

Oxidation of Pristanic Acid in Fibroblasts and Its Application to the Diagnosis of Peroxisomal β -Oxidation Defects

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Abstract

Pristanic acid oxidation measurements proved a reliable tool for assessing complementation in fused heterokaryons from patients with peroxisomal biogenesis defects. We, therefore, used this method to determine the complementation groups of patients with isolated defects in peroxisomal β -oxidation. The rate of oxidation of pristanic acid was reduced in affected cell lines from all of the families with inherited defects in peroxisomal β -oxidation, thus excluding the possibility of a defective acyl CoA oxidase. Complementation analyses indicated that all of the patients belonged to the same complementation group, which corresponded to cell lines with bifunctional protein defects. Phytanic acid oxidation was reduced in fibroblasts from some, but not all, of the patients. Plasma samples were still available from six of the patients. The ratio of pristanic acid to phytanic acid was elevated in all of these samples, as were the levels of saturated very long chain fatty acids (VLCFA). However, the levels of bile acid intermediates, polyenoic VLCFA, and docosahexaenoic acid were abnormal in only some of the samples. Pristanic acid oxidation measurements were helpful in a prenatal assessment for one of the families where previous experience had shown that cellular VLCFA levels were not consistently elevated in affected individuals. (*J. Clin. Invest.* 1996. 97:681–688.) Key words: bile acids and salts • fatty acids • phytanic acid • prenatal diagnosis • Zellweger syndrome

Introduction

Complementation analyses have proved particularly useful in helping to delineate the underlying genetic defects in patients with abnormalities in peroxisomal biogenesis. Initially, patients with these disorders were classified, on the basis of their clinical features, as having the relatively severe disorder of Zellweger's syndrome or the progressively milder syndromes of neonatal adrenoleukodystrophy and infantile Refsum's disease. The reduction in the number and/or changes in the mor-

phology of peroxisomes in their tissues is accompanied by a series of biochemical abnormalities. Subsequent complementation analyses have indicated that the three clinical phenotypes can arise from defects in a single gene (1, 2). In addition, in spite of all of these patients sharing the same series of biochemical abnormalities, it is now clear that the peroxisomal biogenesis disorders, as a group, can result from defects in any of at least 10 genes (2, 3). Recent evidence suggests that three of these complementation groups have defects in the import of peroxisomal matrix proteins via both of the known targeting mechanisms (peroxisomal targeting signals [PTS]¹ 1 and 2), but that for one of the complementation groups the defect is restricted to proteins targeted via PTS1 (4). Recently, the gene (*PXRI*) associated with the latter complementation group has been identified (5). Apart from the difference in uptake of PTS1 and PTS2 targeted proteins, the different complementation groups are currently biochemically indistinguishable. Since several biochemical and immunocytochemical markers are affected in this group of disorders, a range of techniques has been used to assess peroxisomal integrity, and hence complementation, in fused heterokaryons. These include the activity of dihydroxyacetonephosphate acyltransferase (DHAPAT) and the percentage of catalase that is in the particulate fraction (6), the immunocytochemical localization of catalase (7, 8), the oxidation of phytanic acid (9) and very long chain fatty acids (VLCFA, i.e., fatty acids with more than 22 carbons) (10), plasmalogen biosynthesis (1), and sensitivity to pyrene fatty acid-mediated ultraviolet damage (11).

It is now clear that some patients with the clinical features of a peroxisomal biogenesis disorder actually have an isolated defect in peroxisomal β -oxidation. This can result from a defect in one of the enzyme proteins of the peroxisomal β -oxidation pathway, namely acyl CoA oxidase, the bifunctional protein, with its enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities, or peroxisomal 3-oxoacyl-coenzyme A thiolase. Initially, the defective gene in such patients was identified on the basis of a lack of immunologically cross-reacting material to one of these proteins, namely thiolase (12), acyl CoA oxidase (13), and the bifunctional protein (14), in the patient's tissues. However, many patients with an isolated defect in the peroxisomal β -oxidation pathway still have normal levels of cross-reacting material for the three proteins (15–19). Since there are technical difficulties in assaying the individual peroxisomal β -oxidation enzymes in cultured fibroblasts, com-

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1. *Abbreviations used in this paper:* C22:0, docosanoic acid; C24:0, tetra-cosanoic acid; C26:0, hexacosanoic acid; C22:6, docosahexaenoic acid; C28:5, octacosapentaenoic acid, C29-dicarboxylic acid, 3 α ,7 α ,12 α -trihydroxy-27-carboxymethyl-5 β -cholestan-26-oic acid; DHAP, dihydroxyacetonephosphate; DHAPAT, dihydroxyacetonephosphate acyltransferase; DHCA, dihydroxycoprostanic acid, 3 α ,7 α -dihydroxy-5 β -cholestan-26-oic acid; PTS, peroxisomal targeting signal; THCA, trihydroxycoprostanic acid, 3 α ,7 α ,12 α -trihydroxy-5 β -cholestan-26-oic acid; VLCFA, very long chain fatty acids.

plementation assays are proving invaluable in pinpointing the defective enzyme in such patients. Initial studies (20, 21) used the β -oxidation of VLCFA in complementation analyses, while Suzuki et al. (22) also determined the resistance to ultraviolet damage after treating with 1-pyrene dodecanoic acid and the normalization of peroxisomal morphology determined using catalase immunofluorescence in their complementation studies.

An elevation in plasma pristanic acid (2,6,10,14-tetramethylpentadecanoic acid) was first observed in patients with defects in peroxisomal biogenesis (23). More recently increased pristanic acid levels have been reported for patients with isolated defects in peroxisomal β -oxidation due to defects in the bifunctional protein and/or peroxisomal thiolase (24) but not when the defect in peroxisomal β -oxidation has been at the level of acyl CoA oxidase (25). The latter observation is consistent with the identification of two acyl CoA oxidases in human liver and kidney, one which oxidizes the CoA esters of straight chain fatty acids and prostaglandins and the other which oxidizes the CoA esters of 2-methyl-branched fatty acids and also bile acid intermediates (26). Not surprisingly, Poll-The et al. (13) found no bile acid abnormalities in their patients with acyl CoA oxidase deficiency, and detection of bile acid precursors in patients with isolated β -oxidation defects is indicative of a defect at the level of the bifunctional protein or thiolase (14, 27).

In this study on Australian patients with peroxisomal disorders we have investigated the use of pristanic acid oxidation estimations in the diagnosis of these patients and the application of this assay for complementation analyses using cells from patients with peroxisomal biogenesis and β -oxidation defects. While cells from the latter patients all had reduced rates of pristanic acid oxidation, considerable heterogeneity in the other biochemical findings for this group of patients was noted. In addition, we found that the rate of pristanic acid oxidation provided a useful additional test for prenatal diagnosis of a fetus from one of the families affected by a bifunctional protein defect.

Methods

Patient cell lines and tissue samples

Two of the Zellweger's syndrome cell lines (GM00228 & GM04340) used for the complementation assays were obtained from the NIGMS Human Genetic Cell Repository (Camden, NJ) and their complementation groups have already been reported (8). All of the other cell lines used for this study were fibroblast cell lines established from Australian patients referred to the Women's and Children's Hospital, Adelaide, for diagnostic investigation of peroxisomal or other disorders. All of the fibroblast cultures were established from skin biopsies except for cell line 3 which was established from post-mortem lung tissue. Control cell lines were from patients without peroxisomal disease. Clinical findings for the nine patients (from seven families) with isolated defects in peroxisomal β -oxidation are provided in Table I.

While we were undertaking our own complementation studies, a number of our cell lines were independently assessed in the laboratory of Dr. Nobuyuki Shimozawa of The Gifu University School of Medicine, Gifu, Japan. Using the distribution of catalase immunofluorescence as an indicator of whether complementation had occurred between two cell lines (8), cell line 1 was placed in their complementation group A, cell line 3 in their group C, and cell lines 6, 7, 8, 9, and 10 in their group E (Shimozawa, N., personal communication). Cell lines 11 and 12 belonged to a newly identified complementation group (3). In addition, Dr. Shimozawa's group identified cell line 13

Table I. Clinical Findings in Patients with Isolated Defects in Peroxisomal β -Oxidation

| Clinical feature | Patients | | | | | | | | | |
|---------------------------|----------|----|------|----|------|----|------|-------|------|---|
| | FA | RS | TS | AE | WE | JG | CN | JS | IC | |
| | sibs | | sibs | | sibs | | sibs | | sibs | |
| Seizures | + | + | + | + | + | + | + | + | + | + |
| Dysmorphic features | + | - | | + | + | + | - | | | + |
| Hypotonia | + | | | + | + | + | + | + | + | + |
| Hepatomegaly | | + | | | | | + | + | + | |
| Development delay | | + | | + | + | | + | + | | |
| Poor feeding | + | | + | + | + | | | | | |
| Retinal haemorrhages | | | | | | | + | | | + |
| Cataracts | | | | | | | + | | | |
| Adrenal abnormalities | | + | | + | + | | | + | | |
| Neuronal migration defect | | | | | | | | + | | |
| Demyelination | | | | | | | | + | | |
| Parents consanguineous | + | | | + | + | + | | | | + |
| Survival time in months | 6 | 6 | 2 | 4 | 8 | 11 | 16 | > 198 | 5 | |

+, indicates feature observed; -, indicates feature not present; blank, indicates no information supplied; sibs, siblings.

as having a bifunctional protein defect (Shimozawa, N., personal communication). Another of our cell lines (No. 14) was assessed in Professor Hugo Moser's laboratory at the Kennedy Krieger Institute, Baltimore, MD, and was also shown to have a bifunctional protein defect (Moser, H., personal communication).

The liver samples used for the immunoblotting experiment were collected post-mortem. Patients D₁, D₂, and D₃ had clinical features of neonatal adrenoleukodystrophy, infantile Refsum's disease, and Zellweger's syndrome, respectively. The biochemical findings for these three patients were consistent with a peroxisomal biogenesis defect, with elevated levels of VLCFA in plasma and fibroblasts, and a reduced activity of DHAPAT in fibroblasts. The proportion of catalase in their fibroblasts that was sedimentable was also below the control range. Patients D₁ and D₃ correspond to fibroblast cell lines 1 and 3, respectively, in our complementation assays. Patient D₄ had the hallmarks of another peroxisomal disorder, rhizomelic chondrodysplasia punctata. His plasma and fibroblast VLCFA levels were normal, as was the proportion of catalase in his fibroblasts that was sedimentable. However, both alkyl DHAP synthase and DHAPAT activities in his fibroblasts were deficient. The control tissues were from children who died of other causes.

Biochemical assays

Routine diagnostic tests for cultured fibroblasts. Fibroblast VLCFA were measured as previously described (28). The method for measuring the activity of DHAPAT was based on the methods of Schutgens et al. (29) and Singh et al. (30), and alkyl DHAP synthase activity was measured in fibroblast extracts following the method of Singh et al. (30). The percentage of catalase in fibroblasts that was sedimentable was determined after treating fibroblasts with digitonin (31) at a concentration of 0.08 mM for 10 min at 4°C. The activity of catalase in the sedimentable and soluble fractions was then assayed spectrophotometrically (32).

Pristanic acid and phytanic acid oxidation. For pristanic acid oxidation, fibroblasts in 25-cm² flasks, were first incubated for 24 h with 5 ml basal Eagle's medium containing 0.5% FCS. The medium was then replaced with 5 ml fresh medium containing [1-¹⁴C]pristanic acid (55 mCi/mmol, prepared essentially as described by Singh et al. (33); 120,000 dpm/flask was used in earlier experiments, but this was later increased to 480,000 dpm/flask) for 24 h. The amount of radioactivity in CO₂ was determined as described by Poulos (34) except that the

liberated CO₂ was collected for 5 min and trapped in 2 ml 1 M KOH and counted using scintillant (OptiPhase HiSafe 3; Wallac, Gaithersburg, MD). Radioactivity in water-soluble products was determined as described by Singh et al. (33). Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) oxidation experiments were carried out as for pristanic acid oxidation, except that the cells were incubated with the substrate for 96 h before determining the amount of radioactivity in carbon dioxide and water-soluble products. The specific activity of the substrate was 55 mCi/mmol, and 200,000 dpm was added per flask.

Bile acid determinations in plasma. Total bile acids from plasma (0.5–1.0 ml) were extracted using reverse phase octadecylsilane-bonded cartridges. Bile acid conjugates were then hydrolyzed with 4.5 M NaOH at 120°C for 16 h. Neutral sterols were removed by alkaline extraction with hexane, and, after acidification, the bile acids were recovered with ether. The free bile acids were converted to their methyl ester trimethylsilyl derivatives and finally purified on Lipidex 5000 (Packard Instrument B.V., Groningen, The Netherlands) minicolumns. Gas chromatography was carried out on a 5890 series 2 gas chromatograph (Hewlett-Packard Co., Palo Alto, CA) fitted with an SGE BPX-5 capillary column (25 m × 0.22 mm × 0.25 μm film). Bile acids were quantitated using hyodeoxycholic acid as internal standard, and their identity was confirmed by gas chromatography–mass spectrometry.

Fatty acid determinations in plasma. Plasma VLCFA (expressed as the ratio of hexacosanoic acid [C26:0] to docosanoic acid [C22:0] and as the ratio of tetracosanoic acid [C24:0] to C22:0), the polyenoic VLCFA octacosahexaenoic acid (C28:5), and docosahexaenoic acid (C22:6) were determined as outlined in Poulos et al. (28). Phytanic acid and pristanic acid were assayed by the same procedure except that the gas chromatograph was fitted with a 50 m × 0.33 mm i.d. × 0.25 μm film SGE BPX-70 capillary column.

Complementation tests

Complementation tests were carried out using the rate of pristanic acid oxidation as a marker for peroxisomal function. Fusion and enrichment of hybrid somatic cells was based on the method of Nelson and Carey (35) except that the Ficoll gradients consisted of 3, 6, 9, 12, and 15% Ficoll layers. After harvesting the fractions, cells from the 6, 9, and 12% fractions were cultured in 25-cm² flasks for 5 d with basal Eagle's medium 2% FCS before measuring the oxidation of pristanic acid as described above. The 5-d incubation was included because Brul et al. (36) and Stanczak et al. (37) found that it may take a few days for correction of the defect to take effect when peroxisomal biogenesis defect cell lines from different complementation groups are fused. Complementation was deemed to occur if the oxidation of pristanic acid (assessed by either the production of radioactively labeled CO₂ or water-soluble product) was markedly increased (at least four-fold) in cells from the 12% (largely cells with several nuclei) and/or 9% fraction (largely cells with a few nuclei) compared with cells from the 6% fraction (largely mononuclear cells). Cells from the 9% fraction, were also tested, as only a small number of cells were recovered in the 12% fraction in some experiments. Results for the water-soluble product always paralleled the results obtained for CO₂ production. Pristanic acid oxidation was also measured in the original unfused patient cell lines for comparison.

Immunoblotting of peroxisomal proteins

Preparation of tissue extracts. Samples of liver from human controls or patients with peroxisomal disorders were stored at –70°C and maintained in dry ice during transport. Mouse liver was from freshly killed animals. Pieces of liver (~50 mg) were thawed (except mouse liver) in, and then homogenized in, 0.2 ml cold 0.25 M sucrose/0.1% ethanol/5 mM Hepes buffer, pH 7.2, containing the following protease inhibitors: leupeptin, 50 μg/ml; pepstatin, 10 μg/ml; chymostatin, 10 μg/ml; antipain, 10 μg/ml; PMSF, 200 μM. An aliquot of each sample was immediately diluted with sample buffer for SDS-PAGE and heated in a boiling water bath for 3 min. Protein in the samples

dissolved in SDS sample buffer was determined according to Petersen (38), after prior heating of samples to remove interference by 2-mercaptoethanol (39).

Electrophoresis and immunoblotting. SDS-PAGE was carried out in 7–15% gradient gels as described previously (40). Electrophoretic transfer of proteins from gels to nitrocellulose was performed according to the method of Towbin et al. (41) but using the carbonate blot buffer described by Dunn et al. (42). Nitrocellulose membranes were subsequently washed and incubated with antibody, followed by ¹²⁵I-labeled protein A and autoradiography as previously described (40).

Preparation of antibodies. Antibodies were raised in rabbits against mouse liver catalase purified according to Price et al. (43), and affinity purified using the method described by Lazarow and de Duve (44). Antiserum raised against the bifunctional protein has been characterized (45).

Results

Cell lines from all of the patients with peroxisomal disorders used in the complementation assays had elevated VLCFA (measured as the ratio of C26:0 to C22:0 fatty acid). 10 of the patients (cell line numbers 1, 3, 4, and 6–12) had biochemical findings consistent with a peroxisomal biogenesis defect. In particular, the amount of catalase in the cells that was sedimentable and the activity of DHAPAT were both reduced. The other seven patients (cell lines 13–19) investigated in this study had biochemical findings consistent with an isolated defect in peroxisomal β-oxidation, since the amount of sedimentable catalase was normal. Activities of DHAPAT and alkyl DHAP synthase, if measured, were also normal in these patients.

As expected, all of the cell lines from our patients with peroxisomal biogenesis disorders that were included in the complementation study had reduced activities of pristanic acid oxidation (results not shown). We, therefore, investigated the possibility that the oxidation of this substrate could be used when doing complementation analyses on these patients. Of 19 cell fusions between cell lines from patients with peroxisomal biogenesis defects, complementation did not occur in 6 of the heterokaryons, but did occur in the remaining 13 fusions (Table II). While our complementation studies were not exhaustive, the results we obtained were entirely consistent with the parallel studies performed in Japan. The particular peroxisomal biogenesis defect patients included in this study fell into four complementation groups. Using the nomenclature used by the Gifu University, groups A, C, E (2), and a newly identified group (3) were represented in this sample of patients.

When pristanic acid oxidation was assayed in the cell lines from our patients with isolated defects in peroxisomal β-oxidation they all showed considerably lower rates of oxidation than control cell lines (Table III), thus excluding the possibility that any of the patients had an acyl CoA oxidase defect. The broad range of values for pristanic acid oxidation in this group of patients (0.2–85.3 pmol CO₂ plus water soluble product · h⁻¹ · mg protein⁻¹; Table III) was similar to that observed for patients with peroxisomal biogenesis disorders (from 0.1 pmol · h⁻¹ · mg protein⁻¹ for a patient with Zellweger's Syndrome to 43.0 pmol · h⁻¹ · mg protein⁻¹ for a patient with infantile Refsum's disease, when using 480,000 dpm of substrate per flask [20 determinations on 15 different cell lines]).

Since all the β-oxidation defect cell lines had reduced pristanic acid oxidation, the oxidation of this substrate was used in

Table II. Results of Complementation Analyses for Cell Lines from Patients with Peroxisomal Biogenesis and β -Oxidation Defects Using Pristanic Acid Oxidation as the Indicator of Peroxisomal Function

| | Biogenesis Defects | | | | | | | | | β -Oxidation Defects | | | | | | | | | |
|------------------|--------------------|---|---|----|---|---|-----|---|---|----------------------------|---|---|---|---|---|---|---|---|---|
| | I | | | II | | | III | | | IV | | | V | | | | | | |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| 1* | | | | | | | | | | | | | | | | | | | Y |
| 2‡ | | | | N | Y | Y | | | | Y | Y | | | | | | | | Y |
| 3‡ | | | | | Y | Y | | | | | | | | | | | | | |
| 4 | | | | | Y | Y | | | | | | Y | | | | | | | |
| 5§ | | | | | | N | N | | | | | Y | Y | Y | | | | | |
| 6§ | | | | | | | | | | | | | Y | | | | | | |
| 7§ | | | | | | | N | N | | | | | | | | | | | |
| 8§ | | | | | | | | | | | | | | | | | | | |
| 9§ | | | | | | | | | | | | | | | | | | | |
| 10§ | | | | | | | | | | | | | | | | | | | |
| 11 | | | | | | | | | | | N | | | | | | | | |
| 12 | | | | | | | | | | | | | | | | | | | |
| 13 | | | | | | | | | | | | | N | | N | | | | |
| 14 | | | | | | | | | | | | | | N | | N | | | |
| 15 | | | | | | | | | | | | | | | N | | | | N |
| 16 | | | | | | | | | | | | | | | | | | | |
| 17 | | | | | | | | | | | | | | | | | | | N |
| 18 | | | | | | | | | | | | | | | | | | | |
| 19 | | | | | | | | | | | | | | | | | | | |

N, indicates that, when fused, the two cell lines did not complement; Y, indicates that, when fused, the two cell lines did complement. Patient/cell line identification: 2, GM04340; 5, GM00228; 13, FA; 14, TS; 15, WE; 16, JG; 17, CN; 18, JS; 19, IC. The following cell lines were independently assigned to the following complementation groups (for details see Methods section). For the peroxisomal biogenesis disorders the classification system of Shimozawa et al. (2) is used. *Group A = Kennedy-Krieger Institute Group 8; ‡Group C = Kennedy-Krieger Institute Group 4; §Group E = Kennedy-Krieger Institute Group 1, ^{||}new complementation group, ^{||}bifunctional protein deficiency.

complementation studies. In the seven cell fusions undertaken no complementation was observed (Table II), indicating that all of the cell lines belonged to the same complementation group. Since one of the cell lines (No. 13) had been shown to have a bifunctional protein defect in studies at the University of Gifu, and another (No. 14) to belong to the same complementation group in studies at the Kennedy Krieger Institute, all of the patients must have bifunctional protein defects. We independently confirmed that cell lines 13 and 14 belonged to the same complementation group. As expected, complementation did occur when cell line 13 was fused with two different peroxisomal biogenesis defect cell lines (Table II).

In addition to determining the β -oxidation of pristanic acid, we also measured α -oxidation of phytanic acid in the cell lines with isolated defects in peroxisomal β -oxidation. This activity was reduced in four of the patients, but was normal or only marginally reduced in the remaining three patients (Table III). In control cells the proportion of the radioactive product appearing as water-soluble product was much higher for phytanic acid oxidation than pristanic acid oxidation, but the overall rate of oxidation of phytanic acid was much lower than for pristanic acid oxidation (Table III).

Plasma samples were still available from six of the patients with bifunctional protein defects. Consistent with the findings on pristanic and phytanic acid oxidation, and with the previous observations of ten Brink et al. (24, 46), the ratio of pristanic to phytanic acid in plasma was elevated in all of the samples (Table IV), although for two of the patients (RS and JS) the values were relatively low (compare with the other patients studied here and the ratios reported for seven patients with bifunctional protein or thiolase defects by ten Brink et al. [46]). The same two patients had relatively minor elevations in plasma C26:0/C22:0 and C24:0/C22:0 ratios (Table IV). Indeed, a separate plasma sample from JS contained normal ratios of these fatty acids (result not shown). Two of the patients (JG and IC) also had elevated levels of the polyenoic VLCFA, C28:5, and three of the patients (FA, RS, and AE) were deficient in plasma docosahexaenoic acid (Table IV).

Plasma samples from four of the patients (FA, AE, JG, IC) had elevated levels of trihydroxycoprostanic acid (THCA; 3 α ,7 α ,12 α -trihydroxy-5 β -cholestan-26-oic acid) (Table IV).

Table III. Pristanic and Phytanic Acid Oxidation in Fibroblasts from Patients with Peroxisomal β -Oxidation Defects

| Patient | Pristanic acid oxidation | | | | Phytanic acid oxidation | | |
|---------------|--|--|-------------|-----------|--|--|--------------------|
| | [1- ¹⁴ C] CO ₂ | [¹⁴ C] Water-soluble product | Total | | [1- ¹⁴ C] CO ₂ | [¹⁴ C] Water-soluble product | Total |
| | <i>pmol · h⁻¹ · mg protein⁻¹</i> | | | | <i>pmol · h⁻¹ · mg protein⁻¹</i> | | |
| FA | 0.4–0.7 | 0.0–0.7 | 0.4–1.2 | (n = 3) | 1.2 | 8.1 | 9.3 |
| TS | 9.7–26.6 | 19.4–60.8 | 29.1–85.3 | (n = 4) | 2.4 | 14.5 | 16.9 |
| WE | 0.2–0.4 | 0.0–2.0 | 0.2–2.3 | (n = 4) | 2.2 | 7.1 | 9.3 |
| JG | 0.2–0.3 | 0.0–0.2 | 0.2–0.5 | (n = 2) | 0.8 | 6.9 | 7.7 |
| CN | 10.2–12.6 | 12.5–17.8 | 23.6–30.4 | (n = 3) | 3.1 | 12.5 | 15.6 |
| JS | 17.3–21.7 | 25.1–39.5 | 46.8–57.7 | (n = 3) | 3.8 | 18.7 | 22.5 |
| IC | 0.1–0.3 | 0.0–1.2 | 0.3–1.5 | (n = 3) | 1.9 | 6.7 | 8.6 |
| control range | 75.5–189.9 | 77.4–312.0 | 152.4–489.6 | (n = 13)* | 3.6–6.3 | 17.0–27.9 | 22.6–34.2 (n = 4)‡ |

Oxidation of [1-¹⁴C]pristanic acid and [1-¹⁴C]phytanic acid were measured as described in the methods. Pristanic acid oxidation was determined after incubating fibroblasts with ~ 480,000 dpm of pristanic acid for 24 h. Phytanic acid oxidation was determined after incubating fibroblasts with ~ 200,000 dpm of phytanic acid for 96 h. *13 determinations on 7 different cell lines; ‡determinations on 4 different cell lines.

Table IV. Plasma Bile Acids and Fatty Acids in Patients with Bifunctional Protein Deficiency

| Patient | Pristanic acid /phytanic acid | C24:0/C22:0 | C26:0/C22:0 | C28:5 | | THCA |
|----------|----------------------------------|-------------|-------------|---|---------|---|
| | | | | $\mu\text{mol} \cdot \text{liter}^{-1}$ | % total | $\mu\text{mol} \cdot \text{liter}^{-1}$ |
| FA | 2.2 | 2.095 | 0.311 | ND | 0.2 | 2 |
| RS | 0.2 | 1.215 | 0.071 | ND | < 0.1 | ND |
| AE | 1.4 | 1.782 | 0.245 | ND | < 0.1 | 2 |
| JG | 1.4 | 2.521 | 0.785 | 2 | 2.2 | 2 |
| JS | 0.5 | 1.158 | 0.041 | ND | 0.9 | ND |
| IC | 1.9 | 2.481 | 0.660 | 4 | 0.9 | 18 |
| controls | ≤ 0.1 ($n = 25$) | < 1.15 | < 0.035 | ND | 0.9–3.3 | ND |

ND, not detected.

Dihydroxycoprostanic acid (DHCA; $3\alpha,7\alpha$ -dihydroxy- 5β -cholestan-26-oic acid) was also elevated in plasma from these patients (results not shown). By contrast, in plasma from RS and JS no THCA or DHCA could be detected. None of the plasma samples contained detectable levels of the C29-dicarboxylic acid ($3\alpha,7\alpha,12\alpha$ -trihydroxy-27-carboxymethyl- 5β -cholestan-26-oic acid) commonly found in patients with peroxisomal biogenesis defects (27).

Immunoblotting studies on liver samples from two of the patients with bifunctional protein defects (patients WE and RS) indicated the presence of immunoreactive bifunctional protein (Fig. 1). In liver from patient RS there was an apparent increase in the amount of labeling of the bifunctional protein whether it was compared directly to that in control samples or if its labeling relative to the labeling of catalase was compared with that in control samples. Liver from a patient with rhizomelic chondrodysplasia punctata also contained immunoreactive bifunctional protein. By contrast, immunoreactive bifunctional protein was deficient in liver samples from patients with peroxisomal biogenesis defects.

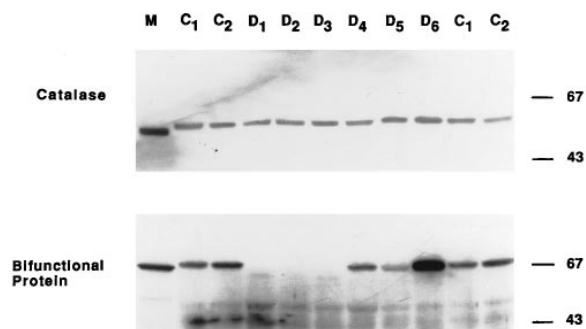


Figure 1. Immunoblot analysis of peroxisomal proteins in liver. Proteins in liver homogenates were separated by electrophoresis in 7–15% gradient SDS-polyacrylamide gels, electrophoretically transferred to nitrocellulose membrane, and probed with antibodies raised against catalase or peroxisomal bifunctional protein. A protein loading of 400 μg liver homogenate protein was used. The bound antibodies were visualized after incubation with ^{125}I -labeled protein A and autoradiography, and the relevant section of each blot is shown. The relative mobilities of the 67- and 43-kD molecular mass markers are indicated. M, mouse liver; C₁, C₂, control human liver; D₁–D₆, livers from patients with peroxisomal disorders; D₁, neonatal adrenoleukodystrophy; D₂, infantile Refsum's disease; D₃, Zellweger's syndrome; D₄, rhizomelic chondrodysplasia punctata; D₅–D₆, patients with bifunctional protein defects; D₅, patient WE; D₆, patient RS.

Over the years, the parents of patients RS and TS have undergone a number of prenatal assessments. Studies on cells from members of this family have demonstrated that the level of VLCFA are not always diagnostic in cells from affected individuals. Indeed cell lines from RS and TS, as well as from one of the affected fetuses, have at times given normal VLCFA results and at other times grossly abnormal results. The reason for the large fluctuations in VLCFA levels is unknown, but clearly, as reported by Carey et al. (47), it presents a problem when carrying out prenatal assessments for this and possibly other families. Since we had now shown that pristanic acid oxidation was defective in cells from TS, both VLCFA levels and rates of pristanic acid oxidation were measured in amniotic fluid cells from the most recent pregnancy. The level of VLCFA in cells from the first subculture was borderline normal with a C26:0/C22:0 (hexacosanoic/docosanoic) ratio of 0.206 corresponding to the top of the control range, but VLCFA were clearly elevated in cells from subculture 3 (C26:0/C22:0 = 0.615). Meanwhile cells from subculture 2 and 3 both had deficient rates of pristanic acid oxidation (Table V). Thus, the inclusion of the pristanic acid oxidation assay gave greater confidence in the diagnosis of an affected fetus.

Discussion

Although [^{14}C]pristanic acid is not available commercially, it is readily synthesized (33) and the product can be stored indefinitely. In addition, there are a number of advantages in using this substrate for the diagnosis of peroxisomal disorders (see below) which easily justify its synthesis and use. Pristanic acid oxidation estimations proved a useful diagnostic tool for patients with either peroxisomal biogenesis or isolated β -oxidation defects. Indeed, at least for the current group of patients with peroxisomal β -oxidation defects, it proved a more reliable indicator of defective oxidation of 2-methyl-substituted substrates (e.g., bile acids and pristanic acid) than plasma bile acid determinations. Also, although we were able to detect an elevated pristanic acid/phytanic acid ratio in the patients, we feel interpretation of results obtained with this method is more problematic than using oxidation measurements, as in some patients the absolute amounts of pristanic acid and phytanic acid were very low (results not shown). The relatively high rates of oxidation of pristanic acid by fibroblasts in culture (33) means that relatively shorter times can be used for incubations than for other substrates such as lignoceric acid (tetracosanoic acid). In addition, it allows good discrimination between high,

Table V. Prenatal Diagnosis of Bifunctional Protein Deficiency Using Pristanic Acid Oxidation

| Cell line | Pristanic acid oxidation | | |
|---------------------------------|--|--|-----------------------------|
| | [1- ¹⁴ C] CO ₂ | [¹⁴ C] Water-soluble product | Total |
| | <i>pmol • h⁻¹ • mg protein⁻¹</i> | | |
| Amniotic fluid cells | | | |
| Test, subculture 2 | 4.9 | 7.3 | 12.2 |
| Test, subculture 3 | 5.0 | 7.7 | 12.7 |
| Controls | 122.6–212.9 | 57.2–139.4 | 179.8–310.9 (<i>n</i> = 5) |
| Skin fibroblast cells | | | |
| Affected fetal sib | 13.4 | 23.2 | 36.6 |
| Father | 181.9 | 149.0 | 330.9 |
| Mother | 129.4 | 145.6 | 275.0 |
| Controls | 150.0, 182.0 | 122.3, 125.9 | 272.3, 307.9 |
| Peroxisomal biogenesis defects* | 1.4–22.7 | 0.0–15.2 | 1.4–37.9 (<i>n</i> = 3) |

*The peroxisomal biogenesis defect cell lines used as positive controls in the prenatal diagnosis of bifunctional protein deficiency came from patients with elevated VLCFA and reduced sedimentable catalase in their fibroblasts.

low and intermediate activities. This has particular relevance for complementation tests, where one has to assess whether there has been any change in activity after cell fusion. A pristanic acid oxidation test also proved helpful for prenatal assessment in a family where VLCFA determinations had proved an unreliable diagnostic indicator of the bifunctional protein defect, and we recommend that the measurement of pristanic acid oxidation be considered when undertaking prenatal assessments in other families with a history of peroxisomal bifunctional protein or thiolase defects.

To our knowledge, this has been the first time that β -oxidation of a branched chain fatty acid has been used for complementation analyses on patients with peroxisomal disorders. We feel the technique has particular application for patients with isolated peroxisomal β -oxidation defects. Indeed, patients with acyl CoA oxidase defects should be able to be identified on the basis of a normal rate of pristanic acid oxidation, rendering subsequent complementation analyses unnecessary for such cell lines. Our complementation tests indicated that all of our patients with isolated peroxisomal β -oxidation defects fell into the same complementation group and that they all had a defect in the bifunctional protein. We did not find any evidence of subsets within this complementation group, as reported by McGuinness et al. (21), however, our studies on fusions between the different cell lines were not exhaustive. Our findings, and those of McGuinness et al. (21), indicate that, among patients with disorders involving individual proteins of the peroxisomal β -oxidation pathway, defects in the bifunctional protein are the most frequent. Although complementation analyses have been performed between cell lines with acyl CoA oxidase and bifunctional protein defects (20–22), we are unaware of any cases where complementation tests have been undertaken between cell lines with proven thiolase and bifunctional protein defects. Thus, as a caveat to the diagnosis of bifunctional protein defects by complementation analysis, the possibility that, for some unknown reason, complementation does not occur when cells from patients with bifunctional protein and thiolase deficiency are fused cannot be totally excluded at this stage.

Since the clinical history of our patients with bifunctional protein defects varied considerably, further biochemical tests were carried out on this group of patients. We found that the activity of phytanic acid oxidation was reduced in some, but not all, of our patients with peroxisomal β -oxidation defects, whereas McGuinness et al. (21) found that phytanic acid oxidation was reduced in the patients they studied and Wanders et al. (48) noted normal activities of phytanic acid oxidation in their patients. For our group of patients the rate of oxidation of phytanic acid reflected the severity of the defect in pristanic acid oxidation and the deficiency, when present, probably represents a secondary effect of the β -oxidation defect. The ratio of pristanic acid to phytanic acid in plasma from the patients with bifunctional protein defects also reflected the severity of the defect in pristanic acid oxidation, such that patients with the lowest rates of pristanic acid oxidation had the highest pristanic acid/phytanic acid ratios.

The finding of undetectable THCA and DHCA levels in two of our patients with isolated β -oxidation defects was unexpected given that the corresponding cell lines showed reduced activities of pristanic acid oxidation. It may reflect a relatively mild defect in peroxisomal β -oxidation, as evidenced by pristanic acid oxidation measurements in fibroblasts from JS and the sibling of RS (TS). Plasma from the same patients showed the mildest elevations in VLCFA and pristanic acid/phytanic acid ratios. Our failure to detect bile acid precursors in the plasma of these two patients, whether due to their complete absence or a lack of sensitivity in our assay, should be heeded. Our results suggest that a bifunctional protein defect should still be considered for the β -oxidation defect patient reported by Espeel et al. (49), which had both a normal acyl CoA oxidase activity and bile acid pattern. Likewise, we recommend that an absence of THCA/DHCA in plasma should not be used as the sole criterion for assigning a patient with a peroxisomal β -oxidation defect to the acyl CoA oxidase defect group. Whereas plasma samples from all of the patients with bifunctional protein disorders had elevated saturated VLCFA, the level of polyenoic VLCFA was raised in samples from only two of the patients. Again this seemed to reflect the overall se-

verity of the peroxisomal β -oxidation defect in these patients, since the same two individuals had the highest plasma levels of saturated VLCFA. In addition they had relatively high plasma pristanic acid/phytanic acid ratios, and very low rates of pristanic acid oxidation in their fibroblasts.

In future studies it will be interesting to see if the severity of the biochemical defect can be related to the presence of particular mutant alleles of the bifunctional protein gene. The peroxisomal bifunctional protein in mammals is known to have Δ^3, Δ^2 -enoyl CoA isomerase activity, which is required for the β -oxidation of polyunsaturated fatty acids, in addition to hydratase and dehydrogenase activities (50). It is, therefore, possible that, depending on the particular mutation present, the efficacy of the different enzyme activities towards the various substrates (saturated VLCFA, polyenoic VLCFA, branched chain fatty acids, or bile acid precursors) might be differentially affected.

Our failure to detect any C29-dicarboxylic acid in our patients with bifunctional protein defects is consistent with the findings of Clayton et al. (27) for patients with peroxisomal β -oxidation defects. In patients with peroxisomal biogenesis defects the C29-dicarboxylic acid is thought to arise by elongation of THCA or its CoA derivative. It is, therefore, curious that in patients with β -oxidation defects and with elevated THCA levels, that the C29-dicarboxylic acid is not detected. It may be that the intracellular site where the THCA (or THCA-CoA) accumulates is different in patients with peroxisomal biogenesis defects, where uptake of the bile acid intermediate into the peroxisome might be impaired, compared to those with isolated defects in the β -oxidation pathway, where the anomaly is distal to uptake of THCA into the peroxisome. If so, this might affect accessibility of THCA to the elongation system.

Interestingly, four novel peroxisomal 3-hydroxyacyl-CoA dehydrogenases, one of which also has hydratase activity similar to the known bifunctional protein, have recently been described in rat liver (51). This raises the possibility that in rats, and possibly other species, there are distinct bifunctional proteins with different substrate specificities involved in peroxisomal β -oxidation. While defects in different bifunctional proteins might account for our failure to observe bile acid abnormalities in two of our patients, this explanation is contraindicated by our complementation studies which suggest that defects in a common gene product are involved in all of our patients.

A deficiency of docosahexaenoic acid has been reported in patients with peroxisomal biogenesis defects (52), and three of our patients with bifunctional protein defects also had a gross deficiency in docosahexaenoic acid, however, the deficiency was not correlated with the severity of the β -oxidation defect. It may result from a poor nutritional intake, which can occur in such severely affected infants, rather than as a direct consequence of the metabolic defect.

Immunoblotting studies on liver samples from two of the patients with bifunctional protein defects indicated the presence of immunoreactive bifunctional protein. Of particular note, the liver from RS had higher than normal levels of immunoreactive bifunctional protein, suggesting that the defect present affects the turnover of the protein. Consistent with these immunoblotting studies, electron microscopy studies on liver from an affected fetal sibling of RS showed increased immunocytochemical labeling of bifunctional protein (Case PD1

in Hughes et al. [53]). It is possible that the elevated levels of defective bifunctional protein may, in part, account for the fluctuations in VLCFA levels found in affected members of this family, particularly if there are any regulatory changes in the amount of enzyme present at different times. Interestingly, the number and size of peroxisomes in the liver of the affected fetus were normal (53) in contrast to other patients with peroxisomal β -oxidation defects where peroxisomes were enlarged in liver (13, 16) or fibroblasts (22). Nevertheless, the morphology of the peroxisomes in the fetal tissue was altered with electron-dense nucleoids present in peroxisomes (53). Similar structures have been reported in other patients with peroxisomal disorders (49, 53, 54).

Although the biochemical findings for the affected siblings RS and TS and patient JS were remarkably similar, there was a large disparity in their clinical phenotype. The poor correlation between biochemical and clinical phenotype, suggests that other secondary factors may have a bearing on clinical phenotype in bifunctional protein deficiency.

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