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FIVE YEARS' EXPERIENCE OF PRENATAL DIAGNOSIS OF CYSTIC FIBROSIS IN THE FORMER U.S.S.R.

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SUMMARY

From a total of 490 cystic fibrosis (CF) high-risk families under supervision (mostly Russian Slavs from the European part of the country), DNA data including both direct screening for some CF gene (CFTR) mutations (delF508, G551D and 1677delTA) and allelic polymorphism studies with tightly CF linked DNA markers were collected from 261 families. All full families (129) and 86 CF families with a deceased index child were found to be either fully (42 per cent) or partially (40 per cent) informative for DNA analysis. Prenatal diagnosis (PD) was carried out in 161 CF families. Microvillar enzyme (MVE) assay was applied to all 140 PD at the second trimester either as a single test (88) or in conjunction with DNA analysis (52). The frequency of false-negative results of the MVE assay was 1·3 per cent and that of false-positive results, as judged by the albumin meconium test, was 5·0 per cent. Ambiguous results of MVE analysis were found in 30 cases, 12 of which were verified by DNA analysis. Molecular diagnosis of CF at the first trimester was carried out in 21 cases and four pregnancies were terminated. Altogether, 39 pregnancies with a predicted high risk of CF fetuses were terminated. The low average frequency of delF508 in CF chromosomes of Russian Slavs (50 per cent), its remarkable inter-population variation, and the significant proportion of at-risk families without an affected child determine the necessity of combined molecular and biochemical (MVE assay) approaches for efficient prenatal diagnosis of CF in the former U.S.S.R.

KEY WORDS Cystic fibrosis CFTR gene mutations RFLP Prenatal diagnosis Microvillar enzymes

INTRODUCTION

Cystic fibrosis (CF) is one of the most common autosomal recessive diseases, affecting about 1 in 2500 livebirths in Caucasian populations. The actual incidence of CF in different populations and ethnic groups of the former U.S.S.R. still remains rather obscure. According to some pilot clinical observations, it varies in different Caucasian communities from 1 in 2000 to 1 in 4500 (Kapranov, 1986). This means that 3500–4000 CF children are born annually in this country. Meanwhile, the absence of proper screening programmes with the common sweat test or immunoreactive trypsin does not permit an estimation of the true genetic load of CF gene mutations in our populations. Prenatal diagnosis (PD) of CF in the former U.S.S.R.

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Table 1. Ethnic distribution of CF high-risk families under supervision of the All-Union Centre by 1 January 1991

Population (nationality)	No. of families
Slavs	444
Russians	402
Ukranians	33
Belorussians	8
Polish	1
Moldovians	12
Tatars	6
Georgians	7
Jewish	6
Armenians	3
Udmurdians	2
Buriates	2
Komi-permiaky	2
Chuvashians	1
Mordovians	2
Lithuanians	1
Uzbeks	1
Gypsies	1
Total	490

was launched in 1986. Since then, owing to advances in molecular analysis of CF, the PD of this severe disease in the former U.S.S.R., as well as throughout the world, has passed three successive stages: biochemical analysis of microvillar intestine enzymes (MVE) in amniotic fluid (Brock *et al.*, 1984, 1988), RFLP analysis with closely linked DNA markers (Spence *et al.*, 1987; Strain *et al.*, 1988) and direct identification of CF gene (CFTR gene) mutations, especially delF508 (Carey *et al.*, 1990).

We summarize here the results of molecular analysis of CF high-risk families and PD of CF in the former U.S.S.R. for a 5-year period up to January 1991 with the assistance of diagnostic tests available at the time of management.

MATERIALS AND METHODS

Families and sampling

The ethnic distribution of CF high-risk families under the supervision of our centre is shown in Table 1. Of the 490 families at risk (predominantly (402) of Russian Slavs by origin), 387 couples were considered amenable for PD. Blood samples were collected from both parents in 132 and from the mother, father, and index child in 129 CF families.

DNA preparations were made by routine methods and were used for allelic polymorphism studies and for CF gene mutation (delF508, 1677delTA, G551D, R553X, R334W) detection. For PD, 161 pregnancies were tested in this study: 21 at the first and 140 at the second trimester.

Table 2. RFLPs detected by Southern blotting (Sb), polymorphic sites studied by the polymerase chain reaction (PCR), and DNA probes used in this study

Locus	Probe	RFLP	Method of detection	Alleles	Size (kb)
MET	Met H	Msp 1	Sb	1	6·5
				2	2·3
				3	1·8
			PCR	M1	0·414
				M2	0·220/0·194
D7S8	J3.11	Msp 1	Sb	1	4·2
				2	1·8
IRP	XV.2c	Taq 1	Sb	1	2·1
				2	7·4
			PCR	X1	1·0
				X2	0·6/0·4
D7S23	CS7	Hind 1	PCR	C1	0·95
				C2	0·65/0·30
	KM-19	Pst 1	PCR	K1	0·330
				K2	0·165/0·165
D7S399	pMP6d9	Msp 1	PCR	P1	0·377
				P2	0·175/0·200
			Sb	1	13·0
				2	8·5/4·5

Chorionic villus samples were taken by either the transcervical or the transabdominal route under ultrasonic guidance, at an average gestational age of 10·3 weeks. Transabdominal placentocentesis with concomitant amniocentesis and withdrawal of about 25–30 ml of amniotic fluid was performed between 18 and 21 weeks of gestation.

DNA analysis of blood samples

Both Southern blot analysis and the polymerase chain reaction (PCR) were used for the RFLP studies of the polymorphic sites given in Table 2. DNA probes J3.11, CS7, KM-19, MP6d9, XV.2c as well as oligosequences and digest information for the primers used (D7S8, KM19, CS7, Met H and mutations G551D, R553X, R334W) were generously provided by Professor R. Williamson (London), and the probe Met H was a gift from Dr Ray White (U.S.A.). Oligonucleotide sequences for MP6d9 as well as for detection of delF508 were kindly supplied by Dr Michael Dean (Frederick, U.S.A.). The latter primers were found to be quite suitable for the detection of our native CFTR gene mutation 1677delTA as well (Ivaschenko *et al.*, 1992). DNA extraction, restriction digestion, agarose gel electrophoresis, Southern blotting, and hybridization were performed according to conventional protocols. The technique used for PCR and mutation detection has been described previously (Baranov *et al.*, 1991a,b). Allelic polymorphism studies performed with the Southern blotting technique or by PCR gave quite identical results, which are combined.

Microvillar intestinal enzyme (MVE) assay

The microvillar enzyme activities tested on amniotic fluid (AF) were gamma-glutamyltranspeptidase (GGTP), aminopeptidase (APM), and phenylalanine-inhibitable alkaline phosphatase (I-ALP). All measurements were performed on freshly withdrawn cell-free AF according to protocols suggested by Brock (1985). MVE activity for each sample was compared with a standard curve based on 100 measurements of control amniotic samples at equivalent stages of pregnancy (negative control) as well as those of AF samples from CF fetuses (positive control). The latter were initially provided through the courtesy of Professor David Brock (Edinburgh, U.K.). For optimal diagnostic discrimination, the three enzyme values for each sample were combined into a single linear discrimination function (Mulivor *et al.*, 1987). To exclude false-positive results, the postmortem examination of a fetus thought to have CF included measurement of albumin in meconium extract (Brock and Barron, 1986).

DNA analysis of CVS and amniocytes

An original modification of PCR amplification directly from chorionic villus (CV) samples, amniotic cells, and scrapings of cytological preparations was used throughout for studies of allelic polymorphism and mutation identification (Ivaschenko *et al.*, 1991). Briefly, a small fragment of CV (about 1 mm) was washed twice in 1 ml of solution 1 (SSC, 10 mM EDTA) and introduced directly into the PCR mixture (25 μl: 16·6 mM $(NH_4)_2SO_4$; 67 mM Tris–HCl, pH 8·8; 6·7 mM $MgCl_2$; 10 mM β-mercaptoethanol; 6·7 μM EDTA, pH 8·0; 1·7 μM SDS; 1–1·5 U Taq-DNA polymerase (Thermus thermophilis); 1·25 mM each dNTP). For amniotic fluid, 1 ml of a freshly withdrawn sample was spinned down for 5 min; the pellet was washed twice with 1 ml of solution 1 (see above); the cells were gently resuspended in a few drops of the last washing solution; and 1 μl of this suspension was used for PCR. The scrapings of nuclei from cytological preparations were obtained by a small syringe needle under visual control of a stereomicroscope. The tip of the needle was plunged into the PCR mixture just after each scraping. About 300–500 scraped nuclei were usually enough for one PCR. The amplification was performed as on purified DNA using a Perkin-Elmer Cetus Thermal Cycler or a PRS-1 Dry-Block (Techne, Cambridge, U.K.).

RESULTS

Molecular analysis of CF families

All 261 at-risk families with some of them (88) previously subjected to PD by MVE assay alone (see below) were used for molecular studies which included both major mutation identification and RFLP analysis.

The identification of delF508 provided the major impact for both fully and partially informative CF families (28 and 44 per cent, respectively, Table 3). The overall frequency of delF508 in this cohort of CF chromosomes is thus very close to the expected 50 per cent (259 out of a total of 522). The contribution of some other known major CFTR gene mutations (G551D, R553X, R334W, 1677delTA) to disease in our CF families is rather small (Table 3). Each of them was found in only

Table 3. Informativeness of 261 CF families as judged by mutation identified

Informativeness	ΔF508	1677delTA	G551D or R553X	R334W	ΔF508 and R334W and 1677delTA and G551D (R553X)
Fully	73	1	—	—	77
Partially	113	2	3	1	116
Not found	75	258	258	260	68

Table 4. Number of families fully informative with different RFLP systems alone and in combination with ΔF508 deletion

RFLP probe/enzyme	Total No. of families	Single RFLP	In combination with other RFLPs	RFLPs + ΔF508
Met H/Msp 1	27	2	14	25
J3.11/Msp 1	18	2	10	13
XV.2c/Taq 1	11	3	11	11
CS7/Hind 1	59	19	32	49
KM-19/Pst 1	42	16	22	30
MP6d-9/Msp 1	30	8	22	30

one parent of the family. 1677delTA was demonstrated in both parents of the same couple of Georgian origin. As might be inferred from Table 3, altogether 74 per cent of all CF families studied are fully or partially informative by direct mutation tests.

Out of 92 at-risk families with an index child subjected to RFLP analysis of polymorphic sites indicated in Table 5 (11 of them were tested for one polymorphic site, 32 for two, 23 for three, 14 for four, 8 for five, and 6 for six sites), 69 were found to be fully and 29 partially informative. As shown in Table 4., the informativeness of each particular polymorphic site tested varied significantly (from 7·4 per cent for Met H-Msp 1 to 35·7 per cent for KM-19/Pst 1). If applied in combination, the informativeness of RFLP analysis increased dramatically, with a significant proportion of the families (60 per cent on average) being totally informative. All the families got full or partial informativeness if RFLP analysis was applied in combination with delF508 detection.

Altogether, 215 out of a total of 261 CF families under supervision were actually valid for PD by molecular methods. This group included 129 full CF families with an index child as in more than a half (86) of at-risk couples the index child was already deceased. Meanwhile 46 CF families of the latter group did not reveal any major CF mutations tested so far and thus still remain non-informative for PD by molecular methods. Subsequent RFLP analysis of these families disclosed a significant non-random distribution of marker alleles for almost all polymorphic sites studied (Baranov *et al.*, 1992) with an obvious preponderance of haplotype B (allelle 2—restriction site plus for KM-19-Pst 1; allele 1—absence of restriction site – for

Table 5. Prenatal diagnosis of CF in 161 at-risk families of the former U.S.S.R.

Year	MVE test alone	MVE test and RFLP analysis	MVE test and mutation detection	Major mutation detection	Mutation and RFLP analysis	RFLP analysis alone
1986	5					
1987	23	4				
1988	34	4				1
1989	18	6	5	2	1	2
1990	8	—	33	9	6	—
Total	88	14	38	11	7	3

XV.2c/Taq 1). The strong linkage disequilibrium between haplotype B and the CF gene (Strain *et al.*, 1988) was taken into account in the PD of all pregnancies at risk with equivocal results of the MVE assay.

PD of CF in couples at risk

Table 5 lists 161 prenatal diagnoses (140 in the second trimester and 21 in the first trimester of pregnancy) performed in our centre since 1986 and up to 1 January 1991. Since 1987 the standard MVE assay has been supported by RFLP analysis and the latter in 1989 by direct mutation detection as well. In spite of definite fluctuations, there is an obvious increase in the number of CF families admitted for PD each year, with 56 cases of PD in 1990. Altogether, 39 (24·2 per cent) fetuses at risk were found to be affected and 37 of them were aborted. Two women with a predicted CF fetus refused termination of pregnancy and gave birth to affected children. CF diagnosis was confirmed clinically as well as by meconium and sweat tests at age 6 months in both cases.

PD with MVE assay

MVE assay for PD of CF has been used in our centre since 1986. Its application as a single test gradually decreased after 1988 when it was routinely used in combination with molecular analysis (Table 5). In most cases of this group (70 out of 88), the three MVE patterns evaluated showed a high degree of concordance and were either all above or all below the cut-off level of 0·5 times the median at the relevant gestational week (Brock *et al.*, 1988). These data were in full agreement with the corresponding values of a single linear discrimination function Zb ($<2{\cdot}60$ presumed CF; $>2{\cdot}60$ presumed normal), thus indicating the expected prediction confidence of about 95–96 per cent. All the latter women but one gave birth to normal healthy children. Meanwhile, one pregnancy with quite normal values of MVE assay ended in the birth of a live child with very early (age of 6 months) manifestations of the chest form of CF without any obvious pancreatic involvement. Predictive values of the test were less conclusive in 18 other prenatal diagnoses (confidence values below 95 per cent). The absence of DNA methods during that period at our centre made molecular verification of these cases impossible. In one of them, normal values of APM and slightly raised levels of I-ALP were associated with twice as low a value of

GGTP. This pregnancy also led to a CF child. Up to now, these are the only two cases of a false-negative prenatal diagnosis of CF at our centre.

MVE assay plus genotyping diagnosis

Since 1987, some and usually not perfectly conclusive MVE assays have been complemented with DNA analysis. Initially, the latter was confined exclusively to RFLP studies; however, both RFLP studies and mutation identification techniques have been applied since later 1989 (Table 5).

Of the total 52 PD in this group, 32 showed good concordance of MVE assay values and genotyping analysis. In 12 other cases with equivocal MVE assay values, PD relied exclusively on DNA analysis (Table 6). One fetus presumed to be CF-affected with MVE assay was actually found to be 100 per cent normal by DNA analysis. Six more false-positive prenatal diagnoses out of a total of 140 second-trimester PD were registered with the assistance of the albumin meconium test (Brock and Barron, 1986). Thus, the actual frequencies of false-negative and false/positive results of PD with MVE assay in our studies were $1{\cdot}3 \pm 0{\cdot}6$ and $5{\cdot}0 \pm 2{\cdot}8$ per cent, respectively.

DNA analysis could not be applied in eight other pregnancies because the affected child was already deceased or because the major CFTR gene mutations were absent in both parents. MVE assays of these samples were substantiated with RFLP analysis. Two fetuses with a 90 per cent probability of CF by the MVE test were found to be homozygous for haplotype B (see above). Because of the high correlation of this particular haplotype with CF (Strain *et al.*, 1988; Holloway and Brock, 1989), the pregnancies were terminated and the CF diagnosis was confirmed by the albumin meconium test.

Genotyping diagnosis in first-trimester pregnancies

The first successful attempt of PD or CF by molecular tests alone was achieved at our centre in 1988 (Table 5). Table 7 lists 21 PD performed so far at 9–11 weeks of gestation by 1 January 1991. Four fetuses, including one case of a twin pregnancy with both fetuses affected, were ascertained to have CF with confidence close to 100 per cent and they were all aborted. In the remaining 17 cases, the fetus was predicted to be unaffected, with seven of them being obligatory heterozygous carriers. Fifteen presumed normal fetuses have been born and confirmed to be free of CF. Two more pregnancies with presumably normal fetuses were aborted after transcervical CVS which was applied almost exclusively by 1989.

DISCUSSION

Spectacular achievements in the molecular biology of CF (identification of the CFTR gene and its numerous mutations, including the common delF508) (Rommens *et al.*, 1989; Kerem *et al.*, 1989), RFLP analysis of CF-linked DNA markers, and their linkage disequilibrium with the CFTR gene (Estivill *et al.*, 1988) have made a major impact on our understanding of the molecular entity of CF as well as its prenatal diagnosis.

According to our prospective and retrospective (after PD with MVE assay alone) DNA studies of all 261 families at risk of CF, combined application of tests for

Table 6. Prenatal diagnosis of CF with equivocal MVE assay values verified by DNA analysis in the second trimester of pregnancy

Case No.	Gestational age (weeks)	MVE values			Tentative risk of CF (%)	DNA analysis RFLP	Informativeness CFTR-gene mutations	Final diagnosis predicted	
		GGTP (U/l)	APM (U/l)	I-ALP (U/l)				Normal	Affected
1. AK	17	214	73	17	10	−	+	+	−
2. MA	17–18	144	63	6	10	−	+	+	−
3. PI	17–18	142	66	12	10	−	+	+	−
4. RA	18	148	36	4	20	−	+	−	+
5. MU	18	200	65	8	10	−	+	+	−
6. TR	18–19	108	26	3	50	−	+	+	−
7. JO	18–19	96	60	42	50	+	−	+	−
8. PE	19	144	38	4	10	+	−	−	+
9. USH	19–20	103	30	10	10	−	+	+	−
10. LY	20	71	73	16	10	+	+	−	+
11. AB	20–21	98	25	2	50	+	+	−	+
12. RE	24	46	69	29	50	−	+	+	−
13. CA	21–22	53	9	1	50	−	+	+*	

*DNA analysis of aborted fetus (false-positive MVE test results).

Table 7. Results of prenatal diagnosis by DNA analysis in the first trimester of pregnancy

Total No. performed 21	100% informative 16	Partially informative 5	Predicted normal	Predicted affected
Affected (mutations)	2	—	—	2
Affected (RFLP + mutations)	1	1	—	2
Not affected (RFLP)	1	1	2	—
Not affected (mutations)	9	3	12	—
Not affected (RFLP + mutations)	3	—	3	—

major mutations supplemented, if necessary, with complex RFLP analysis can provide information for PD in practically all families with a live index child, and in a significant proportion (over 60 per cent) of families with a deceased affected child. Meanwhile, many families of the latter group remain non-informative and thus cannot be subjected to PD by DNA methods alone. The absence of an index child in more than a half of all at-risk families known so far still remains a major problem for the efficient PD of CF in the early stages of pregnancy. This problem stems from existing drawbacks in the early diagnosis of CF (mainly due to the absence of an adequate screening programme of newborns) and from the poor survival (life span less than 5 years) in a significant proportion of CF children in the former U.S.S.R.

Another obvious limitation in the molecular diagnosis of CF arises from the relatively low rate of delF508 in CF chromosomes of Russian Slavs (only about 50 per cent) and its even much smaller frequencies in some other populations and ethnic groups of the former U.S.S.R. studied so far (Baranov *et al.*, 1991). The search for the other major mutations in the native populations of our country is now in progress. Only a few major mutations (G551D, R553X, R334W) have been detected so far (Table 3), indicating them to be rather rare in CF chromosomes of our patients. On the other hand, the new mutation 1677delTA has been found in at least three non-related Georgian families as well as in two of 45 population samples of Georgian origin. A few more cases of 1677delTA have been recently reported in some Turkish and Bulgarian CF patients (Kaladjieva *et al.*, 1991). Thus, 1677delTA seems to belong to the group of major mutations destined to the Black Sea populations (Ivaschenko *et al.*, 1992; Kaladjieva *et al.*, 1991). The search for the other major CFTR gene mutations in different ethnic groups of the former U.S.S.R. thus seems to be important and highly promising. High precision and the possibility of early diagnosis, even before implantation (Strom *et al.*, 1990), are great advantages of the molecular diagnosis of CF. Meanwhile, as our experience shows, so many couples at risk of a CF child are submitted for PD only at the second trimester of pregnancy. Both MVE assay and DNA tests have been applied for these cases at our centre since 1987. The good concordance of both tests is quite evident in almost

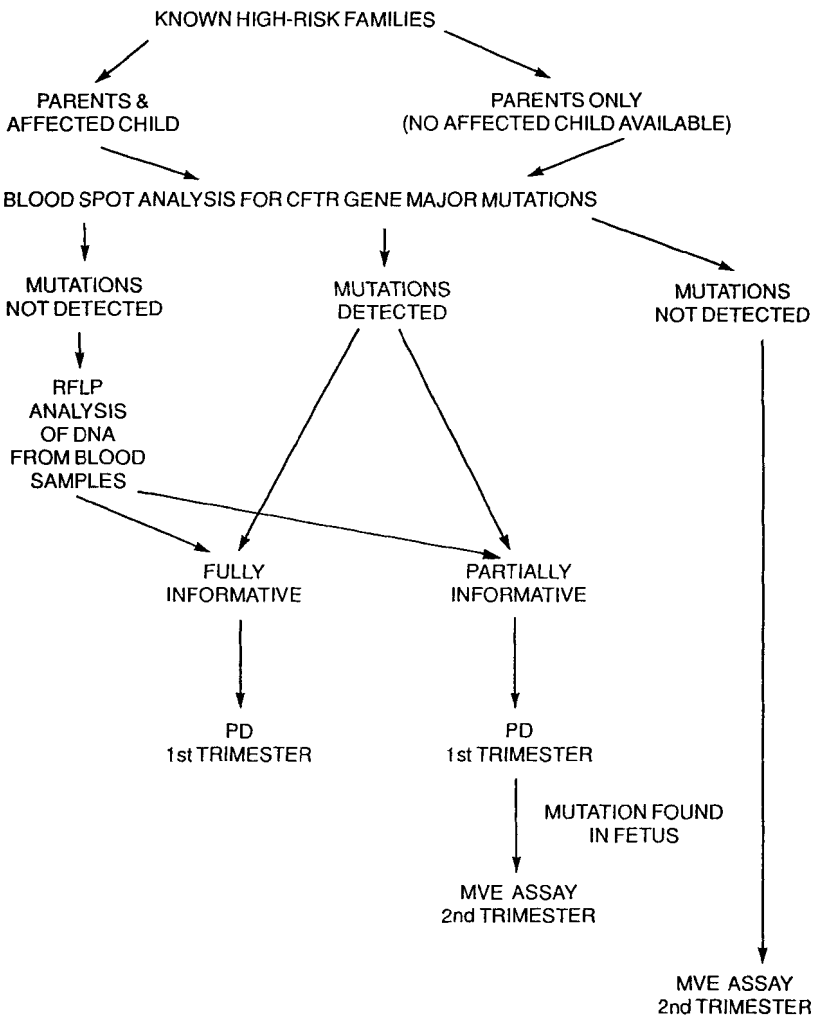

Figure 1. Principal strategy of prenatal diagnosis (PD) of CF in the former U.S.S.R.

two-thirds of our cases. The results of MVE assay in the remaining pregnancies were ambiguous and need careful verification by DNA analysis.

As there are some indications of unfavourable manifestation of delF508 even in some heterozygous carriers (Dumur *et al.*, 1990) with chronic pulmonary diseases and with elevated immunoreactive trypsin (IRT) (Laroche and Travert, 1991), we have suspected that fetuses at risk of CF bearing genotype delF508/+ might also be prone to somewhat abnormal levels of their MVE in amniotic fluid. Actually, according to the present data, delF508/+ genotype was found in 5 out of 6 presumed CF unaffected fetuses with equivocal MVE test at 17–20 weeks of gestation compared with 15 out of 21 fetuses of the same age with clear-cut negative results of CF by the MVE test. Thus, the heterozygosity for the delF508 mutation by itself does not seem to be directly related to the values of MVE assay. This suggestion, however, remains open for the further verification on statistically more representative material.

The proportion of apparently affected fetuses in our series (24·2 per cent) is more in line with the expected Mendelian proportion (25 per cent for autosomal recessive diseases) than reported elsewhere (Boué *et al.*, 1986; Brock *et al.*, 1988). This discrepancy with reported data may be explained most probably by the uncertainty of the albumin meconium test. Actually, only one of our false-positive results has been ascertained by DNA analysis. The frequency of false-negative results (1·3 per cent) in our studies is almost two times smaller than that reported in the literature (Boué *et al.*, 1986; Brock *et al.*, 1988). Both false-negative cases relate to our initial experience with MVE assay as a single predictive test. One of these cases (low level of GGTP, normal level of APM, and elevated level of I-ALP) favours Brock's suggestion on actual correlation of this enzyme pattern as being highly indicative of fetal CF (Brock *et al.*, 1988). The absence of obvious deviations of MVE assay in the second child (fetus) presumed to be normal by MVE assay correlates with early manifestation of the clear-cut chest form of CF in this individual.

Taking into account the definite limitations for efficient DNA diagnosis in the former U.S.S.R. as mentioned above, as well as the rather late application of PD for our at-risk couples, we find that the demand for MVE analysis is still rather high. That is why we suggest the following strategy for the PD of our families at risk of CF as depicted in Figure 1. Up to now and at least for the time being, it is limited to already known high-risk families. The management of the latter depends on the availability of an index child and includes three successive steps—mutation detection, RFLP analysis, and MVE assay. Depending on the level of family informativeness, PD is accomplished at the first or second trimester of pregnancy.

A screening programme for the identification of delF508 as well as some other major CFTR gene mutations in all at-risk couples for the subsequent efficient molecular PD early in gestation is now in progress.

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