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

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Summary. The glycoprotein structure of two pregnancy-associated murine proteins, \( \alpha_1 \)-PAMP and \( \alpha_2 \)-PAMP, was analysed by using concanavalin A (Con A) affinity electrophoresis: \( \alpha_2 \)-PAMP was completely precipitated by free Con A, whereas \( \alpha_2 \)-PAMP showed heterogeneity. The isoelectric points of \( \alpha_1 \)-PAMP and \( \alpha_2 \)-PAMP were determined at 4.2 and 4.0 respectively by using crossed immunoelectrofocusing. The \( \alpha_2 \)-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 \( \alpha_1 \)-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 & Svehag (1978). One protein, \( \alpha_1 \)-PAMP, with \( \alpha_1 \)-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, \( \alpha_2 \)-PAMP, with \( \alpha_2 \)-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 \( \alpha_2 \)-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 α₁-PAMP and α₂-PAMP

Antibodies against α₁-PAMP and α₂-PAMP were raised in conventional albino rabbits (State Serum Institute, Copenhagen) as previously described (Hau et al., 1978). The antiserum with reactivity against α₁-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 α₁-PAMP by crossed immunoelectrophoresis using serum of males, non-pregnant and pregnant females. The antiserum reacting against α₁-PAMP and α₂-PAMP was absorbed with 30% volume of a pool of serum of non-pregnant females to remove activity against α₁-PAMP. The supernatant was tested for monospecificity against α₁-PAMP by crossed immunoelectrophoresis using serum of pregnant and non-pregnant females.

Crossed immunoelectrophoresis

Crossed immunoelectrophoresis was performed on 7 x 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 µl 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 µl 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 µg 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 α₂-PAMP serum level during pregnancy was determined by rocket immunoelectrophoresis (Laurell, 1972) on 11 x 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 µl antibody preparation per cm². The wells received 5 µl samples. One arbitrary unit (AU) of α₂-PAMP refers to the amount of α₂-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 x 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 $\alpha_1$-PAMP. The antigen was non-pregnant female serum diluted 1:12.5, without Con A (Fig. 1) and with 100 $\mu$g Con A/cm$^2$ (Fig. 2) in the first dimension gel.

Figs 3 and 4. Crossed immunoelectrophoresis with the rabbit antiserum against $\alpha_2$-PAMP in the second dimension gel. The antigen was serum from pregnant mice diluted 1:5, without Con A (Fig. 3) and with 200 $\mu$g Con A/cm$^2$ (Fig. 4) in the first dimension gel. The areas marked x and y apparently represent two different molecular forms of $\alpha_2$-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 µl 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 α1-PAMP and α2-PAMP

At a Con A concentration of 100 µg/cm² gel in the first dimensional gel, α1-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 α2-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 α2-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 µg Con A/cm² did not cause any further precipitation of α2-PAMP in the first dimensional gel or alteration of the precipitates in the second dimensional gel.

Neither α1-PAMP nor α2-PAMP were stainable with the lipoprotein staining method.

Cross-reactivity between α2-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 α2-PAMP by using the rabbit antiserum to α2-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 β1-glycoprotein and pregnancy zone protein.

Table 1. The mean ± s.e.m. (no. of samples in parentheses) concentrations of α2-PAMP in serum, urine and amniotic fluid of mice

<table>
<thead>
<tr>
<th></th>
<th>Conc. of α2-PAMP (arbitrary units/ml)</th>
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<tbody>
<tr>
<td>Non-pregnant adult females</td>
<td>0 (80)</td>
</tr>
<tr>
<td>Early pregnant females (Days 1-7)</td>
<td>0 (37)</td>
</tr>
<tr>
<td>Late pregnant females (Day 17)</td>
<td>1526 ± 94 (21)</td>
</tr>
<tr>
<td>Females at term (Day 20)</td>
<td>55 ± 7 (28)</td>
</tr>
<tr>
<td>Post-partum females</td>
<td>0 (76)</td>
</tr>
<tr>
<td>Adult males</td>
<td>0 (66)</td>
</tr>
<tr>
<td>Fetus</td>
<td>Trace (90)</td>
</tr>
<tr>
<td>Amniotic fluid</td>
<td>0 (20)</td>
</tr>
<tr>
<td>Newborn young (2 days)</td>
<td>0 (34)</td>
</tr>
<tr>
<td>Juvenile females (3-4 days)</td>
<td>0 (18)</td>
</tr>
<tr>
<td>Urine of pregnant females</td>
<td>0 (8)</td>
</tr>
</tbody>
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Isoelectric point of \( \alpha_1\)-PAMP and \( \alpha_2\)-PAMP

The peak of the precipitate indicating the isoelectric point of \( \alpha_1\)-PAMP was at pH 4.2 of the gradient. The isoelectric point of \( \alpha_2\)-PAMP was pH 4.0.

Concentration of \( \alpha_2\)-PAMP

It was possible to detect \( \alpha_2\)-PAMP by rocket immunoelectrophoresis only in the serum of pregnant mice and fetuses (Table 1).

The results of daily \( \alpha_2\)-PAMP determinations are shown in Text-fig. 1. The \( \alpha_2\)-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 \( \alpha_2\)-PAMP. On Day 2 post partum the protein could no longer be detected in serum by rocket immunoelectrophoresis.

![Text-fig. 1. The concentrations of \( \alpha_2\)-PAMP, measured by rocket immunoelectrophoresis, in 318 serum samples from 30 primigravid NMRI mice. Values are mean ± 1 s.e.m. for 9–28 samples at each point. P is the day of parturition, for litters born early in the morning.](image)

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 \( \alpha_1\)-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 \( \alpha_1\)-PAMP is a homogeneous glycoprotein with two or more binding sites for concanavalin A per molecule. The \( \alpha_2\)-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 \( \alpha_2\)-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 $\alpha_1$-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, $\alpha_2$-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 $\alpha_1$-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 $\alpha_2$-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 $\alpha_1$-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 $\alpha_1$-sialoglycoprotein is identical to $\alpha_1$-PAMP or $\alpha_2$-PAMP. The $\alpha_2$-PAMP pattern during pregnancy resembles those of the human pregnancy-associated Pregnancy Zone Protein (PZP or SP$_3$) (Bohn, 1974a), and human steroid-binding $\beta$-globulin (SB$\beta$G or SP$_2$) (Bohn, 1974a, b). These, however, are present in low concentrations in non-pregnant women. The occurrence of $\alpha_2$-PAMP is also similar to that of human pregnancy-specific $\beta$-glycoprotein (PS$\beta$G, SP$_1$, 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 $\alpha_2$-PAMP is rapid and similar to the decline of human placental lactogen and pregnancy-specific $\beta_1$-glycoprotein (Lin, Halbert, Spellacy & Gall, 1976), while steroid-binding $\beta$-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 $\alpha_1$-PAMP suggest that this protein may be a possible analogue to human steroid-binding $\beta$-globulin or human placental lactogen, although attempts to demonstrate immunological cross-reactivity or partial identity between the two human proteins and $\alpha_2$-PAMP were unsuccessful using antibodies raised in rabbits.

References


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