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Novel Subtype of Peroxisomal Acyl-CoA Oxidase Deficiency and Bifunctional Enzyme Deficiency with Detectable Enzyme Protein: Identification by Means of Complementation Analysis

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Summary

We describe four infants with a novel subtype of an isolated deficiency of one of the peroxisomal β-oxidation enzymes with detectable enzyme protein. The patients showed characteristic clinical and biochemical abnormalities, including hypotonia, psychomotor retardation, hepatomegaly, typical facial appearance, accumulation of very-long-chain fatty acids, and decreased lignoceric acid oxidation. However, β-oxidation enzyme proteins were detected by immunoblot analyses, and large peroxisomes were identified by immunofluorescence staining. In order to identify the underlying defect in these patients, complementation analysis was introduced using fibroblasts from these patients and patients with an established deficiency of either acyl-CoA oxidase or bifunctional enzyme, as identified by immunoblotting. In the complementing combinations, fused cells showed increased lignoceric acid oxidation, resistance against 1-pyrene dodecanoic acid/UV selection, and normalization of the size and the distribution of peroxisomes. The results indicate that two patients with a more severe clinical course were suffering from bifunctional enzyme deficiency and that the other two infants, who were siblings and had a less severe clinical presentation, were the first patients with acyl-CoA oxidase deficiency with detectable enzyme protein.

Introduction

Fatty-acid β-oxidation in peroxisomes (Lazarow and deDuve 1976) proceeds via a similar mechanism as in mitochondria and involves the sequential action of acyl-CoA oxidase (Osumi and Hashimoto 1978), bifunctional enzyme with enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activity (Furuta et al. 1980), and finally 3-ketoacyl-CoA thiolase (Miyazawa et al. 1980). Recent studies have shown that the peroxisomal bifunctional enzyme also harbors isomerase activity (Palosaari and Hiltunen 1990). Furthermore, peroxisomes seem to contain two additional acyl-CoA oxidases with distinct substrate specificities (Casteels et al. 1988; Van Veldhoven et al. 1991, 1992; Wanders et al. 1992*a*). An important distinction between the mitochondrial and peroxisomal β-oxidation system involves their substrate specificities. Mitochondria catalyze the β-oxidation of the bulk of fatty acids taken up via our daily diet, whereas peroxisomes are involved in the β-oxidative chain-shortening of a distinct set of substrates including very-long-chain fatty acids (VLCFA) (Singh et al. 1984) and di- and trihydroxycholestanoic acid (Kase et al. 1985).

In recent years, a growing number of patients have been reported with an isolated deficiency of one of the peroxisomal β-oxidation enzymes. In these patients, there is accumulation of VLCFA in the absence or pres-

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Table 1

Biochemical Data

	Patient 1	Patient 2	Patient 3	Patient 4	Control
VLCFA (serum sphingomyelin):					
C24:0/C22:0	1.11	1.52	1.61	1.90	.71 ± .10 (n = 18)
C25:0/C22:0	.06	.09	.06	.11	.016 ± .004 (n = 18)
C26:0/C22:0	.04	.08	.04	.03	.012 ± .005 (n = 18)
Dicarboxylic aciduria	±	+	−	+	
Lignocerate oxidation (pmol/mg protein/h)	25	24	35	27	283 ± 98 (n = 5)
DHAP-AT (nmol/mg protein/h)	.69	1.33	.67	1.19	1.0 ± .4 (n = 6)
Immunoblot analysis:					
Acyl-CoA oxidase	+	+	+	+	
Bifunctional enzyme	+	+	+	+	
3-Ketoacyl-CoA thiolase	+	+	+	+	
Peroxisomes	Large	Large	Large	Large	

ence of elevated di- and trihydroxycholestanoic acid levels. Clinical abnormalities include hypotonia, psychomotor retardation, hepatomegaly, and facial dysmorphism resembling those described in peroxisome-deficient Zellweger syndrome (McKusick 214100) or neonatal adrenoleukodystrophy (McKusick 202370). Most of the reported patients with an isolated deficiency of β-oxidation enzyme were identified by showing the lack of enzyme protein on immunoblot analysis. Peroxisomal thiolase deficiency (Schram et al. 1987), first reported as pseudo-Zellweger syndrome (McKusick 261510) (Goldfischer et al. 1986), and bifunctional enzyme deficiency (Watkins et al. 1989; Wanders et al. 1990*b*, 1992*b*) were identified in infants with severe clinical manifestations resembling Zellweger syndrome, whereas acyl-CoA oxidase deficiency seems to be associated with a less severe phenotype (pseudoneonatal adrenoleukodystrophy [McKusick 264470]) (Poll-The et al. 1988; Wanders et al. 1990*a*).

However, there have been many reports on patients with defective β-oxidation activity and detectable enzyme proteins (Clayton et al. 1988; Naidu et al. 1988; Stephenson 1988; Barth et al. 1990; Mandel et al. 1992). They were considered to have an enzyme protein without catalytic activity. Elucidation of the primary enzyme defect in these patients has proved to be difficult, since measurement of each peroxisomal β-oxidation enzyme activity separately from those of their mitochondrial counterparts is problematic.

In this paper, we have used complementation analysis to identify the primary defect in patients with an impairment of peroxisomal β-oxidation of unknown etiology. This approach has previously been shown to be useful for genetic grouping of peroxisome-deficient disorders (McGuinness et al. 1990; Yajima et al. 1992) and for the diagnosis of bifunctional enzyme deficiency (Wanders et al. 1992*b*). We first identified patients with acyl-CoA oxidase deficiency with detectable enzyme protein.

Patients and Methods

Patients

Patient 1.—This female child of nonconsanguineous parents of Japanese origin was delivered at 40 wk of gestation. She weighed 3,160 g, and mild asphyxia was present. Soon after delivery, profound hypotonia, feeding difficulty, and intractable convulsions occurred. Craniofacial dysmorphism, including large fontanelle, frontal bossing, low nasal bridge and upward-slanting of palpebral fissures, hepatomegaly with normal transaminases and bilirubin, funnel chest, and talipes equinovarus were present. A subdural hematoma, possibly due to vitamin K deficiency, occurred on the 49th day after birth. A cranial computed tomography at 3 mo showed mild colpocephaly. Calcific stippling of the patella was present. Development was poor, and the patient died of pneumonia at 12 mo of age.

Patient 2.—The second female child of consanguineous (second-cousin) Japanese parents was delivered at term and weighed 2,464 g. From the neonatal period, feeble cry and poor activity were present. Scaphocephaly, frontal bossing, micrognathia, high-arched palate, delayed closure of the anterior fontanel, mild hepatomegaly with decreased coagulation factors, and calcific stippling of the shoulder and knee joints were

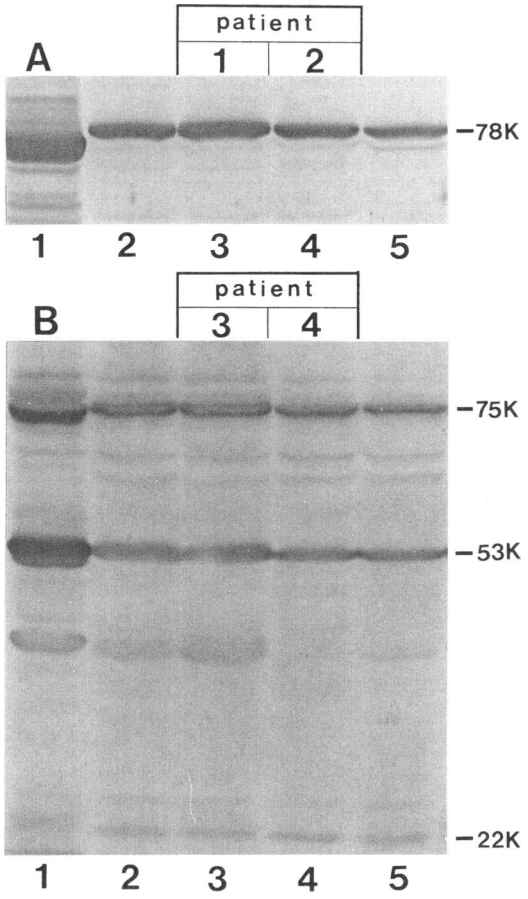

Figure 1 Immunoblot analysis of peroxisomal β-oxidation enzymes. *A*, Bifunctional enzyme in liver tissues. Lane 1, Purified rat bifunctional enzyme (2 ng; slightly smaller than human enzyme). Lanes 2 and 5, Controls (10 μg protein). Lanes 3 and 4, Patients 1 and 2 (10 μg protein). *B*, Acyl-CoA oxidase in fibroblast homogenates. Lane 1, Purified rat acyl-CoA oxidase (2 ng). Lanes 2 and 5, Controls (30 μg protein). Lanes 3 and 4, Patients 3 and 4 (30 μg protein).

also present. Multifocal tonic-clonic convulsions started at age 2 mo, and myoclonus-like movements appeared at 6 mo. Development was maximal at 3 mo of age, when she could smile and follow a moving person. Diarrhea, hyponatremia, and skin pigmentation due to adrenocortical insufficiency were observed from age 11 mo. The patient died of airway obstruction by vomitus at 21 mo of age.

Patient 3.—The first male child of consanguineous (first-cousin) Japanese parents was delivered at 40 wk of gestation and weighed 2,464 g. Profound hypotonia, dysmorphic features including hypertelorism, epicanthus, low nasal bridge, low-set ears, and polydactyly were present from the neonatal period. There was no hepatomegaly or calcific stippling. At age 4 mo there were focal seizures of the left arm. There was head control at 5 mo and crawling at 12 mo. At 32 mo of age the patient could walk without assistance. Since then, his motor ability has regressed. At age 41 mo he could not sit, but he did respond to members of his family by smiling or crying. Fundoscopic examination suggested retinal degeneration. Magnetic resonance imaging revealed symmetrical T1 and T2 elongation of pyramidal tract in the brain stem and cerebellar medulla. He is living at age 7 years.

Patient 4.—This infant is the younger sister of patient 3, and clinical manifestations were similar to those of patient 3. She could control her head at age 6 mo, crawled at 18 mo, and walked with support at 22 mo; then regression followed. She can speak a few words and is living at age 4 years.

Biochemical Analysis

Peroxisomal β-oxidation activity in fibroblasts was assessed by the degradation of [1-^{14}C]lignoceric acid (CEA, Gif-Sur-Yvette, France) (Suzuki et al. 1991). Immunoblot analysis was performed as described elsewhere (Suzuki et al. 1986), by using antibodies raised against peroxisomal enzyme proteins as purified from rat liver (Osumi and Hashimoto 1978; Furuta et al. 1980; Miyazawa et al. 1980). Biosynthesis of β-oxida-

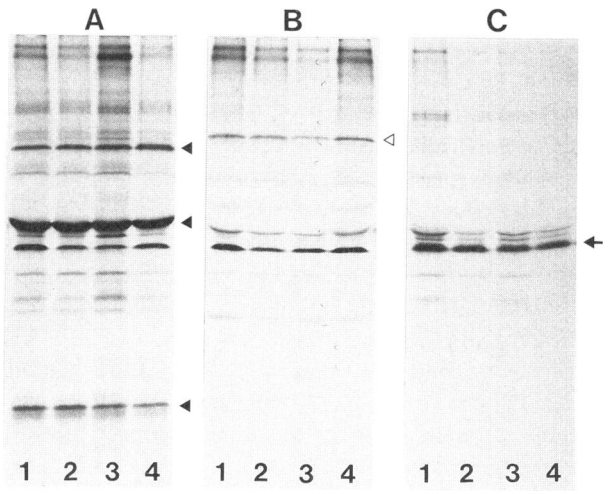

Figure 2 Continuous labeling of peroxisomal β-oxidation enzymes in fibroblasts from patients 1 (lane 1), 2 (lane 2), 3 (lane 3), and 4 (lane 4). *A*, Acyl-CoA oxidase. The blackened arrowheads indicate subunits A (75 kD), B (53 kD), and C (22 kD). *B*, Bifunctional enzyme. The unblackened arrowhead indicates bifunctional enzyme (78 kD). *C*, 3-Ketoacyl-CoA thiolase. The arrow indicates mature subunit (41 kD).

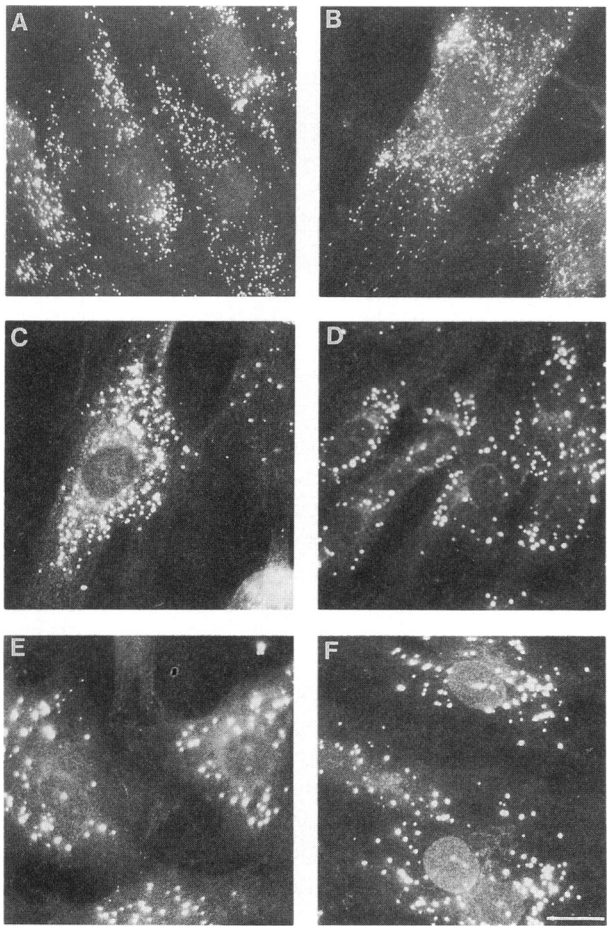

Figure 3 Indirect immunofluorescence light microscopy of fibroblasts. *A*, Control, stained with anti-human catalase IgG. *B*, Control, stained with anti-rat acyl-CoA oxidase IgG. *C*, Patient 1, stained with anti-human catalase IgG. *D*, Patient 2, stained with anti-rat acyl-CoA oxidase IgG. *E*, Patient 3, stained with anti-human catalase IgG. *F*, Patient 4, stained with anti-rat acyl-CoA oxidase IgG. Scale bar = 10 μm.

tion enzymes was evaluated by continuous labeling of the cells for 48 h with [^{35}S]methionine (ICN Biomedicals, Costa Mesa, CA) and immunoprecipitation of peroxisomal proteins from cell lysates (Tsukamoto et al. 1990). 1-Pyrene dodecanoic acid/UV (P12/UV) selection (Zoeller et al. 1988), measurement of dihydroxyacetone phosphate acyltransferase (DHAP-AT) activity (Schutgens et al. 1984), and VLCFA analysis in serum sphingomyelin (Tsuji et al. 1981) were performed as described elsewhere.

Morphological Analysis

Peroxisomes in fibroblasts were detected by indirect immunofluorescence light microscopy (Suzuki et al. 1990), by using rabbit antibodies against human erythrocyte catalase and rat liver acyl-CoA oxidase (Osumi and Hashimoto 1978). Antigen-antibody complex was detected with fluorescein isothiocyanate–conjugated goat anti-rabbit IgG antibody (TAGO, Burlingame, CA).

Complementation Analysis

A mixture of two cell lines (10^6 cells each) was cocultivated for 1 d before fusion in minimal essential medium (MEM) supplemented with 10% FCS. The cells were treated with 41% polyethylene glycol (PEG), 10% dimethyl sulfoxide, MEM, and 25% PEG, MEM for 1 min each, then PEG was gradually diluted, and this was followed by washing of the cells with MEM. The next day, multikaryons were separated from monokaryons essentially according to the method described by Nelson and Carey (1985), with minor modifications. Cells were trypsinized and layered on top of a stepwise density gradient of Ficoll 400 (2.5%, 5.0%, 10.0%, 12.5%, and 15% [w/v]) in MEM supplemented with 10% FCS. After 3 h, cells in the 12.5% fraction were collected, recultivated, and used for the measurement of lignoceric acid oxidation, P12/UV selection, and morphological study of peroxisomes. Fibroblasts from patients with an established deficiency of either acyl-CoA oxidase (Wanders et al. 1990*a*) or bifunctional enzyme (Watkins et al. 1989) were used as reference cell lines.

Results

Biochemical and Morphological Analysis

Results of biochemical analyses in these patients are summarized in table 1. Both accumulation of VLCFA in serum sphingomyelin and decreased activity of [1-^{14}C]lignoceric acid oxidation in fibroblasts were observed in all these patients. However, the activity of DHAP-AT was not deficient. Immunoblot analyses revealed the presence of all peroxisomal β-oxidation enzyme proteins. Figure 1 shows the presence of (1) immunoreactive material of bifunctional enzyme in autopsied liver tissues from patients 1 and 2 and (2) acyl-CoA oxidase in fibroblasts from patients 3 and 4.

Continuous labeling of fibroblasts with [^{35}S]methionine and immunoprecipitation supported the results of the immunoblotting. Three different subunits of acyl-CoA oxidase—subunits A (75 kD), B (53 kD), and C (22 kD)—were clearly detected in all fibroblasts from the patients (fig. 2*A*), as in the fibroblasts from the control (data not shown). Both the 78-kD bifunctional enzyme protein, which was difficult to detect by means of im-

Table 2

Restoration of Lignoceric Acid Oxidation by Cell Fusion

	A. Cocultivation	B. Fused Cells	B/A	Judgment[a]
Patient 1 × bifunctional enzyme deficiency	16[b]	37[b]	2.3	−
Patient 2 × bifunctional enzyme deficiency	26[b]	15[b]	.6	−
Patient 3 × bifunctional enzyme deficiency	35[b]	212[b]	6.0	+
Patient 4 × bifunctional enzyme deficiency	53[b]	344[b]	6.5	+
Patient 1 × acyl-CoA oxidase deficiency	12	141	11.8	+
Patient 2 × acyl-CoA oxidase deficiency	21	106	5.0	+
Patient 3 × acyl-CoA oxidase deficiency	38	52	1.4	−
Patient 4 × acyl-CoA oxidase deficiency	35	51	1.5	−
Patient 1 × Patient 2	16	21	1.3	−
Patient 2 × Patient 3	53	480	9.1	+
Patient 2 × Zellweger syndrome	35	301	8.6	+
Bifunctional enzyme deficiency × acyl-CoA oxidase deficiency	15[b]	164[b]	11.0	+

[a] − = not complemented; and + = complemented.
[b] Expressed as pmol/mg protein/h.

munoblotting using fibroblasts (fig. 2*B*), and the mature size (41 kD) of 3-ketoacyl-CoA thiolase subunit were also evident (fig. 2*C*).

Peroxisomes in fibroblasts from the patients, which were visualized by indirect immunofluorescence light microscopy with the use of anti-human catalase or anti-rat acyl-CoA oxidase, were larger and fewer than those in fibroblasts from the controls (fig. 3). Peroxisomes in fibroblasts from patients 3 and 4 were much larger than those in fibroblasts from patients 1 and 2.

Complementation Analysis

Efficiency of selection of multikaryons from monokaryons was first examined. Cells from each fraction were collected, reseeded, and stained with Giemsa solution. About 90% of the cells in the 12.5% fraction were multikaryons.

In the next series of studies, activities of lignoceric acid oxidation of the fused cells were compared with those of the cocultivating monokaryons (table 2). The activities increased about 5–12-fold either when fibroblasts from patients 1 or 2 were fused with the acyl-CoA oxidase–deficient fibroblasts or when fibroblasts from patients 3 or 4 were fused with the bifunctional enzyme–deficient fibroblasts. The activities also increased in the following pairs: patient 2 × patient 3, acyl-CoA oxidase deficiency × bifunctional enzyme deficiency, and patient 2 × Zellweger syndrome. On the other hand, the activities did not increase in the following combinations: patient 1 or 2 × bifunctional enzyme deficiency, patient 3 or 4 × acyl-CoA oxidase deficiency, and patient 1 × patient 2. These results suggest that the defect in the fibroblasts of patients 1 and 2 is at the level of bifunctional enzyme, whereas acyl-CoA oxidase seems to be deficient in fibroblasts from patients 3 and 4.

The results of P12/UV selection correlated well with those of lignoceric acid oxidation. Fused cells of control fibroblasts were resistant to P12/UV selection (fig. 4*A*). The unfused fibroblasts from the patients (data not shown), and the fused cells of either patient 1 × patient 2 (fig. 4*C*) or patient 3 × patient 4 (fig. 4*D*), which all remained defective in VLCFA oxidation, were sensitive to P12/UV treatment, and few survived after selection. However, the fused cells of either patient 2 × patient 3 (fig. 4*B*) or patient 1 × patient 4 (data not shown) with restored activities of lignoceric acid oxidation were resistant and did survive. This is in agreement with the finding of restoration of lignoceric acid β-oxidation in these combinations.

Morphological changes of peroxisomes after fusion were analyzed by the indirect immunofluorescence staining. In the fused cells of either patient 1 × patient 2 or patient 3 × patient 4, peroxisomes remained large and few, as in the parental cells (fig. 5*A* and *B*). However, in the fused cells of either patient 2 × patient 3 or patient 1 × patient 4, the size and distribution of the peroxisomes were similar to those in the control (fig. 5*C* and *D*, *arrows*).

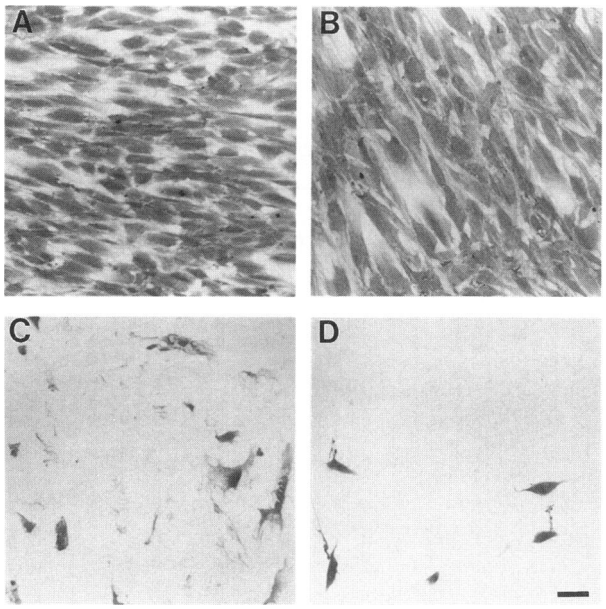

Figure 4 P12/UV selection of multinuclear cells. Fused cells were collected by the stepwise density gradient procedure using Ficoll 400. After P12/UV selection, cells were reseeded and stained with Giemsa solution. *A*, Control × control. *B*, Patient 2 × patient 3. *C*, Patient 1 × patient 2. *D*, Patient 3 × patient 4. Scale bar = 100 μm.

Discussion

Our patients had clinical and biochemical characteristics described for peroxisomal disorders, including hypotonia, psychomotor retardation, typical face, accumulation of VLCFA, and decreased VLCFA oxidation. Findings including normal DHAP-AT activity, large peroxisomes as identified by immunofluorescence staining, and detectable enzyme proteins of peroxisomal β-oxidation suggested a deficient activity of one of the peroxisomal β-oxidation enzyme proteins.

Measurement of each enzyme activity of peroxisomal β-oxidation is difficult, for the following reasons: (1) The activities of the corresponding mitochondrial β-oxidation enzymes are much higher than the activity of the peroxisomal enzymes. (2) The elimination of mitochondrial enzymes is not an easy task even if either immunotitration of mitochondrial enzymes (Suzuki et al. 1986) or subcellular fractionation of peroxisomes (Wanders et al. 1990*b*) is used. (3) The activity of each peroxisomal enzyme in fibroblasts is below the limit of detection, when conventional methods are used.

On the other hand, each peroxisomal enzyme protein is detectable by means of immunoblotting using specific antibodies (Osumi and Hashimoto 1978; Furuta et al. 1980; Miyazawa et al. 1980). Thus, most patients heretofore reported were diagnosed on the basis of the lack of the enzyme protein (Schram et al. 1987; Poll-The et al. 1988; Watkins et al. 1989; Wanders et al. 1990*a*). However, there are an increasing number of reports on patients who had peroxisomal β-oxidation enzyme protein but in whom a deficiency of one of these enzymes was suspected because of both the accumulation of VLCFA and decreased VLCFA oxidation (Clayton et al. 1988; Naidu et al. 1988; Stephenson 1988; Barth et al. 1990).

In attempts to resolve this problem, we used complementation analysis, which was earlier found to be useful for the elucidation of the genetic defects among peroxisome-deficient disorders (McGuinness et al. 1990; Yajima et al. 1992). Wanders et al. (1992*b*) first introduced this approach to diagnose a patient with bifunctional enzyme deficiency with detectable enzyme protein. Primary etiology of cell lines used for cell fusion is considered to be the same when a metabolic abnormality is not restored. If an abnormality is restored, responsible genes of cell lines used are thought to be different. In our study, restoration of VLCFA oxidation was not observed in the following pairs: patient 1 or 2 × bifunctional enzyme deficiency and patient 3 or 4 × acyl-CoA

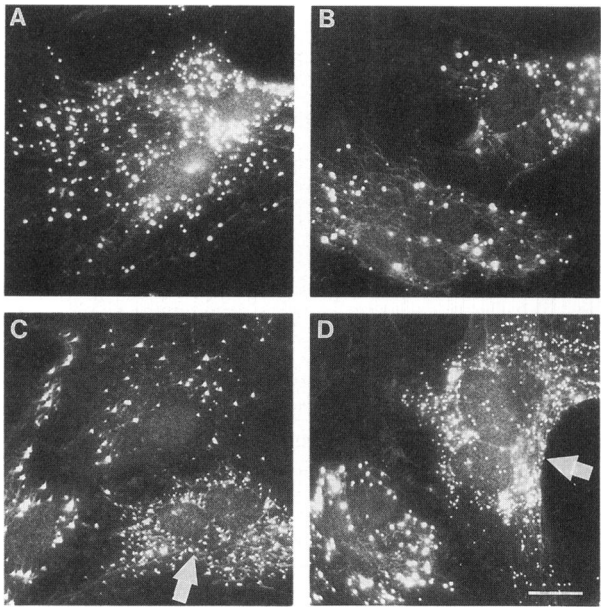

Figure 5 Indirect immunofluorescence light microscopy of multinuclear cells after cell fusion. Peroxisomes were stained with anti-human catalase IgG. *A*, Patient 1 × patient 2. *B*, Patient 3 × patient 4. *C*, Patient 2 × patient 3. *D*, Patient 1 × patient 4. Scale bar = 10 μm.

oxidase deficiency, thereby indicating that patients 1 and 2 had bifunctional enzyme deficiency and that patients 3 and 4 had acyl-CoA oxidase deficiency although the mutations do not appear to result in gross instability of the respective enzyme proteins. P12/UV selection, a useful method to select peroxisome-positive cells among peroxisome-deficient cells (Zoeller et al. 1988), was also effective for the selection of cells with normalized VLCFA oxidation (Hoefler et al. 1991). Morphological normalization of enlarged peroxisomes in the complemented cells was elucidated for the first time in the present study.

Selection of multikaryons by the density gradient of Ficoll-400 was essential to obtain results more clear-cut than those obtained using unselected fused cells. The increase of VLCFA oxidation was marked (5–12 fold), and the corrected activities were 37%–122% of the control values in normal fibroblasts. Both P12/UV selection and morphological analysis of peroxisomes also gave unambiguous results. The increase of VLCFA oxidation was less and depended on efficiency of the cell fusion when the unselected cells were used (Wanders et al. 1992*b;* Yajima et al. 1992).

Findings in our patients and in those in previous reports indicate that the clinical manifestations of acyl-CoA oxidase deficiency are milder than those of either bifunctional enzyme deficiency or peroxisomal 3-ketoacyl-CoA thiolase deficiency. Age at death in the case of acyl-CoA oxidase deficiency was 4–5 years (Poll-The et al. 1988; Wanders et al. 1990*a*), whereas that in the case of bifunctional enzyme deficiency was 5–9 mo (Clayton et al. 1988; Watkins et al. 1989) and that in the case of thiolase deficiency was 11 mo (Goldfischer et al. 1986). This may be attributed to the fact that there is only VLCFA accumulation in acyl-CoA oxidase deficiency, whereas both VLCFA and bile acid abnormalities are present in either bifunctional enzyme deficiency or thiolase deficiency. The clinical courses of our patients seem to be slightly milder than those of patients reported elsewhere, possibly because of the presence of enzyme protein. Genetic analysis of these patients is under way, to shed more light on these questions.

Acknowledgments

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