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Morphometry of peroxisomes and immunolocalisation of peroxisomal proteins in the liver of patients with generalised peroxisomal disorders

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Abstract. Hepatic peroxisomes were studied by morphometric and immunocytochemical techniques in control patients and in four Zellweger syndrome patients, two infantile Refsum's (IRD) patients, one neonatal adrenoleukodystrophy (NALD) patient, and three patients with peroxisomal disorders (PD) which do not fit any currently recognised classification, but have disorders involving a defect in peroxisomal biogenesis. Peroxisomes which were ultrastructurally abnormal and greatly reduced in size and/or number were found in two of the Zellweger syndrome patients, and the NALD and IRD patients. There was variation in their numerical density ranging from none at all in two of the Zellweger syndrome patients to normal numbers in the IRD patients. In most patients there was a decrease in the immunolabelling of catalase over the peroxisomes. In the Zellweger syndrome and NALD patients, the small, abnormal peroxisomes did not label for any of the β-oxidation proteins. The IRD patients and the PD patients however, were heterogeneous with respect to β-oxidation labelling. The ultrastructural heterogenity of peroxisomes in these peroxisomal disorders patients indicates there may be genotypic differences between the major groups and also within each group. The common factor in all the patients in this study where peroxisomes were present was the presence in the hepatic peroxisomes of an electron dense centre which did not label immunocytochemically for catalase or the β-oxidation enzymes. This electron dense centre may indicate a structural abnormality in the peroxisomes in these patients.

Key words: Peroxisomal disorders – Immunocytochemistry – Zellweger syndrome – Neonatal adrenoleukodystrophy – Infantile Refsum's disease

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Introduction

Peroxisomal disorders are a group of inherited diseases affecting children which result in severe clinical consequences including abnormalities of the liver, kidney, brain, adrenal and bone. The disorders are characterised biochemically by a deficiency of one or more peroxisomal enzymes, and an accumulation of substrates in the patient's tissues and plasma (recent reviews see Moser et al. 1991; Wanders et al. 1991; Wilson 1991). The peroxisomes in the liver of these patients show alterations in their size, number and ultrastructural appearance ranging from a complete absence of hepatic peroxisomes in some Zellweger patients (Goldfischer et al. 1973) to extremely enlarged, flocculent organelles in some rhizomelic chondrodysplasia punctata patients (De Craemer et al. 1991; Hughes et al. 1992). Small, abnormal peroxisomes have been found in small numbers in the liver of some patients with generalised peroxisomal disorders i.e. Zellweger syndrome, neonatal adrenoleukodystrophy (NALD) and infantile Refsum's disease (IRD) Goldfischer et al. 1985; Roels et al. 1986; Vamecq et al. 1986; Hughes et al. 1990). These patients have a deficiency of multiple enzyme functions.

Ultrastructural immunolabelling of peroxisomal enzymes in liver biopsies has been demonstrated by Litwin et al. (1987, 1988) in both Lowicryl sections and Epon sections after etching with ethanolic sodium hydroxide. The technique has been applied to human postmortem liver and human fetal liver for the detection of the peroxisomal β-oxidation enzymes (Espeel et al. 1990a, b).

In the present study we have used a similar on-grid immunogold technique to localise the peroxisomal matrix protein catalase; the β-oxidation enzymes; acyl-CoA oxidase, bifunctional protein, and 3-ketoacyl-CoA thiolase; and a 68 kDa peroxisomal integral membrane protein (PMP68). Liver from five control patients, and patients with Zellweger syndrome, NALD, IRD, and three unclassified peroxisomal disorders (PD) was investigated. The results indicate that the peroxisomal mor-

Table 1. Clinical features

Condition	IUGR	FTT	Hepato-megaly	Dysm. F	Ret. Pigm. or Cataract	Deaf	Alive/ Deceased	Stippled Epiphysis	Hypo-tonia	Fits	Devt. Delay	Periph. Neuro-pathy
ZS1	+	+	+	+	N.R.	N.R.	Died 4/12 yr	+	+	−	+	−
ZS2 (fetus TOP)	−	−	−	−	−	−	−	−	−	−	−	−
ZS3	−	+	+	+	+	−	Died 8/12 yr	+	+	−	+	−
ZS4	+	+	+	+	N.R.	N.R.	Died 4/12 yr	N.R.	N.R.	−	+	−
NALD	+	+ + +	+	+	+	+	Died 3.5 yr	−	+	+	+	−
IRD1	+	+ + +	+	+	+	+	Died 2 yr	−	+	+	+	−
IRD2	+	+	+	+	+	+	Alive 11 yr	−	+	−	+	−
PD1 prop	+	+	+	+	N.R.	N.R.	Died 5/12 yr	−	+	+	+	−
PD1 (fetus TOP)	−	−	−	−	−	−	−	−	−	−	−	−
PD2	−	−	−	−	+	+	Died 1.5 yr[a]	−	+	−	+	−
PD3	−	−	−	+	−	+	Alive 7 yr	−	−	−	+	+ +

IUGR, Intra-uterine growth retardation; FTT, failure to thrive; Dysm. F, dysmorphic features; Ret. Pigm., Retinitis Pigmentosa; Devt. Delay, developmental delay; Periph. Neuropathy, peripheral neuropathy; N.R., not recorded

ZS, Zellweger syndrome; NALD, Neonatal Adrenoleukodystrophy; IRD, Infantile Refsum's Disease; PD, Peroxisomal Disorder, not otherwise classified

[a] accidental death

Table 2. Biochemical findings

Case	Plasma			Cultured skin fibroblasts			
	C26/22	C24/22	Phytanic acid	C26/22	Phytanic acid oxidase	DHAP–AT	Catalase %sedimentable
ZS1	Elevated	Elevated	–	Elevated	Decreased	Reduced	Decreased
ZS2[a]	–	–	–	–	–	Reduced[b]	–
ZS3	Elevated	Elevated	Normal	Elevated	–	Reduced	–
ZS4	Elevated	Elevated	–	Elevated	–	Reduced	–
NALD	Elevated	Elevated	Elevated	–	Decreased	Reduced	–
IRD1	Elevated	Elevated	Elevated	Elevated	Decreased	Reduced	Decreased
IRD2	Elevated	Elevated	Elevated	–	Decreased	Reduced	–
PD1[d]	Elevated	Elevated	Normal	Elevated	–	Normal	–
PD1[a]	–	–	–	Elevated[c]	–	–	–
PD2	Elevated	Elevated	Elevated	Normal	Decreased	Normal	Decreased
PD3	Elevated	Elevated	Elevated	–	Decreased	Normal	–

[a] Fetal

[b] Chorionic villus direct and chorionic villus cultured cells

[c] Chorionic villus direct and chorionic villus cultured cells, and cultured amniotic cells

[d] Findings in the propositus

–, Not measured

phology and subcellular localisation of peroxisomal proteins is heterogeneous within the generalised peroxisomal disorders.

Control tissue was obtained from three patients undergoing biopsy for conditions not related to peroxisomal disorders. Autopsy tissue was obtained from one patient without a peroxisomal disorder where the post-mortem interval was approximately 8–9 h. Control fetal tissue was obtained from a 17–18 week old fetus.

The clinical features of the patients in this study are documented in Table 1 and the details of the biochemical findings are recorded in Table 2. The full clinical and biochemical details of three of the patients in this study have been published elsewhere (IRD2 see Hughes et al. 1990 and Robertson et al. 1988; NALD see patient 3 in Hughes et al. 1990; PD3 see patient 2 in Hughes et al. 1990). The clinical criteria employed for the diagnoses of Zellweger syndrome, IRD and NALD have been reported earlier (Poulos et al. 1988).

PD1 fetal tissue was received after prenatal assessment of a pregancy at risk. Chorionic villus biopsy and cultured amniotic cells showed an affected child. Clinical features of the index case are shown in Table 1, and the biochemical information relating to the index case and to the fetus are in Table 2. Tissue from the index case was not available for ultrastructural examination.

Materials and methods

Electron microscopy. Liver was obtained at needle biopsy or at autopsy and fixed immediately by immersion in a mixture of 4% formaldehyde and 1% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.3. After fixation for 2 h at room temperature, the

tissue was washed twice in 0.1 M sodium cacodylate buffer, post-fixed in 1% osmium tetroxide, washed in cacodylate buffer again and stained en bloc with 2% aqueous uranyl acetate. The tissue was then dehydrated in a graded series of ethanol and embedded in Spurr's low-viscosity epoxy resin. Polymerisation was carried out at 70° C under vacuum for at least 12 h. Silver-gold thin sections were cut on a Reichert Ultracut microtome, stained with uranyl acetate for 3 min and lead citrate for 3 min, and examined in an Hitachi 7000 electron microscope.

Fixation for immunocytochemistry. Liver tissue for immunocytochemical investigations was fixed in a mixture of 4% freshly prepared paraformaldehyde and 0.25% glutaraldehyde in 0.1 M sodium phosphate buffer at pH 7.4 for 2 h at room temperature. For transport or storage the tissue was retained in 2% paraformaldehyde in 0.1 M sodium phosphate buffer at 4° C.

Resin embedding. After fixation the tissue was rinsed in phosphate buffer containing 50mM ammonium chloride, dehydrated in ethanol and embedded in Lowicryl K11M resin at −25° C. Polymerisation was carried out by UV irradiation at −25° C for several days. Silver-gold sections were mounted on celloidon coated nickel grids.

Antisera. Antisera to catalase, bifunctional protein and PMP68 were raised and purified as previously described (Hughes et al. 1992). The PMP68 from mouse liver is homologous to PMP70 from rat liver and human liver. Chen and Crane (1992) compared a partial cDNA for mouse PMP68 with rat PMP70 (Kamijo et al. 1990) and found 95% nucleotide homology and 98% amino acid homology.

Antisera to rat liver peroxisomal 3-ketoacyl CoA thiolase and acyl-CoA oxidase were generous gifts from Professor T. Hashimoto, Shinsu University, Japan.

Immunolabelling. The grids containing the sections were washed in three washes of phosphate buffered saline with 50mM glycine for 15 min followed by three washes of D&WB for 20 min. D&WB consisted of phosphate-buffered-saline pH 7.3 containing 0.5% ovalbumin, 0.1% gelatin, 0.05% Tween 20 and 0.2% Teleostean gelatin. They were then incubated in the primary antibody diluted in D&WB for 16 h at 4° C. The grids were washed in six washes of D&WB for a total time of 30 min and then incubated in a protein A gold conjugate (11 nm) for 60 min at room temperature. The protein A gold conjugate was diluted in D&WB to an optical density at 520 nm of 0.14. Following this the grids were washed in D&WB for a further 30 min, washed in clean water and then counterstained with uranyl acetate for 30 s and lead citrate for 15 s.

Controls were carried out on adjacent sections by omitting the primary antibody, and by incubating in normal rabbit serum instead of the primary antibody.

When quantitation was to be carried out, grids containing sections of each liver sample were immunolabelled at the same time in order to reduce variations due to different batches of antibody or gold probe.

Quantitation of immunogold labelling. Twenty electron micrographs from sections of liver which had been similarly treated were randomly selected. Five micrographs of each of the control sections were selected to determine the background density. The labelling density (gold particles per μm^2) was calculated by measuring the area of the peroxisomes and counting the number of gold particles over each peroxisome. For quantitation of the peroxisomal membrane protein, the number of gold particles per μm of membrane was measured. The background density calculated from the controls was automatically subtracted. A Hewlett-Packard 9847 A digitizer linked to a Model 9816 microcomputer was used for these evaluations. The results were compared using a Student's *t* test.

Measurements of peroxisomal size and number. Morphometry was carried out as described earlier (Hughes et al. 1993). In brief, twenty electron micrographs of each section of liver were randomly selected at a magnification of × 10,000 and enlarged photographically to × 22,000. The magnification factor was calculated from a carbon grating replica which was photographed and enlarged at the same magnification as each batch of micrographs. The area, perimeter and diameter of the peroxisomes, and the area of the hepatocyte cytoplasm were measured by manual tracing on a digitiser linked to a computer using a Video Trace software package (Leading Edge Technology, Adelaide, Australia). The volume density, numerical density and surface density of the peroxisomes was calculated according to Weibel (1979).

Results

Morphometry. In two of the Zellweger syndrome patients no peroxisomes could be identified at all. In the other generalised peroxisomal disorders including two of the Zellweger syndrome patients and the NALD patient small, morphologically abnormal structures were identified in biopsy, autopsy and fetal liver (Fig. 1b, c). These organelles were surrounded by a single membrane and contained electron dense centres which often filled the entire organelle except for a clear area just inside the membrane. The area and diameter of these structures was greatly reduced compared to normal peroxisomes, as was their volume, numerical and surface densities (see Table 3). In the two patients to whom a clinical diagnosis of IRD had been assigned, small, abnormal organelles with a similar ultrastructural appearance were seen. These structures were also reduced in size from normal peroxisomes, however their numerical densities were similar to those of normal peroxisomes in control patients. The volume and surface densities were reduced. In the IRD2 patient, a few organelles which approximated the size of normal peroxisomes were also present in some cells. These peroxisomes also had an electron dense centre along with some finely granular matrix of normal appearance.

In the biopsies from the PD2 and PD3 patients, similar small, peroxisomes with electron dense centres were seen. The size of the peroxisomes was reduced in these two patients (0.03 μm^2 and 0.06 μm^2 respectively) and their volume and surface densities were reduced. However in these cases, as in the IRD patients, the numerical density was within normal limits (see Table 3 and Fig. 2).

The other unclassified peroxisomal disorder patient PD1 had peroxisomes which were normal in size, volume, numerical and surface densities. The peroxisomes in this patient did vary from normal in that they also had electron dense centres (Fig. 1d).

Immunocytochemistry. In control liver the labelling for catalase was evenly distributed over the peroxisomal matrix with an average labelling density of 203.1 gold particles per μm^2. In the fetal control tissue the labelling for catalase was much less than this (62.68 gold particles per μm^2). In all the peroxisomal disorder patients in this study the labelling for catalase was considerably reduced or absent (see Table 4) except for the PD1 patient where it was increased compared to the age-matched control fetal tissue. In the Zellweger syndrome, NALD and PD2 patients, there was no labelling for catalase over the

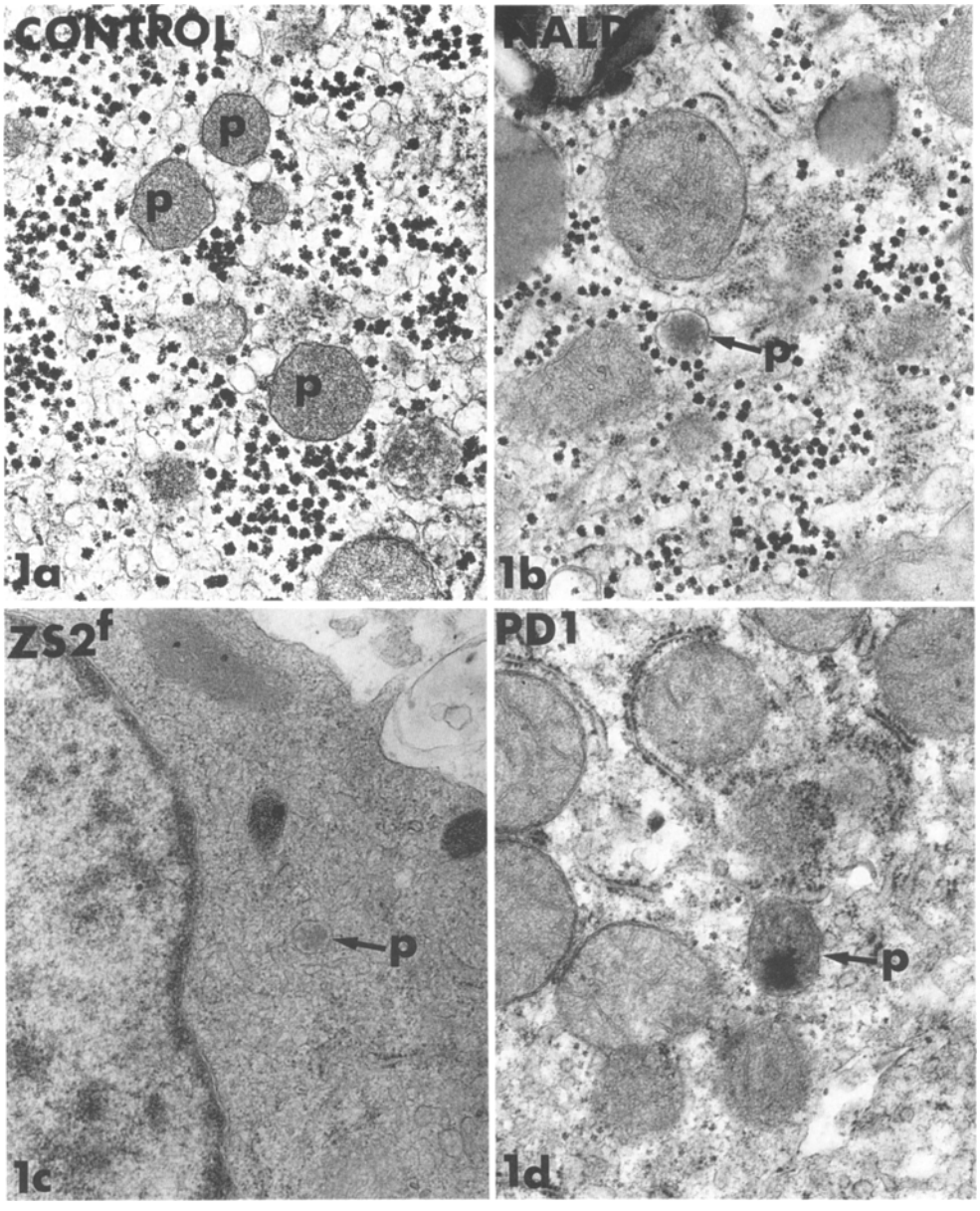

Fig. 1a. Normal peroxisomes (*p*) in the liver from a control patient. **b.** Small, abnormal peroxisome (*p*) with an electron dense centre in the liver from the neonatal adrenoleukodystrophy (NALD) patient. **c.** Small, abnormal peroxisome (*p*) in the liver of a fetus with Zellweger syndrome. **d.** Peroxisome (*p*) in the liver of PD1 patient. These peroxisomes are normal in size and number but have an electron dense centre. × 40,000

Table 3. Morphometry of peroxisomes. The age of the patient, the mean area (μm²) ± standard deviation, the mean diameter (*d*-circle in μm), the relative volume density (%), the numerical density (μm-3) and the surface density (μm-1) of hepatic peroxisomes

Patient	Age	Area	Diameter	V_V	N_V	S_V
Control[b]	17 wks	0.19 ± 0.06	0.49	0.38	0.035	0.040
Control[a]	4 mths	0.37 ± 0.14	0.69	1.6	0.056	0.126
Control[c]	–	0.22 ± 0.11	0.56	1.67	0.125	0.161
Range	(3 mth–18 yr)	(0.17–0.29)	(0.51–0.63)	(0.7–3.2)	(0.057–0.188)	(0.073–0.262)
ZS1	2 wks	0.03 ± 0.008	0.20	0.01	0.014	0.003
ZS2[b]	12 wks	0.03 ± 0.009	0.20	0.02	0.021	0.004
ZS3[a]	8 mths	No peroxisomes	No peroxisomes	No peroxisomes	No peroxisomes	No peroxisomes
ZS4[a]	3 mths	No peroxisomes	No peroxisomes	No peroxisomes	No peroxisomes	No peroxisomes
NALD	2 yrs	0.03 ± 0.02	0.20	0.03	0.051	0.012
IRD1	5 mths	0.03 ± 0.008	0.20	0.05	0.065	0.013
IRD2	5 yrs	0.04 ± 0.03	0.26	0.13	0.122	0.029
PD1[b]	17 wks	0.17 ± 0.12	0.47	0.8	0.086	0.086
PD2	11 mths	0.05 ± 0.04	0.25	0.09	0.065	0.020
PD3	2 yrs	0.06 ± 0.03	0.28	0.2	0.078	0.028

[a] autopsy tissue [b] fetal tissue [c] mean of 16 control pediatric liver biopsies (see Hughes et al. 1993)

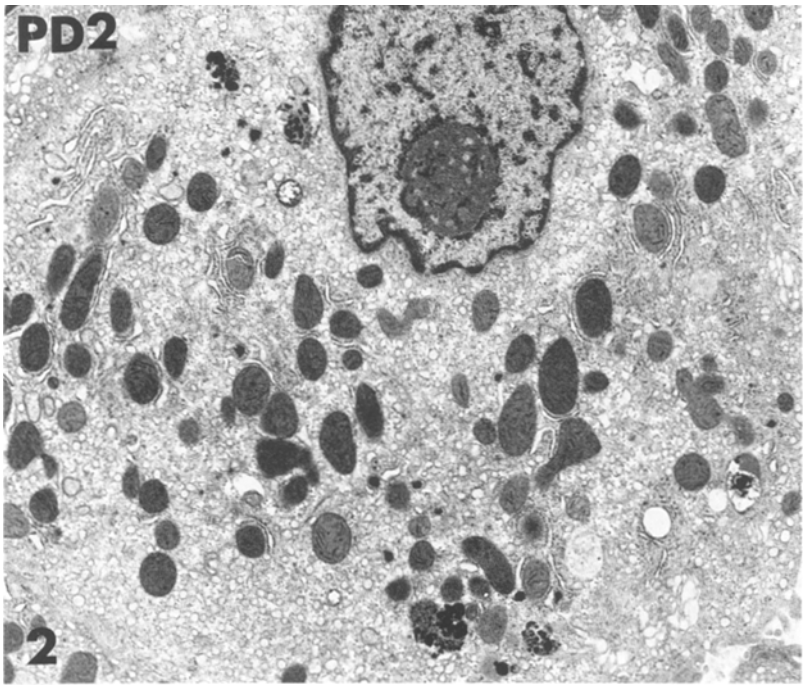

Fig. 2. Hepatocyte from patient PD2 showing normal numbers of small peroxisomes with electron dense centres. × 8,750

Table 4. Immunocytochemistry of peroxisomal enzymes in control and patient livers. Labelling density (number of gold particles per μm^2 of peroxisome) for the matrix enzymes catalase, acyl-CoA oxidase (ACoAOx), bifunctional protein (BFP) and 3-ketoacyl-CoA thiolase (Thiolase); and the integral membrane protein PMP68 (number of gold particles per μm of peroxisomal membrane) ± standard deviation

Case	Catalase	A-CoAOx	BFP	Thiolase	PMP68
C2[b]	62.68 ± 39.60	46.23 ± 23.89	25.99 ± 10.99	82.72 ± 58.55	0.70 ± 0.54
C4	189.42 ± 139.88	106.15 ± 88.28	49.14 ± 87.39	115.75 ± 73.19	3.28 ± 2.15
C5[a]	180.00 ± 122.17	92.85 ± 51.53	34.53 ± 40.10	139.31 ± 91.77	1.14 ± 0.81
C18	220.77 ± 128.43	72.46 ± 49.73	30.74 ± 30.05	119.36 ± 84.39	1.67 ± 1.04
C19	222.22 ± 148.68	94.43 ± 76.29	46.21 ± 35.59	141.59 ± 94.86	2.33 ± 1.68
ZS1	0.00[c]	0.00[c]	0.00[c]	0.00[c]	0.00[c]
ZS2[b]	0.00[c]	0.00[c]	0.00[c]	0.00[c]	0.00[c]
ZS3[a]	px not found	px not found	px not found	px not found	px not found
ZS4[a]	px not found	px not found	px not found	px not found	px not found
NALD	0.00[c]	0.00[c]	0.00[c]	0.00[c]	0.00[c]
IRD1	2.90[c] ± 5.30	0.00[c] ± 0.00	3.24[c] ± 10.31	2.71[c] ± 7.81	0.31[c] ± 0.71
IRD2	31.52[c] ± 50.52	92.11 ± 88.56	34.44 ± 41.97	210.32[d] ± 181.43	1.65 ± 1.28
PD1[b]	143.69[d] ± 60.64	93.66[d] ± 44.77	114.13[d] ± 40.1	135.96[d] ± 19.15	2.56 ± 1.65
PD2	0.00[c]	0.00[c]	0.00[c]	0.00[c]	3.76[d] ± 2.83
PD3	2.93[c] ± 7.22	52.62 ± 62.48	0.00[c]	430.75[d] ± 805.44	2.78 ± 1.29
PD3[e]	72.27 ± 62.77	43.93 ± 23.37	27.77 ± 12.73	48.27 ± 31.10	1.52 ± 0.40

[a] Autopsy tissue
[b] Fetal tissue
[c] Significantly decreased at $p < 0.001$
[d] Significantly increased at $p < 0.001$
[e] Population of large peroxisomes

small, abnormal peroxisomes. However in the IRD and PD3 patients some labelling was found although greatly reduced in density.

In control liver the label for all three β-oxidation enzymes was distributed over the peroxisomal matrix with an average labelling density of 91.47 gold particles/μm^2 for acyl-CoA oxidase, 40.16 gold particles/μm^2 for the bifunctional protein, and 129 gold particles/μm^2 for thiolase. As with catalase, the labelling density for the β-oxidation enzymes was much less in the control fetal tissue (Table 4). The labelling density for acyl-CoA oxidase was virtually undetectable for Zellweger syndrome, NALD and IRD1 patients, but normal for the IRD2 patient. In the PD patients, labelling for acyl-CoA oxidase was zero for PD2, increased for PD1, and normal or decreased for PD3 (see next paragraph). A similar result was observed for the labelling of bifunctional protein, although a very small amount of labelling for this enzyme was also present in the IRD1 patient. The labelling density for thiolase was zero for the Zellweger syndrome and NALD patients. In the IRD patients a very small amount of label was present in one patient (IRD1)

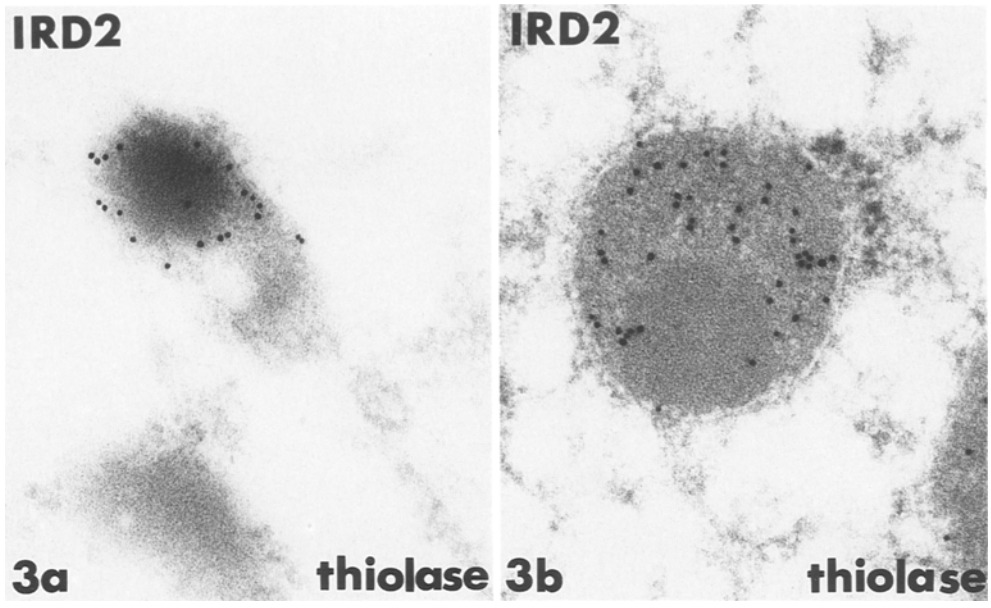

Fig. 3. Immunolabelling of thiolase in peroxisomes from a patient with IRD. **a**. A small peroxisome with an electron dense centre which is relatively free of label. The labelling density of thiolase is increased and labelling is concentrated in the outer rim of matrix. **b**. Larger peroxisome from the same patient with label for thiolase over the matrix but excluding the electron dense centre. × 96,000

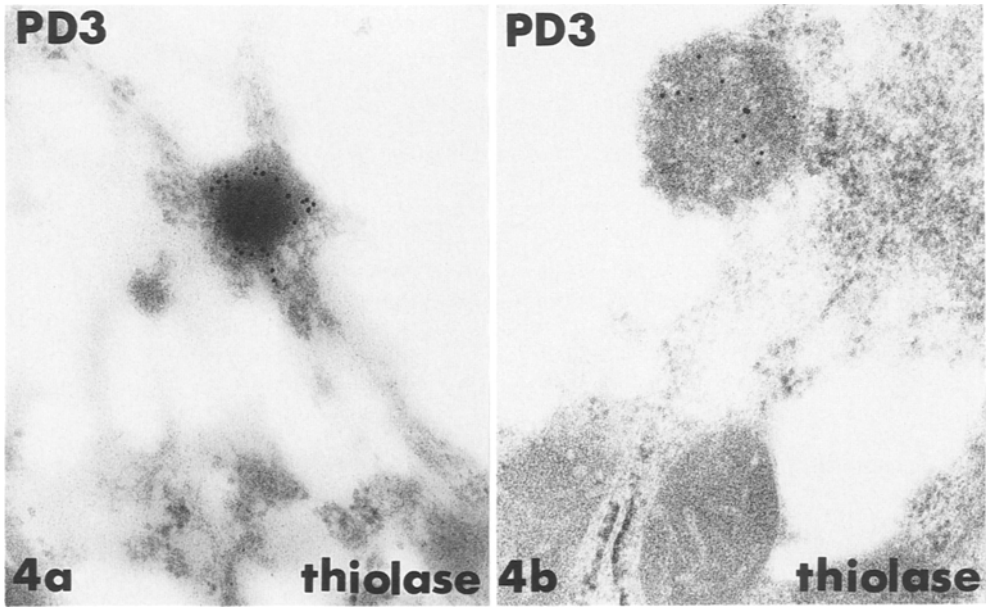

Fig. 4. Immunolabelling of thiolase in patient PD3. **a**. A small peroxisome with an electron dense centre which is relatively free of label. The labelling for thiolase is concentrated in the outer rim of matrix just inside the membrane. **b**. A large peroxisome of normal morphological appearance which also labels for thiolase although the labelling density is decreased. × 64,000

but in the other patient (IRD2) the labelling density for thiolase was increased approximately two fold (Table 4 and Fig. 3a). In this case the labelling for thiolase was concentrated in the thin "halo" just inside the membrane of the small peroxisomes. Labelling was not present over the electron dense centres of these organelles. In this patient some peroxisomes were almost normal in size, but still had an electron dense centre. In these larger peroxisomes there was little label over the electron dense centre even though there was labelling of the matrix (Fig. 3b). This phenomenon was also observed in the PD1 patient, where peroxisomes of normal size and number had electron dense centres which did not label with any of the antibodies used. The rest of the matrix in these peroxisomes had a normal appearance and did label for the matrix proteins.

In the PD3 patient two populations of peroxisomes were identified in the tissue examined for immunocytochemistry. Small peroxisomes with electron dense centres were found which showed very reduced labelling density for catalase and no labelling for bifunctional protein. These small peroxisomes however had a normal labelling density for acyl-CoA oxidase and a greatly increased labelling density for thiolase. As in the IRD2 patient the labelling for thiolase and acyl-CoA oxidase was concentrated in the narrow halo just inside the peroxisomal membrane (Fig. 4a).

Larger peroxisomes with a normal ultrastructure were also seen in this PD3 patient. This population of organelles had a reduced labelling density for catalase, acyl-CoA oxidase and thiolase but the labelling density for bifunctional protein was normal (see Table 4 and

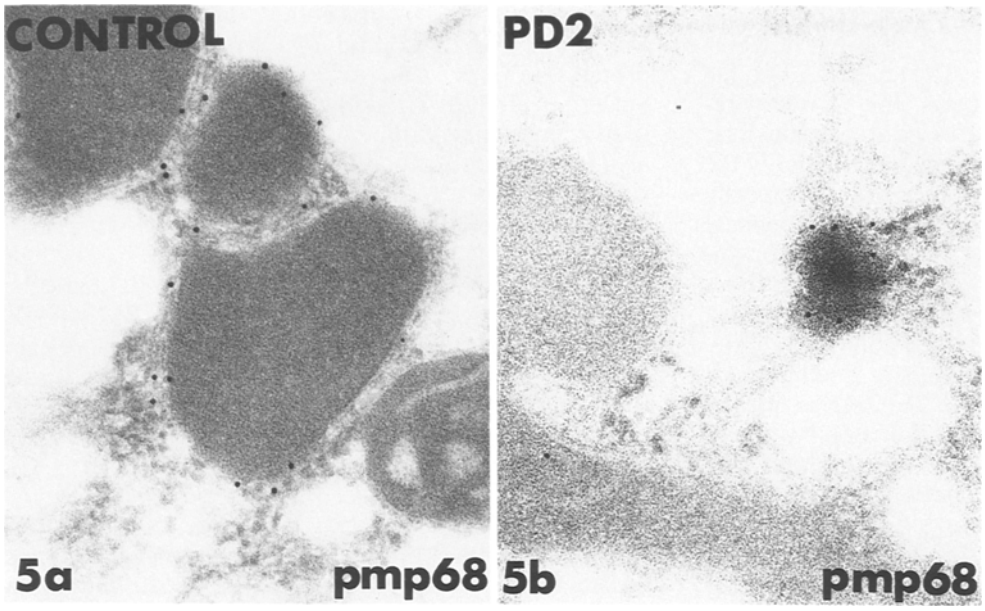

Fig. 5. Immunolabelling of PMP68. **a.** In control liver the label is concentrated in the peroxisomal membrane. **b.** A small peroxisome with an electron dense centre in an unclassified peroxisomal disorder (PD2). These peroxisomes did not label for catalase or any of the β-oxidation enzymes. × 87,000

Table 5. Summary of results. The change in size and structure of the peroxisomes relative to the control group is indicated as well as the labelling density of each of the antibodies for each group of peroxisomal disorders

Group	PX size	PX structure	Catalase	A-CoAOx	BFP	Thiolase	PMP68
ZS	Reduced[a]	Abnormal	Not detectable	Not detectable	Not detectable	Not detectable	Not detectable
NALD	Reduced	Abnormal	Not detectable	Not detectable	Not detectable	Not detectable	Not detectable
IRD	Reduced	Abnormal	Reduced	Reduced/normal	Reduced/normal	Reduced/increased	Reduced/normal
PD1	Normal	Abnormal	Increased	Increased	Increased	Increased	Increased
PD2	Reduced	Abnormal	Not detectable	Not detectable	Not detectable	Not detectable	Increased
PD3	Reduced	Abnormal	Reduced	Normal	Not detectable	Increased	Normal
PD3[L]	Normal	Normal	Reduced	Reduced	Normal	Reduced	Normal

[a] In patients where peroxisomes were detected

Fig. 4b). Both the large and small peroxisomes identified in this biopsy had a normal labelling density for PMP68, thus confirming their peroxisomal nature.

In the control livers the label for PMP68 was found over the membrane of the peroxisomes with an average number of 2.11 gold particles per μm of peroxisomal membrane (Fig. 5a). In the Zellweger syndrome and NALD cases, there was no labelling over the small, abnormal peroxisomes. However in IRD, a very small amount (0.31 particles/μm) was observed in the first patient (IRD1), and the second patient (IRD2) had normal labelling. In the PD2 patient, the label for PMP68 was increased, despite a complete absence of labelling for any of the matrix proteins (Fig. 5b).

One of control livers was autopsy tissue and the labelling density for all proteins was similar to the values in biopsy tissue even though the peroxisomes were larger than normal due to post mortem change (see Tables 3 and 4). A summary of the morphometric and immunocytochemical results for each peroxisomal disorder group is given in Table 5.

Discussion

The small peroxisomes with electron dense centres are associated with the generalised peroxisomal disorders i.e. Zellweger syndrome, NALD and IRD. They have been previously reported in the liver of NALD and IRD patients (Goldfischer et al. 1985; Beard et al. 1986; Roels et al. 1986; Vamecq et al. 1986; Hughes et al. 1990). We have found these abnormal peroxisomes also in two of our Zellweger syndrome patients, including one 12 week old fetus which was diagnosed as Zellweger syndrome by biochemical analysis of chorionic villus biopsy. It should be noted, however, that these peroxisomes are not found in all Zellweger syndrome cases. The difference between an absence of peroxisomes, as observed in some Zellweger syndrome patients, and peroxisomes with a V_v of only 0.01 or 0.02% is not great, and it may be that in those cases described as having no peroxisomes the peroxisomes are so small and scarce that they are not detected by normal morphometric and ultrastructural means.

The V_v of peroxisomes in these generalised peroxisomal disorders is also greatly reduced. The N_v, however is within normal limits for both the IRD patients, but due to the small size of the peroxisomes, the V_v is still reduced. This suggests that in these cases at least the liver is still able to produce small peroxisomes in roughly the right number, but these peroxisomes are structurally abnormal and unable to carry out all the biochemical functions. This is also suggested by the immunocytochemical results where there is no labelling for catalase or the β-oxidation enzymes over any of the small peroxisomes observed in Zellweger syndrome or NALD. In one IRD patient (IRD1) there is a very small amount of labelling, and in the other (IRD2), there is normal labelling for acyl-CoA oxidase and bifunctional protein, and an increased labelling density for thiolase. The peroxisomes in this case have a reduced amount of label for catalase. The increased labelling for thiolase can be explained by the concentration of this enzyme in the narrow "halo" of normal matrix just inside the peroxisomal membrane. The electron dense centre observed in these peroxisomes does not label for any of the matrix enzymes. This suggests that it may consist of a dense material which does not react with antibodies to catalase and the β-oxidation enzymes. We speculate that this electron dense centre may represent a storage product as a result of impaired β-oxidation, or alternatively may represent the breakdown of the internal structure of the peroxisomes. It is also possible that this electron dense material represents an aggregate of a protein or proteins which have been imported but are unable to be properly localised within the organelle. In NALD and Zellweger syndrome and some IRD patients the small peroxisomes consist almost entirely of this abnormal electron dense material, and there is no "normal" matrix to support the matrix enzymes. This is one explanation for the severe deficiency of peroxisomal metabolic functions leading to the rapid demise of these patients.

In our IRD2 patient and the PD3 patient the labelling for thiolase and acyl-CoA oxidase was concentrated in a thin rim between the peroxisomal membrane and the electron dense centre. This suggests that these peroxisomes are still able to import these enzymes but that they are misplaced within the peroxisomes due to the presence of the electron dense centre. These patients both have a biochemical defect in β-oxidation, despite the presence of immunologically reactive β-oxidation enzymes in these abnormal peroxisomes.

Of the small peroxisomes observed in the generalised peroxisomal disorders, only those in the IRD patients labelled for PMP68. In one IRD patient (IRD1) the labelling for peroxisomal membrane protein was reduced, but in the other (IRD2) it was normal. This IRD patient was an older patient who had some larger peroxisomes present in the liver, as well as the small peroxisomes. This data is in conflict with that found by immunoblotting of autopsy liver where three peroxisomal membrane proteins (22, 53 and 68 kDa) were present in normal amounts in the liver of three Zellweger syndrome and one IRD patients (Small et al. 1988). However, Wiemer et al. (1989) found the 69 kDa membrane protein was greatly reduced in autopsy liver of one Zellweger patient but not in another. Aikawa et al. (1987) also found the 22, 26 and 70 kDa integral membrane proteins were deficient in one Zellweger patient; and Suzuki et al. (1987) found deficient 70 kDa protein in four Zellweger syndrome patients. However, Gartner et al. (1992) recently found normal amounts of normal sized PMP70 mRNA in fibroblasts from eleven Zellweger probands, suggesting normal amounts of PMP70 protein.

Labelling for PMP68 was found in the 3 PD patients. In the PD2 patient, the labelling density for PMP68 was increased oven the small, abnormal peroxisomes, despite a lack of labelling for any of the matrix proteins. In the PD3 patient both the large and small populations of peroxisomes, labelled for PMP68. This confirms the peroxisomal nature of these abnormal organelles.

Peroxisomal "membrane ghosts". Large empty vesicles which react with antibodies to peroxisomal membrane protein have been described in cultured skin fibroblasts from Zellweger syndrome and rhizomelic chondrodysplasia punctata patients (Santos et al. 1988a, b; Wiemer et al. 1989; Balfe et al. 1990). We did not find any evidence of these peroxisomal "membrane ghosts" in the liver biopsies from our peroxisomal disorder patients. Certainly many of the structurally abnormal peroxisomes described in our patients displayed labelling for PMP68, with or without concurrent labelling for peroxisomal matrix enzymes. However, there was no evidence of the very large structures described in cultured skin fibroblasts. These "membrane ghosts" have recently been reported to co-label with lysosomal enzymes (Heikoop et al. 1992), and they may represent the degradation of peroxisomes. This may be a phenomenon peculiar to cultured skin fibroblasts.

Heterogeneity of peroxisomes. Complementation analysis experiments have identified at least eight different complementation groups from patients deficient in peroxisomes (Brul et al. 1988; Poll-The et al. 1989; Roscher et al. 1989; Yajima et al. 1992). This suggest that at least eight genes may be involved in the assembly of functional peroxisomes, and a mutation in any one of these genes can lead to the biochemical and clinical phenotype of a generalised peroxisomal disorder. Furthermore, studies in cultured skin fibroblasts from patients belonging to the same complementation group, have shown further phenotypic variation (Wiemer et al. 1991). Our studies of the morphological characteristics of peroxisomes in the liver of peroxisomal disorders patients clearly supports the complementation analyses where there is genetic heterogeneity between and within the major groups of peroxisomal disorders. We have found heterogeneity with regard to number of peroxisomes within our Zellweger syndrome patients, and heterogeneity between the IRD patients with regard to the size and number of peroxisomes and the labelling for peroxisomal proteins. We have also identified three patients who do not fit any current classification of peroxisomal disorders, who display heterogeneity in the size, number, and immunocytochemical labelling of hepatic peroxisomes.

Table 6. Morphological/immunocytochemical types of peroxisomes identified in generalised peroxisomal disorders

Normal size and morphology peroxisomes, with moderately reduced labelling of matrix proteins (PD3-large)
Normal size peroxisomes with electron dense centres and normal labelling of peroxisomal matrix and membrane proteins (PD1)
Small peroxisomes with electron dense centres and reduced labelling of some matrix proteins (i.e. catalase, IRD2; catalase and bifunctional protein, PD3 small)
Small peroxisomes with electron dense centres and markedly reduced labelling of all matrix proteins, and reduced labelling of PMP68 (IRD1)
Small peroxisomes with electron dense centres with undetectable labelling for matrix proteins but normal labelling for membrane proteins (PD2)
Small peroxisomes with electron dense centre with undetectable labelling for matrix and membrane proteins (Zellweger syndrome, NALD)

Table 7. Cellular phenotypes seen in generalised disorders of peroxisomal dysfunction

Normal numbers of normal size peroxisomes with electron dense centres (PD1)
Heterogeneous populations of large and small peroxisomes, some with electron dense centres and normal numerical density (IRD2, PD3)
Small peroxisomes with electron dense centres and normal or near normal numerical density (IRD1, PD2, NALD)
Small peroxisomes with electron dense centres and greatly reduced numerical density (Zellweger syndrome)
No peroxisomes detected (Zellweger syndrome)

Conclusion. The different morphological types of peroxisomes that we have identified in these patients with generalised peroxisomal disorders are summarised in Table 6 and the various cellular phenotypes in Table 7.

The measurement of morphometric values of peroxisomes provides a more accurate description of the types of peroxisomes in peroxisomal disorders. However, qualitative as well as quantitative descriptions are required in order to avoid misinterpretation. This is emphasised by the occurrence of electron dense centres which are immunocytochemically negative, in some peroxisomes of normal size (e.g. patient PD1). All the patients in this study had peroxisomes in the liver which were ultrastructurally abnormal, in some respect. There was heterogeneity with regard to size and number, but all patients had peroxisomes with electron dense centres. At least six different types of peroxisomal abnormalities based on morphological and immunocytochemical criteria, were detected in patients with generalised disorders of peroxisomal dysfunction. We conclude that synthesis of even abnormal peroxisomes must take place in all patients.

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