Absence of linkage between idiopathic dilated cardiomyopathy and candidate genes involved in the immune function in a large Italian pedigree

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Abstract

Idiopathic dilated cardiomyopathy (IDC) is a heart disease of unknown aetiology characterised by impaired ventricular function usually associated with dilatation of the cardiac chambers. In order to test the hypothesis of an immunological cause for the disease at the genetic level, we performed linkage analysis between the putative disease locus and some of the potential candidate genes involved in the immune response or coding for the targets for autoantibodies in a large multi-generation family (63 members) from southern Italy with autosomal dominant transmission of the disease. Twenty-nine polymorphic markers on 18 different chromosomal locations were investigated, including markers linked to the genes coding for the HLA antigens, the immunoglobulin heavy and light chains, the receptors for the immunoglobulin Fc fragments, the subunits of the T cell receptor and the associated CD3, CD4, CD8, and CD45 antigens, interleukins 1, 3, 4, 5, 6, 9, and 11, the interleukin 1 and 2 receptors, and the genes coding for the β_1 adrenoreceptor, the adenine nucleotide translocator-1, and the cardiac α and β myosin heavy chains.

No evidence for genetic linkage to IDC was found at any of these candidate loci. These results indicate that the still unidentified IDC gene maps outside several loci involved in the regulation of immune reactivity.

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Idiopathic dilated cardiomyopathy (IDC) is a chronic heart muscle disease of unknown aetiology,¹ characterised by impaired systolic function usually associated with ventricular dilatation, with a prevalence of 36.5 affected persons per 100 000 in the USA population.² Progressive deterioration is common during the clinical course of the disease, with symptoms resulting from heart failure, low output state, and arrhythmia. The disease is usually associated with a very poor prognosis,^{3,4} and represents the major indication for heart transplantation in the world.⁵

The aetiology of the disease is unknown. A coxsackie virus type B infection, with previous

myocarditis and subsequent development of chronic myocardial damage, has long been considered.⁶ However, the search for enteroviral genomes in endomyocardial biopsies by the extremely sensitive polymerase chain reaction technique has led to disappointing results, allowing several groups, including ours, to rule out the persistence of these viruses in the myocardium as the major cause of the disease.⁷⁻⁹

Among the several pathogenetic hypotheses formulated,^{10,11} the involvement of the immune system in the development of the disease is supported by the finding of several immunological abnormalities in patients with IDC, including decreased natural killer cell activity,¹² functional deficit in suppressor cell activity,¹³ and cellular and humoral auto-immune reactivities against myocytes.¹⁴ In particular, an increased presence of organ specific autoantibodies against myocardial antigens has been shown in patients with IDC as compared to the normal population or control patients¹⁵; these autoantibodies are directed against several cardiac proteins including the α and β cardiac myosin heavy chain isoforms,¹⁶ the β_1 adrenergic receptor,^{17,18} the muscarinic acetylcholine receptor-2,¹⁹ and some mitochondrial antigens such as the M7²⁰ and the adenine nucleotide translocator²¹ proteins. Whether these serological reactivities are the primary cause of the disease or represent specific markers of cardiac damage²² is not known at present.

Another indirect support to the hypothesis of the involvement of the immune system in the pathogenesis of the disease derives from several studies which evaluated the association of IDC with the presence of specific antigens of the major histocompatibility complex, in particular of class II, in patients with the disease. A positive association between IDC and HLA-DR4 has been described in several serological studies from different countries.²³⁻²⁶ Since class II antigens are involved in the presentation of foreign peptides to T helper lymphocytes, it is conceivable that an abnormal antigen presentation could lead to an autoimmune response. In addition, it has been shown that a specific IgG heavy chain constant region (Gm) phenotype is overrepresented in IDC patients compared with normal controls, and that a potentiating interactive effect is found between the Gm phenotype and HLA-DR4.²⁷

Finally, an increasing number of patients

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with IDC (more than 20%) has recently been acknowledged to be affected by the familial form of the disease,^{3,28} with different patterns of transmission. At present, it is not known whether the sporadic and the familial forms, which are indistinguishable by clinical and morphological criteria, represent different nosological presentations of the same disease.

The availability of DNA samples from members of families with IDC offers the possibility to study the genetic transmission of known chromosomal markers and to define their linkage to the disease. In this work, we investigated the linkage of the putative disease gene with several candidate genes involved in the immune function, as well as genes encoding some IDC autoantigens, in a large Italian family with IDC.

Materials and methods

PATIENTS AND SAMPLES

To avoid genetic heterogeneity, a single, large, four generation family from the south of Italy was used in this study. Sixty-three members (mean age 31 years) were clinically evaluated, six of whom were affected. Clinical, electrocardiographic, and echocardiographic assessments were performed as previously described.^{3,11} The diagnosis of IDC was based on the demonstration of poor contractility of

the left ventricle or of both ventricles, associated with dilatation of the cardiac chambers, in the absence of any known cause of myocardial disease.¹ In this family, the disease is inherited as an autosomal dominant trait with age dependent penetrance.

DNA samples from each available member of the family were extracted by standard procedures²⁹ either from fresh blood samples or from continuous B lymphocyte cell lines immortalised with the Epstein-Barr virus.³⁰

MICROSATELLITE ANALYSIS

Polymerase chain reaction (PCR) analysis was performed using highly polymorphic markers with a high percentage of heterozygosity (table), chosen for their localisation either intragenic or very close to the gene of interest, based on published chromosomal maps.^{45,48} These chromosomal markers were constituted by di-, tetra-, or pentanucleotide polymorphisms (microsatellites), with the exception of the immunoglobulin heavy chain gene cluster, which was analysed by the study of a VNTR region. The references, locus, and name for each of the markers used are given in the table. The chromosomal locations of the candidate genes were based on data reported at the 11th International Workshop on Human Gene Map-

Linkage of IDC to candidate genes involved in the immune function

Gene	Location	Locus	Marker	θ_1	θ_2	Ref
HLA	6p21.3	D6S265	AFM101xa1	0.045	0.002	31
Immunoglobulins and Fc receptors						
Heavy chain (IGH)	14q32.33	IGHJ	Ig-J _H	0.070	0.090	32
Light chain κ (IGLK)	2p12	CD8A	CD8APCR1	0.026	0.003	33
Light chain λ (IGLL)	22q11.1-11.2	D22S275	AFM164ze3	0.036	0.002	31
Fc receptor for IgA, IgG, IgE	1q23	D1S104	Mfd67	0.118	0.014	34
Fc receptor for IgEII	19p13.3	LIPE		0.037	0.015	35
T cell receptor and coreceptors						
α cluster (TCRA), δ cluster (TCRD)	14q11.2	MYH7	MYH7PCR1	0.108	0.064	36
β cluster (TCRB)	7q35	D7S495	AFM168xc3	0.037	0.015	31
		D7S498	AFM183ya3	0.025	0.014	31
		D7S509	AFM203wg1	0.085	0.060	31
γ cluster (TCRG)	7p15-p14	D7S528	AFM248ve5	0.098	0.050	31
CD3 δ , ϵ , γ (CD3D, CD3E, CD3G)	11q23	CD3D	Mfd69	0.057	0.018	37
CD3Z (CD3Z)	1q22-q25	D1S104	Mfd67	0.118	0.014	34
CD4	12p12-pter	vWF	vWF	0.056	0.025	38
CD8A	2p12	CD8A	CD8APCR1	0.026	0.003	33
CD45	1q31-q32	D1S158	MLOW1	0.102	0.069	39
Interleukins and interleukin receptors						
IL-1 α (IL1A), IL-1 β (IL1B), IL-1 receptor (IL1R)	2q12-q21	D2S121	AFM087xa1	0.055	0.025	31
IL-2 receptor β (IL2RB)	22q13	D22S283	AFM262vh5	0.081	0.034	31
IL-3, IL-4, IL-5, IL-9	5q23-q31	D5S210, FGFA	Mfd122	0.021	0.006	40 41
		D5S421	AFM210vd6	0.093	0.078	31
IL-6	7p21-p14	D7S528	AFM248ve5	0.098	0.050	31
IL-11	19q13.3-13.4	D19S49	Mfd11	0.122	0.054	42
Autoantigens						
Cardiac α , β myosin heavy chain (MYH6, MYH7)	14q11.2-q12	MYH7	MYH7PCR1	0.108	0.064	36
ATP-ADP carrier (ANT1)	4q35-qter	D4S171	Mfd22	0.061	0.016	43 44
β_1 adrenoceptor (ADRB1)	10q24-q26	D10S259, D10S173, D10S88	JS8001, MIT-M6, Mfd7	0.054	0.060	45 46 47
		D10S187, D10S168	AFM042xe9, Mfd175	0.042	0.030	31 47
		D10S108	Mfd100	0.027	0.014	47
		D10S192	AFM049tc9	0.047	0.053	31
		D10S169	Mfd187	0.027	0.024	47
CD antigens						
CD1, CD48	1q21.3-q23	D1S104	Mfd67			
CD14, CD49B	5q22-q32	D5S210, FGFA	Mfd122			
		D5S421	AFM210vd6			
CD28	19q13.3	D19S49	Mfd11			
Complement components						
r and s subcomponents	12p13	vWF	vWF			
C2, C4A, C4B, CH	6p21.3	D6S265	AFM101xa1			
C4 binding proteins α , α -like1, and β ; 3b/4b receptors 1, 1-like, and 2	1q32	D1S158	MLOW1			

The CD antigens and complement components listed in the bottom part of the table are localised in the same chromosomal regions reported above for other genes. θ_1 and θ_2 represent the recombination fractions giving a lod score of -2 as evaluated by two different models (age related penetrance and "affecteds only" for the fourth generation, respectively - see text).

ping⁴⁵ as well as on published high resolution maps.^{43 49 50}

PCR amplifications were carried out in 50 µl of a solution containing 10 mmol/l Tris (pH 8.0), 50 mmol/l KCl, 1.5 mmol/l MgCl₂, 0.01% gelatin, 200 µmol/l each dNTP, 0.1 µmol/l both primers, 200 ng of template DNA, and 2.5 units of *Taq* DNA polymerase (Perkin-Elmer), with 40 repetitions of the following cycle: 45 seconds at 94°C, 30 seconds at the annealing temperature, and 30 seconds at 72°C, in a Perkin-Elmer 480 Thermal Cycler. Annealing temperatures varied from 53 to 68°, according to the different primer pairs. PCR products were resolved by a long run on a 10–12% polyacrylamide native gel and visualised by ethidium bromide staining. All the oligonucleotides were synthesised by the IC-GEB Oligonucleotide Synthesis Service on an Applied Biosystem 380B synthesiser.

LINKAGE ANALYSIS

Linkage analysis was conducted on 21 key members of the family, which is shown in the figure, panel A; this subset of the family was proven by the Simlink computer program version 4.1⁵¹ to have appropriate linkage power.

Two point linkage analyses were performed between each marker and IDC phenotype using the PC version of the MLINK option of the LINKAGE package (version 5.10).⁵² Analysis was carried out under the model of autosomal dominant disease with age related penetrance and the following calculated values¹¹: age 30 to 40, penetrance 0.70; age 40 to 50, 0.92; age >50, 0.96; persons below the age of 30 were not included in the study. No sex difference in recombination events was assumed. This model corresponds to the one suggested by Pastores *et al.*⁵³

Additionally, computations were also performed according to the “affecteds only” model, by recoding the sibs of affected persons of the fourth generation as unknown.

A gene frequency of 0.0003 was calculated from a population prevalence; changes in the gene frequency of the markers made little difference in the computed lod score values.

The calculations were performed at recombination fractions of 0, 0.01, 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 between each marker and the putative disease gene, and then further refined with a recombination fraction increment value of 0.01 in order to find the widest chromosomal region excluding linkage with a lod score value ≤ -2 . The values in cM were obtained by the Haldane mapping function (Utility programs for analysis of genetic linkage⁵⁴).

Results

The linkage between IDC and 29 chromosomal markers was investigated in a large family with autosomal dominant transmission of the disease.³ The pedigree of the key members of this family, which were analysed in this work, are reported in the figure, panel A.

All the markers, with the exception of IGHJ

containing a VNTR mapping in the immunoglobulin heavy chain gene cluster, were represented by di-, tetra-, or pentanucleotide polymorphisms (microsatellites), which were analysed by PCR amplification. All these markers were chosen for their localisation within, or very close to, candidate genes involved in the immune function. When significant lod scores were obtained only for a narrow region, more than one polymorphism was analysed for the same gene.

The immune function genes investigated, their chromosomal localisation, and the results obtained from the linkage studies are reported in the table. The figure (panels B, C, and D) shows the microsatellite amplification results obtained for markers close to the β_1 adrenoreceptor, the β chain of the IL-2 receptor, and the β chain of the T cell receptor, respectively.

A pivotal role in the normal immune function, and in its dysregulation leading to autoimmunity, is played by the interaction between the T cell receptor (TcR) multicomponent complex and the HLA gene products.⁵⁵ The multicomponent protein structure of T cells involved in this function includes the hypervariable α : β or γ : δ chains of the TcR (which confer antigen and HLA specificity) as well as five other invariant molecules constituting the CD3 complex (γ , δ , ϵ , and the ζ : ζ or ζ : η dimers).⁵⁶ Furthermore, the coreceptors CD4 (in CD4 T cells recognising the antigen within HLA class II molecules) or CD8 (in CD8 T cells recognising the antigen within HLA class I molecules) participate in this function, together with a specific isoform of the CD45 gene product.⁵⁶ All the genes encoding for these proteins were included in the list of the candidate genes in this work. The differentiation process of T cells which follows antigen recognition is mediated also by the function of several soluble factors.⁵⁷ Among these, the genes encoding for the two subunits of interleukin-1 (IL-1) and IL-1 receptor, IL-2 receptor β chain, IL-3, IL-4, IL-5, IL-6, IL-9, and IL-11 were included in the study. We also investigated the loci encoding for the immunoglobulin (Ig) heavy and κ and λ light chains, and those encoding for the receptors for the IgA, IgE, IgG Fc fragments (which also belong to the Ig supergene family and mediate different Ig triggered functions on the target cells⁵⁸). Finally, to test the hypothesis that some of the proteins for which autoimmune reactivity was reported in patients with IDC could be the products of specific gene alleles, we also investigated the linkage between the disease and the genes encoding for the β_1 adrenergic receptor, the adenine nucleotide translocator-1, and the cardiac α and β myosin heavy chains.

For all the loci analysed, no segregation of any allele of the marker with the disease was observed (table). Lod scores were in all cases clearly negative. In order to define precisely the region of exclusion of linkage (considered as the widest region where the lod score is still equal to, or less than, -2), linkage analysis was performed at different recombination fractions (θ) with an increment value of 0.1, and sub-

(A) Pedigree of the subset of the family used for the linkage study. Affected persons are shown by black symbols; persons dead before the beginning of the study (belonging to the third generation) in which the clinical history is strongly suggestive of IDC are shown by grey symbols. (B–D) Microsatellite amplification for selected markers. The PCR amplification products obtained for the markers indicated on the left side of the panels were resolved by gel electrophoresis, and stained with ethidium bromide. Below each lane, the family member is indicated according to the numbers reported in (A). On the right of each picture, the position of the different alleles identified is indicated by letters (arbitrarily chosen). On top of each lane, the genotype of each person is shown.

sequently of 0.01. The results were interpolated and the recombination fractions yielding a lod score of exactly -2.0 are reported in the table, column θ_1 . No one of the markers investigated is linked to the disease in this family in a range of θ equal to 0.026 to 0.122 on both sides of the markers, which corresponds to a total genetic distance of 5 to 28 cM. Furthermore, in the possibility that sibs of affected subjects in the fourth generation could be affected at any age, the values of recombination fractions with a lod score of -2 under the "affecteds only" model were also evaluated (table, column θ_2). No linkage could be detected in this case either (although with a more conservative magnitude of exclusion).

These results indicate that in this family the involvement of the investigated genes in the pathogenesis of IDC can be ruled out.

Furthermore, some of the investigated markers map in chromosomal regions containing several other genes coding for proteins involved in some immunological functions. These genes, and the corresponding chromosomal markers, are listed in the bottom part of the table. They include white cell differentiation antigens, such as CD1 and CD48 in 1q21.3–q23, CD9 in

12p12–pter, CD14, CD49 in 5q22–q32, CD28 in 19q13.3, and several complement components and receptors. The involvement of these genes in the development of IDC in this family can also be excluded.

Discussion

The presence of autoimmune reactivities (namely the production of antibodies or reactive T cell clones against self antigens) has often been invoked to explain the pathogenetic mechanisms underlying several diseases of unknown aetiology, including IDC. These studies are based on the presence of organ specific antibodies or T cells reacting against different self antigens,^{14–21} and on association studies indicating an increased frequency of some HLA haplotypes (mainly including the DR4 allele)^{23–26} or TcR polymorphisms⁵⁹ in patients with IDC. Altogether, these data suggest that the inappropriate presentation of foreign antigens (possibly of viral origin) or abnormal self antigens could lead to an aberrant form of activation of cells of the immune system, with consequent autoimmune reactivity against myocardial cells.

In this work, we have investigated the segregation of alleles of several candidate genes involved in the immune function with IDC in a large Italian family by using highly informative microsatellite repeats. Segregation of the disease within this family is consistent with a model of autosomal dominant transmission with age related penetrance.³¹¹

The analysis of allele segregation for the 6p21.3 region, containing the 3 Mb area encompassing class I and class II HLA loci, is consistent with the exclusion of linkage of this region with the disease. Similarly, exclusion of linkage to HLA was also recently obtained in other diseases of suspected autoimmune aetiology, such as Graves's disease and Hashimoto's thyroiditis.⁶⁰ It should be considered, indeed, that, although some data support the direct participation of class II genes in autoimmune disease susceptibility, most persons with disease associated class II alleles do not develop autoimmune disease.⁶¹ This observation, which can be explained by a low penetrance of the disease, nevertheless suggests that other causes should be identified as the ones mainly contributing to disease development. According to this view, the association of IDC with some HLA, Gm, or TcR haplotypes could represent a marker of more severe forms of the disease, characterised by earlier onset and accelerated progression of heart dysfunction.²⁷

In the family investigated, the genes encoding for structural components of the TcR (TCRA, TCRB, TCRG, TCRD, and the D, E, G, W, and Z chains of CD3) and associated proteins (CD4, CD8, CD45) all failed to show linkage. The total region of exclusion of linkage ranged from 12 to 27 cM for all the loci tested, with the exception of CD8, where a significant value of exclusion was obtained only at 2.6 cM on each side of the marker; in this case, however, the marker was chosen to be intragenic. Also, for the TcR genes, association studies have been conducted in several autoimmune diseases,⁶² including IDC.⁵⁹ However, the overall outcome of these studies is difficult to evaluate,⁶³ possibly because of the limited polymorphism of the markers investigated.⁶²

Other genes, coding for several mediators of T cell function such as IL-1 and IL-1 receptor, IL-2 receptor β chain, IL-3, IL-4, IL-5, IL-6, IL-9, and IL-11, are clearly not linked to IDC in this family. Similarly, negative results were also obtained for genes possibly involved in humoral autoimmunity (such as the Ig heavy and light chain genes, and the genes encoding the Ig Fc fragment receptors), and for genes which were indicated as major autoantigens (β_1 adrenoreceptor, adenine nucleotide translocator-1, and cardiac α and β myosin heavy chains) (table). The gene for the β_1 receptor was tested with eight polymorphic PCR markers as it maps to 10q24-26; its recent localisation to a narrower region⁴⁵ makes the exclusion of linkage to the gene even more evident, as loci D10S88, D10S259, D10S173, D10S187, and D10S192 are all in close proximity to the gene.³¹⁴⁵ The gene for the adenine nucleotide translocator-1 is located at 4q35-qter, closely

linked to locus D4S171, where the microsatellite marker is also mapped.⁴³⁴⁴ The less informative results were obtained when testing the gene for IGLK. However, since recent mapping data define the localisation of IGLK on chromosome 2 about 2 Mb apart from CD8,⁶⁴ the κ light immunoglobulin gene cluster can also be excluded from being involved in the development of IDC. The lack of association of these genes with IDC in the family investigated is not surprising, since the data on the serological reactivities against myocardial antigens, although of considerable interest, should be considered primarily phenomenological,⁶⁵⁶⁶ and are unable to provide support for an aetiological role of these antibodies in disease development.²²⁶⁷

In conclusion, taken altogether, these data exclude, at least in the large family examined, the linkage of candidate genes involved in immune reactivity with disease transmission. It is likely that only the extension of linkage studies to other regions of the genome will eventually lead to the identification of the gene primarily involved in the development of IDC.

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