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Diagnosis of Pseudo-Arylsulfatase A Deficiency with Electrophoretic Techniques

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Summary

Deficient arylsulfatase A activity in man has long been associated with the neurodegenerative disease, metachromatic leukodystrophy. However, similar deficiency has been noted in clinically normal individuals, and is referred to as the pseudo-arylsulfatase A deficiency condition. Although direct quantitative analysis of arylsulfatase A activity failed to differentiate between these two conditions, analysis of residual arylsulfatase A activity with either Cellogel electrophoresis or isoelectric focusing in polyacrylamide gels now has been shown to distinguish between them unequivocally. With both techniques, cultured fibroblasts from patients with pseudo-arylsulfatase A deficiency showed faint but clear bands of arylsulfatase A activity. Under identical conditions, fibroblasts from patients with metachromatic leukodystrophy showed no trace of activity. These methods can be adapted easily for general laboratory analysis in cases when results from quantitative arylsulfatase A assays are noninformative.

Abbreviations

ARSA, arylsulfatase A
MLD, metachromatic leukodystrophy
4-MUS, 4-methylumbelliferyl sulfate
***p*-NCS, *p*-nitrocatechol sulfate**
PD, pseudo-arylsulfatase A deficiency

Metachromatic leukodystrophy is a hereditary neurodegenerative disease characterized by accumulation of cerebroside sulfatides in tissues and is associated with deficient activity of ARSA (EC 3.1.6.1) (1, 15). The biochemical diagnosis of this condition can be accomplished by assaying for this enzyme activity in cultured fibroblasts (17) or white blood cells (16) with artificial substrates such as *p*-nitrocatechol sulfate (3) or 4-methylumbelliferyl sulfate (5) and the natural substrate cerebroside sulfatide (15, 18). Prenatal diagnosis, which is the only available method for prevention of this severe and untreatable disease, is carried out by assay of ARSA activity in cultured amniotic fluid cells (22, 24).

Since 1975, through screening of the general population or family members of MLD probands, individuals have been identified who were similarly deficient in ARSA activity, regardless of whether natural or artificial substrates were used for the assays (4, 7, 8, 13). The paradox is that these individuals, now called PD, were free from any clinical manifestations of the disease.

It thus became critical to differentiate between MLD and PD when both types of individuals were identified in the same family for which prenatal diagnosis of a fetus at risk for MLD had to be made. Although conventional assay methods failed to distinguish between these two conditions, the diagnosis was accomplished by means of the sulfatide-loading test (11). In this test, radioactive cerebroside sulfatides were supplied to the cultured amniotic cells. In MLD cells, the exogenous substrate simply accumulated inside the cell without degradation. In PD cells, the substrates did not accumulate but were degraded normally.

We now report a simple electrophoretic method for the diagnosis of MLD and PD in cultured fibroblasts that also allows for unequivocal differentiation between these two conditions.

MATERIALS AND METHODS

Tissue culture. Cultured fibroblasts were maintained in minimal essential medium-4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid supplemented with 10% fetal bovine serum and 2 mM L-glutamine, and kept under 5% CO_2 in air and 100% humidity at 37°C. Cultured cells at confluency were harvested by trypsinization and washed twice with 0.9% NaCl, and the cell pellets were stored at −70°C until use.

Fibroblasts used as controls were established from skin biopsies of individuals without any known metabolic disease. Fibroblasts from PD and MLD patients were established from skin biopsies. Diagnosis was based on the clinical finding of progressive deterioration of neural motor functions in MLD cases, on assays for arylsulfatase A activity, excessive urinary excretion of sulfatides, and/or sulfatide loading tests (9).

For preparing the soluble extract containing ARSA, cell pellets were suspended in 0.05 M acetate buffer, pH 5.6, disrupted by sonication for three bursts at power setting 4 with the microultrasonic cell disruptor (Kontes, Vineland, NJ), and centrifuged at 103,000 × *g* for 10 min at 4–10°C. The supernatants were obtained for ARSA and protein determinations before using for electrophoresis with Cellogel or isoelectric focusing with polyacrylamide electrophoresis.

ARSA activity was determined according to the method of Chang *et al.* (5) with 4-MUS and the method of Baum *et al.* (3) with *p*-NCS as the substrate. A unit of enzyme activity was defined as nanomoles of 4-MUS or p-NCS hydrolyzed per h at 37°C.

Electrophoresis with cellulose acetate (Cellogel, 17 × 17 cm, Chemetron, Italy) was carried out at pH 7.0 according to the

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method of Rattazzi *et al.* (19) and isoelectric focusing was in polyacrylamide gels as described by Chang and Davidson (6). Briefly, 100 or 300 μg of protein from cell extracts in 70 μl containing 10 μl of 80% glycerol and 10 μl of Ampholine (pH 3.5–10) were loaded onto each tube gel (5 × 115 mm). The composition of the separating gels was as follows. For six gels, mix 3.75 ml of 20% (w/v) acrylamide (10 g of acrylamide and 0.4 g of bisacrylamide in 50 ml of distilled water); 1.875 ml of 80% (v/v) glycerol in distilled water; 0.75 ml Ampholines (pH 3–10:4–6, 1:4); 1.116 ml of distilled water. Deaerate for 2 min and then add 9 μl of *N,N,N′,N′*-tetramethylethylenediamine and 7.50 ml of fresh deaerated ammonium persulfate (0.5 mg/ml of distilled water). The cathode buffer was 0.04 M NaOH and the anode buffer was 0.01 M H_3PO_4 (prepared by 0.68 ml of 85% H_3PO_4 in 1 liter of distilled water). Each gel was focused under refrigeration at 2–4°C with 0.5 mA for 0.5 h, then 1 mA until the voltage reached 1000 V, and then continued at constant voltage for a total of 4.5 h. The gels were stained for arylsulfatase activity with 4-MUS and photographed without further enhancement with alkaline buffer.

RESULTS

Twenty cultured cell strains obtained from individuals who were normal, or diagnosed to have PD or MLD, were coded so that all the analyses were performed without knowledge of the identity of the samples.

ARSA activity was assayed in the soluble extracts. Each group of assays was performed with controls from normal, known PD, and known MLD cell strains (Table 1). The specific ARSA activity of these 20 cell strains fell into two groups. One showed the normal range of activity (group A, Table 1), 167 ± 74 units/mg protein when assayed with 4-MUS and about 935 units/mg protein with *p*-NCS. Strain 624 was not as clearly defined: its ARSA activity was slightly higher than the values in the deficient range, yet it had the lowest activity of the normal group, *i.e.* 105 units/mg protein (4-MUS) and 260 units/mg protein (*p*-NCS). The second group showed deficient activity, ranging from 24–28% of normal when assayed with 4-MUS or 5–10% of normal with *p*-NCS. The results were compatible with the diagnosis of MLD (groups B and C, Table 1). However, the ARSA activity of three cell strains in this deficiency group (619, 630, 577) was somewhat higher than the usual MLD range (41 ± 21 units/mg) when assayed with the 4-MUS substrate, *i.e.* 81.0, 87.0, and 74.0 units/mg protein, respectively. When assayed with *p*-NCS as the substrate, they were within the usual deficient range of 5–10% of normal.

Table 1. *Summary of quantitative assays and qualitative electrophoresis*

Cell strain	ARSA* 4-MUS	ARSA* *p*-NCS	Cello-gel†	Isoelectric focusing†	Diagnosis
Normal control	167 ± 74	935	+++	+++	
PD control	47.0 ± 28.0	96	+	+	
MLD control	41.0 ± 21.0	38	−	−	
Group A					
579	176 ± 67	612	++	++	N
623	150 ± 23	660	+++	+++	N
624	105 ± 1	260	++,+	+	N
631	158 ± 11	ND	+++	+++	N
632	181 ± 1	578	+++	+++	N
633	277 ± 15	1180	+++	+++	N
Group B					
619	81.0 ± 25.0	76.3	+	+	PD
630	87.0 ± 36.0	84.4	+	+	PD
3475	44.0 ± 37.0	70.2	+	+	PD
3519	13.0 ± 2.0	101	+	+	PD
4087	19.0 ± 3.0	66.0	+	+	PD
4835	33.0 ± 17.0	86.3	+	+	PD
Group C					
576	39.0 ± 32.0	32.5	−	−	MLD
577	74.0 ± 8.0	45.7	−	−	MLD
1353	23.0 ± 13.0	29.0	−	−	MLD
3072	27.0	33.9	−	−	MLD
3149	37.0 ± 2.0	38.1	−	−	MLD
3585	20.0 ± 7.0	ND	−	−	MLD
4839	48.0 ± 22.0	40.7	−	−	MLD
5171	20.0 ± 4.0	31.9	−	−	MLD

* Specific activity is measured as units/mg protein. With 4-MUS, each value is averaged from two (±range) to six (± SD) separate assays. Each assay was performed in duplicate. With *p*-NCS, each value is the average from duplicate determinations. The range of variation is about ±10%.

† −, +, ++, and +++: not detectable, faint, medium, and strong, respectively.

The 20 cell strains again were examined for ARSA activity with Cellogel electrophoresis. The normal group (A), as expected, showed prominent ARSA activity bands migrating towards the anode (Fig. 1, *lane N*). When the same amount of protein was loaded on each lane (15 μg), the ARSA-deficient groups (B and C) showed two types of results. The first (group B) showed faint bands of ARSA activity with electrophoretic mobility similar to the normal ARSA but with much lower activity (*lanes P, 1, 2* in Fig. 1). The second type (group C) did not show any fluorescent activity bands in this region corresponding to ARSA (*lanes M* and *3* in Fig. 1), which was the classical pattern for MLD. The arylsulfatase B activity bands at the cathodic end appeared normal in all cell strains studied. The results of Cellogel electrophoresis on all cell strains were summarized from two experiments in Table 1. When the two runs gave discordant results, both were indicated (see 624, Table 1).

The same cell strains were further examined with isoelectric focusing at pH 4–6 in polyacrylamide gels (Fig. 2). A pattern similar to that in Cellogel electrophoresis was observed. In group A (normal ARSA specific activity and intensity on Cellogel electrophoresis), loading each gel with 100 μg of protein allowed the ARSA activity to resolve into at least six bands. These were of varying intensity, focusing between pH 4 and 6. If the same amount of protein (100 μg) from the remaining cell strains (groups B and C) was loaded, no distinction could be observed among them and none showed any remarkable activity, as expected of ARSA-deficient cell strains.

When these ARSA-deficient cell strains were examined after overloading with 300 μg of protein instead of 100 μg, two patterns emerged. The first type (*lanes P, 1,* and *2*, Fig. 2), showed discernible bands of ARSA activity focusing at a pH range similar to the normal ARSA. The bands, however, were slightly shifted towards the alkaline range, *i.e.* the cathodic end. The number of activity bands in different cell strains varied. In some, only three bands were clearly visible (*lane P* in Fig. 2). In others, five to six bands were observed (*lanes 1* and *2*, Fig. 2). The cell strains that showed ARSA activity bands on overloading coincided with group B with trace activity on Cellogel electrophoresis. The only exception was cell strain 624 from the group A normal subjects. It behaved as the group B cell strains in demonstrating activity only on overloading the gel.

The second type showed no discernible activity bands in this range of the pH gradient even when the gels were overloaded with 300 μg of protein (*lanes M* and *3*, Fig. 2). These cell strains corresponded to group C with deficient ARSA activity by quantitative assays and no activity on Cellogel. The isoelectric focusing results of all cell strains are summarized from two separate experiments in Table 1. They are completely concordant.

DISCUSSION

Based on the above results, fibroblast extracts from normal controls were identified on the basis of normal ARSA specific activity, normal electrophoretic mobility and normal intensity

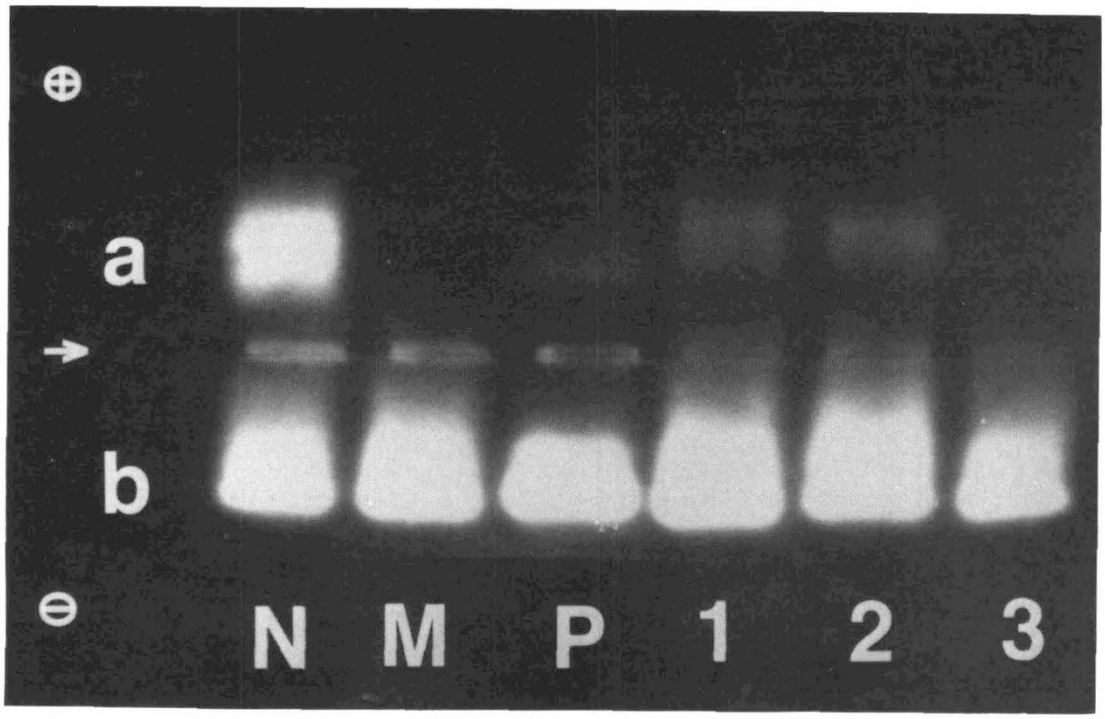

Fig. 1. Electrophoresis of arylsulfatases A and B from normal, PD, and MLD fibroblast extracts on Cellogel. 15 μg of protein from fibroblast extracts of normal, PD, and MLD were applied. After electrophoresis for 3 h at 15 mA, the gel was stained for arylsulfatase activity. *N*, normal control; *M*, MLD control; *P*, PD control; *1*, 4835 (PD); *2*, 3519 (PD); *3*, 5171 (MLD). *a*, arylsulfatase A; *b*, arylsulfatase B; +, anode; −, cathode; →, origin.

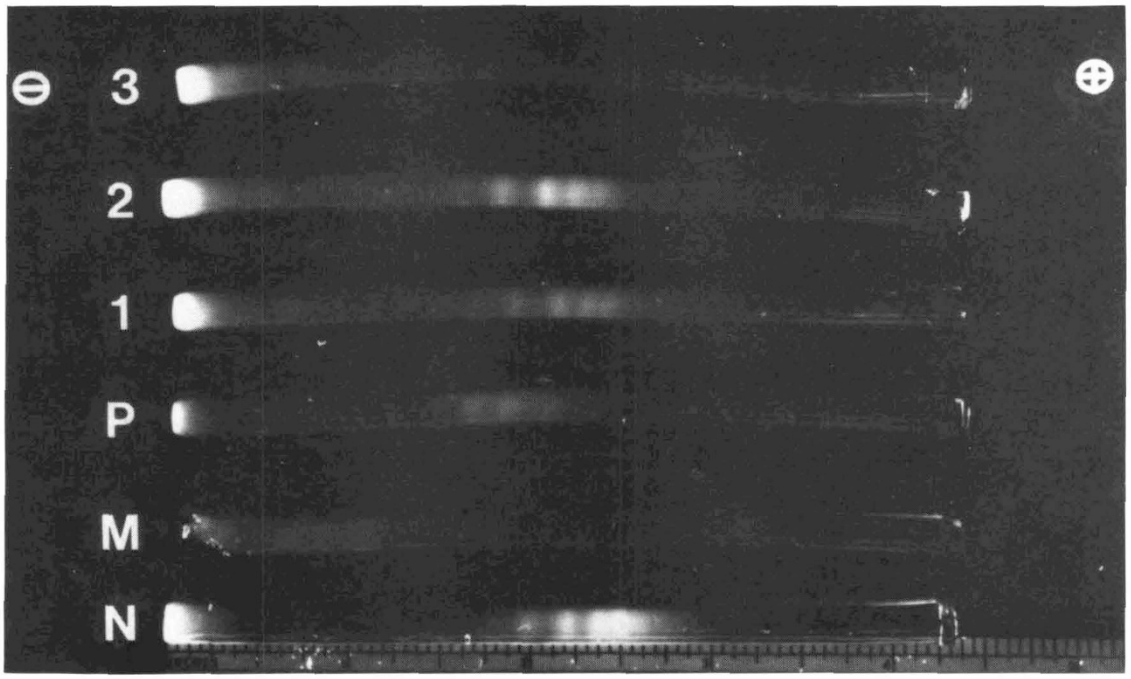

Fig. 2. Isoelectric focusing of arylsulfatase A from normal, PD, and MLD fibroblast extracts. Fibroblast extracts were prepared and subjected to isoelectric focusing as described in "Materials and Methods." The final pH gradient was from about 3.8 (+) to 7.4 (−). *N*, normal control (100 μg protein); *M*, MLD control (300 μg protein); *P*, PD control (300 μg protein); *1*, 4835, PD (300 μg protein); *2*, 3519, PD (300 μg protein); *3*, 5171, MLD (300 μg protein). +, anode; −, cathode.

on both Cellogel and polyacrylamide isoelectric focusing. The diagnosis of PD was based on deficient ARSA specific activity, trace activity discernible on Cellogel electrophoresis, and well defined activity bands by overloading the isoelectric focusing gel with 300 μg of protein instead of 100 μg. The diagnosis of MLD was based on deficient ARSA specific activity, no discernible activity in Cellogel electrophoresis, and no activity in isoelectric focusing even on overloading (Table 1). The only equivocal case

was 624. It may be classified as either normal because of its slightly higher ARSA activity than those of the deficient cell strains or as PD because of its isoelectric focusing behavior. In no case was there any ambiguity between MLD and PD.

Recent reports from Fluharty *et al.* (10) and Bach and Neufeld (2) showed that a band(s) of immunologically cross-reacting material to ARSA was found in PD cultured fibroblasts. Under denaturing conditions, these had the smaller molecular weight of about 56,000 and 59,000, compared to the normal subunit sizes of 59,000 and 63,000 (20). Therefore, the PD condition appears related to a structural mutation in the ARSA subunits.

Under the nondenaturing conditions of isoelectric focusing in polyacrylamide, we observed more heterogeneity in the residual arylsulfatase activity bands among different PD cell strains. Among the normal subjects, ARSA usually focused into at least six bands at pH values of about 5.36, 5.26, 5.11, 4.99, 4.91, and 4.86 (6) although the relative intensities of these bands varied, depending on the cell strain (unpublished observation). In some PD strains, there were only two to three major bands near the cathodic end (*lane P*, Fig. 2) while in others, there may be five to six bands (*lanes 1* and *2* in Fig. 2), all of which seemed to be slightly shifted towards the alkaline range but by only <0.1 pH unit. Thus, it is possible that the structural mutation in PD not only involves a reduction in the size of the subunits (2, 10) but also changes in charge groups. The exact changes may vary in different PD individuals.

Even in the "normal" population (as defined by those not affected with MLD), there seems to be two allelic variant forms of the ARSA subunits (23). In some individuals, only a 59.5-kDa precursor form was synthesized while in others, only a 62-kDa precursor form was synthesized. Individuals who have both forms were considered heterozygotes. Such allelic variations of the normal enzyme subunits may account for the heterogeneous residual ARSA bands observed among the different PD individuals.

The genetic status of the PD condition now appears to be clarified. Both family studies (12, 21, 25) and somatic cell hybrid complementation studies (6) indicate that PD and MLD result from allelic variations, arising from structural mutations of the ARSA locus. However, the possible genotypes of the individuals, as described by Schaap *et al.* (21), cannot be ascertained in the present method because the various permutations of the three alleles, *normal*, *PD*, or *MLD*, can give rise to overlapping values of ARSA activity. The chief benefit with these electrophoretic analyses appears only to distinguish between the clinically affected and nonaffected phenotypes.

Although the exact molecular defect of the PD condition remains to be defined, it is clearly related to changes in charge and size of the ARSA molecule. This condition can be distinguished now from MLD in cultured fibroblasts, with relatively simple techniques and reagents that are readily available commercially. Its application to prenatal diagnosis in cultured amniotic fluid cells still needs to be confirmed.

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