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# *A Comparison of the Caffeine Halothane Muscle Contracture Test with the Molecular Genetic Diagnosis of Malignant Hyperthermia*

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Malignant hyperthermia (MH) is currently diagnosed by the caffeine-halothane contracture (CHC) test. In a previous study, this test was used to establish linkage between the human gene for MH susceptibility and the ryanodine receptor (RYR) gene. The current study extends the genetic linkage analysis to a large French-Canadian kindred. In this family, genetic linkage between RYR and MH genes was not demonstrable using the currently recommended limits of normal for the CHC test in the identification of MH-susceptible individuals. With CHC test threshold limits below those currently recommended, however, complete linkage between the RYR and MH

genes was seen. Comparisons of CHC test results with genetic linkage studies will increase the diagnostic accuracy of both tests as well as generate new insights into the biology of MH. (Key words: Malignant hyperthermia/genetic linkage: ryanodine. Receptor gene measurement techniques: caffeine halothane contracture test.)

MALIGNANT HYPERTHERMIA (MH) is an inherited disorder of skeletal muscle in which susceptible individuals may develop muscle rigidity, hyperthermia, and acidosis in response to certain anesthetic agents.<sup>1</sup> Different modes of MH inheritance have been reported, and the condition has been observed in association with a variety of other illnesses.<sup>2,3</sup> Thus, the MH phenotype may result from a variety of metabolic disorders. In most families, however, MH susceptibility is inherited as an autosomal dominant trait. Because the predisposition to MH is inherited, it is important to identify other family members who carry the MH gene. The caffeine-halothane contracture (CHC) test, which measures the concentration of halothane and caffeine required to trigger contracture in freshly biopsied skeletal muscle, has been used for the past two decades in the diagnosis of MH susceptibility.<sup>4</sup> The high sensitivity of the CHC test is reflected by the absence of any reported MH crises in individuals with negative test results.<sup>5</sup> However, positive or even ambiguous results in any component of the test are likely to yield a positive diagnosis because of the hazards associated with a false negative diagnosis of MH susceptibility. This may lead to a reduction of the specificity of the CHC test.

The primary derangement that causes the dominant form of MH appears to lie in the calcium metabolism of skeletal muscle.<sup>1,2,6</sup> The protein that comprises the calcium release channel of skeletal muscle sarcoplasmic reticulum is called the ryanodine receptor (RYR).<sup>7,8</sup> Recent biochemical studies have implicated a defect in the ryanodine receptor in the pathogenesis of autosomal recessive porcine halothane sensitivity.<sup>9</sup> Using the standard CHC test for the diagnosis of MH susceptibility, we have recently demonstrated close genetic linkage between the MH phenotype and polymorphisms in the RYR gene.<sup>10</sup> In the current study, we have extended the genetic linkage to a large French-Canadian kindred.

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## Materials and Methods

Blood samples were collected from 16 members of a three-generation French-Canadian MH kindred. The method of Miller *et al.*<sup>11</sup> was used for deoxyribonucleic acid (DNA) extraction. Restriction enzyme digestion and Southern blot hybridization conditions were performed as described previously.<sup>12</sup> DNA linkage studies have been approved by the Children's Hospital of Eastern Ontario ethics committee.

### LINKAGE ANALYSIS

If alleles at two distinct genetic loci are observed to travel together in all families studied, the loci are said to co-segregate and are genetically linked. Loci that are genetically linked are situated on the same chromosome, usually relatively close to one another. If the loci always co-segregate, then they are completely linked and have a recombinant fraction (RF) of 0.00; if they travel together 19 times of 20, then they are linked and have an RF of 0.05. The degree of certainty that two loci are linked is given by the "lod" score, which is the logarithm of the odds that genetic linkage exists between two given loci. Therefore, a lod score of 3 indicates odds of 1,000:1 for genetic linkage between two loci. Lod scores were obtained by linkage analysis with the MLINK program.<sup>13</sup>

A portion (or subclone) of DNA called HRR1-1600, which codes for the 1,000 amino acids comprising the COOH-terminus of the RYR protein, was used for the Southern hybridization experiments. DNA was isolated from the leukocytes of MH family members and digested with the DNA cutting restriction enzyme BanI. The digested DNA was separated electrophoretically on an agarose gel, transferred to a nylon membrane and hybridized with radioactively labeled HRR1-1600 (Southern blotting).

The radiolabeled DNA marker hybridized to two different genomic DNA fragment size classes, as outlined previously.<sup>10</sup> These different-sized fragments result from DNA sequence alterations and are examples of restriction fragment length polymorphisms (RFLPs). RFLPs allow the tagging and following of specific chromosomal regions from generation to generation in a given family. Humans contain two copies of all genes. Consequently, if a certain gene is postulated to cause a given illness, then by RFLP analysis, one can assay for the presence of the same copy of the gene in all individual members of a given family affected with the disease and its absence in unaffected individuals. The presence of such genetic linkage is essential in establishing that a given gene defect causes a certain illness.

### PROBAND

At the age of 8 yr, the proband (IV-1, fig. 1) was admitted to hospital for the removal of a trauma-induced

cataract. Upon administration of succinylcholine, masseter spasm occurred, precluding intubation, and the rectal temperature increased from 98.5 to 100.2° F. At this stage surgery was cancelled, and dantrolene (20 mg intravenous) was administered. The patient's temperature returned to normal, and he recovered uneventfully although he complained of severe chest wall soreness the following day. The creatine kinase concentration immediately after the attempted anesthesia was 71.5 IU/l and increased to 577 IU/l the following day (normal range 30–250 IU/l).

### CHC TEST PROTOCOL

Eleven family members underwent quadriceps muscle biopsies during 1985 and 1986. The CHC test was performed according to the recommendations of Rosenberg and Reed.<sup>14</sup> Tests performed on the fresh muscle included 1) caffeine specific concentration (CSC), the concentration of caffeine needed to generate a 1-g contracture; 2) halothane-caffeine specific concentration (HCSC), the concentration of caffeine needed to generate a 1-g contracture when used in combination with 1% halothane; 3) the muscle tension generated with exposure to 2 mM caffeine (2 mM CAF); and 4) the tension generated with exposure to 1% halothane (1% HAL).

### Results

The pedigree of the French-Canadian kindred is presented in figure 1. Those members of the family considered MH-susceptible are the proband (IV-1) and his mother's two first cousins (III-8 and III-9), who died while receiving general anesthesia. Another first cousin (III-14) suffers from recurrent muscle cramping, necessitating ongoing oral dantrolene therapy. Obligate MH gene carriers are the proband's mother (III-3), grandmother (II-2), and great uncle (II-3, deceased). The results of analysis of inheritance of the BanI polymorphism in the RYR gene also are presented in figure 1. The B allele was inherited by those individuals who were either MH positive or MH obligate carriers and by individuals III-2, III-11, III-12, and III-14.

The results of the CHC test performed on 11 muscle biopsies are presented in table 1 and figure 2, together with the segregation of the RYR gene polymorphism. All but one (III-13) of the biopsied individuals were diagnosed as MH-susceptible according to the definition of a positive CSC as  $\leq 4$  mM.<sup>15</sup> In this case, no consistent co-segregation is seen between the RYR gene polymorphism and the MH phenotype. This is reflected in a maximum lod score of 0.02 at an RF = 0.45. If a positive CSC test is redefined as 2 mM caffeine or less, however, only the biopsied individuals carrying the RYR B allele (III-2, III-11, III-12, and III-14) would be identified as MH susceptible (fig. 2A). The co-segregation of MH with RYR gene markers under this condition is 100% and generates a lod score of 3.84 (RF = 0.00).

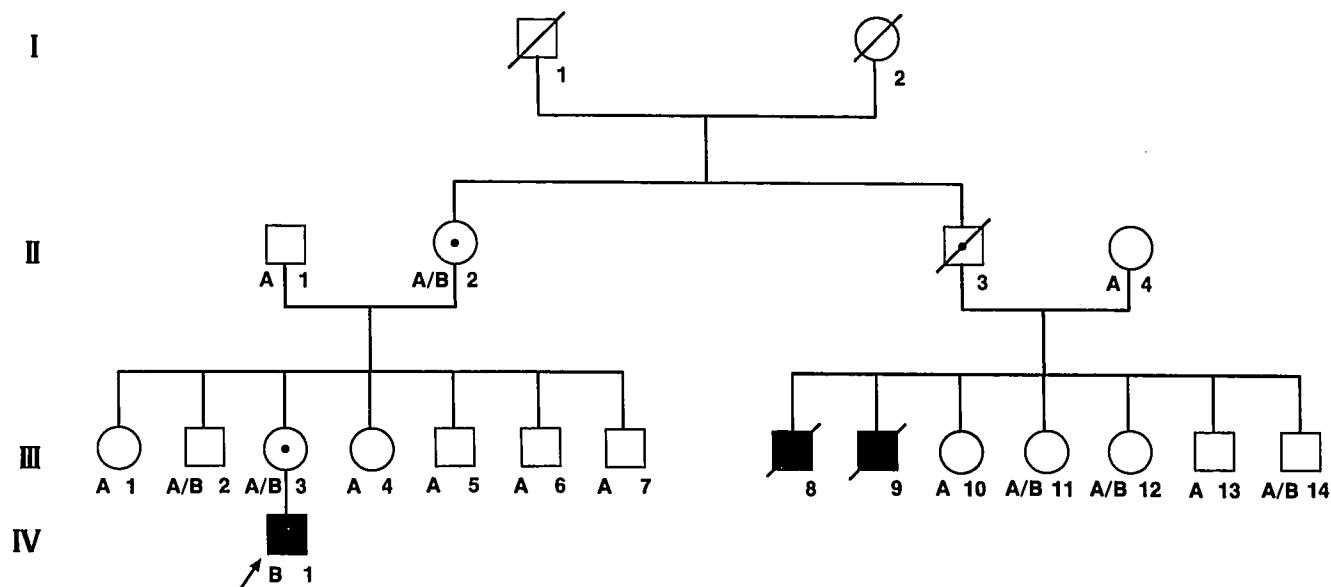


FIG. 1. Pedigree of MH kindred illustrating segregation of a RYR gene polymorphism. The letters A and B refer to the 13- and 6-kb polymorphic fragments detected by probing *Ban*I-digested genomic DNA with the RYR DNA marker HRR1-1600.<sup>9</sup> Individuals with histories of unequivocal MH crises are indicated by a darkened symbol, and obligate heterozygotes are indicated with a central dot (see text).

A positive 2 mM CAF result has been recommended to be the development of a contracture  $\geq 0.2$  g.<sup>15</sup> This would identify individuals III-2, III-4, III-7, III-11, III-12, and III-14 as MH-susceptible, again resulting in no significant MH-RYR gene linkage (lod score = 1.25 and RF = 0.14). Defining a positive 2 mM CAF result as the development of tension  $> 1.0$  g, however, would once again result in complete RYR gene-MH susceptibility linkage (fig. 2B, lod score = 3.84, RF = 0.00).

A positive HCSC is defined as the development of a 1-g contracture after exposure to 1 mM or less caffeine in the presence of 1% halothane.<sup>15</sup> Because all individuals tested developed 1-g contracture at caffeine concentrations well below 1 mM, all would be deemed to be MH susceptible, resulting again in no significant MH-RYR linkage (lod score = 0.03 and RF = 0.45). Defining the

lower limit of normal as 0.3 mM caffeine, however, would result in complete MH-RYR gene linkage (fig. 2C).

Testing with 1% halothane is not currently included in recommended North American protocols.<sup>15</sup> Nevertheless, complete co-segregation was also seen between the development of any contracture to 1% halothane (*i.e.*,  $> 0.0$  g) and the RYR gene polymorphism (fig. 2D).

### Discussion

This study extends our previous analysis of linkage between RYR gene polymorphisms on chromosome 19 and the MH gene.<sup>10</sup> The linkage established in the earlier study (lod score = 4.2 and RF = 0.00) suggests that these genes are located within a few million base pairs of each other and may be identical.

In this study complete genetic linkage between the MH phenotype and the *Ban*I RYR RFLP is observed (lod score = 3.84 and RF = 0.00) if positive muscle biopsy results are defined to be CSC  $\leq 2$  mM caffeine, HCSC  $< 0.3$  mM caffeine, 2 mM CAF  $> 1.0$  g, or 1% HAL  $> 0.0$  g. These upper limits of normal for CSC, HCSC, and 2 mM CAF differ from those currently recommended in North America.<sup>15</sup> There is no such recommendation for 1% halothane responses. However, Rosenberg and Reed observed contractures of  $> 0.4$  g to 1% halothane in six individuals with MH and  $< 0.4$  g in 15 controls.<sup>14</sup> Our study is in agreement with their work insofar as the use of 0.4 g as the limit of normal for 1% HAL would also have resulted in complete genetic linkage between the MH phenotype and the *Ban*I RYR RFLP.

The linkage observed between MH and the RYR gene using altered positive CHC test criteria depends upon the accuracy of the test results themselves. Although not

TABLE 1. CHC Test Results and RYR Allele Segregation in 11 Members of an MH French-Canadian Kindred

| Individual | CSC (mM) | 2 mM CAF (g) | HCSC (mM) | 1% HAL (g) | RYR Alleles |
|------------|----------|--------------|-----------|------------|-------------|
| III-1      | 2.54     | 0.00         | 0.51      | 0.0        | A           |
| III-2      | 0.93     | 3.80         | 0.26      | 1.1        | A/B         |
| III-4      | 2.26     | 0.50         | 0.38      | 0.0        | A           |
| III-5      | 2.46     | 0.15         | 0.60      | 0.0        | A           |
| III-6      | 3.00     | 0.00         | 0.79      | 0.0        | A           |
| III-7      | 2.45     | 0.20         | 0.43      | 0.0        | A           |
| III-10     | 2.90     | 0.00         | 0.58      | 0.0        | A           |
| III-11     | 0.67     | 6.15         | 0.13      | 3.6        | A/B         |
| III-12     | 1.17     | 2.70         | 0.16      | 0.7        | A/B         |
| III-13     | 4.10     | 0.00         | 0.66      | 0.0        | A           |
| III-14     | 1.05     | 4.45         | 0.14      | 5.0        | A/B         |

The various tests are described in the text. The A and B alleles of the ryanodine receptor (RYR) gene are described in the legend to figure 1. Note cosegregation of B allele and strong CHC test responders.

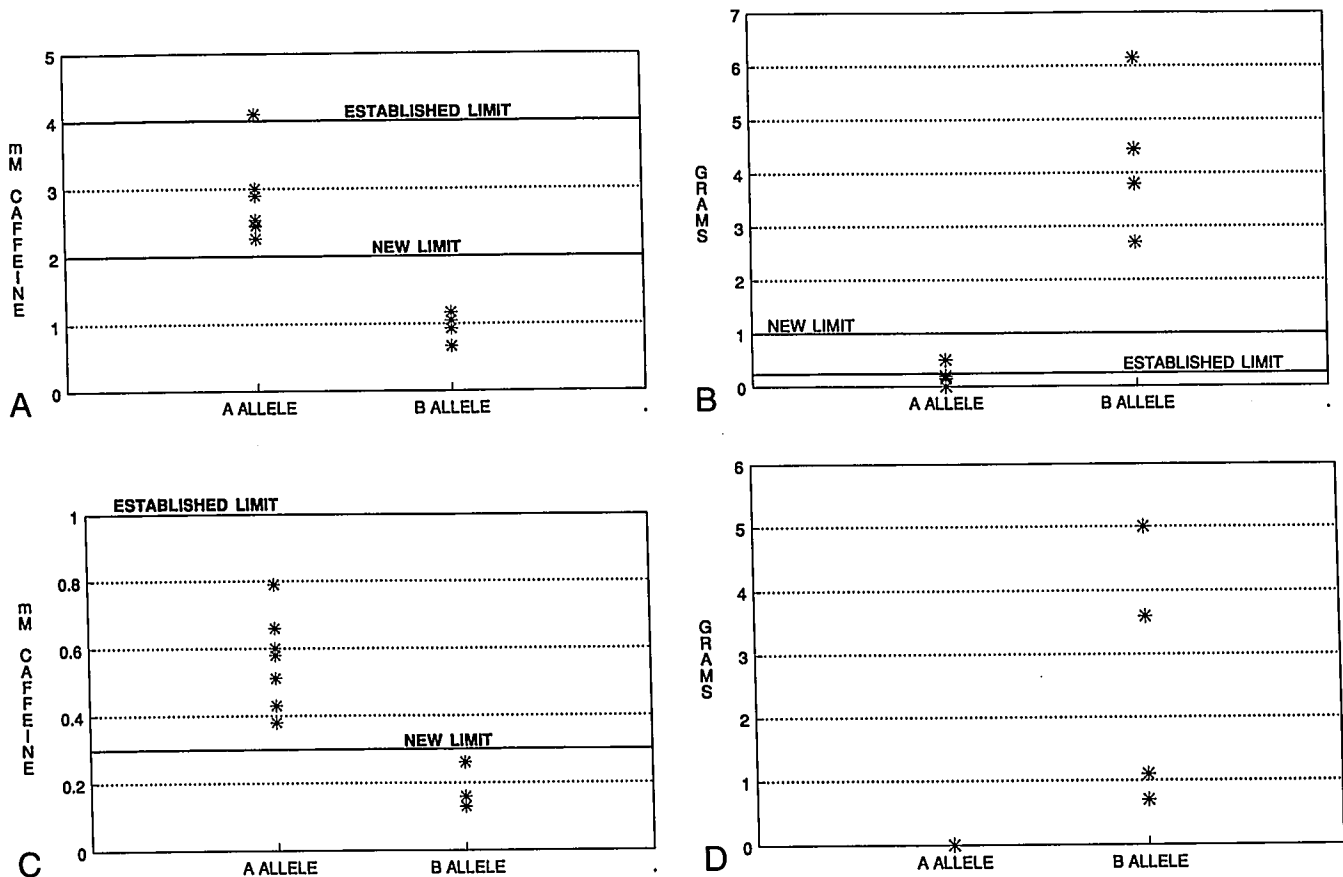


FIG. 2. CHC test results obtained for MH kindred members. A: Caffeine specific concentration. B: 2 mM caffeine. C: Halothane-caffeine specific concentration. D: 1% halothane. The results have been grouped for those individuals who inherited the A RYR RFLP allele and those who inherited the B allele. Where possible, the recommended limit of normal for a CHC subtest (established limit) as well as a limit that distinguishes the two different groups (new limit) are given.

identical, the recently published suggested protocol for diagnostic muscle biopsies and the methodology used in this work were very similar.<sup>15,16</sup> Consequently, we feel that the published CHC test result guidelines can be reliably applied to the data obtained in our laboratory.

It may be that there is no linkage between the MH and RYR genes in this family and that the causative MH mutation lies elsewhere in the genome. This is unlikely, however, in light of 1) the previously established genetic linkage of MH to the region of chromosome 19 containing RYR<sup>10,17,18</sup> and 2) the linkage that can be demonstrated in this family if the CHC test criteria are modified. We estimate the odds of complete A allele-weak CHC result and B allele-strong CHC result co-segregation occurring randomly to be approximately 1:8,000. We believe a much more likely possibility is that the accepted criteria for the positive CHC test<sup>15</sup> in this family leads to false positive results. This interpretation is compatible with Larach *et al.*'s recent finding of significant false positive rates for the CHC test performed on individuals not at risk for MH.<sup>19</sup>

Analyses such as in the current study may lead to a more complete understanding of CHC test results. Ulti-

mately, the identification of the mutation(s) causing MH will allow a more precise correlation between the CHC test and the true MH susceptible state. A critical question is whether the criteria defining a positive CHC test should be changed in light of our findings. Given the relatively small number of individuals in this study, their representation of only one of a potentially large number of MH-causing mutations, and the hazards associated with a false negative diagnosis of MH, we believe that any alteration of the criteria for the CHC test would be premature at this stage. Nevertheless, further studies to determine whether CHC test standards should be altered are clearly warranted.

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