Eur J Pediatr (1989) 149:170–174

European Journal of
Pediatrics

Possible association of sudden infant death with partial complement C4 deficiency revealed by post-mortem DNA typing of HLA class II and III genes

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Abstract. Based on evidence of an increased rate of respiratory infections in sudden infant death (SID) infants as well as the observation of familial occurrence, we analysed in a retrospective study class II and class III genes of the major histocompatibility complex in 40 cases of SID by Southern blot analysis of DNA obtained post mortem from tissue samples. In 24 cases, the parents were interviewed and confirmatory human lymphocyte antigen (HLA) and DNA typing was carried out. Using HLA-DRβ and -DQβ probes, no evidence of an abnormal HLA-DR frequency distribution in SID infants was detected ($P = 0.97$). Using DNA probes for the tandemly arranged complement C4 and steroid 21-hydroxylase genes, an increased number of C4B gene deletions in SID cases was found. The increase in C4 gene deletions was significant ($P = 0.0125$) in infants with recurrent infections. These data indicate a possible role of partial C4 deficiency as a genetically predisposing risk factor in SID.

Key words: SID – HLA-DR – C4 deficiency – Respiratory infections

Introduction

Sudden infant death (SID) contributes significantly to infant mortality, and occurs at a frequency of 2–3 cases per 1000 newborns in the Federal Republic of Germany. The pathogenesis of SID is still not clear. Possible risk factors include premature birth and low birth weight, immaturity of the respiratory and circulatory systems leading to respiratory and cardiac arrest, increased rate of bacterial or viral infections, predominantly of the respiratory system, resulting in hypersensitivity reactions and apnoea, as well as an observation of familial predisposition [1, 36].

Some of these risk factors point to a genetically determined dysfunction of the immune system. The major histocompatibility complex (MHC) provides the genetic basis for the regulation of the immune response. Numerous diseases are associated with different alleles of MHC genes [28, 37]. Among others, systemic lupus erythematosus (SLE) and subacute sclerosing panencephalitis (SSPE) [12, 17, 27] are correlated with the presence of null alleles at the tandemly arranged complement C4A and C4B loci of the MHC class III region [8, 9, 38].

To investigate a possible immunogenetic risk in SID, we analysed the HLA-DR and complement C4 genes of infants who had been classified as SID cases after autopsy. Due to the rapid decay of cell surface proteins and serum components after death, we relied on the typing of MHC genes by restriction fragment length polymorphism (RFLP) analysis of genomic DNA from small tissue samples taken during autopsy.

Using DNA probes for the HLA-DRβ and -DQβ loci [19, 20], the restriction fragment patterns can be correlated to the alleles identified by conventional serology [6]. The C4 gene products are highly polymorphic with more than 35 alleles including null alleles at both C4A and C4B loci [21]. At the DNA level, several RFLP's have been detected [23, 32, 39] using probes for the C4 and 21-OH genes [5, 9]. It has been shown that about half of the C4 null alleles (C4Q0) are due to large structural deletions of the respective genes, whereas the other half is structurally present, but probably not expressed [10, 33].

Using these techniques, we detected a number of structural deletions of C4 genes as evidence for an increased frequency of C4 Q0 in cases of SID, whereas the frequencies of HLA-DR alleles did not differ significantly.

Materials and methods

Selection of SID cases and controls

A total of 40 infants from the western part of the Federal Republic of Germany who died between the ages of 2–13 months were included. They had been classified as SID cases according to the pathoanatomical results after autopsy. In 24 cases, the parents were included in the study to confirm results of in-

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Abbreviations: 21-OHA, 21-OHB = the two genes for the cytochrome P_{450} steroid 21-hydroxylase (21-OHA is a pseudogene); BF = factor B of the alternative pathway of complement; C2 = the second component of complement; C4A, C4B = the two isotypes of the fourth component of complement; C4 Q0 = null allele of C4 (no gene product detectable); EDTA = Ethylendiamintetraacetate; HLA = human lymphocyte antigen; MHC = major histocompatibility complex; RFLP = restriction fragment length polymorphism; SID = sudden infant death; SLE = systemic lupus erythematosus; SSPE = subacute sclerosing panencephalitis

fantile DNA typing. As there are no data available on the frequency of C4 gene deletions in the normal population, a control group of 47 children was included for this part of the study. These children belonged to families from routine paternity cases from the same geographical area.

Complement and HLA typing of parents

Blood from parents of SID cases was typed according to standard procedures. C4 typing was carried out by high voltage agarose gel electrophoresis in a discontinuous buffer system after desialylation of the samples with neuraminidase, followed by immunofixation or haemolytic overlay [3]. C4 Q0 alleles were confirmed by C4 alpha-chain typing after sodium dodecylsulphate polyacrylamide gel electrophoresis [29]. Lymphocytes for HLA-typing were isolated by gradient separation and HLA-A, -B, -C, and -DR antigens assigned by standard assay [35].

Preparation of human genomic DNA and Southern blotting

For parental DNA preparation, individual blood samples were collected into a syringe containing ethylendiamintetraacetate (EDTA) to a final concentration of 7 m*M*. The DNA was extracted from white blood cells by proteinase K digestion and repeated phenol/chloroform extractions according to standard procedures [14]. To obtain DNA from SID cases, small pieces (0.5–1 g) of tissue (muscle and thymus) were taken during autopsy and stored frozen at −20°C. The tissue samples were ground in liquid nitrogen, and the resulting powder lysed in a buffer containing 75 m*M* NaCl, 25 m*M*, EDTA, 10 m*M* Tris, pH 7.5, 1% sodiumdodecylsulfate and 0.2 mg/ml proteinase K (Boehringer Mannheim, Mannheim, FRG). The samples were then treated as described above. Restriction enzyme digestions with the enzyme Taq I (Boehringer Mannheim, Mannheim, FRG), DNA electrophoresis, blotting, hybridization, and autoradiography were carried out as described previously [33]. As hybridization probe, 0.5 µg of purified insert DNA was labelled with 50 µCi alpha-(^{32}P) deoxy cytosine triphosphate (Amersham Buchler, Braunschweig, FRG) in a nick translation reaction [26].

DNA probes and interpretation of fragment patterns

HLA-DRβ-specific restriction fragment patterns were detected with a 520 bp Pst I DRβ_1-cDNA insert [20]. As the alleles DR 3/w6 and 7/w9 cannot be distinguished with this probe, a 630 bp Ava I DQβ_1-cDNA insert [19] was used in these cases to identify the correct allele. The fragment patterns of the DR and DQ probes were assigned to the serologically defined alleles as described [11, 30]. As C4 probe, a 500 bp Bam HI/Kpn I fragment of the full-length C4A cDNA clone pAT-A [5] recognizing the 5′ ends of both C4A and C4B genes was used. The 21-OH genes were detected with a 900 bp Bgl I genomic fragment from the subclone p21-K_4 of cosmid clone cos 1E3 [9]. The calculation of chi-squared values and probabilities for the significance of change was performed with the statistical analysis software package TADPOLE (Ver. 2, Elsevier Biosoft, Cambridge, UK).

Results

HLA-DR typing

The data obtained by RFLP typing of HLA-DR alleles using the HLA-DRβ_1 probe were compared to the results of serological typing in cases where the parents were included. No discrepancy between results of both typing techniques was observed. The HLA-DR frequencies of 38 SID cases are given in Table 1. The observed frequencies in SID are compared to the expected proportions obtained from HLA-DR frequencies of the Caucasian population [4]. The chi square of 1.705 (with Yates' correction for small numbers) and the probability $P = 0.973$ indicate that there is no significant difference in HLA-DR frequencies of SID cases compared to the normal population.

Table 1. Frequencies of HLA-DR in SID

HLA-DR allele	No. observed in SID	Frequency (%)	Frequency (normal)[a]	Chi square	*P*
1	10	13.2	9.5		
2	12	15.8	15.8		
3	10	13.2	12.0		
4	11	14.4	12.7		
5 (11, 12)	12	15.8	14.3		
w6	9	11.8	11.2		
7	6	7.9	12.0		
w8	2				
w9	0	7.9	4.6		
w10	4				
	$n = 76$ alleles			1.705	0.973

[a] HLA-DR frequencies of Caucasian population [29]

C4 gene structure

The structural analysis of complement C4 and 21-hydroxylase genes was carried out to obtain information on the presence of C4 null genes in SID cases. In 24 families, the parents were typed at the protein and the DNA level (data not shown). All

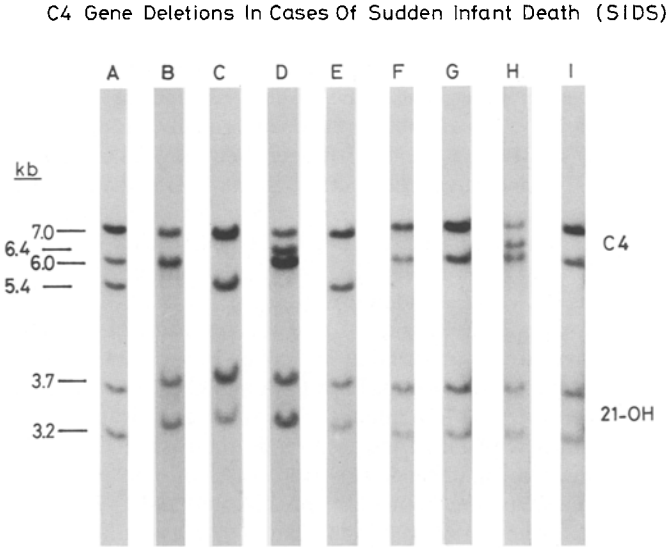

Fig. 1. Restriction fragment patterns of genomic DNA digested with restriction enzyme Taq I and hybridized simultaneously with the C4 and 21-hydroxylase probes of nine cases of SID (A-I). The DNA fragment sizes are indicated on the left: 7.0 kb-C4A gene (22 kb size), 6.4 kb – short C4B gene (16 kb size) in combination with a deletion of the C4A gene, 6.0 kb – long C4B gene (22 kb), 5.4 kb – short C4B gene (16 kb); 3.7 kb – 21-OHB gene, 3.2 kb – 21-OHA gene

Table 2. Frequencies of C4A and C4B gene deletions in SID

Gene	fragment (Taq I)	SID observed		Controls observed	*P* (Fisher)
C4A:	7.0 kb		75	91	
	deletion		5	3	0.473
C4B:	6.4 kb	5			
	5.4 kb	17			
	6.0 kb	47	70	89	
	5.4 kb dupl.	1			
	deletion		10	5	0.109
			$n = 80$	$n = 94$ (haplotypes)	

Table 3. C4 gene deletions and recurrent infections

C4 gene deletions		Recurrent infections		
		Yes	No	*n*
C4 gene deletions	Yes	7	5	12
	No	2	16	18
		$n = 9$	21	(30)

Fisher's exact probability: $P = 0.0125$

structural polymorphisms obtained by DNA typing in these SID cases were confirmed using the parental data.

Examples of C4 and 21-OH restriction fragment patterns in SID cases are given in Fig. 1. Two patterns without deletions are shown in lanes A and B. In lane A, the 7.0 kb C4A band is stronger than both the 6.0 kb and 5.4 kb C4B bands. The 21-OH bands are equally strong. This indicates the presence of a C4A and a C4B gene as well as a 21-OHA and a 21-OHB gene on each chromosome, one having a long and one having a short C4B gene. Deletions are heterozygous in all other examples and only detectable as different strengths of bands. In all cases, the deletion of a C4 gene is reflected by a deletion of an adjacent 21-OH gene, e.g., in lane C, the 7.0 kb C4A band is stronger than the 5.4 kb C4B band (as compared to lane B, 7.0 kb and 6.0 kb bands are equally strong). Also, the 3.7 kb 21-OHB band is stronger than the 3.2 kb 21-OHA band. This indicates a deletion of both a C4B and a 21-OHA gene on one chromosome. A more complex case is shown in lane D. The individual has a C4A/21-OHA deletion on one chromosome, and a 21-OHA/C4B duplication on the other, i.e. three C4 genes of identical size.

All C4-specific fragments and the deletions observed in SID cases are summarized in Table 2 and compared to the results of the control group. Both C4A and C4B gene deletions are increased in SID infants. The value for Fisher's exact probability (two-tailed test) is only in the case of C4B deletions close to significance ($P = 0.109$). Among the parents of 24 SID infants, the frequencies of C4A null and C4B null alleles according to conventional allotyping are significantly increased (30.5% and 25.6% vs. 12.5% and 13.7%, respectively, in the normal population, $P < 0.005$ [4]).

Infections and C4 gene deletions

In 30 cases, the parents were interviewed about their children and the circumstances of death [25]. At the time of the interviews, the C4 status of the infants was not yet established. In 9 cases, the parents reported on recurrent infections of the respiratory tract, mainly rhinitis, sinusitis, and bronchitis, sometimes combined with otitis media, diarrhoea, and fungal infections. In some of these cases, infections could also be confirmed by histological analysis after autopsy (data not shown). The rate of recurrent infections among 30 cases is shown in Table 3 in relation to the presence or absence of C4 gene deletions. The probability value of $P = 0.0125$ (calculated according to Fisher's exact probabiltiy, two-tailed test) shows a significant correlation of C4 gene deletions and the occurrence of repeated infections in SID cases.

Discussion

In contrast to previous studies in which HLA phenotypes of parents of SID children were investigated [15, 34], we directly studied MHC genes of affected children using recombinant DNA methods in post-mortem typing. The data obtained do not indicate an important predisposing role of the HLA-DR genes, since the frequency of DR in SID did not differ significantly from the normal population.

The frequency of complement C4A and C4B genes differed from the normal distribution, since only about 50% of all C4 Q0 alleles are caused by gene deletions [10, 24, 33]. It can be speculated that the actual number of C4 Q0 alleles in SID cases is about twice the number of gene deletions observed by DNA typing. The results on the C4 gene structure in SID infants are supported by a markedly increased frequency of C4 Q0 among the parents of these infants. Although the parents have an even higher C4A Q0 than C4B Q0 frequency (30.5% vs. 25.6%), only the C4B Q0 alleles in SID infants seem to be more clearly increased. This may partly be due to unusual expression of alleles based on homoduplications or gene conversions not detectable by RFLP analysis [7, 41].

Infants with recurrent infections (Table 3) had a significantly increased proportion of C4 gene deletions. It is possible that in cases with one deletion, the other allele is silent due to non-expression leading to homozygous C4A or C4B deficiency in the individual. It has been shown in a recent study of homozygous C4A and C4B deficiency that only 1 of 14 C4B Q0 individuals had a homozygous C4B gene deletion, whereas 7 individuals were heterozygous and 6 had no deletion at all [7].

C4A and C4B differ significantly in their binding properties. Upon activation of the internal thiolester bond, C4A reacts more strongly with amino groups, whereas C4B has a stronger affinity to carboxyl groups (reviewed in [31]). It has been shown by analysing C4A and C4B genes transfected into mouse fibroblasts, that both genes are regulated and expressed differentially [22]. These data indicate an independent and important biological role for the two isotypes of C4.

The peak incidence of SID is at the age of 2–4 months, and during this period, the immunological status of the infant is in transition from dependence on the presence of maternal antibodies to the development of fully self-sustained immune competence. A "window of vulnerability" [18] caused by this labile equilibrium in combination with partial C4 deficiency could exist in infants at this genetic risk. A confrontation with infectious agents, e.g., respiratory syncytial virus [40], influenza virus [42], or other viruses or bacteria of the respiratory tract, may "trigger" obstructive apnoea or damage vital or-

gans without preceding symptoms, and thus lead to death [16]. The infection hypothesis is supported by pathomorphological findings after detailed autopsy of the upper respiratory tract [2]. The observation of grossly raised concentrations of IgG and IgM in lung lavage fluid of SID cases also points to an important role of respiratory infection [13].

Using the approach presented in this study, a subgroup of infants may be identified which is at risk for SID due to genetic predisposition affecting an important effector molecule of the immune system. This is demonstrated by the fact that C4 gene deletions are increased significantly only in the group of infants with recurrent infections. However, only a prospective study including complete data on pregnancy, birth, medical records of the infant as well as the disease history of the family and microbiological and virological findings at the time of autopsy will provide a more solid basis of the role of infections and C4 deficiency in SID.

Acknowledgements. We thank Mrs. P. Everke, Mrs. B. Stradmann-Bellinghausen, and Mrs. A. Susemichel-Hüppner for technical assistance. We gratefully acknowledge the advice of R. Fimmers, Institute of Medical Statistics, University of Bonn, regarding the statistical analysis. We thank Professor Müntefering, Department of Paediatrics, Johannes-Gutenberg-University Mainz, for tissue samples of three SID cases. The DRβ and DQβ probes were kindly provided by Drs. E. O. Long and P. A. Peterson, and the C4 and 21-OH probes were kindly provided by Dr. M. C. Carroll. The study was supported in part by a grant from the Deutsche Forschungsgemeinschaft to C.R. (DFG Ri 164/16-3).

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Received December 22, 1988 / Accepted April 3, 1989