Immunochemical characterization and serum concentrations of pregnancy-associated serum proteins in mice

J. Hau, P. Svendsen, B. Teisner* and G. Thomsen Pedersen†

*Laboratory Animal Unit, and *Institute of Medical Microbiology, Odense University, and*

†Institute of Obstetrics and Gynaecology, Odense University Hospital, Denmark

Summary. The glycoprotein structure of two pregnancy-associated murine proteins, α_1 -PAMP and α_2 -PAMP, was analysed by using concanavalin A (Con A) affinity electrophoresis: α_1 -PAMP was completely precipitated by free Con A, whereas α_2 -PAMP showed heterogeneity. The isoelectric points of α_1 -PAMP and α_2 -PAMP were determined at 4.2 and 4.0 respectively by using crossed immunoelectrofocusing. The α_2 -PAMP was detectable only in the serum of pregnant mice and fetuses. Maternal serum concentrations increased from Day 8 of pregnancy, remained at high values between Days 14 and 18, and could not be detected by 2 days *post partum*.

Introduction

Proteins specific for human pregnancy appear in maternal serum (Lin, Halbert, Kiefer, Spellacy & Gall, 1974a). Lin, Halbert & Kiefer (1974b) demonstrated the existence of 4 murine pregnancy proteins by using double immunodiffusion and a polyspecific rabbit antiserum absorbed with freeze-dried plasma powder from non-pregnant mice. An α_1 -sialoglycoprotein, which increased in concentration during murine pregnancy, was described by Groth & Kaden (1977). The production of monospecific antisera against two serum proteins associated with pregnancy in the mouse has previously been described by Hau, Svendsen, Teisner & Svebag (1978). One protein, α_1 -PAMP, with α_1 -electrophoretic mobility and an estimated molecular weight of 140 000 was present in serum of pregnant and non-pregnant female mice, but could not be detected in the serum of male or juvenile female mice. The serum concentration of this protein increased from the day of implantation, reached a maximum level on Day 11 after conception, and declined to the preconception level just before parturition. The second protein, α_2 -PAMP, with α_2 -electrophoretic mobility and an estimated molecular weight of 70 000, was exclusively found in serum of pregnant mice.

In the present report the results of additional immunochemical investigations of these 2 pregnancy-associated murine proteins are described, and the concentration of α_2 -PAMP in maternal serum during pregnancy, and in fetal serum and amniotic fluid is examined.

Materials and Methods

Outbred 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. Blood samples were obtained randomly 10–11 times during pregnancy from each animal. Samples were taken between 09:00 and 11:00 h by periorbital puncture under light diethyl ether anaesthesia.

Production of antisera against α_1 -PAMP and α_2 -PAMP

Antibodies against α_1 -PAMP and α_2 -PAMP were raised in conventional albino rabbits (State Serum Institute, Copenhagen) as previously described (Hau *et al.*, 1978). The antiserum with reactivity against α_1 -PAMP was absorbed with 30% volume of pooled serum from male mice for 24 h and ultracentrifuged at 70 000 *g* for 1 h. The supernatant was tested for monospecificity against α_1 -PAMP by crossed immunoelectrophoresis using serum of males, non-pregnant and pregnant females. The antiserum reacting against α_1 -PAMP and α_2 -PAMP was absorbed with 30% volume of a pool of serum of non-pregnant females to remove activity against α_1 -PAMP. The supernatant was tested for monospecificity against α_1 -PAMP by crossed immunoelectrophoresis using serum of pregnant and non-pregnant females.

Crossed immunoelectrophoresis

Crossed immunoelectrophoresis was performed on 7 × 7 cm glass slides in a 1% (w/v) agarose gel (Indubiose A 37, L'Industrie Biologique Francaise) with a thickness of 1.5 mm (Weeke, 1973). Tris-barbital buffer, ionic strength 0.020, pH 8.5, was used, and the wells received 5 μ l antigen samples. The first dimension electrophoresis was run at 10 V/cm until a bromphenol blue-labelled albumin marker had migrated 4 cm from the antigen well. The second dimension electrophoresis was performed in an antibody containing gel at 2.5 V/cm for 18 h. The gel contained 1.5 μ l antibody preparation per cm². The crossed immunoelectrophoresis slides were stained with Coomassie Brilliant Blue or with a saturated solution of Sudan Black in 60% ethanol for 16 h.

Crossed affinity immunoelectrophoresis

The crossed immunoelectrophoresis with free concanavalin A (Con A) added to the first dimension gel was performed as described by Bøg-Hansen (1979). The first dimension gel contained 100, 200 or 500 μ g Con A/cm². The gel-buffer system and the antibody concentrations in the antibody-containing gels were the same as the crossed immunoelectrophoresis system. As controls the same serum samples without Con A were tested in crossed immunoelectrophoresis.

Quantitative rocket immunoelectrophoresis

The daily α_2 -PAMP serum level during pregnancy was determined by rocket immunoelectrophoresis (Laurell, 1972) on 11 × 20 cm glass slides in a 1% agarose gel with a Tris-barbital buffer, ionic strength 0.020, pH 8.5, at 2.5 V/cm for 18 h. The gel contained 1.5 μ l antibody preparation per cm². The wells received 5 μ l samples. One arbitrary unit (AU) of α_2 -PAMP refers to the amount of α_2 -PAMP present in 1 ml of a standard serum pool, from 50 19-day-pregnant female mice, diluted 1 : 800 in 0.9% (w/v) NaCl solution.

Crossed immunoelectrofocusing

Isoelectric focusing (Rosén, Ek & Åman, 1979) was performed on 12.5 × 26 cm glass slides covered with transparent film on a Multiphor apparatus (LKB, Bromma, Sweden). Sorbitol 10% and agarose 0.8% (type HSIF, Litex, Glostrup, Denmark) were dissolved in double-distilled water and heated to 98°C under constant stirring, and ampholines (LKB, Bromma, Sweden) were added. The ampholine concentration was 2% (v/v), prepared from a mixture of 1 volume

Figs 1 and 2. Crossed immunoelectrophoresis with the rabbit antiserum against α_1 -PAMP. The antigen was non-pregnant female serum diluted 1:12.5, without Con A (Fig. 1) and with 100 μ g Con A/cm² (Fig. 2) in the first dimension gel.

Figs 3 and 4. Crossed immunoelectrophoresis with the rabbit antiserum against α_2 -PAMP in the second dimension gel. The antigen was serum from pregnant mice diluted 1:5, without Con A (Fig. 3) and with 200 μ g Con A/cm² (Fig. 4) in the first dimension gel. The areas marked x and y apparently represent two different molecular forms of α_2 -PAMP, of which x contains one Con A-binding carbohydrate molecule, and y contains no Con A-binding carbohydrate.

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ampholine pH 2.5–4, 3 volumes pH 3.5–5, and 6 volumes pH 4–6. The agarose–sorbitol–ampholine gel was poured onto the glass plate covered with transparent film, to give a thickness of 1.2 mm. Electrode strips were soaked in 0.5 M-NaOH at the cathode and 0.5 M-acetic acid at the anode. Undiluted serum samples were applied in 5 μ l droplets directly onto the gel surface. The pH was measured with a Multiphor surface electrode 2117–111 (LKB, Bromma, Sweden). The focusing was performed at 100 V, 5 mA for 20 min, followed by 200 V, 10 mA for 30 min, and finally 1000 V, maximum 6.25 W, for 30 min. The pH gradient covered the range from 3.8 to 5.5.

After focusing, crossed immunoelectrophoresis was performed, using the focused gel strip as a first dimensional gel. The conditions were identical to those given above for crossed immunoelectrophoresis.

Results

Carbohydrate and lipid in α_1 -PAMP and α_2 -PAMP

At a Con A concentration of 100 μ g/cm² gel in the first dimensional gel, α_1 -PAMP was completely precipitated and no precipitate appeared in the second dimensional antibody containing gel (Pl. 1, Figs 1 and 2). In crossed immunoelectrophoresis the α_2 -PAMP precipitate was asymmetric and skewed to the cathode side, with the peak appearing 24 mm from the well (Pl. 1, Fig. 3). In crossed affinity electrophoresis α_2 -PAMP exhibited heterogeneity to Con A, resulting in the appearance of a biphasic precipitate with the peaks situated 9 mm and 20 mm from the well (Pl. 1, Fig. 4). Even a concentration of 500 μ g Con A/cm² did not cause any further precipitation of α_2 -PAMP in the first dimensional gel or alteration of the precipitates in the second dimensional gel.

Neither α_1 -PAMP nor α_2 -PAMP were stainable with the lipoprotein staining method.

Cross-reactivity between α_2 -PAMP and proteins present in serum from pregnant individuals of other species

Serum samples from pregnant women, cows, pigs, guinea-pigs and rats were tested for proteins cross-reacting with α_2 -PAMP by using the rabbit antiserum to α_2 -PAMP. No such cross-reacting proteins were detected. No cross-reaction was observed when serum from pregnant mice was tested against monospecific antisera against human placental lactogen, pregnancy specific β_1 -glycoprotein and pregnancy zone protein.

Table 1. The mean $\pm$ s.e.m. (no. of samples in parentheses) concentrations of α_2 -PAMP in serum, urine and amniotic fluid of mice

	Conc. of α_2 -PAMP (arbitrary units/ml)
Non-pregnant adult females	0 (80)
Early pregnant females (Days 1–7)	0 (37)
Late pregnant females (Day 17)	1526 $\pm$ 94 (21)
Females at term (Day 20)	55 $\pm$ 7 (28)
Post-partum females	0 (76)
Adult males	0 (66)
Fetus	Trace (90)
Amniotic fluid	0 (20)
Newborn young (2 days)	0 (34)
Juvenile females (3–4 days)	0 (18)
Urine of pregnant females	0 (8)

Isoelectric point of α_1 -PAMP and α_2 -PAMP

The peak of the precipitate indicating the isoelectric point of α_1 -PAMP was at pH 4.2 of the gradient. The isoelectric point of α_2 -PAMP was pH 4.0.

Concentration of α_2 -PAMP

It was possible to detect α_2 -PAMP by rocket immunoelectrophoresis only in the serum of pregnant mice and fetuses (Table 1).

The results of daily α_2 -PAMP determinations are shown in Text-fig. 1. The α_2 -PAMP did not appear until Day 8 of pregnancy, it increased until Day 14, reached maximum level at Day 17, and remained at a high level until Day 19. Samples obtained the morning after parturition and Day 1 *post partum* contained only trace amounts of α_2 -PAMP. On Day 2 *post partum* the protein could no longer be detected in serum by rocket immunoelectrophoresis.

Text-fig. 1. The concentrations of α_2 -PAMP, measured by rocket immunoelectrophoresis, in 318 serum samples from 30 primigravid NMRI mice. Values are mean $\pm$ 1 s.e.m. for 9–28 samples at each point. P is the day of parturition, for litters born early in the morning.

Discussion

The analytical affinity Con A electrophoresis was used for identification of carbohydrate-containing proteins. Free Con A cross-links and precipitates only molecules with two or more binding sites per molecule, whereas proteins not containing carbohydrate appear in their normal position after migration. Proteins containing one binding site per molecule are retarded in their migration velocity. Heterogeneous glycoproteins are revealed as multipeak precipitates, each peak thus representing a component with a distinct carbohydrate composition (binding site) with a distinct affinity to Con A (Bøg-Hansen, 1979). The α_1 -PAMP precipitate in crossed immunoelectrophoresis (Pl. 1, Fig. 1) was almost symmetrical. Addition of Con A to the first dimensional gel precipitated the protein completely (Pl. 1, Fig. 2). This indicates that α_1 -PAMP is a homogeneous glycoprotein with two or more binding sites for concanavalin A per molecule. The α_2 -PAMP precipitate (Pl. 1, Fig. 3) was asymmetrical and skewed to the cathode side, indicating that the protein appears in at least two different molecular forms. With Con A in the first dimensional gel, the precipitate became biphasic (Pl. 1, Fig. 4), suggesting that α_2 -PAMP appears in one molecular form containing one molecule of Con A-binding carbohydrate, in which the electrophoretic migration is reduced by the addition of Con A, and one molecular form without Con A-binding carbohydrate, in which the electrophoretic mobility is not affected by addition of Con A to the first dimensional gel.

The α_1 -PAMP level increases from the day of implantation, reaches maximum on Day 11 and declines to pre-conception values the day before parturition (Hau *et al.*, 1978). However, α_2 -PAMP did not appear until Day 8 after conception and the plateau reached at Day 14 lasted until Day 18 and was not detectable by the 2nd day after parturition.

The α_1 -PAMP cannot be identical to any of the 4 proteins described by Lin *et al.* (1974b) since it is present in non-pregnant females at a rather high concentration. The α_2 -PAMP, however, is not present in non-pregnant females and could be identical to one of the proteins described by Lin *et al.* (1974b). Groth & Kaden (1977) have described an α_1 -sialoglycoprotein in the mouse which increased in serum concentration during pregnancy, declined before parturition, increased again after birth and fell to pre-conception values within 2–3 weeks. The high post-partum level makes it unlikely that α_1 -sialoglycoprotein is identical to α_1 -PAMP or α_2 -PAMP. The α_2 -PAMP pattern during pregnancy resembles those of the human pregnancy-associated Pregnancy Zone Protein (PZP or SP₃) (Bohn, 1974a), and human steroid-binding β -globulin (SB β G or SP₂) (Bohn, 1974a, b). These, however, are present in low concentrations in non-pregnant women. The occurrence of α_2 -PAMP is also similar to that of human pregnancy-specific β -glycoprotein (PS β G, SP₁ or PAPPC) (Bohn, 1974a; Lin *et al.*, 1974a), the human pregnancy-associated plasma protein A (PAPP A) (Lin *et al.*, 1974a) and human placental lactogen (Towler, Jandial, Horne & Bohn, 1976), all being present during pregnancy only, and with maximum levels in the last third of pregnancy. The post-partum decline of α_2 -PAMP is rapid and similar to the decline of human placental lactogen and pregnancy-specific β_1 -glycoprotein (Lin, Halbert, Spellacy & Gall, 1976), while steroid-binding β -globulin, pregnancy-associated plasma protein A and pregnancy zone protein show a more delayed decline *post partum* (Bohn, 1974c; Lin *et al.*, 1976). The pregnancy specificity and the low molecular weight of the glycoprotein α_2 -PAMP suggest that this protein may be a possible analogue to human steroid-binding β -globulin or human placental lactogen, although attempts to demonstrate immunological cross-reactivity or partial identity between the two human proteins and α_2 -PAMP were unsuccessful using antibodies raised in rabbits.

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