ORIGINAL ARTICLE

Biochemical analysis of cultured chorionic villi for the prenatal diagnosis of peroxisomal disorders: biochemical thresholds and molecular sensitivity for maternal cell contamination detection

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Objectives: The prenatal diagnosis of peroxisomal disorders is most often performed by biochemical analysis of cultured chorionic villus sample (CVS) or amniocytes. We aimed to (*a*) highlight the risk of maternal cell contamination (MCC) in biochemical prenatal diagnosis, (*b*) establish the threshold of these biochemical assays to MCC, and (*c*) document the sensitivity of PCR based genotyping of microsatellites for the detection of MCC in prenatal diagnosis of inborn errors by biochemical analysis.
Methods: The threshold of each biochemical assay was assessed by co-cultivating fibroblasts from known affected and normal individuals. Genotypes for three polymorphic loci were determined by PCR and GeneScan analysis. The sensitivity of the molecular test was determined by DNA mixing experiments and isolation of DNA from co-cultivated fibroblasts.
Results: MCC was detected in 2.5% of at risk CVS cultures (n = 79). Co-cultivation of defective and normal fibroblasts demonstrated that the peroxisomal biochemical assays were accurate at 25% contamination. Very low level DNA or cell contamination (1–5%) was detectable by genotyping, but an allele did not yield a definitive peak based on morphology until ~10% contamination. Furthermore, we demonstrated that other inborn errors of metabolism might be more susceptible to diagnostic error by low level MCC.
Conclusion: The sensitivity of the microsatellite analysis (⩾10%) is well within the threshold of peroxisomal biochemical assays. Although peroxisomal biochemical assays would not be predicted to introduce a false positive or negative result if MCC <10% were present but not recognised by molecular analysis, the same may not be true for other inborn errors of metabolism.

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The severity of and inability to offer effective treatment for peroxisomal disorders makes prenatal diagnosis a common choice for families with a known risk. Prenatal diagnosis is reliable using biochemical methods for the diagnosis of X linked adrenoleukodystrophy (X-ALD, OMIM #300100)[1] and the autosomal recessive peroxisomal biogenesis disorders (PBD) (Zellweger syndrome (OMIM #214100), neonatal adrenoleukodystrophy (OMIM #202370), infantile Refsum disease (OMIM #266510) and rhizomelic chondrodysplasia punctata (RCDP, OMIM # 215100)),[2] and the related isolated enzyme defects that resemble the aforementioned disorders.[3] Measurement of very long chain fatty acid (VLCFA) content and/or plasmalogen synthesis in cultured chorionic villus cells or amniocytes are the most common methods used, but alternative approaches include bile acid analysis of amniotic fluid[4] and enzyme assay of direct chorionic villus biopsy.[5 6] With the identification of *ABCD1*, prenatal diagnosis by DNA mutation analysis is becoming more common for X-ALD. However, the necessity for mutation identification and the associated costs that are frequently not covered by insurance, or the immediacy of testing requirements for an at risk pregnant woman whose familial mutation is unknown, leaves biochemical analysis as the only option in many cases. Although DNA testing for the PBDs is theoretically possible, these methods are only beginning to be offered in certified diagnostic laboratories. Until more resources are dedicated to the development of rapid, reliable, and cost effective molecular testing, biochemical testing will remain an important approach to prenatal diagnosis for peroxisomal disorders.

Using cultured cells for biochemical analysis presents a variety of risks that could have a deleterious impact on the diagnostic outcome, including failure to establish a viable culture, acquired enzyme activity due to contamination by microorganisms,[7]* and maternal cell contamination (MCC). Amniocyte cultures occasionally succumb to MCC, as reported for a pregnancy at risk for Hurler's disease,[8]† but the risk is greater for cultured chorionic villus sample (CVS). This increased hazard is due to the anatomical intimacy of the chorionic villi with maternal tissue. Even careful dissection may leave visually unrecognisable maternal decidua that could proliferate once in culture. Multiple reports have documented the occurrence of MCC in CVS. In a Canadian CVS trial reported in 1989, 20/478 samples (4.2%) from male fetuses had mixed 46XX/46XY karyotypes,[9] although the newborns had normal 46XY karyotypes. The report of the US Collaborative Study several years later[10] identified no cases of MCC in 601 CVS analyses, although a defined protocol to test for MCC was not documented. Although cytogenetic analysis is very effective at detecting low levels of MCC when the fetal sex is male, it is limited by

Abbreviations: CVS, chorionic villus sample; MCC, maternal cell contamination; X-ALD, X linked adrenoleukodystrophy; PBD, peroxisomal biogenesis disorders (PBD); RCDP, rhizomelic chondrodysplasia punctata; VLCA, very long chain fatty acid

*A Moser has observed acquired plasmalogen biosynthetic activity in deficient amniocytes infected with fungus.

† A Moser has documented significant MCC in amniocytes derived from fluid containing a tissue plug.

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its inability to distinguish maternal cells from those of a female fetus. Identity testing by PCR linkage analysis of polymorphic microsatellite markers has numerous applications, and is widely used by diagnostic laboratories to rule out MCC. The alleles in maternal DNA at several polymorphic loci are compared with the alleles in DNA isolated from the cultured CVS to rule out MCC. Antoniadi *et al* reported the usefulness of this approach by testing four loci and identified MCC in 4/85 CVS biopsies (4.7%).[11]

The sensitivity of PCR provides the detection of very low level DNA contamination (down to 1%). However, the threshold of biochemical analysis (the percentage of MCC at which the results are no longer valid) and the sensitivity of molecular analysis have not been reported for the assays used to test for inborn errors of metabolism. As many molecular laboratories guarantee detection of MCC only if it is ⩾10%, a biochemical threshold of only 5–10% would make the sensitivity of the molecular analysis insufficient. To correlate detection of MCC by DNA and biochemical methods, we undertook a systematic evaluation of the impact of varying levels of MCC on biochemical assays used for prenatal diagnosis of peroxisomal disorders and the lysosomal disorder globoid cell leukodystrophy (Krabbe disease, OMIM #245200). In this report we: (*a*) describe our experience with CVS for the prenatal diagnosis of the most common peroxisomal disorders, (*b*) determine the threshold of biochemical methods to MCC, (*c*) document the sensitivity of an identity panel for three polymorphic loci in the detection of low level MCC, (*d*) correlate the sensitivity of the molecular analysis for MCC with the biochemical threshold for MCC, and (*e*) show that a non-peroxisomal biochemical disorder has a different correlation with MCC detected by DNA and by biochemical methods.

METHODS

Microsatellite analysis

Genomic DNA was isolated from cultured cells using the Puregene kit (Gentra). Markers ID1, ID2, and ID3 correspond to the tetranucleotide repeats D21S11, D19S253, and D18S5, respectively. Urquhart *et a.*[12] described amplification of these markers, which reside on chromosomes 21, 19, and 18, respectively, in a multiplex PCR. Each marker is polymorphic for the repeat number, with the majority of variation occurring in 4 bp units, but some ID2 and ID3 polymorphisms occur in 2 bp units. The three markers have a heterozygosity index of 76–91% for white, Afro-Caribbean, and South Asian populations. The combined match probability for all three markers ranged from 0.6 to 1.4 in 10 000. Oligonucleotide PCR primer sequences were: ID1-F: 5′-atatgtgagtcaattccccaag-3′; ID1-R*Ned: 5′-TGTATTAGTC AATGTTCTCCAG-3′; ID2-F*Fam: 5′-ATAGACAGACAGACG GACTG-3′; ID2-R: 5′-GGGAGTGGATATTACCCCt-3′; ID3-F*Hex: 5′-CAAACCCGACTACCAGCAAC-3′; ID3-R: 5′-GAGCCATGTTCATGCCACTG-3′. One primer from each set was labelled with the indicated dye. Each PCR reaction tube had a 50 µl total volume containing 0.2 µmol/l ID1F and ID1R; 0.1 µmol/l ID2F, ID3F, ID2R, and ID3R; 0.8 mmol/l each dNTP; 1.82 mmol/l $MgCl_2$; 100 ng genomic DNA; and 1.25 U Hot Start *Taq* (Qiagen). The PCR had 30 cycles of denaturing at 95℃ for 30 seconds, annealing for 30 seconds, and extension at 72℃ for 30 seconds. The annealing temperature started at 65℃ and dropped by 2℃ every two cycles until it reached 55℃. PCR product (1 µl) was mixed with 12 µl formamide and 0.5 µl ROX size standard (Applied Biosystems), then denatured at 95° for 3 minutes and cooled on ice till ready for analysis on an ABI 3100 analyser. Samples were injected onto POP4 polymer capillary columns (36 cm) for electrophoretic separation. GeneScan and Genotyper software were used for analysis of the peaks detected on each sample.

Biochemical analyses

Biochemical analyses were performed in skin fibroblast cultures maintained in Eagle's minimum essential medium supplemented with 10% fetal calf serum.[13] Peroxisomal analyses of VLCFA content and plasmalogen synthesis were assayed as described elsewhere.[14 15] Enzyme activity of cerebroside-ß-galactosidase was measured using the fluorogenic substrate 6-hexadecanoylamino-4-methylumbelliferyl-ß-D-galactoside (HMU-ßGal; available through Moscerdam Substrates) as originally described.[16]

DNA mixing

Genomic DNA samples from two unrelated individuals were mixed after repeated measurement of the stock genomic DNA concentration at different dilutions. The ''contaminating'' DNA was added in proportions of 1, 5, 10, and 15%. In order to mimic the situation that may arise when maternal cells contaminate a cell culture of fetal origin, we selected for each marker two individuals who shared one allele in common, but had a different second allele. The three alleles for each marker are referred to as: (*a*) S for shared allele; (*b*) C for contaminating allele; and (*c*) N for non-shared, non-contaminating allele. To optimise our ability to recognise low level contamination, we selected DNAs where the C allele for each marker was at least 8 bp larger than the next nearest repeat size. This requirement provided a flat baseline on the electropherogram in the region of interest and would avoid interference by stutter peaks (caused by slippage during amplification of the repeat, and which commonly precede a primary peak) thus enabling detection of a small peak.

Fibroblast co-cultivation for biochemical and molecular analyses

Control (CTL1 and 2) and PBD (PBD1 and 2) fibroblasts were cultivated in several T150 flasks and aseptically harvested. The number of cells in each suspension was determined using a haemocytometer. For cell mixing experiment 1 (CME1), PBD1 was contaminated with 0, 6.25, 12.5, 25, 37.5, 50, 75, and 100% CTL1. Two T25 flasks were set up for each admixture, the first being for biochemical analysis and the second being for molecular analysis. CME1 was set up in duplicate using different batches of the same cell lines, thus there was an A and B flask for both the biochemical and molecular analysis for CME1. CME2 was set up using a single batch of cells (CTL2 and PBD2) in a similar fashion to CME1; in this case, however, both flasks of each admixture were used for biochemical analysis alone. CME3 used strains PBD2 and CTL2, and was set up in the same fashion as CME2. Flasks from CME1 and CME2 marked for biochemical analysis had plasmalogen synthesis measured, whereas CME3 had VLCFA measurement. Genomic DNA was isolated from the flasks marked for molecular analysis from CME1 on the same day the corresponding cells were harvested for the biochemical assay. KD1 (a Krabbe disease proband) had CTL2 added in proportions of 0, 5, 10, 15, and 20% for CME4. Two aliquots of each admixture were prepared, one of which was cultivated for 4 days and then harvested for enzyme analysis and one of which was frozen as a pellet and then assayed.

Cell lines and DNA samples

Fibroblast lines were obtained from the Peroxisomal Diseases Laboratory and the Mental Retardation Research Center cell banks at Kennedy Krieger Institute. Informed consent was obtained to use patient cell lines for research, as approved by the institutional review board at the Office of Human

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Table 1 Biochemical prenatal diagnosis by CVS

Disorder for which fetus is at risk	Biochemical tests		Number tested	Number unaffected	Number (%) affected
	VLCFA	Plasmalogen synthesis			
ZS	X	X	65	47	18 (28%)
ß-Ox defect	X		6	6	0 (0%)
RCDP		X	14	6	8 (57%)
X-ALD*	X		20	12	8 (40%)

ZS, Zellweger syndrome; ß-Ox, ß oxidation; *male fetuses only.

Subjects Research, Johns Hopkins University. Previously isolated DNA samples used for mixing experiments were available in the DNA Diagnostic Laboratory at Johns Hopkins University. Consent to use DNA samples for research purposes had been obtained from patients prior to this study.

RESULTS

Our experience with prenatal diagnosis of peroxisomal disorders and MCC

The Peroxisomal Diseases Laboratory uses VLCFA content measurement and plasmalogen biosynthesis capacity to perform biochemical prenatal diagnosis of the most common peroxisomal disorders. In the past 4 years we received 105 CVS cultures (table 1) from pregnancies at risk for Zellweger syndrome (68%), isolated enzyme deficiencies of peroxisomal ß-oxidation (6%), RCDP (13%), and X-ALD (19%). The proportion of predicted affected Zellweger syndrome fetuses (28%) falls within expectations for an autosomal recessive disorder. In contrast, there was a higher proportion of predicted affected fetuses (57%) at risk for RCDP than would be expected, and there were no predicted affected fetuses in pregnancies at risk for isolated defects in peroxisomal ß-oxidation. However, the total number of pregnancies screened for these disorders was relatively small. Postpartum follow up was obtained on five of the six babies at risk for isolated ß-oxidation defects and confirmed they are all unaffected. We obtained follow up fibroblast cultures on 3/8 fetuses predicted to be affected with RCDP, and in each of those cases confirmed the biochemical defect.

MCC detected during the prenatal diagnosis of peroxisomal disorders

We required referring laboratories to perform molecular or cytogenetic analyses that would be able to recognise MCC. DNA studies to rule out MCC indicated that one fetus at risk for a PBD (case 1) and a second fetus at risk for a ß-oxidation defect (case 2) had 100% and >80% MCC, respectively. It had been noted in case 1 that the cells were morphologically atypical for CVS. A follow up amniocentesis was performed in both instances. Case 1 had biochemical results consistent with a PBD (table 2), whereas case 2 had normal biochemical results in amniocytes (results not shown). The pregnancy was terminated for case 1, and analysis of fetal fibroblasts confirmed the results obtained from the amniocyte culture (table 2). CVS culture for a third fetus that was at risk for X-ALD had cytogenetic evidence of 6–10% MCC. FISH analysis using X and Y probes of later cultures that had normal VLCFA levels identified XY interphase nuclei only, indicating that the fetal cells had overgrown the maternal cells. Overall, significant MCC was identified in two CVS cultures from pregnancies at risk for peroxisomal disorders. This represents 1.9% (2/105) of all CVS cases analysed. Although two cases with obvious MCC were detected and averted the risk of diagnostic error, additional background studies are necessary to understand the effect of low level contamination on the biochemical assays.

Co-cultivation experiments predict a high threshold for MCC when measuring VLCFA content or plasmalogen biosynthesis

PBD fibroblasts were co-cultivated with varying proportions of normal control fibroblasts as a surrogate for MCC. Fig 1A shows results for plasmalogen synthesis in experiments (CME1 and CME2) using two different PBD cell lines mixed with a normal control cell line as detailed above. PBD1 had a more severe deficiency in plasmalogen synthesis, as indicated by the higher ratio of $^3H/^{14}C$. Both PBD cell lines still had $^3H/^{14}C$ ratios consistent with a deficiency of plasmalogen synthesis at 50% contamination. Similarly, PBD2 and a normal control line were co-cultured in different proportions and then assayed for VLCFA content (CME3). Results in fig 1B indicate that at 25% contamination the C26/C22 was definitively abnormal. At 37.5% contamination C26/C22 was still higher than the control range and only at 50% contamination did the C26/C22 approach the upper normal control range. Thus, both of the interrogated biochemical methods had high thresholds for cell contamination.

Microsatellite analysis detects low level contamination in mixed DNA samples

We used DNA mixing to ascertain the sensitivity of PCR based genotyping of microsatellite markers ID1, ID2, and ID3. The ''contaminating'' DNA was added in proportions of 1, 5, 10, and 15% as explained above. The data obtained for all three markers are shown in fig 2. The contaminating (C) allele was consistently detectable at 1% contamination for ID2 only, but was evident for all three markers at 5% contamination. In addition, we determined the area under the curve for each primary allele peak and then calculated the proportion of the C allele to the non-contaminating (N) allele, which theoretically should be increasing and decreasing reciprocally. This calculated percentage (C/(C+N)) roughly correlates with the percentage of DNA contamination (table 3). This experiment demonstrates that low level (5–10%) DNA contamination is within the detection limits of our method.

Table 2 Biochemical analyses in a CVS with MCC

	VLCFA content		Plasmalogen synthesis
	C26:0 (μg/mg protein)	C26:0/C22:0	$^3H/^{14}C$
Case 1, CVS*	0.057	0.162	0.595
Case 1, AF*	0.369	1.212	6.032
Case 1, fetal fibroblasts*	0.416	0.923	6.607
Normal CVS*	0.07 (0.04)	0.12 (0.07)	0.74 (0.20)
Normal AF*	0.10 (0.07)	0.17 (0.10)	0.61 (0.11)

CVS, chorionic villus sample; AF, amniotic fluid cells; *Cultured cells. VLCFA: CVS control n = 20 and AF control n = 23; plasmalogen biosynthesis: CVS control n = 308; AF control n = 240.

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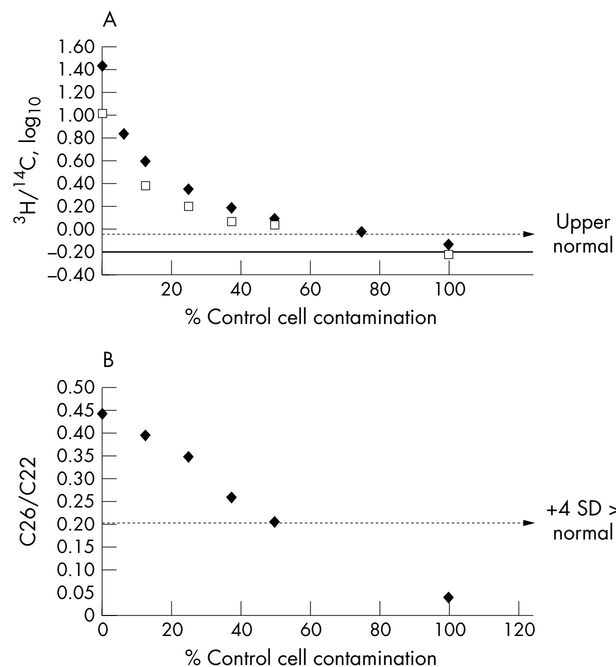

Figure 1 Biochemical analysis of fibroblast admixtures. Plasmalogen synthesis activity (^{3}H/^{14}C) is reported on a log scale. PBD1 (diamonds) is more deficient, and thus has a higher ratio than PBD2 (squares). Both cell lines have a ratio indicative of a moderate deficiency at 50% control cell contamination. (B) PBD2 has a significant elevation of C26/C22 at 25% contamination, consistent with a perturbation of peroxisomal fatty acid metabolism. Although not as compelling, the ratio is significantly higher than the normal control level at 37.5% contamination.

Microsatellite analysis identified ''contaminating'' alleles in co-cultivated fibroblasts

To demonstrate that the sensitivity of the molecular analysis in co-cultivated cells correlated with direct DNA mixing experiments, admixtures from CME1 were harvested for DNA isolation and microsatellite analysis. The alleles labelled C and N (fig 3) were used as markers for detecting the contaminating (CTL1) and the non-contaminating (PBD1) cell lines, respectively. The N and C alleles for ID3 provided optimised conditions for determining the presence or absence of either cell line. In contrast, stutter peaks for ID1 and ID2 made it impossible to demonstrate that at 100% contamination there was no residual DNA from PBD1. We compared the area under the curve of C/(C+N) for each ID marker to estimate the contamination of PBD1 by CTL1 (table 4). These values correlated well with the percentage of contamination based on cell counts (correlation coefficient = 0.998). Most importantly, the DNA test identified the C allele for all three markers at 6.25% contamination, again highlighting the sensitivity of this method for detecting low level contamination.

ß-galactosidase activity in deficient cells is sensitive to low level contamination

Cultured fibroblasts from a Krabbe disease patient (KD1) had ß-galactosylcerebrosidase activity <2% of normal control activity (0.5 *v* 30.8 nmol/17 hours/mg protein). Fibroblasts from the mother of this patient had enzyme activity at only 19% of normal control levels (5.7 nmol/17 hours/mg protein), far below the ~50% expected for a heterozygote. These results show that the carrier range for some families requiring biochemical prenatal diagnosis may be significantly lower than normal control activity. Co-cultivation of fibroblasts from the proband and normal control cells showed that

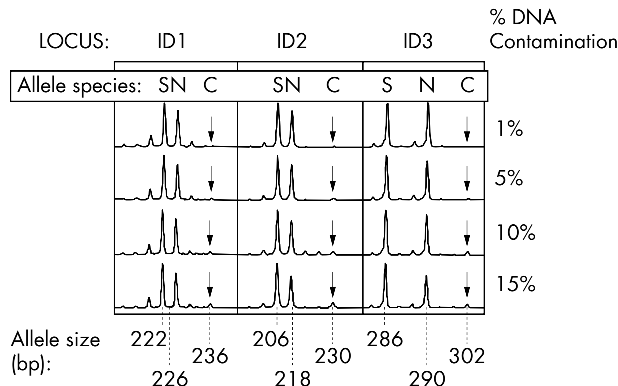

Figure 2 DNA mixing: gene scan analysis at three loci. S, shared allele; N, non-shared allele; C, contaminating allele. Arrows indicate the C alleles. An allele C peak is detected at 5% contamination for all three markers, but ideal peak morphology is not present until 10% DNA contamination.

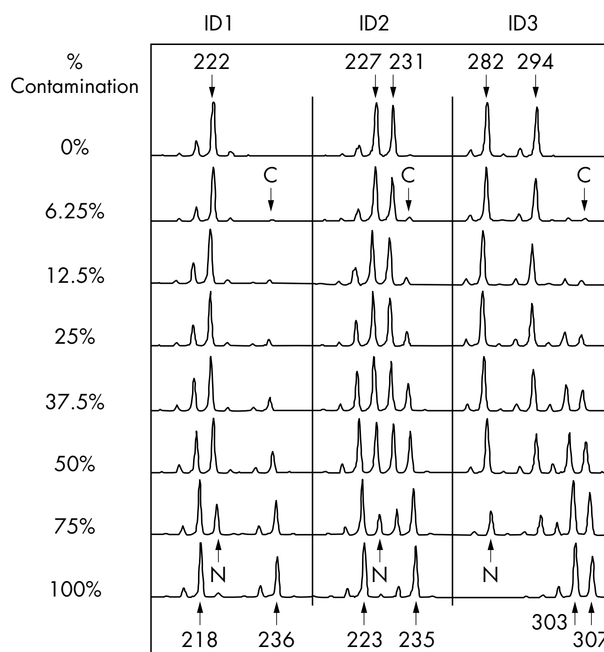

Figure 3 Cell mixing: gene scan analysis at three loci. N, non-contaminating allele; C, contaminating allele. The size of each allele is indicated at 0% or 100%, each representing the genotype of PBD1 and CTL1, respectively. The C and N alleles that were optimal for distinguishing PBD1 DNA from CTL1 DNA are identified in panels for 6.25% and 75% contamination, respectively. Only ID3 has a favourably positioned C and N allele that can distinguish the presence or absence of either cell line at the lowest levels of DNA contamination.

Table 3 Calculated DNA contamination by microsatellite analysis

% contaminating DNA added	% contamination*		
	ID1	ID2	ID3
1	0	0.7	0
5	3.3	6.1	4.1
10	7.5	10.7	7.1
15	10.5	15.9	10

*% cell contamination = $(C_{area}/(C_{area}+N_{area}))\times 100$.

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Table 4 Proportion of cell contamination in co-cultivated fibroblasts

Based on cell counts	Microsatellite markers* ID1	ID2	ID3	Mean of three markers
0	0	1	0	0.3
6.25	5.7	7.2	4.6	5.8
12.5	11.9	12.9	8.7	11.2
25	22.5	22.6	18	21
37.5	33.8	34.8	28.1	32.2
50	45.2	47.1	39.5	43.9
75	70.3	70.1	66.2	68.9
100	97.4	94.1	100	97.2

*% cell contamination = $(C_{area}/(C_{area}+N_{area}))\times100$.

5–10% contamination was sufficient to raise the total activity from the affected range to the carrier level (fig 4). In contrast, when the two cell lines were mixed but not cultured prior to assay, the total ß-galactosylcerbrosidase activity did not reach the carrier level till 20% contamination (fig 4). To determine whether release of soluble enzyme by control cells followed by uptake by the deficient cells accounted for this difference, we added conditioned media (recovered from normal control cell flasks) to KD1 cells and then measured enzyme activity. However, exposure to conditioned media did not increase enzyme activity in KD1 fibroblasts (results not shown). Overall, these studies indicate that the threshold for MCC is much lower in Krabbe disease cells and, thus, a more sensitive molecular method would be required to eliminate the risk of mistaking an affected fetus for a carrier.

DISCUSSION

Questions addressed

Historically, prenatal diagnosis for inborn errors of metabolism has been performed using biochemical analyses. Identification of the genes responsible for many of these disorders permits prenatal diagnosis by DNA analysis. However, limitations in resources for mutation identification, inability to identify disease causing mutation(s), and/or insufficient time to perform molecular studies on an at risk family mean that prenatal diagnosis is still commonly performed by biochemical analyses. Analysis of fetal cells obtained from CVS provides the advantage of yielding a result earlier in the pregnancy. In general, though, CVS is more vulnerable to MCC than amniocentesis. Thus, it is critical to rule out the possibility of MCC, which can be achieved by comparing polymorphic loci in the maternal and fetal DNA samples. In order for both the molecular and biochemical analyses to be most effective, it is important to determine the limits of each assay. Thus, we addressed the following questions. (*a*) What is the sensitivity of the molecular ID marker test—that is, what is the lowest level DNA contamination that can be reliably detected? (*b*) What is the threshold of the two biochemical assays most commonly used for biochemical prenatal diagnosis of peroxisomal disorders (VLCFA content and plasmalogen biosynthesis)—that is, would a low or intermediate percentage of MCC lead to a false negative or positive result?

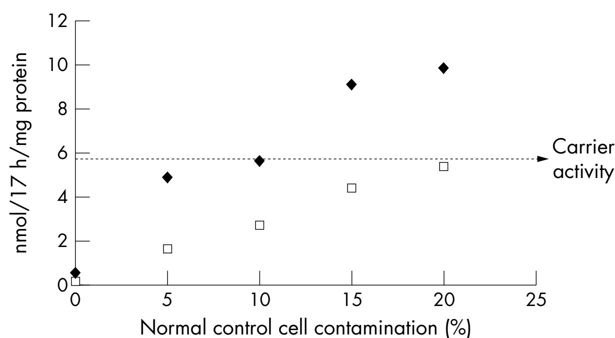

Figure 4 Cell mixing: ß-galactosidase activity. The activity of cultured admixtures (diamonds) are compared with non-cultured admixtures (squares) in fibroblasts from a Krabbe disease proband contaminated with varying proportions of CTL2. At 10% contamination, the cultured cells have the same activity as the carrier mother. In contrast, the non-cultured cells do not yield this much activity until 20% contamination. The activity of the heterozygous fibroblasts did not change between cultured and non-cultured conditions (5.7 *v* 5.9 nmol/17 hours/mg protein, respectively). Normal control cultured fibroblast activity = 23.7 (4.4) nmol/17 hours/mg protein (n = 5, range 20.2 to 30.8).

Types of errors associated with maternal cell contamination and biochemical diagnosis

Two types of errors may occur when cells of maternal origin multiply in a culture that is supposed to be solely of fetal origin. We define type I error as MCC of cultured cells from an affected fetus with biochemically normal maternal cells. Thus, type I error is equivalent to a false negative result. Type II error is defined as MCC of cultured cells from an unaffected fetus with maternal cells expressing a biochemical deficiency. Thus, type II error is equivalent to a false positive result. The type of error for which a given pregnancy is at risk depends partly on the type of analysis performed, and whether carriers for the disorder partially express a defect and if so, to what degree.

PBDs are inherited in an autosomal recessive manner. Carriers for these disorders do not have measurable biochemical deficiencies. Although there may be unrecognised, subtle functional manifestations of *PEX* gene mutations on peroxisome assembly in the carrier state, the biochemical pathways are not measurably perturbed. Thus, biochemical prenatal diagnosis for these disorders is only at risk for type I error. On this basis, the 26 CVS cultures with biochemical results consistent with a PBD did not require testing to rule out MCC because there is no biological basis for MCC introducing a false positive result. Overall, this means that 2.5% (2/79) of cases considered at risk for MCC postbiochemical testing were found to have significant MCC.

In contrast, X-ALD is an X linked disease and thereby at risk for both type I and II errors. This arises because X-ALD carriers are mosaic for two cell populations owing to random X inactivation. 80% of X-ALD heterozygotes have increased VLCFA in plasma and cultured fibroblasts.[17] We have previously demonstrated that carriers of a contiguous deletion syndrome involving *ABCD1* had normal plasma and fibroblast VLCFA and favourable skewing of X inactivation.[18 19] Females with skewed X inactivation would be at greater risk for different types of error in association with MCC, depending upon the direction of skewing and the genotype of the fetus. For example, an X-ALD carrier with 95% inactivation of the normal X would be at greater risk for type II error when carrying a normal male fetus. We have previously observed this phenomenon in a male fetus at risk for X-ALD. The cultured cells derived from CVS biopsy had elevated VLCFA, but a follow up CVS and postnatal plasma samples had normal VLCFA (results not shown).

Sources of error for biochemical prenatal diagnosis

In addition to the variation in activity observed in heterozygotes for X linked disorders, carriers of some autosomal recessive disorders may also express a partial defect. For example, pseudodeficiency alleles for Tay-Sachs disease,[20] metachromatic leukodystrophy,[21] and Krabbe disease[10] genes are associated with significant reductions in enzyme activity. Our cell mixing experiments using Krabbe disease and

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normal control fibroblasts showed that only 5–10% of contamination raised the total activity to the low carrier range (16–18% of normal). Thus, a relatively small proportion of contaminating cells could produce a misleading result for this defect. In contrast, when cells from the proband and normal control were mixed but not cultivated, the total activity did not reach the low carrier range until 20% contamination. This difference could arise due to preferential growth of the normal cells or cross correction of the deficient cells by normal cells releasing the lysosomal enzyme into the media, although we did not find evidence for this. Overall, our findings suggest that the susceptibility of cultured cells to MCC should be greater than direct biopsy tissue, although Harzer and Schuster[22] reported problems with using uncultured villi for the prenatal diagnosis of Krabbe disease.

Biochemical methods for lysosomal storage disorders may have a much lower threshold for MCC and, consequently, highlight the importance of having a method that can detect very low level MCC. A more quantitative approach to detection of MCC may be required for the prenatal diagnosis of these disorders. A large proportion (81%) of the biochemical prenatal diagnosis cases (283 total) reported in the US collaborative CVS study were for lysosomal storage disorders.[10] One of the 11 CVS analyses for Krabbe disease reported in that study had uninformative biochemical tests and resulted in the birth of an affected child. Although the father was reported to have a pseudodeficiency allele, it was not clear if the equivocal biochemical result was due to MCC, higher than usual residual activity in one mutant allele, or a combination of the two. In general, the concerns associated with allelic variation underline several important points: (*a*) the benefits of turning to mutation analysis for prenatal diagnosis of inborn errors of metabolism with well characterised gene defects, (*b*) the increased risk of type I error in the presence of low level MCC when performing prenatal diagnosis using biochemical methods; (3) the need to characterise the biochemical defects in proband and parents prior to prenatal diagnosis, and (4) the necessity of establishing the limits of both the biochemical and molecular assays.

Sensitivity of PCR based genotyping of microsatellite markers

To determine the sensitivity of the microsatellite analysis for each of our three markers, we mixed fixed proportions of DNA from two individuals known to share only one allele at a given locus. The absolute amount of each DNA is equivalent to a particular number of cells. Based on the estimate that 7 μg of genomic DNA is isolated on average from 10^6 cells, then 100 ng of DNA per PCR represents ~14 000 cells. Thus, 1% contamination is equivalent to 1 ng of genomic DNA or about 140 cells. We detected 5% DNA contamination for all three markers (700 cells). However, the peak detected for contaminating alleles did not achieve definitive peak morphology that distinguished it sufficiently from background until 10% contamination (~1400 cells). In a diagnostic situation, the presence of the small peaks associated with <10% contamination would require a qualified assessment such as ''significant contamination not detected'' or ''evidence of minor contamination''.

Assessing the area under the curve can provide only an estimate for the percentage of contamination in a nonquantitative PCR. However, comparing the proportion of the contaminating allele (C) to the reciprocally diminished allele (N) in mixed DNA samples provided a reasonable assessment of the degree of DNA contamination (table 1). Similarly, making these comparisons in the fibroblasts co-cultivated at varying proportions of contamination (table 4) provided a reasonable guide for how much contamination was present. Thus, it could be estimated whether the contamination is relatively minor (<10%) or significant (>25%). This leaves a grey zone of uncertainty between 10 and 25%, and thus highlights the need to know the sensitivity of the biochemical assay being used in conjunction with MCC testing.

Threshold of the biochemical assay

Measurement of both VLCFA content and plasmalogen synthesis are robust assays for prenatal diagnosis. Our cocultivation studies showed that Zellweger syndrome cells contaminated with 25% normal control fibroblasts had results in the affected range for both tests. This information has practical relevance, because PCR linkage analysis reliably detects DNA contamination starting at 10%. Thus, the thresholds of the biochemical assays are well within the sensitivity limitations of the DNA analysis.

Nonetheless, we still advocate careful documentation of the biochemical defects in cultured fibroblasts from the proband prior to using biochemical analysis for prenatal diagnosis. Although we demonstrated that a cell line with a moderate deficiency in plasmalogen synthesis is resilient to ⩾25% contamination, there are PBD patients with normal fibroblast plasmalogen synthesis. Thus, the significance of low level MCC could still be influenced by the extent of the biochemical defect documented in the proband. Although one could be cautiously optimistic about a normal result in the presence of ~20% MCC in CVS from a pregnancy at risk for a classical PBD, it would be prudent to recommend follow up with an amniocentesis for confirmation. On the other hand, if no significant MCC is detected (<10%), it is highly likely that the biochemical results are accurate and one could confidently predict the fetus is not affected with a PBD.

A model for correlating detection of MCC by DNA analysis with biochemical methods

Our experience with the use of cultured CVS for the prenatal diagnosis of peroxisomal disorders highlights the risk of MCC and its potential consequences if unrecognised. However, both of these cases involved MCC ⩾80%. Low level MCC is a potentially more insidious source of diagnostic error, because inadequate testing may be requested to rule it out and its danger may be underappreciated when interpreting the biochemical results. These hazards prevail unless steps are taken to correlate the sensitivity of the DNA test and the threshold of the biochemical method. We demonstrated that the sensitivity of PCR based genotyping of our markers is more than adequate for the threshold of the two biochemical methods we use routinely for the prenatal diagnosis of peroxisomal disorders. However, our comparison of the same molecular approach to the biochemical assay for Krabbe disease failed to establish suitable sensitivity. As little as 5–10% contamination was sufficient to shift the biochemical result of majority deficient cells into the carrier range. Thus, we would not advocate using the microsatellite method for the detection of MCC in a pregnancy at risk for Krabbe disease. It is incumbent upon the laboratory offering prenatal diagnosis for biochemical disorders to establish the threshold of their methods to determine the potential consequences of MCC ranging from 1 to 25%, and on this basis to choose the appropriate approach for detection of MCC.

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