Complementation of arylsulfatase A in somatic hybrids of metachromatic leukodystrophy and multiple sulfatase deficiency disorder fibroblasts

(Publication characteristic polymorphism were Hybrids cells double-nucleated gravity mixture (9). Studies. Enzyme complemented heterokaryons the tation of clinical manifestations. Zyme Many inherited deficiencies. Restored sulfatase deficiency ARSA; aryl-sulfate drolase, ARSA and multi sulfatases are and other sulfatases also reported to be reduced. Somatic hybrid cell clones produced by fusing cultured fibroblasts from patients with these diseases were isolated by a nonselective technique based on unit-gravity sedimentation. Arylsulfatase A activity was restored in these hybrids. The complemented enzyme resembled the normal arylsulfatase A in heat stability, pH optimum, K_m, electrophoretic mobility, and immunologic reactivity. Because a structurally normal enzyme can be restored in a hybrid only through intergeneric complementation, these results indicate that the mutations responsible for the deficiency of arylsulfatase A activity in metachromatic leukodystrophy and multiple sulfatase deficiency disorder are nonallelic and that at least two genetic loci control the expression of arylsulfatase A activity in the human genome. Furthermore, arylsulfatase C activity was also restored to normal in the hybrids, indicating that a common sulfatase inhibitor is not the cause of the multiple sulfatase deficiency.

Many inherited metabolic diseases are caused by specific enzyme deficiencies. However, among diseases characterized by the same enzyme deficiency, many variants exist with diverse clinical manifestations. Frequently, heterokaryons formed by fusing cultured cells from these variants showed complementation of the previously deficient enzyme activity, indicating that the genetic defects in these variants were not identical although the biochemical manifestations were apparently the same. Complementation groups have been found among the variants of GM_1 gangliosidosis (1), the mucopolysaccharidoses (2), xeroderma pigmentosum (3), methylmalonic acidemia (4), galactosemia (5), and the GM_2 gangliosidoses, Tay–Sachs and Sandhoff diseases (6–8). The lack of proper selection medium, however, precluded the isolation of somatic cell hybrids from these heterokaryons for more precise identification of the complemented enzyme and further biochemical and genetic studies.

A nonselective method to circumvent the above limitation in isolating euploid human somatic hybrid clones was recently developed (9). It depended on separating a fusogen-treated mixture of two fibroblast strains according to size by unit-gravity sedimentation. The fractions highly enriched with double-nucleated cells were cloned to isolate the hybrid cells. Hybrids were identified by karyotyping and by an enzyme polymorphism characteristic of the two parent cell strains. This technique was applied to study the genetic defects of two inborn errors of metabolism, metachromatic leukodystrophy (MLD) and its O-variant, multiple sulfatase deficiency disorder (MSDD).

Hereditary metabolic diseases have been useful in providing answers to important basic genetic questions. MLD and MSDD are severe neurodegenerative diseases both characterized by a deficiency of arylsulfatase A (ARSA; aryl-sulfate drolase, EC 3.1.6.1) activity although they are inherited as separate autosomal recessive traits. They have similar clinical symptoms but can be differentiated by subtle differences, such as the presence of Alder–Reilly granules in leukocytes (10) and the accumulation of glycosaminoglycans in addition to cerbrosides in patients with MSDD (11). In MLD, only ARSA activity is affected, but in MSDD, deficiencies of other sulfatases occur—i.e., arylsulfatase B (ARSB), arylsulfatase C (ARSC), and steroid sulfatases (11–14). This raised a number of questions. What is the nature of the genetic defects involved such that two distinct diseases are deficient in the same enzyme activity? Are they due to allelic or nonallelic mutations? Furthermore, how does a single genetic defect in MSDD diminish the biochemical activity of so many apparently unrelated enzymes? Answers to these questions were sought by looking for complementation of ARSA activity in hybrid clones formed between cultured skin fibroblasts from MLD and MSDD patients followed by further definition of the nature of such complementation biochemically and immunologically.

MATERIALS AND METHODS

Cell Culture. Cultured skin fibroblasts from a patient with the juvenile form of MLD were supplied by J. A. Lowden (Hospital for Sick Children, Toronto, Ontario); those from a patient with MSDD were supplied by H. Kihara (University of California, Los Angeles, CA). Skin fibroblasts for controls were obtained from patients with normal karyotypes and no known metabolic disease. Fibroblasts were maintained in Ham’s F-10 culture medium supplemented with L-glutamine (2 mM) and 10–20% (vol/vol) fetal calf serum under 5% CO_2 in air and 100% humidity at 37^°C. Penicillin (100 units/ml) and streptomycin (100 μg/ml) were included in the culture medium during experiments.

Nonselective Isolation and Identification of Hybrids. Hybrid somatic cell clones were produced by fusion of fibroblasts with polyethylene glycol 1000 (15) and isolation by unit-gravity sedimentation as described by Chang et al. (9).

Karyotyping was performed in situ on cells grown on cov-

Abbreviations: MLD, metachromatic leukodystrophy; MSDD, multiple sulfatase deficiency disorder; PGM, phosphoglucomutase; ARSA, arylsulfatase A; ARSB, arylsulfatase B; ARSC, arylsulfatase C.
erslips (2 cm²) by treating the cells with colchicine (5 μg/ml of complete medium) for 4 hr at 37°C, incubating them in 75 mM KCl for 0.5 hr at 37°C, fixing them with methanol/acetic acid, 3:1 (vol/vol), and staining them with Giemsa.

Clones were judged to be diploid if three or more mitotic figures on the coverslip showed a diploid modal number of chromosomes. They were judged to be tetraploid if 10 mitotic figures (or all the mitotic figures on the coverslip if the total was fewer than 10) showed a tetraploid modal number of chromosomes.

Cells were harvested by rinsing once with isotonic saline, followed by trypsinization. Cells were washed twice with isotonic saline and the pellet was kept at −70°C until use. Fibroblast extract was prepared by freeze-thawing in 50 mM acetate buffer (pH 5.0) or lysis buffer (16) (10 μl/cell pellet from a 60-mm culture dish) five times on dry ice and centrifuging at 12,000 × g for 2 min at room temperature. The supernatant was saved for ARSA, ARSB, and phosphoglucomutase (PGM) analyses and protein determinations. The pellet was used for ARSC and protein determinations.

Enzyme Assays. PGM polymorphism was identified by a procedure modified from that of Harris and Hopkinson (17) and Khan (18) by use of Cellogel instead of starch gel for electrophoresis. Samples (1–5 μl) containing 20 μg of protein were placed on Cellogel (17 × 17 cm, Kalex) and electrophoresed in 10 mM phosphate/citrate buffer at pH 7.0 with a constant current of 7 mA for 4 hr at room temperature. After the run, the Cellogel was dipped in the substrate solution, porous side down, for 1 min and incubated at 37°C for 3–5 min in the dark. The reaction was stopped immediately at the appearance of the first intense blue band by dipping the gel in 37% (vol/vol) formaldehyde.

ARS and ARSB were identified by Cellogel electrophoresis by the method of Rattazzi et al. (19). Quantitative assays were performed with 4-methylumbelliferyl sulfate (Koch Light, Colnbrook, England) as the substrate, based on the principle that Ag⁺ selectively inhibits ARSA (20). The total activity of ARSA and ARSB was assayed as follows: an appropriate amount of fibroblast extract was made up in a total of 80 μl with 50 mM acetate buffer (pH 5.6). A freshly prepared 30 mM lead acetate solution (20 μl) was added just before the incubation. The reaction was started by addition of 100 μl of a substrate solution containing 10 mM 4-methylumbelliferyl sulfate in 50 mM acetate buffer (pH 5.6) and the mixture was incubated at 37°C for 15 min. The reaction was terminated by adding 1 ml of 0.2 M carbonate/glycine buffer, pH 10.4, containing 1 mM EDTA. The activity of ARSB was assayed as above except for the inclusion of 0.3 mM AgNO₃ in the assay mixture. The difference between the two determinations was the activity of ARSA. ARSC activity was assayed in the pellet by the method of Eto et al. (13) after it was washed once with 50 mM Tris-HCl (pH 8.4).

Heat Stability and Enzyme Kinetic Studies. Fibroblast extracts were diluted with dialyzed bovine serum albumin (1 mg/ml of 50 mM acetate buffer, pH 5.0), then dialyzed first against 1000 vol of 25 mM acetate buffer (pH 5.0) overnight, followed by 1000 vol of deionized distilled water for 1–2 hr before use. Enzyme preparations were inactivated at various temperatures for 10 min each. The residual activities were transformed into logarithmic functions by the method of Feinstein et al. (21). A straight line was fitted to these transformed functions by linear regression from which the temperature at which 50% of the activity remained (T₅₀) was calculated. For estimations of Kₘ, incubations were for 5 instead of 15 min.

**Immunoelectrophoresis.** Crude fibroblast extracts were electrophoresed in agar plates with a barbitone buffer according to standard procedure. An IgG fraction isolated from rabbit antiserum against a partially purified ARSA preparation from human liver was used to react against the electrophoresed fibroblast extracts. The agar plates were then washed extensively with isotonic saline. Although no precipitin bands were visible, ARSA activity was not inhibited by the presence of the antibody and could be detected in normal fibroblasts when developed with p-nitrocatechol sulfate as the substrate by the method of Manowitz et al. (22).

Protein was determined by the method of Lowry et al. (23) with bovine serum albumin as the standard.

All chemicals were of analytical or reagent grade. Tissue culture reagents were from GIBCO. Polyethylene glycol 1000 for fusing cells was from BDH Chemicals, Toronto, Canada. The antiserum against ARSA was kindly provided by H. L. Nadler (Children's Memorial Hospital, Chicago, IL).

**RESULTS**

Isolation of hybrid clones

After cultured skin fibroblasts from MLD and MSDD patients were fused and separated by unit-gravity sedimentation, 123 clones were successfully identified by karyotyping. Of these, 86 were diploid, 12 were mixed diploid and tetraploid, and 25 were pure tetraploid clones.

The origin of the tetraploid clones was established by the PGM électrophoretic mobility on Cellogel. The MLD fibroblasts had PGM₁₁ and the MSDD fibroblasts had PGM₁₂. Five of the tetraploid clones were found to have the PGM₁₁ pattern (homologous MLD fusion), 17 had the PGM₁₂ pattern (homologous MSDD fusion), and 3 had the hybrid PGM₁₂-1 pattern expected from a fusion between MLD and MSDD cells. All PGM₁ patterns in the tetraploids were confirmed again after 6–11 passages in culture.

**Screening for ARSA activity in tetraploid clones**

The soluble arylsulfatase activities (ARS and ARSB) of these tetraploid clones were monitored by Cellogel electrophoresis and by enzymatic assays on fibroblast extracts with 4-methylumbelliferyl sulfate as substrate. ARSA migrated anomalously during Cellogel electrophoresis and appeared as a bright fluorescent band when stained with 4-methylumbelliferyl sulfate. This band was not seen in the MLD or MSDD diploid fibroblasts or in the tetraploid cells formed from homologous fusions. In the three hybrid tetraploid clones, however, a distinct band of activity corresponding to that of ARSA from a normal fibroblast strain appeared (Fig. 1).

The quantitative assays of ARSA, ARSB, and ARSC activities of normal fibroblasts, the diploid MLD and MSDD parent cell strains, and their tetraploid clones that were vigorous enough to propagate in culture are shown in Table 1. MLD cells had 15% of normal ARSA activity, whereas ARSB and ARSC were within normal range. In the single tetraploid clone from homologous fusion of MLD cells, the same enzyme activity pattern was maintained as in the parent diploid cells. In the MSDD diploid cells, only ARSB was within the normal range; ARSA and ARSC were about 25% of normal. In the five tetraploid clones from homologous fusion of MSDD cells, all three arylsulfatases showed increased activities. ARSA was about 50% of normal. ARSC reached normal levels, and ARSB attained almost 300% of the normal value. In the hybrid clone from the heterologous fusion of MLD and MSDD cells, the activity of ARSA reached normal levels; the activities of ARSB and ARSC were elevated similar to the MSDD self-fusion tetraploid. These
quantitative results were confirmed in a separate experiment (data not shown).

**Characterization of restored ARSA in hybrid clones**

**Heat Stability.** The percentage of ARSA activity remaining after heating at 50, 55, 60, 65, and 70°C for 10 min each in three different normal fibroblast cell strains and one hybrid clone is shown in Fig. 2. The temperature at which 50% of the activity remained (T50) was 59.0 ± 1.4°C for the three normal cell strains and 60.0°C for the hybrid clone.

**Kinetics. pH optimum.** The relative activity of ARSA in the hybrid clone at different pH values was compared with that of a normal fibroblast strain (Fig. 3). The optimal pH for both hybrid and normal strains was identical. With acetate as the buffering ion, the highest activity observed was at pH 5.6 (range tested, 4.5–5.6). With maleate as the buffering ion, the highest activity was at pH 6.0 (range tested, 5.3–6.5).

**Michaelis constant.** The activity of ARSA at different concentrations (1–10 mM) of the substrate 4-methylumbelliferyl sulfate was plotted for three different normal fibroblast strains and the hybrid clone. From a Lineweaver–Burk plot of these data, the K_m was calculated by linear regression to be 2.90 ± 0.77 mM for the three normal fibroblast strains and 1.53 mM for the hybrid clone. The apparent K_m for the normal cell strains measured in different experiments varied from 1.3 to 8.9 mM. Those of the hybrid clone were from 0.815 to 2.09 mM. Within the same experiment, the K_m of the hybrid ARSA activity was usually about half of the normal value.

**Immunological Identification.** Extracts from a normal fibroblast strain and the hybrid clone were electrophoresed and then treated with a partially purified preparation of antibody

<table>
<thead>
<tr>
<th>Strain*</th>
<th>ARSA†</th>
<th>ARSB†</th>
<th>ARSC†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diploid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal (2)</td>
<td>176</td>
<td>203</td>
<td>29.8</td>
</tr>
<tr>
<td>(121–242)</td>
<td>(171–231)</td>
<td>(15.0–44.6)</td>
<td></td>
</tr>
<tr>
<td>MLD (1)</td>
<td>28.4</td>
<td>187</td>
<td>23.3</td>
</tr>
<tr>
<td>MSDD (1)</td>
<td>47.4</td>
<td>189</td>
<td>4.3</td>
</tr>
<tr>
<td>Tetraploid clones</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MLD × MLD (1)</td>
<td>38.2</td>
<td>236</td>
<td>14.0</td>
</tr>
<tr>
<td>MSDD × MSDD</td>
<td>92.6 ± 38.4 (4)</td>
<td>604 ± 159</td>
<td>22.3 ± 12.5</td>
</tr>
<tr>
<td>(5)</td>
<td>(54.8–146)</td>
<td>(362–908)</td>
<td>(4.9–39.8)</td>
</tr>
<tr>
<td>MLD × MSDD (1)</td>
<td>172</td>
<td>532</td>
<td>48.8</td>
</tr>
</tbody>
</table>

All activities are expressed as nmol of 4-methylumbelliferyl sulfate hydrolyzed per mg of protein per hr at 37°C.

* Number of strains or clones used are in parentheses.
† Values in parentheses are the range obtained from different cell strains. Each value was determined in duplicate or triplicate; the means ± SD from different strains are shown.

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**FIG. 1.** Cellgel electrophoresis of ARSA and ARSB activities of diploid fibroblasts and tetraploid clones from MLD and MSDD fusion. Lane 1, normal; lane 2, MSDD; lanes 3–5, MSDD × MSDD; lane 6, MLD × MSDD. O, origin. Arrows, ARSA activity.

**FIG. 2.** Heat stability of ARSA in normal fibroblast and hybrid MLD × MSDD clone. ○, Δ, Δ, and ■, Normal fibroblasts; ○—●, hybrid clone.
The restored ARSA activity exhibited properties indistinguishable from the normal enzyme when tested for heat stability (Fig. 2), optimal pH (Fig. 3), and $K_m$. Its electrophoretic mobility, as shown by Cellogel electrophoresis (Fig. 1), and immunologic reactivity, as tested by immunoelectrophoresis on agar gel (Fig. 4), were also similar to those of the normal enzyme. In assessing the heat stability of this enzyme, it was important to dialyze the samples thoroughly and to inactivate the enzyme over a range of temperatures in order to compute $T_{50}$, the temperature at which 50% of the activity remained. If the heat stability were tested at only a single temperature, there was too much variation even among different normal cell strains to allow for unequivocal identification. In contrast, the $T_{50}$ was a more consistent parameter (see Fig. 2).

Because heat stability is one of the most sensitive techniques for detection of structural changes among enzymes, the identical $T_{50}$ in the normal and complemented ARSA enzymes strongly suggested that the restored enzyme was structurally normal. This was corroborated by the similarities in kinetic, electrophoretic, and immunologic properties of the normal and the complemented ASA activities. These data showed clearly that the criteria for interacistronic (24, 25) complementation were met in that a structurally normal ARSA was restored in hybrids formed between MSDD and MLD cells.

The detection of complementation between MLD and MSDD cells depended on the successful isolation of the hybrid clones. In unsorted mixtures of fused and unfused cells from previous fusion experiments involving MLD cells and two other MSDD cell strains, we failed to detect significant increases in ARSA activity. Absence of complementation between MLD and MSDD cells was also reported by workers in two other laboratories (14, 26) using similar techniques of cell fusion without isolation of heterokaryons or hybrids. This points out the advantage of isolating pure hybrid clones, especially when a negative result is obtained in an unsorted fusion mixture.

The enzyme deficiency in MLD was shown to be a structural mutation in which an abnormal ARSA molecule was immunologically crossreactive with normal ARSA but enzymatically deficient (27). In MSDD, however, various hypotheses have been put forward to explain the multiple sulfatase deficiencies (11, 13). Whatever the genetic defect is, this report has shown that it must involve a locus distinct from that responsible for MLD.

One hypothesis suggested that in MSDD a structural gene coding for a common subunit of all the deficient sulfatases was defective. If that were the case, all the residual sulfatase activities in MSDD would be due to structurally abnormal enzymes. This was not supported by recent studies which showed that the residual ARSA in MSDD was due to a reduced amount of an otherwise normal enzyme (28) and that its level can be modulated by the pH of the culture medium (29).

Another hypothesis suggested that MSDD was due to the presence of a common inhibitor of this group of sulfatases. This was supported neither by the usual type of mixing experiments (13) nor by our results. We showed that although ARSC was deficient in the MSDD diploid parent cells, it was not at all inhibited in the MLD × MSDD hybrids.

A third hypothesis suggested that MSDD was due to a defect of a regulatory gene that controlled the level of all the sulfatases. In this case, the positive complementation of ARSA activity between MLD and MSDD cells may be interpreted as follows. In MLD, the structural gene coding for ARSA is defective but the regulator gene controlling the synthesis of all the other sulfatases is normal. Therefore, structurally normal ARSB and ARSC continue to be synthesized in normal amounts. In MSDD, the regulatory gene is defective but the structural gene coding for ARSA is normal.

**DISCUSSION**

Cultured skin fibroblasts from patients with MLD or MSDD expressed the biochemical defects of these diseases in part as a deficiency of ARSA activity. By fusing these two types of fibroblasts and isolating the hybrid clones, we have shown that ARSA activity was restored by complementation.

**FIG. 4.** Immunoelectrophoresis of ARSA in normal, MLD, and MSDD diploid fibroblasts and a hybrid MLD × MSDD tetraploid clone. Fibroblast extracts in the wells were electrophoresed and then reacted against anti-ARSA IgG in the trough. After extensive washing, ARSA activity was revealed as Hatchett's brown bands. HYB, hybrid; NOR, normal.
for ARSA is intact. Therefore, structurally normal sulfatases were synthesized but in much reduced amounts. In the hybrids, the normal regulator gene from the MLD genome and the normal structural gene from the MSDD genome complement each other to produce ARSA molecules that are normal in structure and amount. We also noted that even in the tetraploids produced by homologous fusion of MSDD cells, ARSA, ARSB, and ARSC were generally elevated compared to the parent diploid cells. It could be argued that in the tetraploid genome of MSDD the amount of defective, or partially active, regulator gene product is now doubled, hence allowing for the increased levels of these enzymes.

Two criteria were proposed for identifying a regulatory gene mutation (30). First, the mutation must alter the rate of enzyme synthesis or breakdown. Second, the regulatory gene locus must be separate from the structural gene locus. The intergenic complementation between MSDD and MLD has fulfilled at least the second requirement. It remains to be shown whether the first requirement will be met in the case of MSDD.

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