Fetal- and uterine-specific antigens in human amniotic fluid

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Summary. Removal of the major maternal serum proteins from second trimester amniotic fluid by antibody affinity chromatography revealed various soluble tissue antigens, of which two were fetal-specific skin proteins and another, of α_2 -mobility, was specific to the uterus, and was therefore designated alpha-uterine protein (AUP). These proteins could not be detected in maternal serum by antibody-antigen crossed electrophoresis. The concentration of AUP in amniotic fluid reached a maximum between 10 and 20 weeks of gestation, suggesting that there is an influx of uterine protein into the amniotic fluid at this stage of pregnancy.

Introduction

Fetal antigens are expressed early in the development of various tissues and their number and function constitute a major problem in developmental biology (see review by Uriel, 1975). They are also of significance in oncological diagnosis (Currie, 1974) and the search for new fetal antigens has been encouraged by the fact that some congenital abnormalities can be detected prenatally by the measurement of alpha-fetoprotein (AFP) in human amniotic fluid and maternal serum (Brock & Sutcliffe, 1972; Brock, 1976). A variety of fetal-specific proteins has been revealed by immunological, enzymological and other methods (Gonano, Pirro & Silveti, 1973; Coggin & Anderson, 1974; Drysdale & Singer, 1974). Study of fetal isoenzymes involves precise and sensitive methodology but, unlike the immunological approach, it can only be applied to selected proteins of known function or effect (see Schapira, Dreyfus & Shapira, 1963; Taylor, Stafford & Jones, 1972; Schapira, 1973; Doellgast & Fishman, 1976). However, immunological methods have revealed only a small number of soluble fetal antigens (Alexander, 1972; Uriel, 1975) and may indicate that current serological methods lack sufficient sensitivity for the raising and subsequent analysis of antisera.

Previous immunological studies on human amniotic fluid used unfractionated protein as immunogen and confirmed that the majority of the soluble protein in amniotic fluid was of maternal serum origin. However, these studies were relatively unsuccessful in detecting fetal- or other pregnancy-specific proteins (see Sutcliffe, 1975, for review). In order to increase the immunogenicity of these antigens, we have removed the major serum proteins and AFP from specially selected samples of human amniotic fluid by means of antibody affinity chromatography. This subtractive, or negative, approach to the search for new antigens (De Carvalho, Lewis, Rand & Uhrick, 1964; Anderson *et al.*, 1974) has been referred to by Sutcliffe (1976) as "negative antibody affinity chromatography".

Materials and Methods

Amniotic fluid was obtained by amniocentesis from patients affected with rhesus disease or whose pregnancies were at risk for a neural tube defect and who had therefore been referred for AFP determinations. Amniocentesis was performed between the 10th and 34th weeks of gestation, and in each case pregnancy continued to term and resulted in the birth of a normal child. The samples of fluid selected for negative antibody affinity chromatography (NAAC) were those which had relatively high concentrations of alpha-fetoprotein (AFP) (>20 $\mu\text{g/ml}$), in the hope that there would be a

parallel enrichment of other fetal, or pregnancy-specific proteins. Adult human tissue was obtained within 12 h of death from two patients (both male) dying of myocardial infarction and pulmonary embolism, respectively. Human fetal tissues were obtained from second trimester fetuses after termination of pregnancy for social reasons or as a consequence of neural tube defect.

Antibody affinity columns were prepared as described by Sutcliffe (1976). The antiserum raised in sheep to whole human serum protein was fractionated in 40% neutral ammonium sulphate to yield an antibody-enriched fraction. Rabbit antibodies to AFP were similarly prepared. These antibody fractions were coupled to separate preparations of CNBr-activated Sepharose (Porath, Axen & Ernback, 1967) at a ratio of 15 mg protein per wet weight of Sepharose 6B. The final size of the anti-adult serum column was 1.9 kg and that of the anti-AFP column 115 g. The antibody fraction was coupled to Sepharose overnight at 4°C with gentle shaking, and then the unbound fraction of antibody (less than 5% of the total protein applied) was washed off with 0.1 M-NaHCO₃. Any remaining active groups on the Sepharose were reacted with 1.0 M-ethanolamine, pH 8.0, for 2 h. Non-covalently associated protein was then dissociated by washing the Sepharose alternately with 0.1 M-acetate buffer containing 1.0 M-NaCl (pH 4.0) and with 0.1 M-borate buffer containing 1.0 M-NaCl (pH 8.0), the whole procedure being repeated three times. Finally, the immunosorbent was poured into chromatography columns and equilibrated with phosphate-buffered saline (PBS: 0.16 M-NaCl + 5 mM-potassium phosphate, pH 7.5), before being washed with 150 ml 2.0 M-KI, 50 mM-Tris, pH 8.6, and then equilibrated in PBS.

Negative antibody affinity chromatography. The pooled amniotic fluid (310 ml) was filtered through Whatman No. 1 paper and passed through a 10 × 2.5 cm column of pure Sepharose 6B in PBS to remove particulate and adherent material. The elution of amniotic fluid was monitored with an LKB Uvicord I (254 nm) and the material was pooled (419 ml) before being divided into four equal aliquots, which were then passed individually over the anti-adult serum column at 4°C. The adsorbed (serum) protein was desorbed with 0.5 M-acetic acid before re-use. The amniotic fluid protein that was not adsorbed was collected, pooled, concentrated by ultrafiltration (Amicon PM-10 membrane, exclusion value for mol. wt = 10 000), and assayed for total protein and albumin. The concentrated material was then divided into five pools and each was passed over a Sepharose 6B column coupled with rabbit anti-AFP. The column was desorbed between cycles with 2 M-KI + 50 mM-Tris, pH 8.4. The processes were monitored by determinations of total protein, albumin and AFP.

Immunization and examination of antisera. Freeze-dried aliquots of the unadsorbed protein (5 mg each) were suspended in Freund's complete adjuvant and injected intramuscularly into two New Zealand White rabbits. Booster doses (4 mg) were injected after 4 weeks. Antisera were examined by antibody-antigen crossed electrophoresis (AAE) as used by Sutcliffe & Brock (1973). All adsorptions of antisera were carried out in solution and incrementally by using either adult male human serum or tissue homogenates in PBS at protein concentrations of 18–40 mg/ml. Similar homogenates were made to examine the specificities of the antisera before and after adsorption.

Albumin and AFP determinations were carried out by one-dimensional AAE (Brock & Sutcliffe, 1972). Determinations of the protein content employed the Folin method of Lowry, Rosebrough, Farr & Randall (1951). SDS slab-gel electrophoresis was carried out by the method of Marsden, Crombie & Subak-Sharpe (1976). The spacer gel was 4.75% acrylamide and the running gel was a gradient of 7–15% acrylamide. The gel was fixed and stained in 0.2% (w/v) Coomassie blue in 50% (v/v) methanol–7% (v/v) acetic acid and destained in the above solvent, followed by 5% (v/v) methanol–7% (v/v) acetic acid. A rabbit antiserum to hCG was obtained from Miles Biochemicals.

Results

Table 1 shows that the NAAC columns removed approximately 97% of the total protein in the original sample of amniotic fluid. The residual protein was analysed by SDS slab-gel electrophoresis (Pl. 1, Fig. 1) and was shown to differ greatly from the original amniotic fluid protein which was very similar to serum protein, although lacking some high molecular weight components. The antisera raised in rabbits against the residual protein from NAAC columns showed a wide range of precipitin

arcs (Pl. 1, Fig. 2). When the antisera were adsorbed with adult male serum protein, 6 or more of the precipitin lines were still detectable (Pl. 1, Fig. 3), whereas no precipitin lines were visible when 5 mg adult human serum protein was tested against the adsorbed antiserum under the same conditions. When a wide variety of adult and fetal tissue extracts were tested against the adsorbed antiserum a number of precipitin lines were found, indicating that some ubiquitous tissue antigens are present in amniotic fluid. A maximum number of 6 individual precipitin lines was found in samples of fluid from 14 to 18 weeks of pregnancy (Pl. 1, Figs 4 and 5).

Table 1. Protein content of human amniotic fluid before and after adsorption by antibody chromatography

	Total protein (mg) (a)	Albumin (mg) (b)	AFP (mg) (c)	Other (mg) (a - {b + c})
Before adsorption	1480	1435	5.8	39.2
After adsorption	34.1	0.4	<0.01	33.7
Protein unadsorbed (%)	2.3	0.03	<0.2	86.0

Because the decidua is potentially a major source of maternal tissue protein, and extracts of the tissue in PBS gave 2 or 3 precipitin lines with antiserum which had first been adsorbed with adult human serum, an aliquot of antiserum was adsorbed with adult human serum and adult liver homogenate (40 mg protein/ml PBS). With this twice-adsorbed antiserum a single precipitin line was observed when extracts of decidua were tested (Pl. 1, Fig. 6). When tested on two-dimensional AACE the precipitin was found to have an α_2 -mobility. It was detectable by AACE in myometrium, endometrium, decidua, amnion, chorion and amniotic fluid; it was not detectable by AACE in other tissues including maternal serum. Since the antigen is present in the uterus irrespective of pregnancy it is subsequently referred to as α_2 -uterine protein (AUP). Although there is a wide scatter of values for AUP concentrations between different samples of amniotic fluid, maximum concentrations were found during the second trimester at about 16 weeks of pregnancy (Text-fig. 1). Cross-reactivity between AUP and hCG could not be detected by AACE.

EXPLANATION OF PLATE

Fig. 1. SDS slab-gel electrophoresis of (a, b) adult human serum protein; (c) adult red cell lysate; extracts of (d) endometrium, (e) decidua, and (f) amniotic fluid protein after NAAC adsorption, compared with (g, h) amniotic fluid protein before adsorption. The spacer gel was 4.75% acrylamide and the running gel was a gradient of 7–15% acrylamide. Stained with Coomassie blue.

Figs 2 and 3. Antibody-antigen crossed electrophoresis (AACE). The antigen well is to the left and a 50 μ g sample of unadsorbed amniotic fluid protein was tested against unadsorbed (Fig. 2) and adsorbed (Fig. 3) antisera. Adsorption was carried out in solution with adult male serum. The first dimension anode is to the right-hand side of each gel, where the single circular mark shows the mobility of albumin detected by dye binding.

Figs 4 and 5. AACE of untreated samples of amniotic fluid from women at 14 (Fig. 4) and 17 (Fig. 5) weeks of pregnancy. The antiserum had been adsorbed with adult male human serum. The mobility of albumin in the first dimension is shown by the circle.

Fig. 6. AACE of an homogenate of decidua obtained at 14 weeks of pregnancy. The antiserum had been adsorbed with both adult liver and serum. There is only one precipitin line. $\bigcirc$ = the albumin mobility in the first dimension.

Fig. 7. AACE of an homogenate of fetal skin at 16 weeks of pregnancy. The antiserum had been adsorbed with homogenized decidua and adult male serum. Two precipitin arcs are visible. $\bigcirc$ = the mobility of albumin in the first dimension.

Text fig. 1. The concentration of α -uterine protein (AUP) in individual samples of human amniotic fluid obtained from normal pregnancies during the 2nd and 3rd trimesters. AUP was measured in 2–10 μ l samples of amniotic fluid by one-dimensional AACE. The antiserum with the properties shown in Pl. 1, Figs 2–5 was incorporated into the gel at a final strength of 2%, and only the AUP precipitin line was visible after staining. The precipitin lines showed reactions of identity with extracts of decidua and endometrium but not with extracts of liver or skin. The area under each precipitin peak was measured and the concentration of AUP was expressed in units of area (mm^2) per ml amniotic fluid. Internal standards were included in each AACE plate to control for the reproducibility of the assay. The concentration of albumin was also measured in each sample. No relationship was found between the concentrations of AUP and albumin in individual samples at any stage of pregnancy.

Extracts of decidua adsorbed the antiserum of all its precipitin arcs when tested with adult skin and other tissues. However, two clear precipitin lines were found when homogenates of fetal skin and amnion were tested against this decidua-adsorbed antiserum (Pl. 1, Fig. 7). The lines were absent from fetal gut and liver and did not cross-react with purified AFP.

Discussion

Although previous studies have indicated that the major antigens in amniotic fluid are of serum type, some non-serum antigens, e.g. AFP, haemoglobin and two proteins present in extracts of amnion have been reported (Lambotte & Salmon, 1962; Lambotte, 1966; von Kleist, Buffe & Burtin, 1968). The present work shows that at least 6 tissue antigens can be detected by AACE in amniotic fluid early in pregnancy. The nature and precise tissue specificity of AUP remains to be determined by more sensitive methods of detection and purification of the protein is in progress for development of a radioimmunoassay. At present it is not possible to decide whether AUP cross-reacts with the rabbit protein known as cone protein (Feigelson, 1976), blastokinin (Krishnan & Daniel, 1967) or uteroglobulin (Beier, 1968), because the antiserum against AUP was raised in rabbit. However, blastokinin has a total molecular weight of 15 800 (Feigelson, 1976), whereas AUP is a dimer composed of two subunits each of mol. wt 25 000 (unpublished observations).

The concentration of AUP during the second trimester of pregnancy is very similar to the specific activity profiles of α -1,4-glucosidase and heat-labile alkaline phosphatase (Sutcliffe, Brock, Robertson, Scrimgeour & Moneghan, 1972; Sutcliffe & Brock, 1972) and remarkably different from the concentrations of the major serum proteins, all of which reach maximum levels between 20 and 30 weeks of pregnancy (Sutcliffe & Brock, 1973). There appears to be no direct relationship between the concentrations or specific activities of these two groups of proteins (see Text-fig. 1). There is evidence that the α -1,4-glucosidase in amniotic fluid is not of fetal origin, because the enzyme is not present in fetuses affected with Pompe's disease but is still detectable in amniotic fluid (Salafsky & Nadler, 1972).

By contrast, the blood group substances (Harper, Bias, Hutchinson & McKusick, 1971) and the enzymes, β -galactosidase, β -hexosaminidase A and B, and arylsulphatase A, which have been found in the amniotic fluid, are largely of fetal origin (O'Brien *et al.*, 1971; Lowden, Cutz, Cohen, Rudd & Doran, 1973; Desnick, Krivitt & Sharp, 1973; Borresen & van der Hagen, 1973). It is possible that AUP, heat-labile alkaline phosphatase and α -1,4-glucosidase all enter the amniotic fluid from the uterus during the second trimester, either because the uterus is enriched in these proteins at this stage of pregnancy, or because of a structural change in the fetal membranes which permits an influx of uterine protein. Although there is no well established histological evidence for such a change in the membranes, there is an influx of maternal serum protein into the fluid at this gestational age and the lack of correlation between the concentrations of AUP and albumin in amniotic fluid may simply reflect a larger available pool size of maternal serum albumin. It may also be that maternal serum proteins enter the amniotic fluid mainly from the placental bed via the chorionic plate and the placental amnion, whereas AUP may diffuse through the reflected membranes (see Sutcliffe, 1975). This large maternal contribution to amniotic fluid protein in man must, to some extent, reflect the organization of the placental membranes. In the mouse, the uterus is separated from the conceptus by the yolk sac splanchnopleur, and Renfree & McLaren (1974) have shown that much higher concentrations of fetal proteins are present in the amniotic fluid of mice than in that of man.

Further advances in prenatal diagnosis by detection of fetal-specific proteins in amniotic fluid may be possible by elaboration of more refined serological methods, particularly by fractionating complex mixtures of antigens before immunization. In his studies on the human placenta, Bohn (1976) first fractionated protein extracts by precipitation in Rivanol and ammonium sulphate in order to raise antisera against 6 placental tissue proteins. However, NAAC has the apparent advantage of being an immunologically specific form of protein fractionation. As others have found (see Pihko, Lingren & Ruoslahti, 1973), the use of affinity columns of antibodies raised against adult serum protein gave minimal cross-reaction with AFP. Such a lack of cross-reactivity cannot be assumed to apply in all cases because different proteins may bear common antigenic determinants. We have attempted to guard against this problem in our experiments by adsorbing the amniotic fluid protein until only 2–3% of it remained in solution after passage over NAAC columns. Further adsorption might have resulted in the removal of cross-reacting non-serum antigens as the concentration of serum protein was reduced to non-competitive levels. Our failure to purify the β -oncofetal antigen described by Fritsché & Mach (1975) was probably because this protein is found in appreciable concentrations in adult human serum.

We are grateful to Dr A. A. M. Gibson and Dr F. Sharp for supplying fetal material and to Dr J. B. Scrimgeour for samples of amniotic fluid. The work was supported by grants from the M.R.C. and The Radiochemical Centre, Amersham.

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Received 21 November 1977