Evidently this method can be used even with gas chromatographs having relatively poor oven-temperature control, although an air current diffuser is probably advisable if such instruments were to be used routinely.

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References

Silver-Stained Phenotyping of α₁-Antitrypsin in Dried Blood and Serum Specimens

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In this technique for determining the electrophoretic phenotype of α₁-antitrypsin in dried blood or serum specimens, the adsorbed material is eluted with a concentrated solution of dithiothreitol, focused on polyacrylamide thin-layer gel, and made visible with silver stain. With this staining technique all normal and pathological α₁-antitrypsin phenotypes can be detected. The procedure is relatively simple, inexpensive, and suitable for use in large-scale screening for α₁-antitrypsin deficiency in selected populations.

Additional Keyphrases: screening · genetic variants · electrophoresis, polyacrylamide gel · pediatric chemistry · heritable disorders

Subjects with congenital deficiency of α₁-antitrypsin (AT) in their serum are prone to lung and liver disorders (1). About half of those with homozygous deficiency of AT (electrophoretic ZZ phenotype, as compared with the MM phenotype of normal subjects) start to develop liver disorders during fetal life; the minority of those affected develop manifest cirrhosis during the first postnatal decade (2). Another 5 to 10% of those with the ZZ phenotype develop liver cirrhosis of unknown etiology after age 50. The clinical evolution of affected individuals who do not show clinical signs of liver involvement in childhood appears to be related to environmental conditions, or life style, or both. E.g., smokers frequently develop chronic obstructive lung disease and die at about age 40–50. Conversely, the life expectancy of nonsmokers with this phenotype is fairly close to that of normal individuals (3). There are several other combinations of variant alleles (SZ, MZ, etc.) besides the ZZ phenotype, all associated with an intermediate quantitative deficiency of AT, but their clinical significance is still uncertain.

To assess the incidence of AT deficiency in the general population and to study the natural history of the related disease, screening has been done. Current screening tests involve dried blood specimens, from which AT is eluted, then semiquantitatively determined (4, 5). We describe here a relatively inexpensive technique for phenotype determination of AT in either dried blood or serum, and illustrate its advantages and results of its application.

Materials and Methods

Samples. No. 3 Whatman paper was used to adsorb specimens of fresh, nonheparinized blood or serum. Instructions for use of such strips were sent to 37 departments of pediatrics, centers of respiratory physiopathology, and clinical chemistry laboratories of northern Italy, where the incidence of AT deficiency is highest. The samples obtained from 30 subjects were mailed to our laboratory and a further 50 samples were obtained in our own laboratories from voluntary donors or patients who were carriers of AT deficiency. The blood or serum was eluted from the paper, either promptly upon arrival or after storage for a week at 4 °C or a month at –18 °C. For the elution, we placed a 0.5-cm sample-impregnated square of the filter paper in a conical test tube and added a few drops of β-th-dithiothreitol (DTT; cat. no. D-0632; Sigma Chemical Co., St. Louis, MO 63178), 20 g/L in doubly distilled water. The quantities of paper or solution are not critical, but the best results were obtained by using 0.5-cm paper squares with 100 µL of solution, and by incubating these at a constant temperature of 37 °C for 1 h. After 30 min the paper at the bottom of the test-tube should be pressed to further release blood or serum into the solution.

Genetic typing of AT. We used the following gel composition: 2.5 mL of a 275 g/L solution of acrylamide (cat. no. 161-0100; Bio-Rad Laboratories, Richmond, CA); 2.5 mL of a 27 g/L solution of bisacrylamide (cat. no. 161-0200, Bio-Rad); 10 mL of doubly distilled water; 200 µL of Ampholine pH 3.5–5 (cat. no. 1509-111; LKB, Bromma, Sweden); 100 µL of Ampholine pH 4–6 (cat. no. 1809-116, LKB); and 1.4 g of
sucrose (cat. no. 161-0720; Bio-Rad). The solution was deaerated in a flask under reduced pressure for 15 min and 150 µL of a 10 g/L solution of ammonium persulfate (cat. no. 420627; C. Erba, Milan, Italy) was added. We used ultrathin-layer gels (6, 7), pouring the mixture between two glass sheets (22.5 × 12.5 × 0.2 cm) separated by rubber lining, 0.3 or 0.6 mm thick and 0.5 cm wide, on each side.

Isoelectric focusing. For the 2-h pre-run, we used a constant 3.5 mA current (2117 LKB Multiphor, 2103 LKB power supply). Samples were adsorbed onto special LKB paper for isoelectric focusing (cat. no. 1850-901) and placed along the cathode. A reference serum of known phenotype, treated with DTT (1 mL of serum per 3 mL of 20 g/L DDT solution, at 37 °C for 1 h), was placed between every two test samples. Electrofocusing was for 2 h at 5 mA constant current. The temperature of the gel was kept constant (5 °C) by use of a circulating cold-water bath (2209 Multitemp, LKB). The electrodes consisted of Whatman No. 1 paper strips dampened with alanine, 0.1 mol/L (cathode), and glutamic acid, 50 mmol/L (anode).

For gel fixation we used an aqueous solution containing, per liter, 130 g of trichloroacetic acid, 120 g of sulfosalicylic acid, and 300 g of methanol, in which the gels were immersed for 15 min. We then stained the gels with a 10 g/L solution of Coomassie Brilliant Blue R (cat. no. B0630; Sigma) in ethanol, acetic acid, and water (5:2:5 by vol). For destaining, we used another mixture of ethanol, acetic acid, and water (13:2:5 by vol) and, if AT phenotypes are not detectable, thoroughly rinsed the gels with doubly distilled water and stained again, this time by the simplified silver staining technique (8), as follows.

We placed the gels in a container into which the various silver staining solutions were consecutively added and withdrawn. First, the gel was immersed in 100 mL of ammoniacal silver solution for 15 min. This solution was prepared as follows: 4 mL of a 200 g/L aqueous solution of AgNO₃ was slowly added, with magnetic stirring, to a mixture of 21 mL of dilute (3.6 g/L) NaOH plus 1.6 mL of NH₄OH. Then water was added to a final volume of 100 mL.

After three 5-min washings in water, the gels were rinsed in a citric acid/formalin solution (100 mg of citric acid and 1.2 mL of 400 mL/L formalin solution in 2 L of water). The stain developed immediately.

The staining reaction was then stopped by adding dilute acetic acid (100 g/L of water) and stored in H₂O at 4 °C. For destaining the overstained gels we used a 30 g/L aqueous solution of Kodak fixative solution (cat. no. 3233865).

In practice the gels can be stained directly with silver, omitting the Coomassie stain, if the trichloroacetic acid solution used as a fixative is first completely washed away with water (three 20-min washings is sufficient).

All solutions used were in doubly distilled water at room temperature.

Results

No major difficulty arises when phenotyping AT from blood or serum eluted from paper. Coomassie staining is usually sufficient if typing is carried out within five days from the collection of specimens. Adsorbed samples stored at −18 °C for a month or delivered by mail after a week from collection require this silver staining technique (Figure 1); in practice, staining with Coomassie can be omitted and the silver stain used directly. Staining with Coomassie alone can be used for individual samples brought quickly to the laboratory.

Assay of controls prepared from fresh serum samples confirmed the reliability of our procedure. Phenotypes MZ, ZZ, and SZ were always easily detectable, especially if a large number of reference sera are placed along side the test samples. This greatly facilitates detection of differences in the phenotype patterns with silver staining. Very dark phenotypes or excessive background can be encountered, but these rarely hinder the diagnosis; if necessary, however, the gel can be progressively destained with Kodak fixing solution or 100 g/L acetic acid.

Although difficulties can arise for rare phenotypes, in no case are these greater than those encountered when using fresh serum samples. In these cases it may be necessary to obtain a fresh serum sample to perform isoelectric focusing followed by immunofixation (9); however, these cases are only rarely of pathological importance.

Dried blood or serum specimens stored at room temperature for more than 15 days or at −18 °C for more than two months are not suitable for genetic typing.

Discussion

Sveger in Sweden (4) and O’Brien et al. in the United States (5) have carried out the widest screenings for AT deficiency in the general population. These authors used dried blood specimens to obtain semi-quantitative measurements of AT or to study its functional activity. Orfanos et al. (10) have recently described a low-cost technique—the fluorescent spot test—for the determination of serum antitryptic activity. The screening tests used so far permit semi-quantitative or functional measurements of serum AT but not genetic typing of the molecule.

As concerns the use of semi-quantitative methods for large screening programs for AT deficiency, the following considerations must be taken into account. The potential benefit of mass screening of newborns for AT deficiency is at present limited to the prevention of lung pathology in Z adults, because liver damage cannot be prevented or foreseen. Some authors have also pointed out that the mere fact of labeling children as “at risk” increases the chance that they or their families may encounter psychological problems later on (11). These disadvantages have discouraged generalized screening for AT deficiency with semi-quantitative assays.

For these reasons, and considering the cost/potential-benefit ratio of a phenotypic rather than a merely semi-quantitative diagnosis, it would seem reasonable to limit a genetic typing to a small number of selected population samples apparently "at risk." Such samples could consist of, e.g., subjects suspected of AT deficiency and their relatives, heavy smokers, and miners. For this purpose a small number of experienced laboratories operating nationwide is sufficient.
Serum Copper Concentration Significantly Less in Abnormal Pregnanecies
Paul K. Buamah, Margaret Russell, A. Milford-Ward, Peter Taylor, and Derek F. Roberts

We estimated copper concentration in maternal serum during 244 normal and 15 abnormal pregnancies. Values were lower in the abnormal pregnancies than in the normal ones, and did not vary with gestational age between 15 to 18 weeks in normal pregnancies.

Additional Keyphrases: anencephaly - spontaneous abortion - trace elements - fetal status

During normal pregnancy the copper concentration in maternal serum may almost double because of increased synthesis of ceruloplasmin (1); the copper content of the maternal liver is also increased. The exact mechanism of these increases in pregnancy is unclear, but hormones produced by the placenta may be responsible (2). Reportedly (3-5), premature infants receiving copper-deficient milk diets develop neutropenia, anemia, and scurvy bone changes, and growth is impaired in these infants, along with profoundly low serum copper and ceruloplasmin.

To examine the possibility that copper deficiency in pregnancy may be related to the development of fetal malformations, we measured serum copper in pregnant women who were being screened for fetal anomalies.

Patients and Method
We examined 259 pregnant women (244 normal pregnancies and 15 abnormal), looking for any association between serum copper and fetal malformation or demise. Serum was sampled from pregnant women referred for measurement of serum alpha-fetoprotein in the geographical areas served by our institutions (1-2). The samples taken at known gestational ages during the second trimester of pregnancy were stored in plain plastic tubes at -30°C until analyzed. Postmortem examinations were carried out on abnormal fetuses to determine the exact fetal malformation.

Maternal serum was diluted fivefold with propanol/copper-free water (10/1 by vol) and its copper content determined by flame atomic absorption (6), in a Pye Unicam SP191 spectrophotometer. Samples were measured in duplicate and the mean value was taken.

Results
As compared with the concentration of copper in maternal serum from 244 normal pregnancies, that in seven of the nine anencephalic pregnancies fell below the mean at the corresponding gestational age (Figure 1), five of them beyond twice the standard deviation. The overall mean maternal serum copper concentrations in anencephalic pregnancies was lower (mean 25.5 μmol/L, 2 SD 7.7 μmol/L) than in normal pregnancies (32.5 μmol/L, 2 SD 5.5 μmol/L). The t-test analysis for the difference between the two groups gave p < .001, which is highly significant.

Similarly, by comparison with 179 normal pregnancies, the serum copper concentrations in four of the six which resulted in spontaneous abortion are more than 2 SD below the mean (Figure 2), and the overall mean serum copper concentration at 15-18 weeks of gestation was lower (25.2 μmol/L, 2 SD 5.4 μmol/L). The t-test analysis of the difference between the two groups gave p < .005, which is significant. In the normal pregnancies, in the 15 to 18 weeks gestation period, maternal serum copper concentration did not vary with gestational age (Figure 1).