

IMMUNOCHEMICAL STUDIES OF AMNIOTIC FLUID: DECREASE OF A 36,000 POLYPEPTIDE IN PREGNANCIES OF FETUSES WITH DOWN'S SYNDROME

Débora D' A. Reis¹, Vânia F. Prado¹, Leila M. Farah² and Sérgio D.J. Pena¹

ABSTRACT

In a search for potential new biochemical markers for prenatal diagnosis of Down's Syndrome, we studied 14 samples of second trimester amniotic fluid obtained from pregnancies of fetuses with this chromosomal disease and an equal number of normal controls matched for gestational age. All samples were analyzed by one and two-dimensional Western Blots using an antiserum specific for the fetal proteins of human amniotic fluid. A fetal polypeptide with an apparent molecular weight of 36,000 (P36) was markedly decreased in ten (71%) of the Down's Syndrome amniotic fluids. Once appropriate quantitative immunoassays are developed, the deficiency of P36 may prove to be a useful biochemical marker for prenatal detection of Down's Syndrome.

INTRODUCTION

Down's Syndrome is the most common congenital cause of mental retardation, with an incidence at birth of about 1.3 per 1000 (Wald *et al.*, 1988). The syndrome is always associated with mental retardation and accounts for between 10 and 30% of all cases of mental deficiency (Hook, 1981). Thus, Down's Syndrome constitutes a public health problem and efficient preventive measures are needed. Presently, the only feasible approach to prevention is prenatal screening of pregnancies, followed by selective abortion of affected fetuses, on request. The most common

¹ Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, UFMG, Caixa Postal 2486, 30161 Belo Horizonte, MG, Brasil. Send correspondence to S.D.J.P.

² Departamento de Genética, Escola Paulista de Medicina, 04023 São Paulo, SP.

current method for screening is to select pregnant women for chorionic villus sampling or amniocentesis on the basis of advanced age. However, since at least 60% of Down's Syndrome patients are born to women under 35 years of age and not all patients at risk actually undergo prenatal tests, such programs have had little effect on the incidence of the disease at birth.

In 1984 Merkatz *et al.* reported that pregnancies with fetal chromosome abnormalities tended to be associated with lower maternal serum alpha-fetoprotein (AFP) levels than unaffected pregnancies. Their data have been confirmed by a large number of studies (reviewed by Wald and Cuckle, 1988), which additionally show a decrease in AFP concentration in the amniotic fluid of Down's Syndrome pregnancies. Both decreases were subtle: the level of maternal serum and amniotic fluid AFP in Down's Syndrome pregnancies were 75 and 68% the median of normal pregnancies, respectively (Wald and Cuckle, 1988). However, when combined with the maternal age risk data, the determination of maternal serum AFP proved to be useful for screening for Down's Syndrome. More recently, determination of the maternal serum concentrations of unconjugated estriol (Canick *et al.*, 1988) and human chorionic gonadotrophin (Wald *et al.*, 1988) have also been found to have predictive value. However, even the joint utilization of all these indices for screening in ideal conditions would allow detection of only 60% of affected pregnancies at an amniocentesis rate of 5% (Wald *et al.*, 1988). Certainly, more efficient screening tests will have to be developed.

In search of potential new biochemical markers for prenatal screening of Down's Syndrome, we have made an immunochemical study of the polypeptide composition of second-trimester amniotic fluids obtained from pregnancies of fetuses with Down's Syndrome and matched normal controls.

MATERIALS AND METHODS

Samples

Amniotic fluid samples were collected by amniocentesis in the second trimester of pregnancy. The pregnant women had been referred for prenatal diagnosis for a variety of reasons, elevated maternal age being the most common. Cells were removed by centrifugation at 2000 x g for 5 min and the samples were stored at -20°C until studied. Maternal plasma was collected from pregnant women in the second trimester and also stored at -20°C. Protein concentration was measured by the coomassie blue G binding method of Bradford (1976) with bovine serum albumin as a standard.

Affinity Chromatography in Blue Sepharose

Albumin, which represents approximately 50-60% of the total protein of

amniotic fluid (AF), was removed by passing 1 ml of amniotic fluid through a 1.5 ml Blue Sepharose CL-6B (Pharmacia) affinity column equilibrated with Tris-HCl buffer (50 mM, pH 7.0) containing 0.1 M of KCl, as described by Burdett *et al.* (1982).

Polyacrylamide Gel Electrophoresis in the Presence of Sodium Dodecyl Sulfate (SDS-PAGE)

SDS-PAGE was performed in 1.5 mm thick slabs using the procedure described by Pena (1982). The separating gel contained 14% acrylamide and 0.1% bisacrylamide and had an effective separation range of 10,000-250,000. Gels were run for 5 hours at 35 mA. Standard protein markers included in every gel were a mixture of myosin (205,000), beta-galactosidase (116,000), phosphorylase b (97,400), bovine serum albumin (66,000), ovalbumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), trypsin inhibitor (20,100) and alpha-lactalbumin (14,200).

Two-dimensional electrophoresis

Two-dimensional macromolecular mapping was done with isoelectric focusing in the first dimension and SDS gel electrophoresis in the second dimension using a modification of the procedure described by Pena *et al.* (1979) as follows:

(a) First dimension: isoelectric focusing: Isoelectric focusing gels were cast in glass tubes (150 mm long x 3 mm i.d.). The gel mixture contained 3.8% acrylamide, 0.2% bisacrylamide, 8 M urea, 2% NP-40, 5 mM aspartic acid and 5 mM glutamic acid (to stabilize the acid end of the gradient), and 2% of a mixture of Ampholines, pH 3-10, 2.5-5, 4-6.5 and 5-8, in the proportions 2:1:1:1. A pH gradient in the range of 3.5-7 was regularly obtained.

(b) Second dimension: SDS electrophoresis: Isoelectric focusing gels were placed horizontally on top of the stacking gel of polyacrylamide slabs. SDS-PAGE was then performed as described above.

Preparation of an antiserum specific for fetal proteins

Antibodies against maternal proteins were raised in New Zealand albino rabbits by subcutaneous and intramuscular injections of maternal plasma. When an adequate titer was obtained, as tested by passive immunodiffusion and immunoelectrophoresis, the rabbits were bled to death by heart puncture. The immunoglobulin fraction was enriched by precipitation with sodium sulphate as described by Kekwick (1940) and bound to CNBr-activated Sepharose 4B (Pharmacia) according to the

instructions of the manufacturer. In order to obtain a fraction enriched in fetal proteins, approximately 10 ml of a pool of albumin-depleted amniotic fluids from several normal pregnancies was passed through this column and recirculated 5 times. This fraction was used to immunize rabbits and the response was monitored by immunoelectrophoresis. The antiserum was then extensively absorbed with maternal plasma proteins immobilized by cross-linking with glutaraldehyde according to the method of Avrameas and Ternynck (1969).

Western blots

After the proteins had been electrophoretically separated in polyacrylamide gels, they were immediately transferred to nitrocellulose using a Bio-Rad Trans-BlotTM cell as described by Gordon and Pena (1982). Transference was at a constant current of 175 mA for 18-20 h. After transfer, the strip containing the molecular weight markers was cut and stained with coomassie blue R-250. For reactions with antibodies, nitrocellulose sheets were soaked in blocking solution (1% casein, 3% calf skin gelatin (Sigma), 0.05% Tween-20, in 0.5 M Tris-HCl, pH 7.5 with 2.5 M NaCl) for 2 h at 37°C. The membranes were then incubated with the antiserum diluted 1/50 in washing buffer (0.05% Tween-20 in PBS) overnight at 4°C. Then the membranes were washed and incubated with goat anti-rabbit IgG conjugated to alkaline phosphatase (Bio-Rad) diluted 1/2000 in washing buffer for 90 min at room temperature. After this incubation, the membranes were again washed and stained with substrate solution (0.17 mg/ml 5-bromo-4-chloro-2-indolyl phosphate and 0.33 mg/ml nitroblue tetrazolium in 0.1 M pH 9.1 Tris-HCl, containing 0.1 M NaCl and 5 mM MgCl₂).

RESULTS

The human amniotic fluid contains a complex mixture of proteins of both fetal and maternal origin. Apparently, more than 95% of the proteins are maternal and probably enter the amniotic cavity as an ultrafiltrate of the plasma (Sutcliffe, 1981). In order to be able to study only the fetal proteins we developed an antiserum, which was made specifically for absorption with maternal plasma proteins. This antiserum was able to recognise some, but not all amniotic fluid polypeptides (Prado, Reis and Pena, unpublished results). To perform the comparative analyses of amniotic fluid polypeptides of normal and Down's pregnancies, we decided to use this antiserum to identify fetal polypeptides in one- and two-dimensional Western blots. However, before running the gel, it was necessary to remove albumin, which constitutes 60% of the protein in amniotic fluid and limits the gel load by distorting the electrophoresis pattern. This removal was efficiently accomplished by affinity chromatography on Blue Sepharose. We then compared fourteen samples of albumin-depleted amniotic

fluid from pregnancies with fetuses with Down's Syndrome and gestation-matched normal controls by using one-dimensional Western blots. Although more than ten bands were visualized in the blots, only one showed differences among the two groups. This was a polypeptide with a molecular weight of 36,000 which was significantly reduced in ten of the fourteen Down's Syndrome samples analyzed. Four of these are shown in Figure 1. The decrease was even more noticeable in two-dimensional blots (Figure 2), where it was also the only abnormality seen.

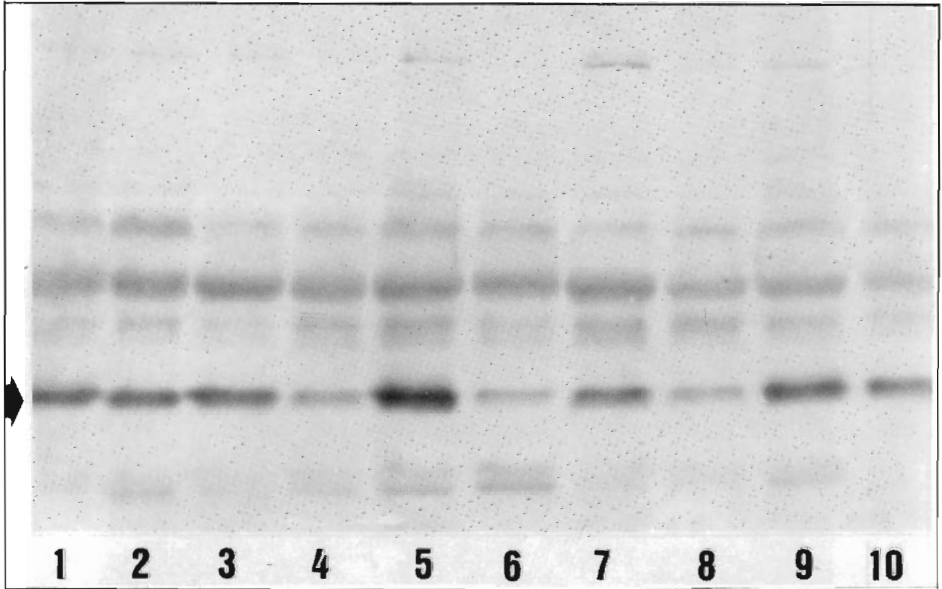


Figure 1 - One-dimensional Western blots of albumin-depleted amniotic fluid samples from four fetuses with Down's Syndrome (tracks 2, 4, 6, 8 and 10) and gestational age-matched controls (tracks 1, 3, 5, 7 and 9). Tracks 4 and 6 show the polypeptide pattern of two samples from the same Down's Syndrome fetus at 16 and 19 weeks of pregnancy, respectively. The migration of the 36,000 polypeptide (P36) is indicated with an arrow. When compared to controls, the levels of P36 were not different from the control in track 2 (patient D1 in Table I), significantly decreased in tracks 4, 6 and 8 (patients D2 and D3) and mildly decreased in track 10 (patient D4).

Details of each of the pregnancies studied are given in Table I together with a visual assessment of the level of P36. With the exception of case D11, which was a mosaic and had a normal level of P36, it is clear that there were no differences between the groups with normal or decreased levels of P36 with respect to the chromosomal type of Down's Syndrome or the indication for amniocentesis. There were also no differences related to the time of storage of the samples at -20°C .

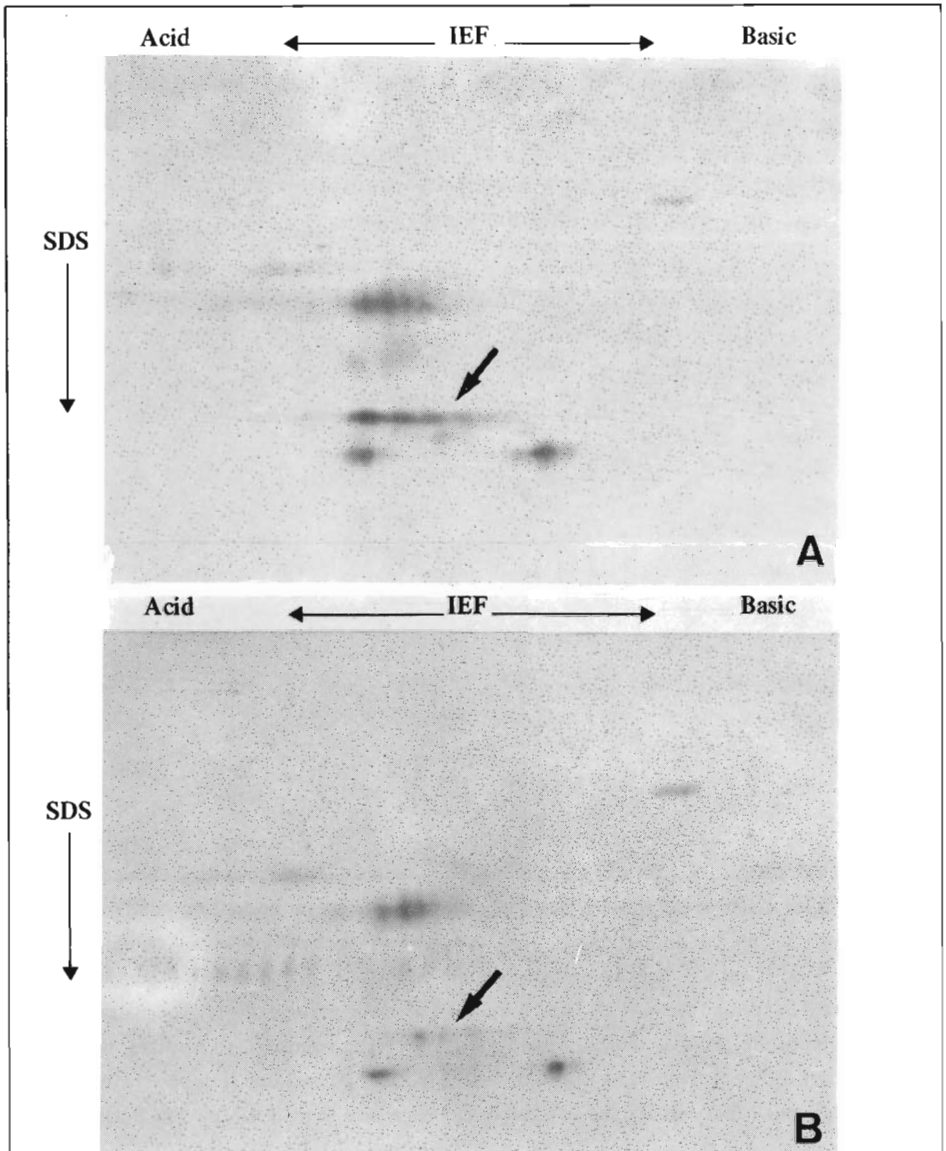


Figure 2 - Two-dimensional Western blots of albumin-depleted amniotic fluid samples from a fetus with Down's Syndrome (B) and a gestational age-matched control (A). The samples were submitted to isoelectric focusing (IEF) in the first dimension and polyacrylamide gel electrophoresis in presence of SDS in the second dimension. The only difference between the two gel patterns is the marked decrease of a polypeptide with a molecular weight of 36,000 (P36; arrow) in the amniotic fluid from the Down's Syndrome pregnancy.

Table I - Details of the cases of Down's syndrome studied.

Case	Gestational age (weeks)	Karyotype	Indication for test	Level of P36
D1	16	47,XY, + 21	A.M.A. *	Normal
D2	19	47,XX, + 21	A.M.A.	Decreased
D3	16 and 18	47,XY, + 21	A.M.A.	Decreased
D4	16 and 18	46,XY,t (14q21q)	Mother 45,XX, t (14q21q)	Decreased
D5	18	47,XY, + 21	A.M.A.	Decreased
D6	18	47,XY, + 21	A.M.A.	Normal
D7	16	46,XY, t (21q22q)	Mother 45,XX, t(14q21q)	Decreased
D8	18	47,XX, + 21	A.M.A.	Decreased
D9	16 and 19	47,XY, + 21	2 sons with Down syndrome	Normal
D10	17	46,XY, t (14q21q)	Father 45,XY, t(14q21q)	Decreased
D11	16	46,XY/47,XY, + 21	A.M.A.	Normal
D12	19	47,XY, + 21	A.M.A.	Decreased
D13	16	47,XY, + 21	A.M.A.	Decreased
D14	15	47,XX, + 21	A.M.A.	Decreased

* Advanced Maternal Age.

DISCUSSION

Our data showed a significantly lower concentration of P36 in ten (71%) of fourteen Down's Syndrome pregnancies. *A priori*, this finding is unexpected, since one would expect increased levels of some proteins in Down's Syndrome because of a gene dosage effect. However, it has become clear that the molecular correlations of aneuploid states are far from simple (reviewed in Epstein, 1988). For instance, Whatley *et al.* (1984), studying the relative amounts of specific mRNA species in the developing brains of Down's Syndrome fetuses and controls, observed that while some mRNA concentrations were increased as predicted, several were significantly decreased, probably representing secondary effects of the trisomy. Stefani *et al.* (1988) studied the expression pattern of four chromosome-21 specific DNA sequences

in Down's Syndrome and control tissues. Thei results showed no clear pattern of expression and were very different from the 3/2 ratio expected. Seen under this light, the decrease of P36 in Down's Syndrome is not so peculiar. It also matches the decreased levels of alpha-fetoprotein observed in amniotic fluid and maternal plasma from pregnancies of fetuses with Down's Syndrome (Wald and Cuckle, 1988).

The observations reported here are semi-quantitative in nature and should be considered preliminary. Obviously the next step should be to purify the polypeptide, obtain specific antibodies against it and establish quantitative immunoassays to confirm more rigorously these results. These experiments are under way in our laboratory. Until this is done, it would not be productive to speculate why P36 levels appear normal in some Down's Syndrome pregnancies and what is the significance of this finding for the biology of the disease. What is clear is that the decrease of P36 is a potential prenatal biochemical marker for Down's Syndrome.

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RESUMO

Nós estudamos 14 amostras de líquidos amnióticos obtidos de gravidezes nas quais os fetos apresentavam Síndrome de Down, assim como um número igual de controles normais ajustados pela idade gestacional. Todas as amostras foram analisadas por "Western Blots" uni e bidimensionais com um anti-soro específico para proteínas fetais do líquido amniótico humano. Em 10 das 14 amostras (71%) havia redução importante de um polipeptídeo com peso molecular de 36.000 (P36). Assim que sejam desenvolvidos imunoenaios quantitativos, a deficiência de P36 pode provar ser de utilidade no diagnóstico pré-natal de Síndrome de Down.

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