Isolation and Partial Characterization of Proteins in Equine Fetal Membranes

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An antiserum against the extract of equine allantoamniotic membrane was absorbed with the colt liver extract. This antiserum gave four precipitations in immunoelectrophoresis with extract of allantoamnion as the antigen. The four precipitates exhibited electrophoretic mobility of prealbumin area and were termed antigens A, B, C and D. The antiserum also reacted with amniotic fluid, whereas no precipitin band was visible when pregnant mare serum (days 120–270) was tested against absorbed antiserum.

The equine allantoamniotic antigens were purified from the extract of allantoamnion by gel filtration, anion exchange chromatography, isoelectric focusing and affinity chromatography. Antigen A had a molecular weight of about 50,000 and an isoelectric point of 3.8–4.0. Antigen B and C had a molecular weight of about 21,500. The isoelectric point of antigen B was 3.7–4.0 and that of antigen C was 3.4–3.6.

Key words: allantoamnion, equine, fetal protein


Each of the equine fetal membrane is a highly specialized organ and produces a variety of biologically active compounds. The pregnancy specific protein of the horse, pregnant mare serum gonadotropin (PM SG), was discovered by Cole and Hart [2]. More recently, Gidley-Baried et al. [5] have detected a new pregnancy protein in pregnant mare serum. We investigated a few proteins, which have been detected in extract of equine allantoamnionic membrane by means of immunoelectrophoresis and immunodiffusion techniques. The present study describes the immunochemical detection and partial characterization of the proteins, which are considered to be synthesized in allantoamnionic membranes, by using antibodies against the proteins.

Materials and Methods

Specimens. The somatic tissues (liver and kidney) from a Thoroughbred colt, 46 months of age, were obtained following its fracture of phalanx proximalis. Allanto-ammions were collected from four full-term spontaneous deliveries (330–340 days gestation). The sample of amniotic fluid were obtained from a pregnant mare who underwent delivery by amniocentesis, and were centrifuged at 10,000 × g for 20 min at 4°C. The supernatants were stored at −20°C. Blood samples were obtained from ten non-pregnant mares, six colts, and fourteen mares during their pregnancies.

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Antisera and tissue extracts. The extracts of allantoamnions were prepared by homogenizing 700 g each of tissues in 700 ml of 0.01 M Tris-HCl buffer, pH 7.5. After centrifugation at 10,000 × g for 30 min at 4°C, the supernatant was assayed for protein and stored at −20°C. Five male rabbits (Body weight: 3.5 kg) received subcutaneous injections of 5 ml soluble extract emulsified in an equal volume of Freund’s complete adjuvant at 10-day intervals for 7 weeks. The rabbit antisera obtained 9 days after the final injection were absorbed with an equal volume of colt liver extracts by incubation at 37°C for 2 h and then 4°C overnight, centrifuged at 10,000 × g for one h and stored. Complete absorption was confirmed by immunoelectrophoresis.

The antisera used for antigen detection were prepared in rabbits (4 male rabbits) by using the antigen-antibody complex as antigen, which precipitated specifically between the extract of allantoamnion and its antiserum. Immunoglobulin of the resulting antiserum, precipitated in 50% saturation ammonium sulfate solution, was separated by the affinity column of cyanogenbromide-activated Sepharose 4B [1] coupled with the extracts of a colt liver. The column (1.5 × 9 cm) of Sepharose 4B trapped 40 mg of liver tissue protein. Dialyzed immunoglobulin against allantoamniotic antigen was applied to the column, which had been equilibrated with 0.03 M phosphate-buffered saline, pH 7.4. The sample was eluted with the same buffer at a flow rate of 15 ml/h at 4°C. The eluate from this column was passed through a UV monitor and was concentrated.

Immunochemical techniques. Immunelectrophoresis in agar or agarose was done by the method of Grabar and Williams [6]. For immuno-diffusion, 1.2% agar in 0.01 M phosphate buffered saline (pH 7.4) was used for the microgel diffusion technique [10].

Biochemical separation techniques

Chromatography. Ion exchange chromatography was done at 4°C on a column (2.2 × 27 cm) of DEAE-cellulose (DE 52, Whatman). Approximately 650 mg of proteins were loaded onto the column in 0.01 M Tris-HCl buffer (pH 7.5) at a rate of 20 ml/h. After equilibration with three column volumes of the same buffer, the proteins were eluted with a 0–0.3 M NaCl gradient prepared in 0.01 M Tris-HCl buffer, pH 7.5. Fractions (5 ml) were collected and the elution of proteins was monitored by absorbance at 280 nm.

Affinity chromatography. Samples were applied to a column (1.5 × 9 cm) of Sepharose 4B coupled with 39 mg of IgG fraction obtained from the rabbit anti-allantoamnion serum. The column was equilibrated with 0.03 M phosphate-buffered saline, pH 7.4. The sample was eluted with the same buffer at a flow rate of 15 ml/h at 4°C. The eluate from this column was passed through a UV monitor. When 280 nm readings began to fall, the column was washed with 0.03 M phosphate-buffered saline, pH 7.4, and then the same buffer was applied to the column until the absorbance at 280 nm of the effluent fell to less than 0.02. The bound allantoamnionic protein was eluted with 150 ml of 0.5 M NaCl-0.1 M glycine-HCl buffer, pH 2.6, at a flow rate of 15 ml/h at 4°C, followed by 400 ml of 0.03 M phosphate-buffered saline, pH 7.4. The protein fractions eluted from the column, 6 ml each, were measured spectrophotometrically at 280 nm and then neutralized with 0.5 M NaCl-0.1 M carbonate-bicarbonate buffer, pH 8.3. The eluted allantoamniotic protein fraction was dialyzed at 4°C against 10 fold excess...
of 0.03 M phosphate buffer, pH 7.4.

**Gel filtration.** Samples were applied to a column (3.2 x 80 cm) of Sephadex-G 200 (Pharmacia) equilibrated and eluted with 0.01 M Tris-HCl buffer (pH 7.5). Fractions were monitored spectrophotometrically at 280 nm.

**Isoelectric focusing.** The preparative electrophoresis was performed on the LKB 8100-1 column with a sucrose gradient containing 2% ampholytes at an operating range of pH 2.5–4.0 and at 3 W and 4°C for 24 h. Fractions from columns were monitored at 280 nm.

**Polyacrylamide gel electrophoresis under non-denaturing conditions.** The electrophoresis was carried out as described by Ornstein [9] and Davis [3]. The gel was stained with Coomassie brilliant blue R-250/0.05% in 10% acetic acid-45% methanol.

**SDS-polyacrylamide gel electrophoresis.** For one-dimensional SDS gradient gel electrophoresis, up to 50 μl volumes of column fractions were mixed with the equal volume of buffer containing 2-mercaptoethanol (10%), SDS (10%), glycerol (20%), Tris-HCl (0.125 M pH 6.8) and bromophenol blue (0.002%). Samples were heated at 100°C for 1 min before analysis. Gels were stained for 30 min with Coomassie brilliant blue R-250/0.05% in 10% acetic acid-45% methanol. Bands were calibrated with marker proteins of known molecular weights (Sigma).

**Protein estimation.** The colorimetric method of Lowry et al. [7] was used to determine total protein content using bovine serum albumin as a standard.

**Results**

Five rabbits were immunized against the soluble antigens of allantoamniotic membranes of post parturition and antibodies were detected first in its seventh week after the first injection in all animals. The antisera were analyzed by immunodiffusion and immunoelectrophoresis before and after absorption by colt liver extracts. Unabsorbed antisera produced numerous precipitin arcs with the soluble extracts of liver and kidney in immunodiffusion tests. However, absorbed antiserum produced
only four precipitin arcs with extracts of allantoamnion. The four antigens thus detected were termed antigens A, B, C and D.

Immunoelectrophoresis indicated that antigens A, B, C and D were distinct species of proteins (Fig. 1). Absorbed antisera also formed precipitation lines with amniotic fluid, which could not be detected with pregnant mare serum (Days 120-270).

Chromatography of the extracts of allantoamnion on DEAE-cellulose resulted in partial separation of A, B, C and D antigens (Fig. 2). The elution profile of allantoamnionic proteins from a Sephadex G-200 column is shown in Fig. 3. The result of isoelectric focusing performed in the pH range between 2.5 and 4.0 is shown in Fig. 4. The molecular weights, estimated by SDS-polyacrylamide gel electrophoresis, were about 50,000 for antigen A (Fig. 5-a) and about 21,500 for antigen B and C (Fig. 5-b).

**Discussion**

By means of immunochemical techniques four antigens have been detected in the extracts of equine allantoamnion. Negative results were also obtained when the absorbed antisera were tested against pregnant mare serum from 120 to 270 days after the last service. The results indicated that equine amniotic epithelial cells secrete a form of allantoamnionic antigen into the amniotic fluid.

Several proteins of non-maternal serum origin have been known for the human amniotic fluid and recent reports [4, 11,13] have indicated the synthesis of secretory proteins by the fetus. The immuno-affinity chromatography that removed vast majority of the nonspecific serum proteins from amniotic fluid with anti-human serum antibody linked to a solid matrix, was introduced for the detection of proteins in amniotic fluid that were not derived from...
maternal serum [12]. Bovine amniotic fluid has been shown to contain a protein that is likely to be of amniotic origin [8].

The measurement of allantoamniotic protein levels in amniotic fluid and in pregnant mare serum provides valuable clinical information as a predictor of fetal risk and compares favorably with other well-established indices of fetal well-being.

It is not known whether this pregnancy-associated proteins has effects on conceptus survival or development. Further precise studies on the purification of the antigens A, B, C and D in parallel with physiological response are necessary.

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References


ウマ胎膜中の妊娠関連蛋白の分離とその一部性状について——松下博治1，矢萩真弓1*，浅野修一1，西田利穂2，藤谷英男2，安藤博文2（1麻布大学獣医学部生理学第一教室，2同分子生物学教室 〒229 神奈川相模原市淵野辺1-17-71，3帝京大学医学部臨床研究棟小動物室 〒173 東京都板橋区加賀2-11-1）

ウマ尿膜羊膜抽出液をウサギに免疫して抗体を作製し、若雄馬肝臓抽出液を用いて吸収操作を行った。ゲル内反応において、吸収抗体血清と尿膜羊膜抽出液との間に4本の沈降線を認めた。寒天免疫電気泳動の結果、4本の沈降線はプレアルブミン位に位置していた。アガロース免疫電気泳動により生じた沈降線で、各の抗原を陰極側より、抗原A、B、CおよびDと呼ぶことにした。

吸収抗体血清を用いて、半水中に共通抗原を検出したが、妊娠（120-270日）ウマ血清では共通抗原が検出されなかった。免疫電気泳動により、吸収抗体血清と尿膜羊膜抽出液間に生じた抗原抗体複合物を切り出し、これを抗原として、ウサギを免疫し、A、B、C、Dの各抗原に対する特異抗体血清を作製した。これらの抗血清を用い、ゲルろ過、イオン交換クロマトグラフィー、等電点電気泳動、アフィニティークロマトグラフィーなどにより、各抗原の分離を試み、抗原の一部性状を明らかにした。抗原Aの分子量は約50,000を示し、抗原BおよびCは約21,500であった。また、等電点については抗原Aが3.8-4.0、抗原Bは3.7-4.0、抗原Cは3.4-3.6と酸性側に位置していた。