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Genetic and Phenotypic Heterogeneity in Disorders of Peroxisome Biogenesis—A Complementation Study Involving Cell Lines from 19 Patients

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ABSTRACT. Disorders of peroxisomal biogenesis include the Zellweger syndrome, neonatal adrenoleukodystrophy, infantile Refsum syndrome, and hyperpipecolic acidemia. These names were assigned before the recognition of the peroxisomal defect and the distinction between phenotypes is uncertain. Recent studies have identified at least four complementation groups, and indicate the presence of at least that number of distinct genotypes. The purpose of the present study was to examine the relationship between genotype and phenotype. We studied cultured skin fibroblasts from 19 patients in whom deficiency of peroxisomes had been established. Complementation analysis was performed with the criterion of complementation being the restoration of the capacity to synthesize plasmalogens when fibroblasts from two patients were fused. Six complementation groups were identified, and consisted of one 13 member group, one two member group, and four groups comprising single cases. The phenotype of each group was examined with respect to age of survival, clinical manifestations, and biochemical alterations. The 13 member group included patients with all of the four currently designated phenotypic entities, while the most common phenotype (Zellweger syndrome) was distributed among five of the six groups. We conclude that the currently used clinical categories do not represent distinct genotypes. Apparently different genes code for a similar phenotype and one defective gene may lead to variant phenotypes. Definitive classification and understanding of these disorders await definition of the specific biochemical defect in each of the genotypes. (*Pediatr Res* 26: 67–72, 1989)

Abbreviation

ALD, adrenoleukodystrophy

There exist a group of genetically determined disorders in which the principal abnormality is the absence or diminution in the number of morphologically detectable peroxisomes (1, 2). Peroxisomal proteins are synthesized on free polyribosomes, released in the cytosol, and then targeted to preexisting peroxisomes (3). Recent studies involving cultured skin fibroblasts from four patients (4, 5) indicate that a defect in targeting mechanisms represents the primary abnormality in several and perhaps all of these peroxisomal deficiency disorders.

The Zellweger cerebro-hepato-renal syndrome was the first disorder in which an absence or deficiency of peroxisomes was recognized (6). Patients with classical Zellweger syndrome display characteristic dysmorphic features, eye abnormalities, profoundly disordered neuronal migration and demyelination, renal cysts, patellar calcific stippling, and liver disease. Psychomotor development is virtually absent and most infants die in the first few months of life (7). Biochemical studies have shown a "panel" of abnormalities. These include cytosolic localization of catalase; the impaired capacity to degrade very long chain fatty acids, phytanic acid, and pipecolic acid; and defective synthesis of plasmalogens and bile acids (1, 2).

More recently it has been shown that peroxisomal deficiency and the abnormalities cited above also are found in somewhat less severe disease states that may be compatible with survival to the mid-teens or later. Currently such patients are designated as having neonatal adrenoleukodystrophy (8), hyperpipecolic acidemia (9), or infantile Refsum disease (10). These names were assigned originally on the basis of a single biochemical abnormality that was thought to be the sole biochemical manifestation. These concepts had to be revised totally when it was recognized that all of these disorders have the full panel of biochemical abnormalities. The relationship between the Zellweger syndrome and the milder disorders is unclear. Possibly most or all of the differences represent a gradation of severity, with Zellweger syndrome the most severe; infantile Refsum the least severe, and neonatal ALD intermediate (11, 12). For reasons discussed elsewhere, hyperpipecolic acidemia is no longer viewed as a separate phenotype, and depending upon severity is assigned to the Zellweger or neonatal ALD category (12).

The challenge to the understanding and classification of the disorders of peroxisome biogenesis has been intensified by recent complementation studies conducted by Brul *et al.* (13) and in our laboratory (14). In complementation analysis, somatic cells derived from patients with the apparent same metabolic defects, but clinically different phenotypes, are induced to fuse. The resulting multinuclear cells are examined for their ability to carry out normal metabolic functions (15). Restoration of activity can

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only occur if each parental cell provides the gene defective in the other. These studies have identified at least five distinct complementation groups and this finding has led to the conclusion that this group of disorders comprises at least an equal number of distinct genotypes.

During the last 5 y our laboratory has studied samples from 261 patients with disorders of peroxisomal biogenesis. The purpose of our study is to examine the relationship between phenotype and genotype. We have performed complementation studies in cultured skin fibroblasts from 19 thoroughly documented patients who represent the broad range of phenotypic variation.

MATERIALS AND METHODS

Patient selection. In all patients included in this study 95% or more of catalase activity in cultured skin fibroblasts was recovered in the cytosolic fraction (5), compared with less than 5% in control cell lines. Experience in our laboratory (Watkins PA, unpublished observation) indicates that cytosolic localization of catalase in cultured skin fibroblasts is associated with a lack or diminution of demonstrable peroxisomes in the liver. Electron microscopic studies of liver biopsy or autopsy specimens (16) were available in only five patients. The previously cited panel of biochemical abnormalities were present in all instances in which these tests were performed.

Table 1 lists the major clinical findings of the patients included in the study, as well as the diagnostic label assigned in accordance with current criteria. Patients who had the characteristic dysmorphic features (7), chondrodysplasia punctata and renal cysts were assigned to the classical Zellweger syndrome category. The history and biochemical findings of one of these cases have been reported (17). The diagnosis of neonatal ALD was applied to patients who had less striking or no dysmorphic features, and who lacked chondrodysplasia punctata or demonstrable renal cysts. Neonatal ALD cases N1, N2, N3, N8, respectively, are cases 7, 1, 5, and 6 in Reference 8 and N7 is the case reported in Reference 18. The patient with hyperpipecolic acidemia has been reported by Thomas *et al.* (19). Case N5 had presented as Leber congenital amaurosis and was subsequently assigned to the neonatal ALD category by Ek *et al.* (20). We diagnosed infantile Refsum syndrome in two more mildly involved patients who had achieved psychomotor milestones even though all were moderately or severely retarded. One of these patients (I2, Table 1) who was described previously (Ref. 21, case 1) also had retinitis pigmentosa, hearing deficits, and elevated levels of phytanic acid in plasma. The sample selected for this study is representative of the 261-member cohort of patients with disorders of peroxisomal biogenesis tested in our laboratory, except that we included a larger proportion of more mildly involved patients since we wished to encompass the full phenotype range.

Materials. Cell culture reagents were from GIBCO Laboratories (Grand Island, NY). [1-^{14}C]Hexadecanol (11.4 mCi/mmol) was obtained from Amersham Corp. (Arlington Heights, IL), and [9′,10′-^{3}H]-sn-hexadecylglycerol (5.5 mCi/mmol) was prepared by chemical synthesis (22). Ficoll 400 was obtained from Pharmacia Fine Chemicals (Piscataway, NJ) and polyethylene glycol 4000 from Merck (Rahway, NJ). All other reagents were of analytical grade and were obtained from commercial sources.

Biochemical assays of peroxisomal function. Catalase was assayed by the method of Peters *et al.* (23). The subcellular distribution of catalase was measured in digitonin-treated fibroblasts as previously described (17). Protein was determined by the

Table 1. *Clinical findings in patients with disorders of peroxisomal biogenesis [cases are listed by complementation groups (1–6) and by diagnostic label]**

Patients	Z1	Z2	Z3	Z4	N1	N2	N3	N4	N5	N6	I1	I2	H1	Group 1 patients showing feature (%)	N7	Z5	Z6	Z7	Z8	N8
Complementation group	1	1	1	1	1	1	1	1	1	1	1	1	1		2	2	3	4	5	6
Sex	F	F	M	F	M	F	M	M	M	M	F	M	M		F	M	M	F	M	F
Age at death (mo)	5	1	15	9		30	72	24					24		4	1	3	3	1	54
Last known survival (mo)					82				79	54	46	45								
Dysmorphism																				
Large fontanelle	1	1	1	1	1	1	1	X	0	1	1	X	1	91	X	0	0	0	X	0
High forehead	1	X	1	1	1	X	0	1	0	X	X	1	X	86	0	0	0	0	0	X
Broad nasal bridge	1	1	1	1	0	1	1	1	0	1	1	1	X	83	0	1	1	1	1	0
Epicanthus	1	X	1	1	X	1	0	1	0	1	1	X	0	70	0	1	1	1	1	X
Micrognathia	1	X	1	1	0	X	0	0	0	X	X	1	1	56	0	1	1	X	X	X
External ear deformity	1	X	1	0	1	X	1	0	0	X	X	X	0	50	0	1	0	X	X	1
High arched palate	0	1	1	1	0	1	0	0	0	X	0	1	X	45	0	1	1	1	X	0
Shallow orbital ridge	X	X	1	1	X	X	0	0	0	X	X	X	X	40	0	1	X	X	X	X
Webbed neck	X	X	X	1	0	X	X	0	X	X	X	X	X	33	0	0	1	X	X	X
Eye																				
Retinal degeneration	X	X	1	1	1	1	1	X	1	1	1	1	1	100	0	X	0	1	X	1
Optic nerve dysplasia	X	X	X	0	0	1	1	1	1	1	0	0	1	60	1	X	0	0	X	1
Cataract	X	X	X	0	0	0	1	X	0	X	0	0	X	14	0	X	1	1	0	0
Corneal clouding	0	1	0	0	0	0	X	X	0	X	0	0	X	11	0	1	0	1	1	X
Nystagmus	1	X	0	X	1	1	1	X	X	1	1	X	1	88	1	X	1	1	X	1
Hepatomegaly	1	1	1	1	1	0	0	1	1	1	1	1	1	85	0	1	1	1	0	1
Loss of skills	0	X	1	X	1	1	1	1	1	0	1	X	1	80	0	X	X	X	X	1
Convulsions	1	X	1	1	1	1	1	1	0	0	0	1	X	73	1	1	1	1	1	1
Kidney cysts	1	1	X	X	X	X	X	0	X	X	X	X	X	67	X	X	1	X	1	X
Cryptorchism	0	1	X	X	X	1	X	X	0	1	X	X	X	60	1	X	X	X	X	0
Polymicrogyria	X	X	1	X	0	1	0	X	1	X	X	X	X	60	X	1	1	X	1	X
Calcification	1	X	1	1	0	X	0	X	0	0	0	X	X	38	0	1	1	1	X	X

* 0, absent; 1, present; X, unknown; Z, Zellweger syndrome; N, neonatal ALD; I, infantile Refsum disease; H, hyperpipecolic acidemia.

method of Lowry *et al.* (24) or by the method of Bradford (25). Concentrations of very long chain fatty acids in fibroblasts and plasma were measured by gas-liquid chromatography as previously described (26, 27). The steps of plasmalogen biosynthesis in peroxisomes were assessed by the method of Roscher *et al.* (28). This method uses a double-label, double-substrate incubation. The peroxisomal component of plasmalogen biosynthesis is measured by the incorporation of [1-^{14}C]hexadecanol into plasmalogens and compared with the microsomal component, assessed by the rate of incorporation of [9′,10′-^{3}H]hexadecylglycerol into plasmalogens.

Cell fusion. Cells were fused in the presence of polyethylene glycol (29). Two parental cell lines (2×10^6 cells each) were seeded in 75-cm^2 flasks in a 1:1 ratio. The next day the confluent monolayer was washed once with Hanks' balanced salt solution and treated with a solution consisting of 42% polyethylene glycol and 15% dimethylsulfoxide in minimum essential medium (Eagle) supplemented with 10% FCS for 90 s followed by addition of 42% polyethylene glycol in Hanks' balanced salt solution for another 90 s. Then 40 ml of Hanks' solution were added for 2 min, the mixture was removed and the cells were washed three times with Hanks' solution and cultured overnight.

To demonstrate the effects of fusion most effectively, we separated multikaryons from monokaryons. We used a modification of the method of Nelson and Carey (30). Cells were harvested and layered on top of a stepped density gradient at 1 U of gravity (5 ml each of 2.5, 5, 10, 12.5, 15% w/vol Ficoll 400 in minimum essential medium supplemented with 10% FCS). After 3 h, monokaryons (2.5% fraction = fraction I) and multinuclear cells (12.5% fraction = fraction II) were collected. Intermediate fractions consisting of a mixed population of cells were discarded. After three washes with Hanks' balanced salt solution, cells in fraction I and II were recultivated and plasmalogen synthesis was measured as described (28) after a 20-h recovery period. After each fusion aliquots of the two fractions were stained with Giemsa and examined under the microscope. This permitted distinction between "clumped" mononuclear cells and multinucleate fused cells. Fraction I contained > 98% mononuclear cells, serving as coculture-control. In all of the studies reported here at least 80% of the cells in fraction II were multinucleate.

Restoration of plasmalogen synthesis was used as the criterion for complementation. Fusion of combinations of cell lines was performed until 1) no correction could be found and the assignment to the same complementation group could be established or 2) correction with each of the groups could be found and the independence of the genetic defect could be ascertained.

Selection of cell lines for complementation studies. We first tested for complementation between cell lines from patients with widely varied phenotypes. However, as studies progressed we also noted complementation in cell lines from patients with similar phenotypes, and as shown under "Results," we demonstrated the existence of one large and five small complementation groups. Because the availability of samples and time constraints made it impractical to perform assays required to test for complementation among all cell lines, we devised a test strategy that would provide reasonably secure confirmation of these groupings. In accordance with this design, a cell line was assigned to the large group if it were shown not to complement with two other members of this same group, except that lines N1, N2, and I2 were tested against only one line. In addition, each member of the small groups was shown to complement at least one member of each of the other groups.

RESULTS

Plasmalogen synthesis was measured in monokaryotic cells recultivated from fraction I of the gradient (equivalent to coculture but having undergone the same treatment as fused cells) and in cells from fraction II with a fusion efficiency of 80 to 90% (Table 2). Values in Figure 1 are expressed as percent of the mean plasmalogen synthesis in control cells ($n = 28$). Figure 1 and Table 3 display the six complementation groups that have been established. Each complementation assay gives the plasmalogen synthesis capacity before and after fusion. The numbers above the diagonal represent co-cultured cells, those below this line, fused cells. For combinations of cell lines that do not complement (indicated by a dashed line) the plasmalogen synthesis did not exceed values expected for cocultivation. A substantial increase or complete restoration to normal values was taken as evidence of complementation and is indicated by a solid line. Fusion of control or disease cells with themselves did not affect the respective rates of plasmalogen synthesis and fusion of control with disease cells resulted in restoration of normal synthesis. Because we assume that each of the complementation groups represents a distinct genotype, we have correlated the information in Table 1 and Figure 1 to determine the relationships between genotype and phenotype. We have used two approaches to evaluate phenotype. The first approach is to correlate genotype with the diagnostic label assigned by the traditional criteria described above. Inspection of Figure 1 suggests that with this approach there is no discernible correlation between genotype and phenotype. All phenotypes are represented in group 1 whereas the most common and consistent phenotype (classical Zellweger syndrome) also occurs in four of the other groups.

Because the current phenotype-based diagnostic labels are somewhat arbitrary and not universally accepted, we developed a second approach in which we attempted to discriminate between the different complementation groups by focusing on certain key phenotypic features. Most of the clinical manifestations did not differ among the six groups. In respect to dysmorphic features, in group 1 there was a wide range from no dysmorphic features to dysmorphism in every listed criterion. One patient in group 2 lacked dysmorphic features (18), whereas the other met almost all of the criteria for Zellweger syndrome. Retinal degeneration was present in all group 1 patients for whom information was available, although it was absent in one case of group 2 and in the single case of group 3 and present in group 4 and 6 (single cases). Only one of the group 1 patients showed cataracts and one had corneal clouding, but corneal clouding was found in one case of group 2 and in the single patients of groups 4 and 5, and cataracts occurred in the single patients of groups 3 and 4. Kidney cysts, commonly regarded as a classical Zellweger syndrome characteristic, are listed as present in Table 1 only when confirmed by autopsy, and information is limited since most of the milder cases are still alive and pathologic examinations were not performed in all patients who died. Figure 2 shows that patients in groups 2–5 died during the first 4 mo of life, whereas the majority of patients in group 1 and the patient in group 6 survived longer. Plasmalogen synthesis was generally less affected in group 1 (Fig. 3) except in patients Z1, Z2, and Z4. Interestingly, those three patients showed the earliest age of death in this group. Patients N7 (group 2) and N8 (group 6) showed a milder deficiency of plasmalogen synthesis whereas in the remaining patients in groups 2 to 5 it was severely impaired. Levels of very long chain fatty acids in plasma and fibroblasts (Fig. 4) were slightly higher in patient Z5 (group 2) and patients in group 3 and 4 than in those of group 1. There was no correlation between hexacosenoic (C26:1) levels and complementation groups (data not shown). When correlated with age at death, markedly elevated very long chain fatty acid levels and severely impaired plasmalogen synthesis are the strongest indicators of severe illness. Nevertheless, they cannot be used to predict the assignment of an individual case to a complementation group.

DISCUSSION

In this study we have used complementation studies to identify six distinct genotypes among the disorders of peroxisomal biogenesis and have compared genotype with phenotype. The sig-

Table 2. *De novo plasmalogen biosynthesis**

1:1	Unfused cells (mononuclear cells) (3H)	(14C)	Ratio (14C/3H)	Fused cells (multinuclear cells) (3H)	(14C)	Ratio (14C/3H)	Complementation
CO × CO	9.10	19.52	2.14	7.02	15.80	2.25	–
ZS × ZS	11.49	1.21	0.11	8.67	0.98	0.11	–
NALD × NALD	7.95	2.26	0.28	9.04	2.53	0.28	–
ZS × CO	10.55	11.28	1.07	6.43	14.47	2.25	+
NALD × CO	8.89	13.22	1.49	5.58	12.68	2.27	+
ZS × NALD	11.58	5.13	0.44	6.87	14.35	2.09	+

* Values represent radioactivity in plasmalogens as percent of total incorporated into phospholipids. Incorporation of the ^{14}C precursor measures peroxisomal and ^{3}H microsomal steps of plasmalogen synthesis (26). Restoration of 14C/3H to normal occurs in complementing cell lines. CO, control; NALD = neonatal adrenoleukodystrophy; ZS, Zellweger syndrome.

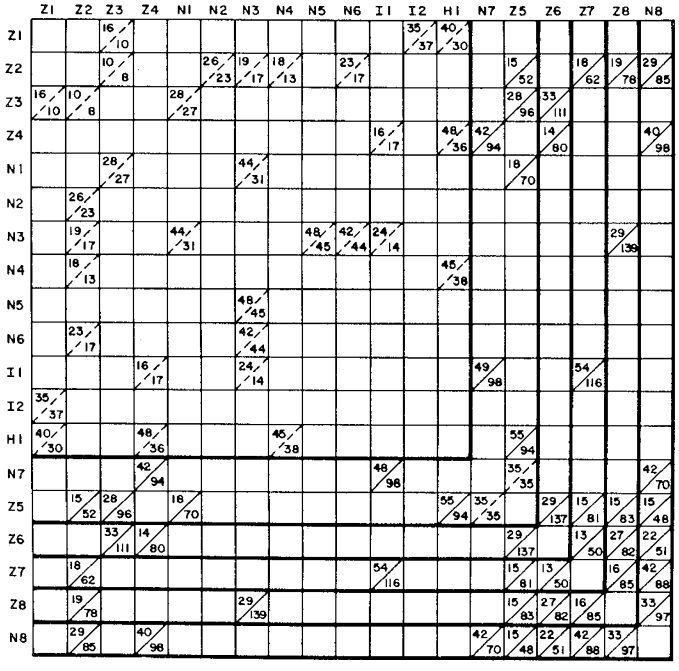

Fig. 1. Complementation analysis in patients with disorders of peroxisomal biogenesis. Values are expressed as percentage of plasmalogen synthesis in control cells (100%, $n = 28$). Numbers above the *diagonal line* represent cocultured cells, those below the line, fused cells. Complementation is indicated by a *solid line*. Six complementation groups were found and are separated by *bold lines*.

Table 3. *Phenotypes in various complementation groups*

Group	*n*	Phenotype
1	13	Zellweger, neonatal ALD, infantile Refsum, hyperpipecolic acidemia
2	2	Zellweger, neonatal ALD
3	1	Zellweger
4	1	Zellweger
5	1	Zellweger
6	1	Neonatal ALD

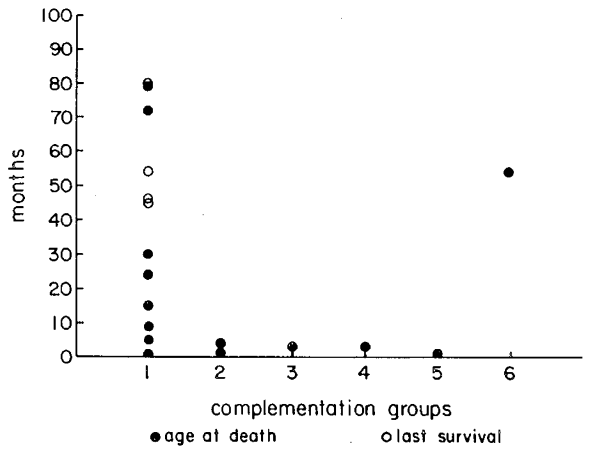

Fig. 2. Age at death or age of last known survival of patients in each complementation group (1–6).

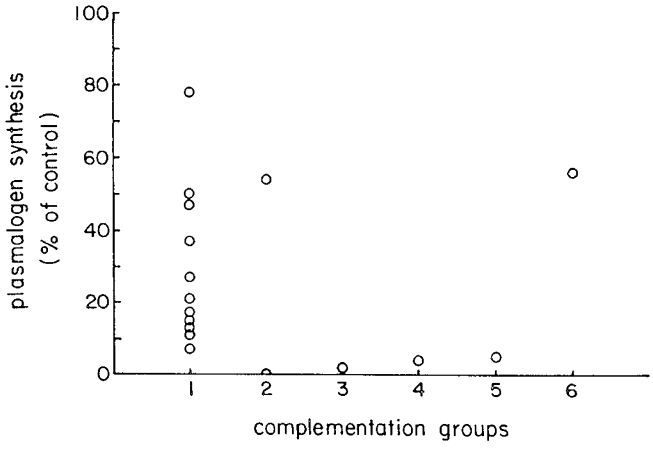

Fig. 3. Plasmalogen synthesis in fibroblasts of patients in each complementation group (1–6).

nificant finding is that there is no discernible correlation: all known phenotypes are represented within the large complementation group, although the most common phenotype is dispersed among five of the six groups. These findings indicate that the historically based nomenclature fails to delineate genotypes. Possibly these phenotypic differences reflect differences in the degree of the impairment of peroxisomal biogenesis, with Zellweger syndrome being the most severe, neonatal ALD intermediate, and infantile Refsum the least severe. Evidence for this point of view was first presented by Arias *et al.* (11). The results reported here, and those of Brul *et al.* (13), indicate the existence of at least six distinct genotypes. Phenotype analysis, at least as currently practiced, does not permit their identification.

The complementation assays reported here were based upon the restoration of the capacity to synthesize plasmalogens. Other criteria of complementation can also be used. Brul *et al.* (13) have demonstrated that subcellular catalase becomes particulate (*i.e.* peroxisomal) in those fused cell lines that increase their level of dihydroxyacetone phosphate acyltransferase, the peroxisomal

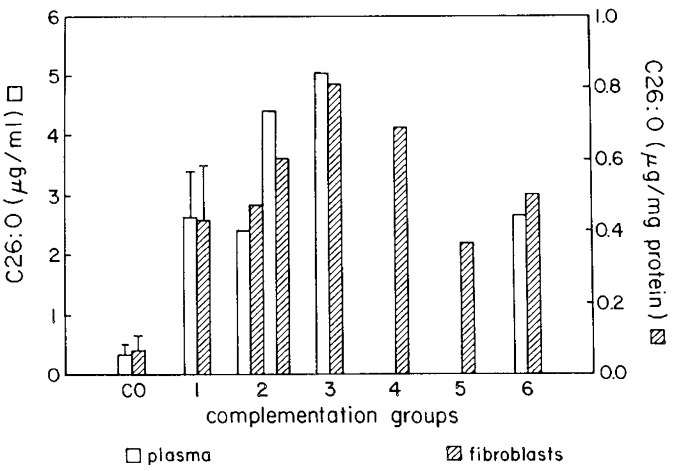

Fig. 4. Level of very long chain fatty acids (C 26:0) in plasma and fibroblasts of patients in each complementation group (1–6). Values for control (*Co*) and group 1 are given ± SD.

enzyme catalyzing the first step of plasmalogen synthesis. Recently, Poll-The *et al.* (31) have demonstrated that complementing cell lines also acquire the capacity to oxidize phytanic acid. The data available so far suggest that these three indices of complementation are congruent and that an unequivocally positive result with any one of them can be taken as evidence that complementation has taken place.

The results presented here are consonant with studies reported previously by Brul *et al.* (13). These authors studied complementation in eight patients and also reached the conclusion that there was phenotypic heterogeneity among the complementation groups. Our groups 1 and 2 appear to be comparable to their groups 2 and 4, respectively.

Our studies suggest that at least six genetically distinct mechanisms can lead to peroxisomal deficiency. Because only a small proportion of known cases have been tested in this way, it is possible, and even likely, that additional groups will be identified. It is important to define the relationship among the groupings represented here with those studied by other investigators. Efforts to accomplish this are in progress.

The identification of complementation groups per se provides the challenge of defining their specific defects. This task will be aided by recent advances in knowledge about the mechanisms that control normal peroxisomal biogenesis. As already noted, most peroxisomal enzymes contain topogenic sequences that target them to this organelle. The topogenic sequence for yeast acyl-CoA oxidase has been identified (32). Miyazawa *et al.* (33) have shown recently that a COOH-terminal ser-lys-leu sequence is essential for the targeting of four rat peroxisomal enzymes. Brul *et al.* (34) have examined the kinetics of complementation and have shown that in some combinations of complementing cell lines complementation is particularly rapid and is unaffected by the addition of cycloheximide, an inhibitor of protein synthesis, whereas in the other three groups in which complementation took place more slowly, this agent abolished complementation. In the study of the mucopolysaccharidoses, the identification of complementation groups provided the impetus and pathway for the definition of the enzyme defects specific for each of these human disease states (35). It is hoped that analogous gains of knowledge will accrue from the complementation study of the disorders of peroxisomal biogenesis.

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