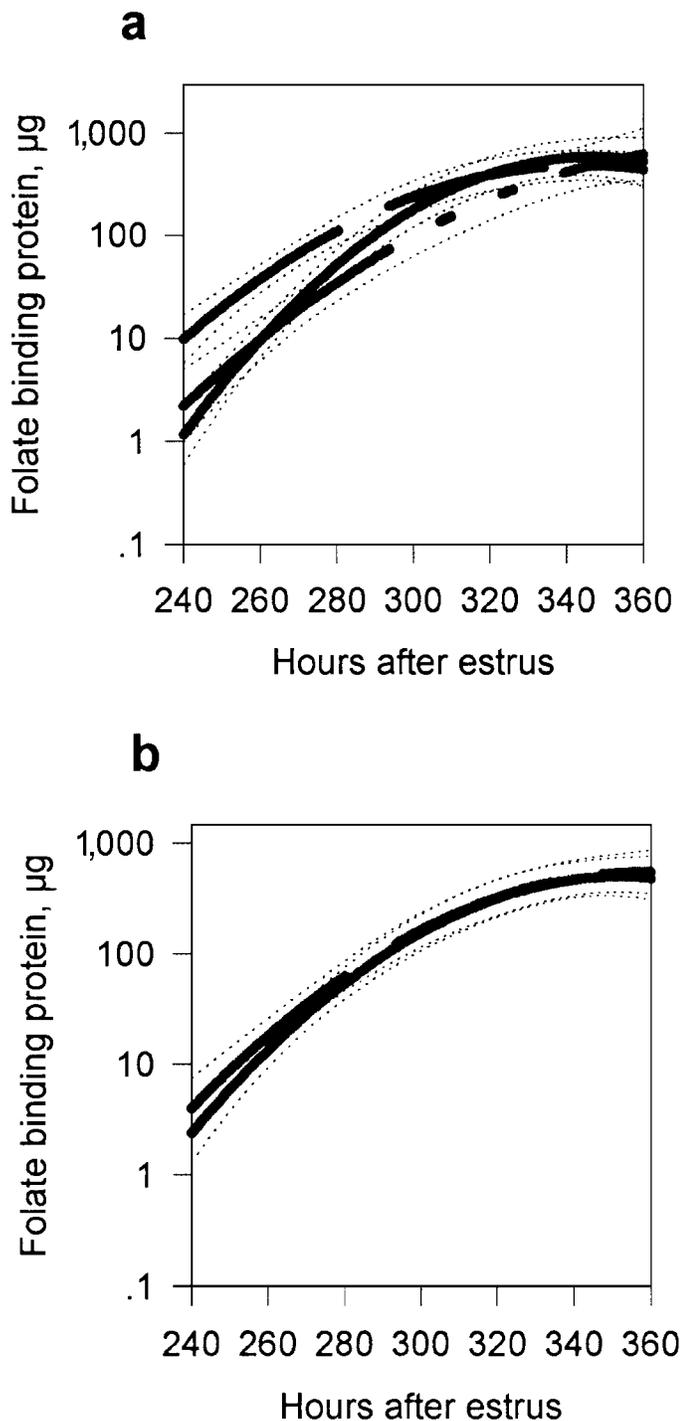


Figure 3. (a) Regression lines from untreated White crossbred (—), progesterone-treated White crossbred (---), and untreated Meishan (····) groups are illustrated. Progesterone treatment advanced ( $P < .01$ ) folate binding protein secretion. The Meishan group was not different from the White crossbred group. (b) Regression lines from nonpregnant (—) and pregnant (---) groups are illustrated. No difference between the two lines was detected. The dotted lines indicate 95% confidence intervals for the regression lines.

tive function, including increased litter size (Matte et al., 1984; Lindemann and Kornegay, 1989; Thaler et al., 1989). Other studies do not confirm this observation (Tremblay et al., 1989; Matte et al., 1990; Harper et al., 1994). One drawback of many of these studies is that it was not known whether giving folic acid increased the delivery of folic acid to developing conceptuses. Mechanisms responsible for transporting folates to the conceptus had not been investigated. However, a folate binding protein is present in uterine flushings and increases at about the time of maternal recognition of pregnancy (Matte et al., 1996; Vallet et al., 1998a). The development of a RIA for this protein improves our ability to study the control of FBP production, including the influence of increased substrate availability on production of FBP by the uterus. These studies may lead to strategies that can increase FBP secretion by the uterus and improve the delivery of this essential vitamin, possibly resulting in increased litter size.

Results indicate that FBP content in the intrauterine environment is not influenced by the presence of the conceptus. Thus, it is unlikely that conceptus estrogen secretion, which occurs during this same period (Robertson and King, 1974; Perry et al., 1976; Vallet et al., 1998b), has an influence on secretion of this protein. However, progesterone given on d 2 and 3 after estrus accelerated the increase in intrauterine FBP content. This suggests that duration of progesterone influence may be the primary factor controlling the onset of FBP production. Also, in contrast to uteroferrin and retinol binding protein (Vallet et al., 1998b), FBP does not differ between Meishan and White crossbred gilts. These data suggest that some of the mechanisms controlling intrauterine FBP content are likely to be similar to mechanisms controlling uteroferrin and retinol binding protein. However, there are also likely to be other components of the control of FBP that are distinct from those controlling uteroferrin and retinol binding protein. Detailed studies of the control of uteroferrin, retinol binding protein, FBP, and other endometrial proteins are needed to more fully understand the control of endometrial protein synthesis during pregnancy.

### Implications

A radioimmunoassay has been developed and validated for measuring folate binding protein in uterine flushings and endometrial culture medium. The dramatic increase in production of this protein, which occurs around the time of maternal recognition of pregnancy, is not influenced by the presence of the conceptus and is influenced by early progesterone treatment. This assay will allow improved study of secretion of this protein, which, in turn, may suggest

treatments that improve folate delivery to the developing conceptus and possibly increase reproductive efficiency.

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