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Pregnancy-Associated Murine Protein 3 (PAMP-3): Characterization and Serum Levels

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ABSTRACT

A pregnancy-associated murine glycoprotein, PAMP-3, with an electrophoretic mobility like that of albumin, a molecular weight of 65,000, and an isoelectric point of 4.1 was present in serum of pregnant mice, nonpregnant mice and male mice.

In serum of male mice, the mean PAMP-3 concentration was 3.0 Arbitrary Units (AU)/ml and in serum of nonpregnant mice the mean concentration was 7.7 AU/ml. During pregnancy there was an increase in PAMP-3 concentration until Day 15 of pregnancy when the PAMP-3 concentration reached 100 AU/ml.

The maternal serum concentration decreased from Day 18, and at 5 days postpartum reached the level in nonpregnant mice. The glycoprotein structure was analyzed by using concanavalin A (Con A) affinity electrophoresis and Con A affinity chromatography.

INTRODUCTION

The existence of two pregnancy-associated murine proteins, PAMP-1 and PAMP-2, has been demonstrated, and their physicochemical and immunochemical properties described (Hau et al., 1978; Hau et al., 1980a). The pregnancy-associated glycoprotein PAMP-1 is also present in serum of nonpregnant mice, whereas the heterogeneous glycoprotein PAMP-2 is detectable in serum of pregnant mice only, and thus is tentatively regarded as a pregnancy-specific protein.

Based on comparisons of physicochemical, immunochemical and biological data, it has been suggested that PAMP-2 is a murine analogue to the human pregnancy-specific β_1-glycoprotein (PSβG, SP-1) (Hau et al., 1980b).

A murine α_1-sialoglycoprotein, which increased in concentration in maternal serum during pregnancy, was described by Groth and Kaden (1977). Lin et al. (1974) reported that production of a polyspecific antiserum against murine pregnancy serum, which, following thorough absorption with lyophilized murine plasma of nonpregnant animals, showed four precipitates in double immunodiffusion.

The present report is a description of a newly detected murine pregnancy-associated glycoprotein, PAMP-3, and its immunochemical properties and serum levels during pregnancy.

MATERIALS AND METHODS

Experimental Animals

Random-bred primigravid SPF-mice of the strain NMRI/BOM were used. Conception time was determined by the presence of a vaginal plug and designated Day 1 of pregnancy. Ten pregnant animals were bled between 0900 and 1100 h on each day of pregnancy. Three to eight juvenile animals were bled on each of Days 0, 1, 2, 3, 4, 5, 6, 8, 10, 13, 16, 20, 26, 29, and 30 after birth.

The animals were anesthetized by i.p. injection of 0.3 mg pentobarbitone sodium (Nembutal:Abbot).

Surgical Procedure

Surgery on mice was performed under general anesthesia. Ovariohysterectomy was performed in six adult females through a midline incision without ligation of blood vessels. The laparotomy was sutured in two layers using Dexon 000. Six animals in which only the laparotomy was performed served as controls. Blood samples were drawn before surgery and at weekly intervals for 7 weeks after surgery. Partial hysterectomy was performed in 16 adult females by ligating the left horn of the uterus a few millimeters from the bifurcation. The mice were killed on Day 17 of pregnancy, the placental and fetal mass was weighed, and the PAMP-3 level measured.

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Production of Antisera

Pooled sera of mice from 11 to 18 days pregnant were fractionated by gel filtration chromatography on a Pharmacia K 26/100 column packed with ACA-34 (L'Industrie Biologique Francaise) as described in Hau et al. (1978). The protein separation profile consisted of three peaks; the fractions from the three peaks were pooled, concentrated, mixed with Freund's incomplete adjuvant, and injected s.c. into goats at doses of 500 μg protein every 2 weeks.

The goat antiserum was absorbed with a 25–40% volume of a pool of sera from male mice for 24 h and ultracentrifuged at 70,000 × g for 1 h. The supernatant was tested for antibody activity by crossed immunoelectrophoresis using sera from male, nonpregnant, and pregnant mice as antigens.

Molecular Weight Estimation

Size chromatography. A 1:1 mixture of late pregnancy serum from women and mice was applied to a Pharmacia K 26/100 column packed with Sepharose G-150 superfine gel (Pharmacia). The column was calibrated and a standard curve established from chromatographic constant (K_{av}) values calculated for the following proteins of known molecular weight: α_2-macroglobulin (MW 800,000, K_{av} = 0.014), immunoglobulin G (MW 150,000, K_{av} = 0.08), pregnancy-associated murine protein-1 (MW 140,000, K_{av} = 0.09), pregnancy-specific β_1-glycoprotein (MW 90,000 K_{av} = 0.12), pregnancy-associated murine protein-2 (MW 70,000, K_{av} = 0.14), and orosomucoid (MW 44,100, K_{av} = 0.25) (Hau et al., 1978).

Sodium dodecyl sulfate (SDS) gel electrophoresis. SDS polyacrylamide gel electrophoresis was performed as described by Maizel (1971) using an acrylamide concentration gradient covering the range from 5 to 15%. The reference proteins were ovalbumin, albumin, bovine serum albumin, transferrin, and immunoglobulin G.

The electrophoresis was performed at 50 V, 20 mA for 0.5 h followed by 300 V for 4 h. After the electrophoresis, the gel was washed in Tris barbital buffer, ionic strength 0.020, pH 0.3, for 12 h. The gel was cut in longitudinal strips, each strip containing one sample of protein. The strips were embedded in 1% agarose gel (Litex type HSA, Denmark) and used as first dimension gels in crossed immunoelectrophoresis, using monospecific antisera in 1% agarose gel (Indubiose A 37, L'Industrie Biologique Francaise). The second dimensional electrophoresis was performed using 4 V/cm for 8 h followed by 2 V/cm for 18 h.

Estimation of Electrophoretic Mobility

The electrophoretic mobility of PAMP-3 was determined by crossed immunoelectrophoresis, using a human reference serum in the same first dimensional run as the murine antigen. The electrophoretic migrations of the human reference proteins α_1-antitrypsin, albumin, and prealbumin were determined by the use of monospecific antisera (DAKO Immunoglobulins, Copenhagen) placed in intermediate gels.

Conventional staining was performed with Coomassie Brilliant Blue. Staining for lipoprotein was performed using a saturated solution of Sudan Black in 60% ethanol for 16 h. As a positive control, precipitates obtained by use of anti-whole-murine serum in the second dimension gel were stained in the same way.

Rocket Line Immunoelectrophoresis

Rocket-line immunoelectrophoresis (Krøll, 1973b) for quantification of PAMP-3 was performed on 11 × 20 cm glass slides in a 1% agarose gel (Indubiose A 37, L'Industrie Biologique Francaise) with a Tris barbital buffer, ionic strength 0.020, pH 8.3, at 2.5 V/cm for 18 h. The antibody-containing gel contained 1.5 μl goat anti-PAMP-1-2-3 preparation per cm^2 in the quantification of PAMP-3 (absorption with 25% volume murine male serum). A neutral gel with wells was placed on the cathodic side of the antibody-containing gel. Cathodic to this gel was the antigen-containing gel which received 1.5 $\mu l/cm^2$ of murine male serum (Fig. 1). One hundred arbitrary units (AU) refer to the amount of PAMP-3 in a serum pool from 18- and 19-day-pregnant mice.

Crossed immunoelectrophoresis, crossed Con A affinoimmunoelectrophoresis, and crossed immunoelectrofocusing were performed as described in Hau et al. (1980a).

Crossed Tandem Immunoelectrophoresis

Two wells cut in the first dimensional gel, 1 cm apart, received two different antigen samples. The first dimensional gel was run at 10 V/cm until a bromphenol-blue-labeled albumin marker had migrated 2 cm from the antigen wells. During the second dimensional electrophoresis, proteins with antigen determinants in common fused to double peaks (Krøll, 1973a). The technique was employed in the identification of PAMP-3 in plates where sera from nonpregnant and pregnant mice were used as antigens (Fig. 3a,b).

Concanavalin A Affinity Chromatography

A pharmacia K 9 column of 5 ml was packed with concanavalin A Sepharose (Con A Sepharose) (Pharmacia Fine Chemicals, Uppsala, Sweden). The column was washed and equilibrated with a pH 6 acetate buffer containing 0.1 M acetate, 1 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM $MnCl_2$. The experiment was performed at 4° C and at a flow rate of 16 ml/h. Samples of 0.5 ml murine late pregnancy sera (a pool of 30 individuals) mixed with 0.5 ml buffer were applied to the column, the column was washed with 76 ml acetate buffer, and 2 ml fractions were collected. The elution of bound substances was performed using 76 ml 0.5 M α-methyl-mannoside in acetate buffer, and fractions of 2 ml were collected.

The fractions were tested for PAMP-3 by using rocket-line immunoelectrophoresis under conditions identical to those given above for rocket-line immunoelectrophoresis.

RESULTS

Identification of PAMP-3

An antiserum with activity against PAMP-1 (Hau et al., 1978), PAMP-2 [suggested murine analogue to human pregnancy-specific β_1-

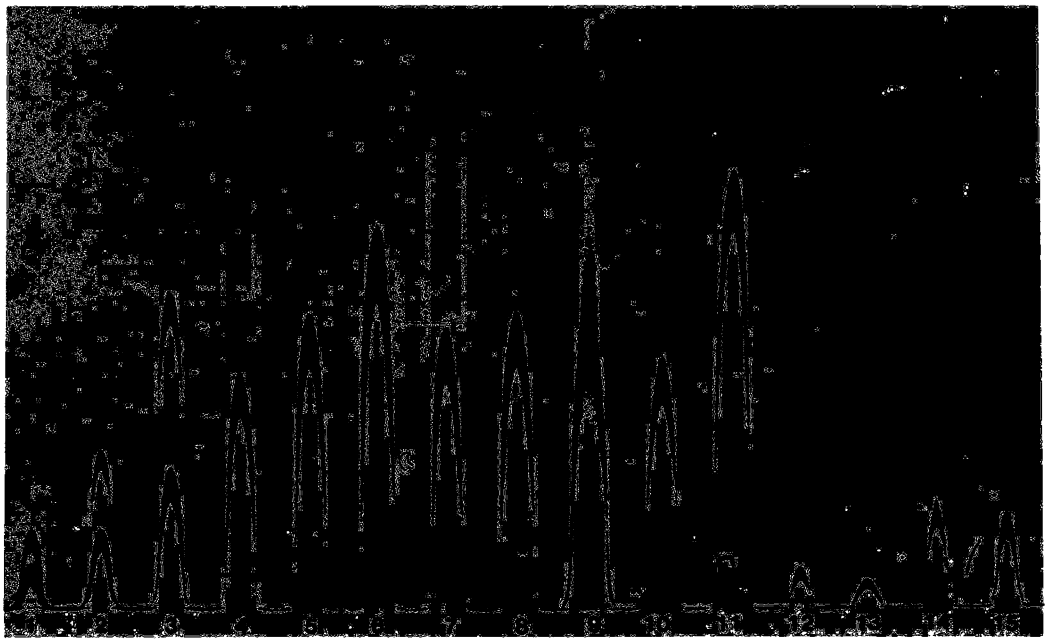

FIG. 1. Line immunoelectrophoresis with goat anti-PAMP-1,-2, and -3 in the antibody-containing gel and with serum from male mice in the line gel. As the antigen-containing gel received male serum, only one line precipitate was formed. Contents of the wells:

1) Late pregnancy serum (LPS) diluted 1:320
2) LPS diluted 1:213
3) LPS diluted 1:160
4) LPS diluted 1:80
5) LPS diluted 1:40
6) PLS diluted 1:20
7) Internal standard, PLS diluted 1:40
8) Serum of nonpregnant mice diluted 1:2
9) Serum of male mice, undiluted
10) Early pregnancy serum, diluted 1:2
11) Late pregnant mouse, diluted 1:20
12) Fetal homogenate, diluted 1:2
13) Placental homogenate, diluted 1:2
14) Liver homogenate, diluted 1:2
15) Kidney homogenate, diluted 1:2

glycoprotein (SP-1); Hau et al., 1980b], and PAMP-3 was produced in goats immunized with the intermediate peak of the separation profile of size chromatography. The antiserum was incompletely absorbed with murine male serum until only one precipitate was visible in crossed immunoelectrophoresis with murine male serum as the antigen (Fig. 2a). The protein responsible for this precipitate was present in a higher concentration in serum of nonpregnant mice and was found further elevated in pregnant mice (Fig. 2b,c, Table 1) and designated PAMP-3. PAMP-3 was identified on the plates which showed all three pregnancy proteins by using the crossed tandem immunoelectrophoresis technique (Figs. 2, 3). In tandem immunoelectrophoresis the completely fused precipitates of PAMP-3 shown in Fig. 3a,b indicate that this protein is identical in males, nonpregnant females and pregnant females.

Electrophoretic Mobility, Isoelectric Point, and Molecular Weight

Using crossed immunoelectrophoresis with intermediate gels, we compared the electrophoretic mobility of PAMP-3 with the electrophoretic mobility of human α_1-antitrypsin, albumin, and prealbumin. The electrophoretic

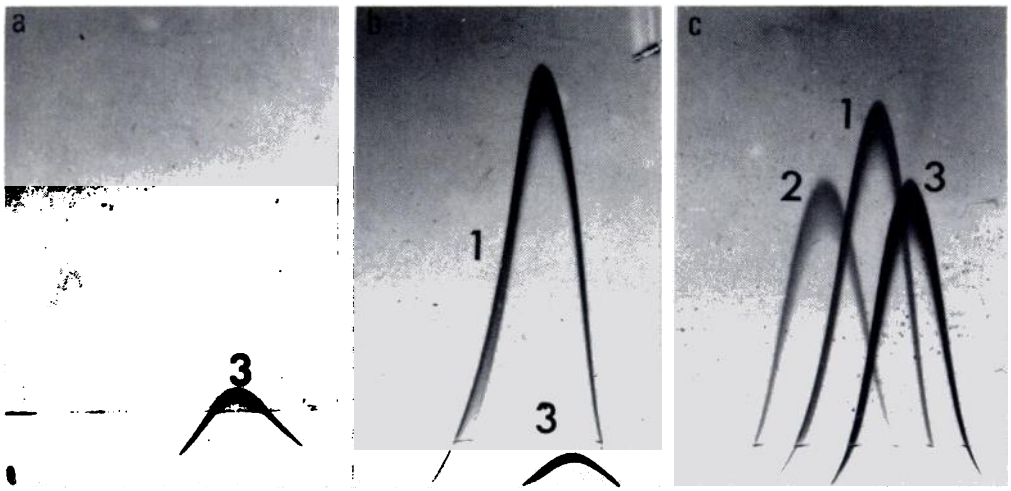

FIG. 2. Crossed immunoelectrophoresis with the goat antiserum absorbed with 33% serum from male mice in the second dimensional gel. The antigens were a) undiluted serum from male mice, b) 1:5 diluted serum from nonpregnant females, and c) 1:15 diluted late murine pregnancy serum. The faint precipitate in (a) is a non-absorbed specificity against a normal serum component.

mobility of PAMP-3 was identical to that of human albumin.

The peak of the precipitate in crossed immunoelectrofocusing, indicating the isoelectric point of PAMP-3, was at pH 4.1 of the pH gradient.

In the calculation of the molecular weight of PAMP-3 by size chromatography, the following values were used: V_t = 515.0 ml, V_O = 155.4 ml, and K_{av} for PAMP-3 = 0.17. The molecular weight of PAMP-3 was estimated by reference to a standard curve based on the six marker proteins at MW 65,000. In the calculation based on migration in SDS gel electrophoresis, the molecular weight of PAMP-3 was found to be 62,000.

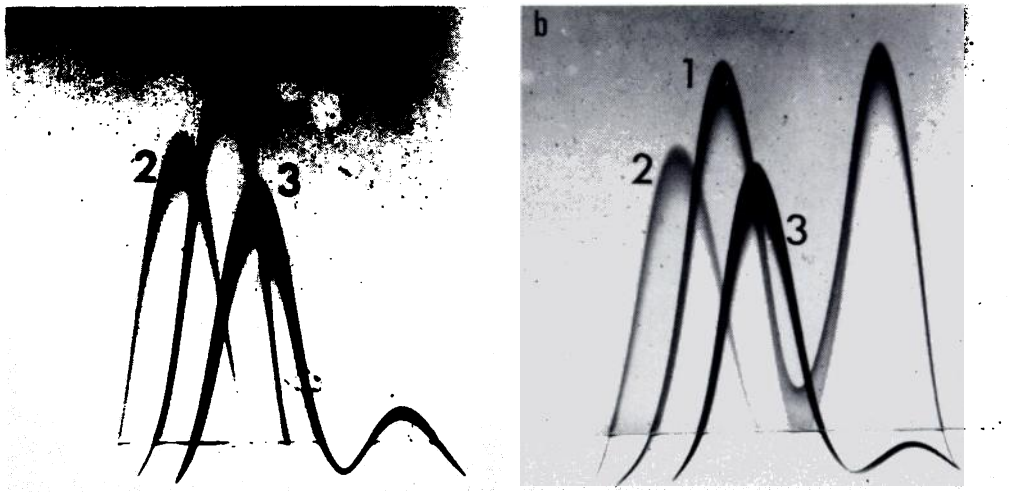

FIG. 3. Crossed tandem immunoelectrophoresis with the same antiserum as in Fig. 1. The antigens were a) 1:15 diluted late murine pregnancy serum in the first well and undiluted serum from murine males in the second well, and b) 1:15 diluted late murine pregnancy serum in the first well and 1:5 diluted serum from non-pregnant females in the second well.

Carbohydrate and Lipid Structure of PAMP-3

The migration velocity of PAMP-3 was retarded by free Con A in the first dimensional gel. Increasing amounts of Con A led to further retardation, but no precipitation, of PAMP-3.

In Con A affinity chromatography, PAMP-3 constantly leaked from the column by the washing procedure, but could be completely eluted by the α-methylmannoside.

PAMP-3 was not stainable by the lipoprotein staining method (Sudan Black in 60% alcohol).

Concentration of PAMP-3

PAMP-3 was detectable by electroimmunoassay in sera from males and nonpregnant and pregnant females, as well as in amniotic fluid and fetal serum, and in small amounts in urine of pregnant females (Table 1).

A total of 220 serum samples was obtained from 220 primigravid mice, and the maternal serum concentration of PAMP-3 was measured by rocket-line immunoelectrophoresis (Fig. 1). Although the antiserum contained specificities against PAMP-1, PAMP-2, and PAMP-3, only a PAMP-3 line was formed since murine male serum was used in the antigen-containing line gel. The results are shown in Fig. 4. By Day 10 after conception, the concentration was significantly higher than values before conception ($P<0.001$, Student's t test). There was an increase in concentration until Day 15 of pregnancy, and the concentration remained at a high level until Day 18 of pregnancy, After which it decreased to preconception values on Day 5 postpartum. The postpartum PAMP-3 levels were similar to the values before conception. Ovariohysterectomy did not lead to significant changes in PAMP-3 serum concentration in nonpregnant animals. The PAMP-3 concentration in 17-day-pregnant, partly hysterectomized mice was not significantly altered compared with the normal 17-day-pregnant animals.

TABLE 1. Concentrations of PAMP-3 in serum, urine, and amniotic fluid of mice (mean ± SEM; number of samples in parentheses).

	Concentration of PAMP-3 (arbitrary units/ml)
Nonpregnant adult females	7.7 (pool of >100)
Females at term	39.0 ± 3.7 (10)
Postpartum females (1–5 days postpartum)	13.0 ± 3.0 (25)
Adult males	3.0 (pool of >100)
Fetal serum	7.5 (pool of >25)
Amniotic fluid	5.5 (pool of >25)
Newborn young (<1 day)	1.6 ± 0.2 (20)
Urine of pregnant females	Trace

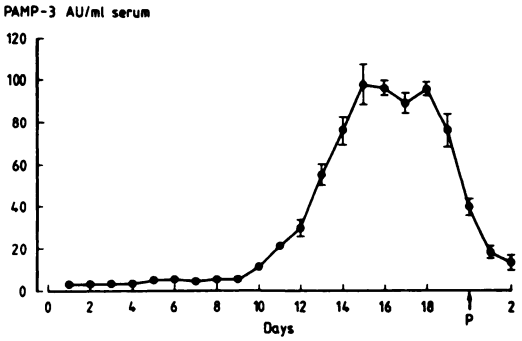

FIG. 4. PAMP-3 in 220 serum samples from 220 mice. Values represent ± 1 SEM; each point represnets mean values of 10 samples on each day.

In serum of newborn mice, the PAMP-3 concentration decreased from 1.6 AU/ml at birth to 0.2 AU/ml on Days 4, 5, and 6. From Day 6 the concentration increased, and the adult level of PAMP-3 was reached about Day 30 (Fig. 5).

Cross Reactivity Between PAMP-3 and Proteins Present in Serum from Pregnant Individuals of Other Species

Serum samples from pregnant women, pigs, dogs, guinea pigs, and rats were tested for proteins crossreacting with PAMP-3 by using the antiserum to PAMP-3 and line immunoelectrophoresis. Crossreacting proteins were detectable in the pregnant rat only. Monospecific rabbit antisera against the human pregnancy-proteins, human placental lactogen (HPL) pregnancy-specific β-glycoprotein (PSβG, SP-1) (Dako Immunoglobulines, Denmark), pregnancy zone protein (PZP, SP-3, α_2-PAG), and pregnancy-associated plasma proteins A (PAPP A, SP-4) did not cause any deflections of the PAMP-3 line in line immunoelectrophoresis.

DISCUSSION

The presence of PAMP-3 in serum of males and nonpregnant females, the striking increase in concentration during pregnancy, and the positive maternal/fetal concentration gradient

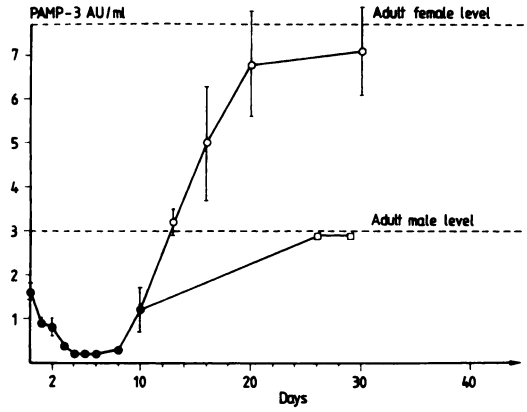

FIG. 5. PAMP-3 in 58 serum samples from 58 juvenile mice. Open circles represent females, open squares represent males, and closed circles represent samples of both sexes. Values represent ± 1 SEM; each point represents mean values of 3–8 samples on each day.

classify this protein in the group of pregnancy-associated serum proteins. The serum concentration of PAMP-3 in adult females is 7.7 AU/ml, whereas that of adult males is 3.0 AU/ml. This difference in concentration is established during puberty when PAMP-3 in serum of juvenile females shows a striking increase in concentration from Day 10 to Day 20 compared with the more moderate increase in male mice.

PAMP-3 is not identical to any of the four murine pregnancy-specific proteins described by Lin et al. (1974), since it is present in nonpregnant females and in males, nor to the α_1-sialoglycoprotein (Groth and Kaden, 1977), which increased after parturition.

Like PAMP-1 (Hau et al., 1978), PAMP-3 shows immunological cross reaction with a protein present in the pregnant rat, but cross reaction with proteins in other species could not be demonstrated using antisera raised in goats and rabbits.

The human pregnancy-associated serum proteins are steroid-binding β-globulin (SBβG, SP-2) (Bohn, 1974a,b) and pregnancy zone protein (PZP, α_2-PAG, SP-3) (Bohn, 1974a). Like the human pregnancy-associated proteins, PAMP-3 seems to be a glycoprotein, and the results of Con A affinoelectrophoresis indicate one Con A-binding structure per molecule (Bøg-Hansen, 1979). This predictive observation was confirmed by Con A affinity chromatography, where PAMP-3 consistantly leaked from the column and could be completely eluted by the eluant, indicating one Con A-binding structure per PAMP-3 molecule.

Ovariohysterectomy and measurements of PAMP-3 in nonpregnant females indicate that PAMP-3 is not a product of the ovaries or uterus and that the presence of ovaries or uterus is not needed in the maintenance of a normal PAMP-3 serum level. Semihysterectomy and measurement of PAMP-3 in 17-day-pregnant females indicate that the PAMP-3 level is not directly correlated to the mass of the placenta or fetus, a normal level being maintained in the semihysterectomized animals in spite of the reduction of the fetal and placental mass.

The PAMP-3 pattern during pregnancy resembles that of human PZP (Bohn, 1974a). Both proteins decline in concentration prior to parturition, whereas human SBβG remains at a high level until parturition (Bohn, 1974b). However, PAMP-3 is a much smaller protein, and the PAMP-3 level shows much less variation from animal to animal than PZP does from one woman to another. This smaller degree of variation might be due to the uniformity of the murine material regarding maternal age, size, feeding, environment, and genetic variation, even though a random-bred strain was used.

It was not possible to demonstrate immunological cross reaction between PAMP-3 and PZP using antisera raised in goats and rabbits.

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