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Involvement of human muscle acetylcholine receptor α-subunit gene (*CHRNA*) in susceptibility to myasthenia gravis

(repetitive DNA/microsatellite repeat/dinucleotide repeat/polymorphism)

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ABSTRACT **The muscle acetylcholine receptor is the major target of the autoimmune response in generalized myasthenia gravis. To investigate the role of the gene encoding the α subunit of the receptor (*CHRNA*), two stable polymorphic d[(GT)·(CA)]dinucleotide repeats, designated HB and BB, were characterized within the first intron of *CHRNA*. The HB*14 allele conferred a relative risk for myasthenia gravis of 2.5 in 81 unrelated patients compared with 100 control subjects. Very significantly, family analysis based on haplotype segregation data indicated that parental haplotypes associated with HB*14 always segregated to the child with myasthenia gravis ($P < 0.0002$ for the comparison with the transmission of haplotypes not bearing HB*14), whereas their transmission to unaffected siblings was equilibrated. Myasthenia gravis patients also showed a high frequency of microsatellite variants unseen in controls. These findings implicate the *CHRNA* in susceptibility to myasthenia gravis.**

Myasthenia gravis (MG) is an autoimmune disease resulting in a postsynaptic blockade of neuromuscular conduction by autoantibodies against the acetylcholine receptor (AChR) (1). The pathogenicity of anti-AChR antibodies is demonstrated by the passive transfer of the disease to rodents by immunoglobulin isolates from sera of MG patients (2) or by murine anti-AChR monoclonal antibodies (3) and by the observation of transient disease (neonatal MG) caused by placental transfer of autoantibodies from MG mothers (4). The central role of the AChR as the target and possibly as the stimulus of the autoimmune response is further supported by the fact that immunization of rabbits and rodents with purified *Torpedo* AChR or with recombinant AChR α subunit induces a disease that closely resembles human MG (5, 6).

The origin of this abnormal production of anti-AChR autoantibodies is presently unknown. However, genetic factors must play an important role in the susceptibility to MG as suggested by the 40% concordance rate for the disease in pairs of monozygotic twins and by the increased incidence of disease in relatives of MG patients (7, 8). The high frequency of autoimmune diseases in families of myasthenic patients, notably including thyroid autoimmune diseases (9), and the high incidence of anti-AChR autoantibodies in the sera of first-degree relatives of the patients (10, 11) also argue in favor of a marked genetic component in MG pathogenesis.

As is the case for most autoimmune diseases, the genetic predisposition to MG is likely to involve multiple genes (for review, see ref. 12). Among the possible susceptibility loci, genes of the major histocompatibility complex (MHC) (13–15) and those coding for lymphoid cell antigen receptors, including immunoglobulin genes (16, 17) and T-cell antigen-receptor genes (18), have logically attracted much attention because their protein products are involved in the processing and in the recognition of antigens, including self-antigens. Strikingly however, very little is known about the contribution of the autoantigen gene itself to the autoimmune process. The study of MG provides an opportunity to investigate the genetic polymorphism of the target autoantigen, here the AChR, with regard to disease susceptibility.

The AChR is a pentameric molecule consisting of four chains under the stoichiometry $\alpha_2\beta\delta\varepsilon$ (in adult muscle) or $\alpha_2\beta\gamma\delta$ (in immature muscle) (19). The α chain appears to focus an important part of the autoimmune response of both the B- and the T-cell lineages (20–22). In the present work, we describe two highly polymorphic and tightly linked genetic markers,¶ based on simple sequence repeats (23, 24), in the gene encoding the α subunit of AChR (*CHRNA*) and we find evidence for a significant contribution of one of its allelic forms to MG susceptibility.

MATERIALS AND METHODS

Subjects. Eighty-one unrelated Caucasoid patients with generalized MG were studied. Their clinical characteristics are shown in Table 1. Only patients with detectable levels of autoantibodies against AChR were included in the study. Anti-AChR autoantibodies were measured with a radioimmunoprecipitation assay (4). Eight of the patients were affected by another autoimmune disease (a thyroid autoimmune disease in five patients; systemic lupus, rhumatoid arthritis, and psoriasis in three other patients). Fifty-three of the 81 patients were part of a family study undertaken with the help of the Association Française contre les Myopathies. Their 228 relatives were typed for HB and BB microsatellites in order to ascertain haplotypes and marker stability. The control population consisted of 100 healthy individuals randomly obtained from the French Bone Marrow Donor Registry.

Cloning and Amplification of d[(GT)·(CA)] Repeats in *CHRNA*. Nine plasmid clones encompassing *CHRNA* (generously provided by S. Numa; ref. 25) were screened for the presence of d[(GT)·(CA)] repeats by using labeled poly[d(GT)·d(CA)] (Pharmacia). Small DNA fragments that gave the hybridization signal were subcloned by established procedures (26). The nucleotide sequences of these subclones were determined by the dideoxy chain-termination technique (27) with modified T7 DNA polymerase (28). The following oligonucleotide sequences were chosen for priming microsatellite amplification: HB upstream primer, 5′-ACTA-

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Abbreviations: AChR, acetylcholine receptor; MG, myasthenia gravis.

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¶The nucleotide sequences reported in this paper have been deposited in the GenBank data base (accession nos. X74625 and X74626).

Table 1. Clinical, immunological, and histopathological characteristics of 81 MG patients (59 females and 22 males)

Characteristic	No. of patients (female/male ratio)
Age at onset of disease	
≤15 years	11 (9/2)
>15 years and ≤40 years	44 (36/8)
>40 years	26 (14/12)
Associated autoimmune diseases*	8 (7/1)
Anti-AChR antibody titer	
High (>100 nM)	22 (18/4)
Intermediate (>10 nM and ≤100 nM)	35 (23/12)
Low (>1 nM and ≤10 nM)	24 (18/6)
Thymus histopathology	
Hyperplasia	28 (24/4)
Thymoma	14 (8/6)
Normal/involution	16 (12/4)
Not determined	2 (2/0)
Not thymectomized	21 (13/8)

*See text.

GTGGATCTCTTAAGGAT-3′; HB downstream primer, 5′-TGACTCTGTCCAGCCAAAGC-3′; BB upstream primer, 5′-AGTCCAAGATTTCATACA-3′; BB downstream primer, 5′-TGACATTCCTTCATACAGC-3′.

Human DNA was extracted from fresh nucleated blood cells or Epstein–Barr virus-transformed cell lines. Aliquots (100 ng) were amplified as previously (29), in a total volume of 50 μl containing 0.8 μM unlabeled primer plus 20 nM 5′-end-labeled ([γ-^{32}P]ATP) primer, 200 μM each dNTP, 1.5 mM $MgCl_2$, 20 mM Tris·HCl (pH 8.3), 50 mM KCl, and 1 unit of *Taq* DNA polymerase (30). Twenty-five cycles, each consisting of three segments of 1 min each at 94°C, 50°C, and 72°C successively, were performed.

Samples (2 μl) of PCR mixtures were then mixed with an equal volume of loading buffer, heat-denatured, and electrophoresed in a 6% polyacrylamide/8 M urea sequencing gel. After electrophoresis, gels were dried and autoradiographed. Alleles were initially sized by use of a plasmid sequencing ladder. Subsequently, a set of three or four control heterozygous DNA samples with known alleles was included in each experiment. These experimental conditions yielded sharp bands with little background (Fig. 1), permitting unambiguous typing of unrelated individuals. All genotypes

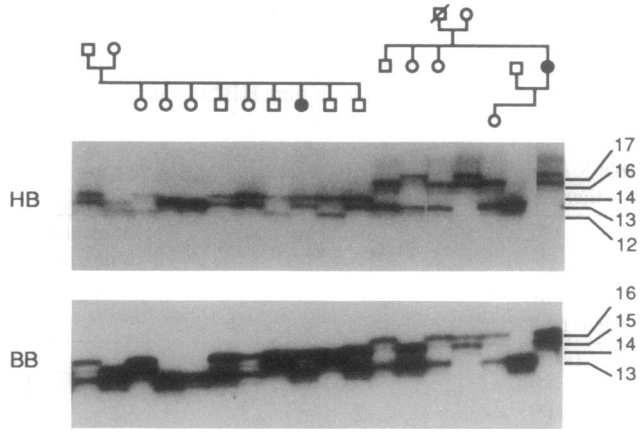

FIG. 1. Autoradiograms of PCR products for HB and BB markers. Genomic DNA from individuals belonging to the pedigrees shown at the top were PCR-amplified with primers corresponding to HB (upper gel) and BB (lower gel) microsatellite markers and the PCR products were electrophoresed in a sequencing gel. Alleles (numbers at left) were determined by comparison with already typed DNA samples (not shown here).

were confirmed by double blind reading of the autoradiograms. New alleles were typed at least twice.

Statistical Analysis of the Data. The weighted average number of repeats was calculated as indicated by Weber (31). The relative risk conferred for disease by each allele was calculated with Woolf's formula (32). Significance of 2 × 2 tables was determined with Fisher's exact test.

RESULTS

Identification of Two Dinucleotide Repeats in *CHRNA*. Two d[(GT)·(CA)] dinucleotide repeats were identified within the first intron of *CHRNA* (25) by hybridization with a poly[d(CA)·d(GT)] probe (Fig. 2). The first of these two repeats was found in a 300-bp *Hin*dIII–*Bgl* II fragment (hence its designation of HB) ≈800-bp downstream of exon P1. The second microsatellite was localized in an 800-bp *Bgl* II–*Bam*HI fragment (and therefore named BB), close to the *Bgl* II site, immediately 5′ of exon P2. The two fragments (HB and BB) were subcloned and sequenced, revealing the existence of two perfect repeats made of 14 (TG) and 13 (GT) dinucleotides, respectively (sequence of the upper strand). Oligonucleotides matching the sequence surrounding these repeats were synthesized and used as primers for PCR amplification. Genomic DNA from 100 unrelated healthy individuals was typed for both of these simple sequence repeats. As shown in Table 2, six and four alleles (designated after the number of CA repeats) differing in size by two or four nucleotides were detected for HB and BB. The weighted average numbers of repeats were 13.2 and 13.8 and polymorphism information content (PIC) values of 0.59 and 0.56 were calculated. Heterozygozities were 0.61 and 0.63, respectively. No allele had a relative frequency >0.5.

Distribution of *CHRNA* Microsatellite Alleles in MG Patients. Eight-one unrelated MG patients (Table 1) were typed for both HB and BB markers (Table 2). Because *CHRNA* was taken as a candidate gene, only patients with detectable serum levels of anti-AChR antibodies were included in the study, to ensure an involvement of the AChR in the disease. Four new alleles (two for each marker) were detected in 6 patients. Two individuals accumulated two new alleles, one being BB*16 homozygous, the other being HB*15/BB*18. The frequency of 6 individuals out of 81, with alleles not seen among controls at two loci, was significantly different from zero ($P < 0.02$). In addition, MG patients showed an increased frequency of the HB*14 allele (relative risk = 2.59; $\chi^2 = 6.51$; uncorrected $P = 0.011$), although the increase was not significant after correction for the number of tests performed. These results nevertheless suggested a trend and the role of *CHRNA* was also investigated by means of a family analysis.

Preferential Transmission of HB*14-Associated Parental Haplotypes to Their Myasthenic Children. Because few families with multiple cases of MG are available, usual techniques are not applicable to the genetic analysis of MG. An alternative approach based on analysis of the segregation of parental marker alleles was taken (34–39). Preferential segregation of a particular allele to myasthenic children thus suggests a role for this allele in disease susceptibility. Parental alleles that are not transmitted to MG children provide internal controls that minimize heterogeneity due to ethnic or geographic diversity.

By using the two HB and BB markers, *CHRNA* haplotypes could be determined in 33 families of MG patients, representing 56 heterozygous parents. As could be expected from their close physical linkage, the two microsatellites were in linkage disequilibrium and a limited number of distinct haplotypes were identified (Table 3). The knowledge of these haplotypes was then used to ascertain the transmission of each parental allele for both HB and BB markers (Table 4).

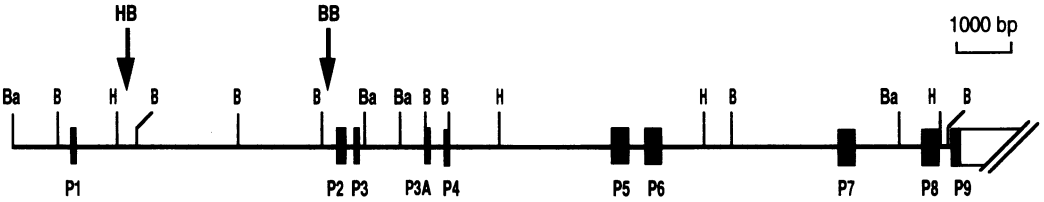

FIG. 2. Localization of HB and BB dinucleotide repeats (arrows) in the first intron of *CHRNA* (25, 33). Boxes show exon coding sequences (P1–P9). Restriction sites: B, *Bgl* II; Ba, *Bam*HI; H, *Hin*dIII.

The transmission of each allele to the myasthenic children was compared with that of other alleles (+ and − row for each allele shown in Table 4). The number of chromosomes bearing a given allele and transmitted to the MG children was compared with the number of chromosomes bearing this allele and not transmitted to the diseased children (columns 3 and 4 of Table 4, respectively). Finally, allele transmission to unaffected siblings was analyzed (columns 5 and 6 of Table 4).

Remarkably, the HB*14 allele was always transmitted to the MG children [$P < 0.0002$ for the comparison with the transmission of other alleles; $P < 0.01$ for the comparison with the expected (6:6) Mendelian segregation]. Importantly, transmission of HB*14 to unaffected sibs remained balanced, ruling out a segregation distortion in MG children unrelated to the disease. The two patterns of HB*14 transmission, to the MG children and to their unaffected sibs, also differed at a highly significant level ($P < 0.0025$ by Fisher's exact test).

One potential limitation of this method of analysis comes from the fact that it ignores homozygous parents. In our study, results for 23 such parents, homozygous for the 13-13 and 12-14 haplotypes (16 and 7 parents, respectively), were discarded. These numbers were those expected given the frequencies of heterozygous parents. Since there was no evidence for an involvement of marker alleles associated with 13-13 and 12-14 haplotypes, the exclusion of homozygous parents introduced no bias in our analysis. Taken together with the results of the population study, these haplotype transmission data therefore pointed to HB*14-associated haplotypes as disease-promoting haplotypes.

DISCUSSION

In this work, we have first characterized highly polymorphic DNA markers within *CHRNA*, a gene whose protein product, the AChR α subunit, plays a central role in the pathogenesis of MG. No polymorphism of the AChR protein is presently known. At the DNA level, restriction fragment length polymorphisms associated with the AChR α-, γ-, and δ-chain genes were described previously (40). However, these markers were of limited use because they were only biallelic and because one of the alleles was largely predominant. We chose to look for d[(GT)·(CA)] dinucleotide repeats, which provide an abundant source of often extremely polymorphic PCR-amplified markers. These repeats seem to be evenly distributed throughout the genome, ensuring that in a given stretch of DNA of 30–40 kb, one of them is likely to be found. We identified and mapped two of these d[(GT)·(CA)] microsatellites 4 kb apart from each other in the first intron of *CHRNA*. Both of them displayed a useful level of allelic polymorphism, permitting an efficient haplotyping of the myasthenic patients and of their relatives. Furthermore, the two markers were reasonably stable in the germline. No mutation was apparent in 224 meioses analyzed. Thus, these two markers could also be used to type unrelated individuals in a case-control study.

The population study and the family-based analysis provided converging arguments that support a role for the HB*14-associated haplotypes of *CHRNA* in MG susceptibility. However, the haplotype segregation analysis was more sensitive for detecting this effect than the population study. As now known from the study of the insulin gene in insulin-dependent diabetes mellitus (34–39), the former method obviates the difficulties arising from population heterogeneity, because the control data set is obtained from the same population sample as the "affected-haplotype" set. In addition, MG is a complex disease with a multigene control and *CHRNA* is only but one susceptibility gene beside *HLA* genes, antigen-receptor genes, or other genes. The HB*14 allele may therefore occur in the general population with limited pathologic consequences, as the probability of presenting all the required disease genes simultaneously is low. In contrast, in myasthenic families, a disease-predisposing genetic background is available and HB*14-bearing individuals are at an increased risk of developing the disease which

Table 2. Distribution of BB and HB alleles in 100 control subjects (n = 200) and in 81 MG patients (n = 162)

Allele	No. of alleles (%)	
	Control subjects	MG patients
HB		
18	0	2 (1.2)
17	24 (12)	14 (8.6)
16	2 (1)	2 (1.2)
15	0	1 (0.6)
14	12 (6)	23 (14.2)*
13	97 (48.5)	65 (40.1)
12	62 (31)	52 (32.1)
11	3 (1.5)	3 (1.8)
BB		
18	0	2 (1.2)
17	17 (8.5)	7 (4.3)
16	0	3 (1.8)
15	18 (9)	23 (14.2)
14	66 (33)	55 (33.9)
13	99 (49.5)	72 (44.4)

Alleles are designated by their number of CA repeats.
*Relative risk = 2.59; $\chi^2 = 6.51$; $P < 0.05$.

Table 3. Transmission of *CHRNA* parental haplotypes to myasthenic children and to their siblings in 33 MG families

HB-BB parental haplotypes	Transmission to MG children		Transmission to unaffected siblings	
	+	−	+	−
18-18	3	0	0	0
17-17	3	7	14	10
17-15	2	3	3	4
16-15	0	1	0	0
14-15	6	0	14	13
14-14	4	0	1	0
14-13	2	0	6	5
13-13	18	20	35	38
12-14	15	23	36	40
11-13	3	2	10	9

Table 4. Preferential transmission of parental HB*14 alleles to myasthenic children

Allele	Presence/ absence	Transmission to MG children +	Transmission to MG children −	Transmission to unaffected sibs +	Transmission to unaffected sibs −
HB					
18	+	3	0	0	0
	−	53	56	119	119
17	+	5	10	17	14
	−	51	46	102	105
16	+	0	1	0	0
	−	56	55	119	119
14	+	12	0*	21	18†
	−	44	56‡	98	101
13	+	18	20	35	38
	−	38	36	84	81
12	+	15	23	36	40
	−	41	33	83	79
11	+	3	2	10	9
	−	53	54	109	110
BB					
18	+	3	0	0	0
	−	53	56	119	119
17	+	3	7	14	10
	−	53	49	105	109
15	+	8	4	17	17
	−	48	52	102	102
14	+	19	23	37	40
	−	37	33	82	79
13	+	23	22	51	52
	−	33	34	68	67

For each allele, parental haplotypes bearing this allele (upper row, +) and those not bearing this allele (lower row, −) were enumerated by using the haplotyping data (see Table 3).
*$P < 0.01$ for Fisher's exact test of the comparison of HB*14 transmission with the expected (6:6) Mendelian pattern.
†$P < 0.0025$ for Fisher's exact test of the comparison of HB*14 transmission to MG children and to their unaffected siblings.
‡$P < 0.0002$ for Fisher's exact test of the comparison of the transmission of HB*14-associated haplotypes to MG children with that of non-HB*14.

can be efficiently detected by the method of haplotype relative risks. In this view, the existence of HB*14-positive unaffected siblings would be explained by the absence of susceptibility alleles of other disease genes in these individuals. Extensive typing of these families for the above-mentioned candidate genes will help determine whether a particular combination of these genes actually predisposes to MG.

Although CA repeats were reported to affect chromatin conformation and to influence gene expression (41–43), the HB*14 allele could also be involved in MG susceptibility by means of its linkage disequilibrium with another sequence polymorphism of *CHRNA* that now remains to be identified. Basically, two non-mutually exclusive mechanisms can account for a role of AChR genes in the susceptibility to MG. The first one is an immunological mechanism whereby a polymorphism of the protein product results in an immunogenic presentation of an AChR-derived self-peptide by HLA molecules to the immune system. Work showing self-reactivity of T lymphocytes from myasthenic patients against oligopeptides derived in particular from the N-terminal portion of the α chain (21, 22), which is precisely encoded by exons closely linked to HB and BB markers, is compatible with this pathogenic mechanism.

However, at this stage of our investigation, a second, nonimmunological type of mechanism cannot be ruled out. In this hypothesis, the functioning of the AChR molecular complex is impaired by itself and the autoimmune aggression sensitizes this malfunction. For example, altered transcriptional regulation of *CHRNA* could result in poor resynthesis of its protein product following crosslinking and internalization of AChR molecules by autoantibodies (44). It is hoped that detailed analysis of sequence polymorphisms in the vicinity of the microsatellite markers will yield decisive information that will support one of these hypotheses.

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