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Identification and Biochemical Characterization of a Novel Ryanodine Receptor Gene Mutation Associated with Malignant Hyperthermia

Ayuk A. Anderson, Ph.D., Rosemary L. Brown, Ph.D.,† Brenda Polster, B.S.,‡ Neil Pollock, M.B., Ch.B.,§ Kathryn M. Stowell, Ph.D.‖*

Background: **Mutations in the skeletal muscle ryanodine receptor gene may result in altered calcium release from sarcoplasmic reticulum stores, giving rise to malignant hyperthermia (MH). MH is a pharmacogenetic skeletal muscle disorder triggered by volatile anesthetics and depolarizing muscle relaxants. Diagnosis of MH is by *in vitro* contracture testing of quadriceps muscle. DNA analysis of causative mutations is limited by the large number of mutations that cosegregate with MH and the relatively few that have been biochemically characterized.**

Methods: **DNA sequence analysis was used to screen the skeletal muscle ryanodine receptor gene in MH-susceptible individuals. A diagnostic test using real-time polymerase chain reaction was developed to detect the mutation in individuals diagnosed as MH susceptible by *in vitro* contracture testing. The functional relevance of this mutation was examined in Epstein-Barr virus–immortalized B-lymphoblastoid cells.**

Results: **A novel ryanodine receptor mutation (cytosine 14997 thymine resulting in a histidine 4833 tyrosine substitution) was identified in pathology specimens from two patients with fatal MH reactions. B lymphocytes from patients with this mutation were approximately twofold more sensitive than MH-negative cells to activation with 4-chloro-*m*-cresol. The amount of $^{45}Ca^{2+}$ released from B lymphocytes of MH-susceptible patients was significantly greater than that released from cells of family members without this mutation. Haplotype analysis suggests that both families had a common ancestor.**

Conclusions: **DNA analysis to detect mutations which cosegregate with MH as well as biochemical assays on cultured lymphocytes obtained from blood can serve as useful diagnostic tools for MH susceptibility and genotype–phenotype correlations.**

MUTATIONS in the skeletal muscle *RYR1* gene have been reported to cause malignant hyperthermia (MH) and central core disease (CCD).[1–3] MH, a disorder that is often inherited as an autosomal trait, is a pharmacogenetic disorder of skeletal muscle triggered by inhalational anesthetics and depolarizing muscle relaxants.[4] It is characterized by hypermetabolism, hypercapnia, tachycardia, hyperthermia, hypoxemia, muscle rigidity, and metabolic acidosis, which can lead to death of the patient if unabated.[5] MH is provoked by an increase in myoplasmic calcium concentration resulting from an abnormal release of calcium from the sarcoplasmic reticulum[6] through the ryanodine receptor calcium channel (RyR). With an incidence of 1 in 15,000 to 1 in 60,000 anesthetics administered,[7,8] the actual proportion of MH-susceptible (MHS) individuals in the community has been reported to be 1 in 8,500[9] and may be as high as 1 in 2,000.[10] Molecular genetic studies have identified more than 100 mutations in the *RYR1* gene associated with MH and/or CCD,[11–13] with at least 28 of these having been functionally characterized and reported to be causative of MH.[13]#

In this study, we describe the use of DNA analysis to identify a novel *RYR1* mutation in pathology specimens from patients with fatal MH reactions that occurred 20–25 yr previously. We also comment on two other unexplained deaths where MH susceptibility was subsequently confirmed by DNA analysis. Several studies have demonstrated that human B lymphocytes express *RYR1*[14–17] with the gene product functioning as a calcium release channel. Therefore, we investigated the effect of the H4833Y transition on calcium release induced by 4-chloro-*m*-cresol (4-C*m*C), a potent and specific activating agent of the skeletal muscle ryanodine receptor,[18] in B lymphocytes from MH-positive family members related to the deceased individuals harboring the same mutation.

Materials and Methods

Patients and Samples

Blood and tissue samples were obtained after informed consent from all individuals involved in each aspect of this study or from relatives of deceased individuals. Ethical approval was obtained from the Manawatu-Whanganui (Palmerston North, New Zealand) and Massey University (Palmerston North, New Zealand) human ethics committees.

Case 1. A 15-yr-old girl (fig. 1, V:16) underwent a cortical mastoidectomy in 1980 after a diagnosis of bacterial meningitis secondary to a suppurating ear infection treated with antibiotics. She subsequently had two anesthetics, using succinylcholine and halothane, over a 2-week period lasting 20 and 40 min for suction clear-

* Postdoctoral Fellow, † Graduate Student, ‖ Senior Lecturer, Institute of Molecular BioSciences, Massey University. ‡ Research Technician, Institute of Molecular BioSciences, Massey University. Current position: Molecular and Medical Genetics Department, Oregon Health and Science University, Portland, Oregon. § Specialist Anesthetist, Department of Anesthesia and Intensive Care, Palmerston North Hospital, Palmerston North, New Zealand.

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Address correspondence to Dr. Stowell: Institute of Molecular BioSciences, Massey University, Private Bag 11-222, Palmerston North, New Zealand. k.m.stowell@massey.ac.nz. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue.

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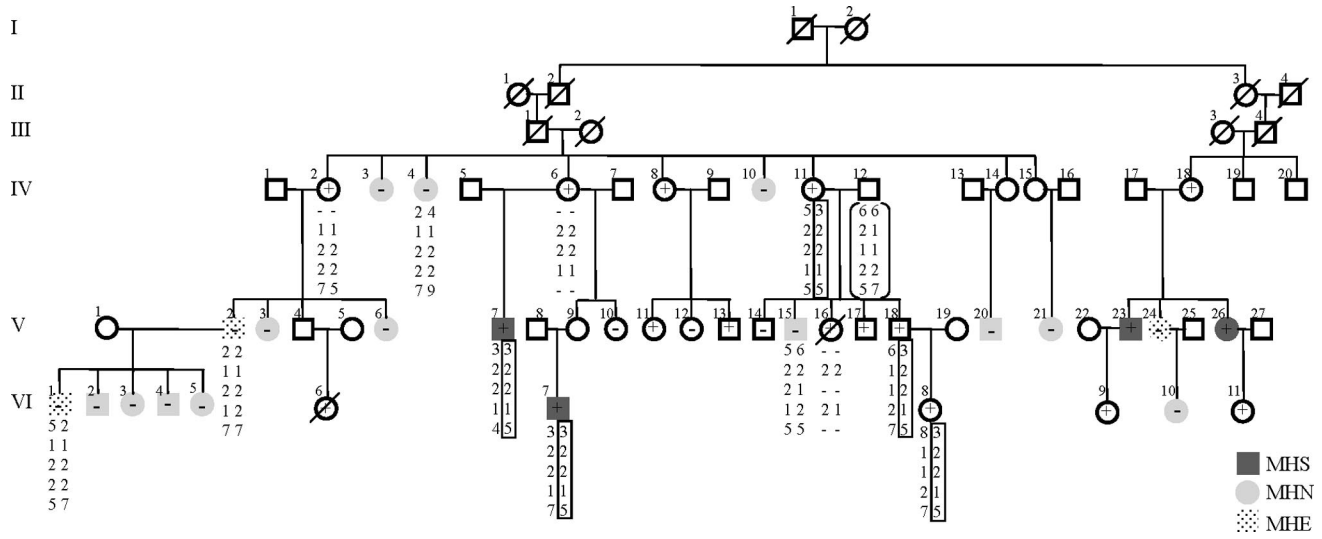

Fig. 1. Pedigree diagram. New Zealand Maori kindred related to case studies 1 (V:16) and 2 (IV:6). The chromosome 19 haplotype (D19S220, Ile1151, Asp2729 Ser2862, D19S47) cosegregating with malignant hyperthermia is shown *boxed*. The haplotypes are represented as alleles. (+) and (−) refer to presence or absence, respectively, of the C14997T mutation. MHE = malignant hyperthermia equivocal; MHN = malignant hyperthermia negative; MHS = malignant hyperthermia susceptible.

ance and further review, respectively. Apart from a brief episode of bigeminal rhythm in the second procedure, which responded rapidly to 50 mg lignocaine intravenously, there were no problems. Anesthesia for the cortical mastoidectomy was induced with 40 mg alfaxalone, intubation facilitated with 80 mg alcuronium, and anesthesia maintained with nitrous oxide, oxygen, and halothane. The surgery was performed in semidarkness, with light provided only by the microscope. Monitoring consisted of manual palpation of the radial pulse. Darkness in the blood of the skin flaps was the first abnormality, noted after approximately 2.5 h; nitrous oxide was discontinued, and oxygen flow was increased to 6 l/min. The procedure was completed 15 min later. After removing the drapes, the patient appeared stiff, with pupils dilated and no respiratory effort, and twitching of the face muscles was apparent. Cardiac arrest occurred after a rapid deterioration. Resuscitation was unsuccessful. Core temperature of greater than 40°C was noted postmortem. Autopsy findings showed no intracranial or cardiac pathology. Subsequent questioning of the family indicated that a cousin had died 4 yr previously (case 2) with a presumptive diagnosis of MH.

Case 2. An 11-yr-old girl (fig. 1, IV:6) underwent appendicectomy in May 1976. She received 200 mg thiopentone and 50 mg succinylcholine, she was intubated, and anesthesia was maintained with halothane. Within 10 min of induction, her pulse rate increased from 94 to 155 beats/min and remained at about this rate. Preoperatively, the temperature was recorded at 37.5°C but was not measured intraoperatively. The procedure took 35 min, and the patient arrived in recovery tachypneic with rigid limbs. She woke 10 min later, reporting stiffness and painful feet and legs. A succinylcholine reaction was considered to be the cause. She was noted to be mildly cyanosed, with a pulse rate of 152 beats/min, blood pressure of 185/100 mmHg, and grunting respiration. She arrested in ventricular fibrillation 15 min later and was defibrillated twice unsuccessfully. Postmortem examination showed no significant findings.

In Vitro *Contracture Testing*

In vitro contracture testing (IVCT) of muscle biopsies was performed according to the European Malignant Hyperthermia Group (Leeds, United Kingdom) protocol.#

Extraction of RNA and Genomic DNA

Total RNA was extracted from 30–100 mg frozen skeletal muscle tissue using Trizol RNA extraction reagent according to the manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, CA). Genomic DNA was isolated from leukocytes using the Wizard DNA extraction kit according to manufacturer's instructions (Promega Corporation, Madison, WI). Paraffin-embedded autopsy tissue samples were obtained from the two individuals with suspected MH (cases 1 and 2). DNA was prepared from deparaffinized tissue with prolonged treatment with proteinase K followed by phenol–chloroform extraction.[19]

Mutation Screening by Reverse-transcription Polymerase Chain Reaction and DNA Sequencing

First strand synthesis was performed using the Superscript reverse transcriptase preamplification system (Invitrogen Life Technologies) with 4 μg total RNA and either 50 ng random hexamers or 500 ng oligo (dT) in a 20-μl volume. Hot-start polymerase chain reaction was performed using 1 μl of a 20-fold dilution of the first strand complementary DNA reaction in 50-μl reactions with 0.32 μM of each primer, 0.3 mM dNTPs, 1.5 mM

$MgCl_2$, and 1.5 U *Taq* polymerase (Invitrogen Life Technologies). Exon 100 of the *RYR1* gene was amplified in one 208–base pair (bp) fragment using forward and reverse primers ACCTGGGCTGGTATATGGTG/TTATCCCTTCACCACCCACT, respectively. Sequencing was performed from a CCCTCTTGGGACACTACAACA primer using an ABI 377 with Big-Dye terminator chemistry (Applied Biosystems, Foster City, CA). The three mutational hot spots for MH/CCD were amplified and sequenced as previously described.[20]

Single-stranded Conformational Polymorphism Analysis

Exon 100 of *RYR1* was amplified from genomic DNA and screened for the presence of the C14497T mutation by single-stranded conformation polymorphism analysis as previously described.[21]

Diagnostic Testing Using Allele-specific Polymerase Chain Reaction

A diagnostic test for the C14497T mutation was developed using hybridization probes and real-time polymerase chain reaction using a Light Cycler (Roche Applied Science, Mannheim, Germany)[22,23] as follows. The allele-specific probe (CACCCACAATGGGAAACAGC) was labeled with Cy5.5, and the anchor probe (GCGCACCATCCTGTCCTC) was labeled with fluorescein. The sequences of the flanking primers were 5′-CACAGTCCTTCCTGTACC-3′ (forward) and 5′-GCCCTTATCCCTTCACC-3′ (reverse). The oligonucleotide primers and probes were designed using Light Cycler Primer Design software (Roche Applied Science). The following thermocycling protocol with the Light Cycler Hybridization probe kit (Roche Applied Science) was used for amplification: denaturation at 95°C for 120 s, followed by 55 cycles of 95°C for 0 s, 55°C for 10 s, and 72°C for 12 s, each with a temperature transition rate of 20°C/s and data acquisition at the annealing step. Primers were used at 0.5 μM, and probes at 0.2 μM, and $MgCl_2$ at 4 mM. Melting curve analysis was as follows: 95°C for 0 s and 45°C for 30 s, each with a temperature transition rate of 20°C/s followed by 75°C for 0 s at a temperature transition rate of 0.2°C/s with continuous data acquisition. For each step in the protocol, the fluorescence display was F3/1.

Haplotype Analysis

Haplotype analysis was performed using the D19S220[24,25] and D19S47[26] chromosome 19q microsatellite repeat markers that flank the *RYR1* locus and three intragenic restriction fragment length polymorphism markers,[27] Ile1151 (*Taq*I), Asp2729 (*Fok*I), and Ser2862 (*Cfo*I), as described previously.[20] Allele frequencies were as published.[24–26]

Establishment of Lymphoblastoid Cell Lines

Peripheral mononuclear cells were isolated by Ficoll-Hypaque (Amersham Biosciences, Amersham, United Kingdom) density gradient centrifugation, from whole blood. The isolated mononuclear cells were then transformed with Epstein-Barr virus.[28] Cells were grown in OptiMem medium (Invitrogen Life Technologies) supplemented with 2% fetal calf serum (Invitrogen Life Technologies), 2 mM L-glutamine (Invitrogen Life Technologies), and 100 U penicillin and streptomycin (Invitrogen Life Technologies), at 37°C, 5% CO_2.

Membrane Preparation and Western Blot Analysis

Total microsomes were isolated from HEK293 cells, rat skeletal muscle, and Epstein-Barr virus–transformed B lymphocytes.[28] *RYR1* expression was analyzed using 6% SDS-PAGE and Western blotting. Immunostaining of proteins was performed using the ryanodine receptor–specific monoclonal antibody, 34C (Sigma, St. Louis, MO) and peroxidase-conjugated antimouse immunoglobulin G (Sigma) as secondary antibody followed by chemiluminescent detection (Roche Applied Science).

Radioactive $^{45}Ca^{2+}$ Uptake and Release by B Lymphocytes

$^{45}Ca^{2+}$ uptake assays were performed in Hanks balanced salt solution (HBSS) containing 140 mM NaCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 10 mM HEPES (pH 7.4) as described previously.[29] Briefly, approximately 1×10^7 cells/ml were incubated with 5–10 μCi $^{45}CaCl_2$ (Amersham Biosciences) at 37°C for 60 min in HBSS buffer, into which 0.5 mM Na–adenosine triphosphate, 5 mM $K_2C_2O_4$, and 100 μg/ml heparin were added. Aliquots of 200 μl containing 0.8×10^6 cells were removed from the incubation medium at appropriate time intervals and washed four times with HBSS buffer. The final wash was performed in calcium-free HBSS buffer containing 2 mM EGTA. Cell pellets were either resuspended and lysed in 200 μl Triton, 0.1%, or resuspended in 200 μl calcium-depleted HBSS buffer and incubated with 400–800 nM thapsigargin to release incorporated $^{45}Ca^{2+}$. Radioactivity of the loaded/released $^{45}Ca^{2+}$ was determined by liquid scintillation counting.

$^{45}Ca^{2+}$ release assays were performed using 4-C*m*C, after lymphocytes had been actively loaded for 1 h, with radioactive $^{45}Ca^{2+}$. Cells were washed as above. Supernatant (200 μl) from the final wash was reserved for scintillation counting as prestimulated released $^{45}Ca^{2+}$. Cell pellets were resuspended in 1 ml calcium-depleted HBSS buffer, and 200-μl aliquots of approximately 0.8×10^6 viable cells were transferred to separate microcentrifuge tubes and incubated with increasing concentrations of 4-C*m*C. Each sample was briefly centrifuged, and the resulting supernatant was collected and radioactivity determined by liquid scintillation counting. Counts per minute obtained for prestimulation were subtracted from those obtained after stimulation of cells with 4-C*m*C. The concentration of 4-C*m*C causing half-maximal release (EC_{50}) of $^{45}Ca^{2+}$ was determined by curve

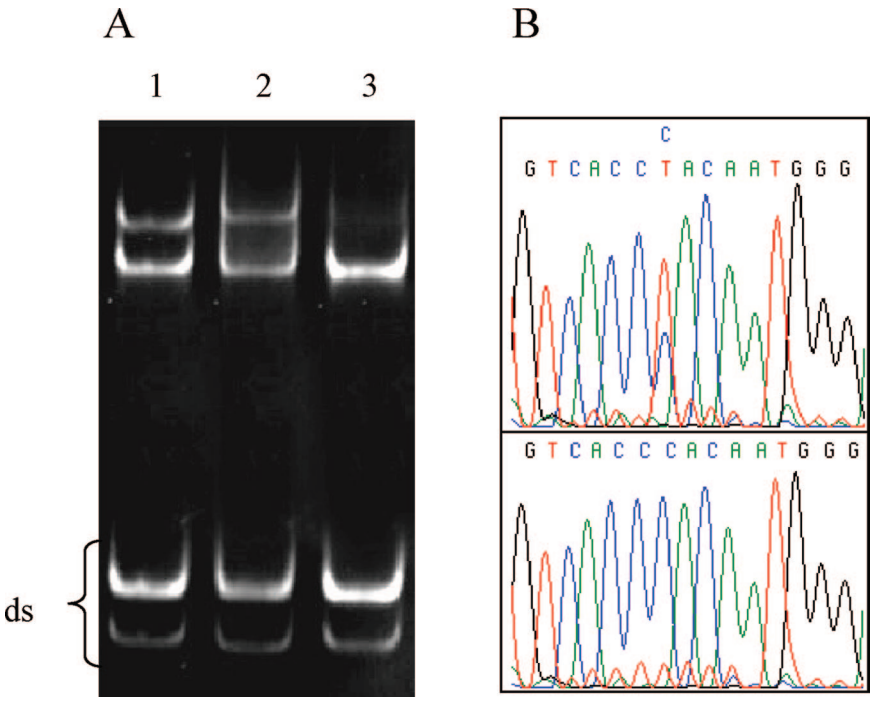

Fig. 2. Identification of a novel RYR1 mutation. (*A*) Polyacrylamide gel stained with ethidium bromide showing single-stranded conformation polymorphism analysis of DNA sample with T4826I mutation (*lane 1*), H4833Y mutation (*lane 2*), and normal sample (*lane 3*). The *top band* in *lanes 1* and *2* represent the mutated allele, whereas the *second to top bands* represent the normal allele. (*B*) DNA sequence analysis of exon 100 of *RYR1*. The *top panel* is from a malignant hyperthermia–susceptible patient with the H4833Y mutation; the C to T transition is indicated. The *bottom panel* is from a malignant hyperthermia–negative subject. ds=double stranded.

fitting. Parallel experiments were performed where thapsigargin[14] was used to establish a reference standard for complete release of $^{45}Ca^{2+}$ from endoplasmic reticulum stores. A time-dependence study of 4-C*m*C–stimulated calcium release was also performed[29,30] to compare the amount and the rate of $^{45}Ca^{2+}$ release from B lymphocytes of both MHS and MH-negative (MHN) individuals over a period of 20 min. Except where indicated, all manipulations including loading and release assays were performed at 37°C and washes in ice-cold HBSS buffers.

Statistical Analysis

Statistical analysis was performed using a paired *t* test and one-way analysis of variance (repeated measures). Analysis was performed using Analyse-it** (Microsoft, Redmond, WA) software for general and clinical laboratory statistics Excel version 1.73.

Results

Identification of the Novel H4833Y Mutation

A T4826I mutation had previously been identified in exon 100 of *RYR1* in a large Maori MHS pedigree.[21] As part of screening for the presence of this mutation in other MHS families, a 208-bp fragment encompassing exon 100 was amplified from genomic DNA of MHS individuals from other families and subjected to single-stranded conformation polymorphism analysis. An additional variant was identified, in a relative of case 1 (fig. 1, IV:17), by the appearance of an additional single-stranded DNA species that migrated slightly slower than the band associated with the T4826I mutation (fig. 2A, lane 2). DNA sequence analysis of the 208-bp polymerase chain reaction product revealed a novel C14997T transition that substituted tyrosine for histidine 4833 (fig. 2B). The three MH/CCD mutation regions were then sequenced, and no additional mutations were identified. Two hundred twenty chromosomes from unrelated (no family history of MH) control subjects of both Maori and Caucasian origin were analyzed by single-stranded conformation polymorphism for the presence of the C14997T polymorphism. The mutation was not detected in any of these families. H4833 is conserved across 14 ryanodine receptor sequences from human to *Drosophila melanogaster*.

Diagnosis by In Vitro *Contracture Testing*

Relatives of both individuals have had IVCT using the European Malignant Hyperthermia Group Protocol. Four had MHS test results, 3 had MH-equivocal results, and 12 had MHN results and are indicated in figures 1 and 3.

Diagnostic Test

A diagnostic test from genomic DNA was developed to facilitate analysis of additional family members. The heterozygote was clearly distinguished from the homozygote with melting temperatures of the wild-type and mutant alleles of 64°C and 55°C, respectively (data not shown). The *RYR1* C14497T mutation was identified in DNA extracted from postmortem specimens of both case studies. The same mutation was

** Available at: www.analyse-it.com. Accessed June 1, 2007.

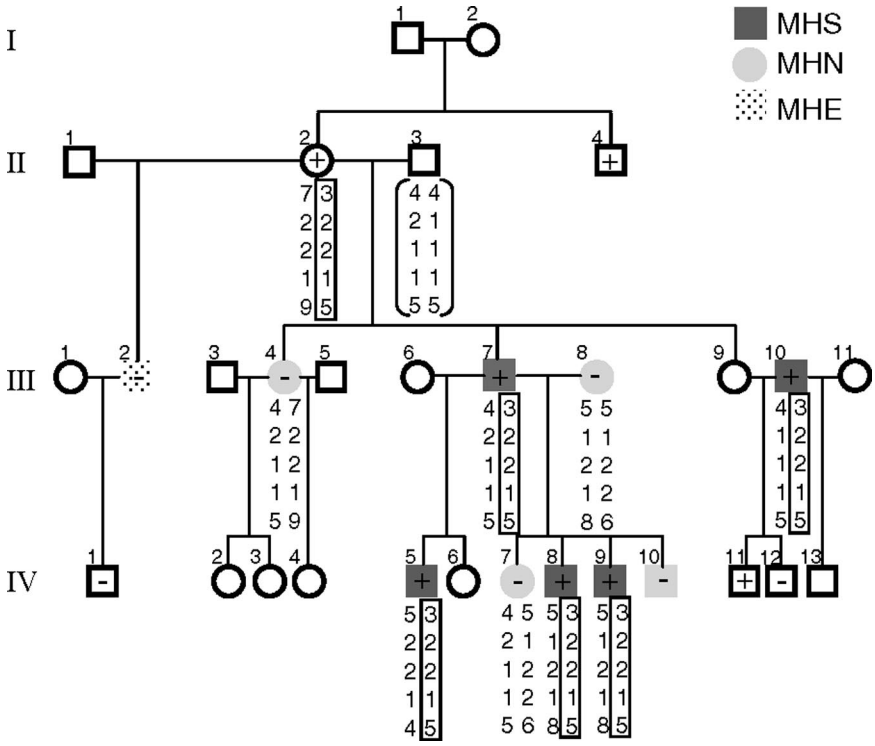

Fig. 3. Pedigree diagram. New Zealand Maori kindred apparently unrelated to case studies 1 and 2. The proband is III:7. The chromosome 19 haplotype (D19S220, Ile1151, Asp2729 Ser2862, D19S47) cosegregating with malignant hyperthermia is shown *boxed*. The haplotypes are represented as alleles. (+) and (−) refer to presence or absence, respectively, of the C14997T mutation. MHE = malignant hyperthermia equivocal; MHN = malignant hyperthermia negative; MHS = malignant hyperthermia susceptible.

identified in extended family members (fig. 1) as well as in a second family previously suspected to be susceptible to MH (fig. 3). There was complete concordance between the presence of the mutation and MHS in both families. These data suggest that the H4833Y mutation is causative of MH in the two families. The mutation was not detected in any of the MH-equivocal patients. We have yet to identify an MH-equivocal patient in any New Zealand family with an *RYR1* mutation.

Haplotype Analysis

Haplotype analysis was performed on selected samples to examine the possibility that the two families had a common ancestor. Microsatellite markers flanking the *RYR1* locus and three intragenic restriction fragment length polymorphism markers were used to analyze the chromosome 19q13.1 genotype in both families.[24–26] This analysis revealed a common haplotype (3-2-2-1-5) that segregated with MHS in each family (figs. 1 and 3). Allele 3 for the D19S220 dinucleotide repeat is one of the least common reported in the Caucasian population.[24,25] Allele 5 for the D19S47 dinucleotide repeat has an intermediate frequency in an undefined population.[26] These results suggest that both families have inherited the H4833Y mutation from a common ancestor.

RYR1 *Expression in B Lymphocytes*

The expression of *RYR1* in immortalized lymphocytes was investigated by immunoblotting analysis on subcellular membrane fractions. A distinct immunoreactive band of approximately 565 kd representing the ryanodine receptor was identified in rat skeletal muscle (fig. 4, lane 2) as well as in B lymphocytes (lane 4) but not in HEK293 cells (lane 3), which do not express the gene for the ryanodine receptor. Although the 34C antibody does recognize all three RyR iso-

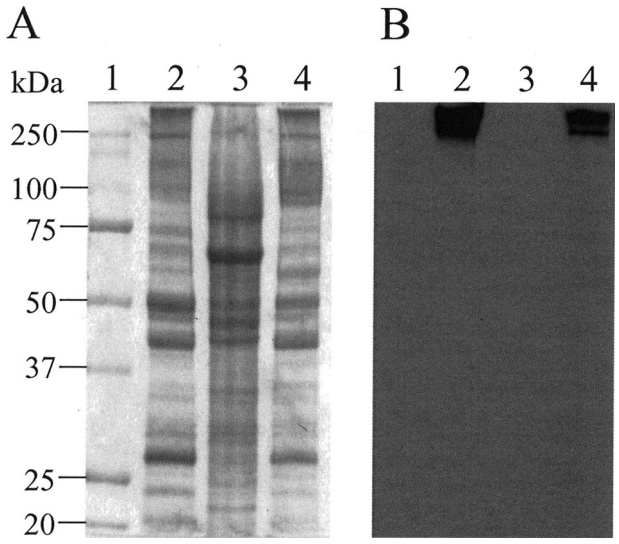

Fig. 4. Expression of RYR type 1. Total microsomes prepared from rat skeletal muscle, HEK293 cells, and human B lymphocytes were electrophoresed in a denaturing 7.5% polyacrylamide gel. *A* represents a Coomassie brilliant blue stain of a gel containing the following amounts of protein: 35 μg from rat skeletal muscle (*lane 2*), 35 μg from HEK293 cells (*lane 3*), and 40 μg from B lymphocytes (*lane 4*). *B* represents Western blot analysis of the same amount of protein transferred to a nitrocellulose membrane and stained with monoclonal antibodies (34C) specific for the ryanodine receptor.

forms, it has a lower affinity for RyR2 and RyR3, and RyR1 has been shown to be the predominant isoform expressed by B lymphocytes.[16]

Adenosine Triphosphate–dependent $^{45}Ca^{2+}$ Uptake by Epstein-Barr Virus–immortalized B Lymphocytes

Lymphocytes were loaded with $^{45}Ca^{2+}$ and total Ca^{2+} stores estimated after lysis with Triton X-100. The total $^{45}Ca^{2+}$ incorporated into the cells and released by lysis with 0.1% Triton (results not shown) was only 2% greater than that released by 800 nM thapsigargin. Therefore, the amount released by 800 nM thapsigargin (considered to be approximately 100%) was used as a reference for $^{45}Ca^{2+}$ release.[14,31] Cells from MHS (fig. 1, IV:2 and V:13, and fig. 3, II:4) and MHN (fig. 1, V:10 and V:15, and fig. 3, III:4) patients were treated with 400–800 mM thapsigargin, which specifically blocks the sarco(endo) plasmic reticulum Ca^{2+}–adenosine triphosphatase,[32,33] to stimulate Ca^{2+} release from stores. No significant differences were found between MHS and MHN lymphocytes (data not shown). Therefore, the H4833Y mutation does not affect the thapsigargin-sensitive Ca^{2+} pool.

*4-Chloro-*m*-Cresol Concentration–Response Activation of B Lymphocytes*

Drug-induced $^{45}Ca^{2+}$ release was initiated in a concentration-dependent manner with 4-C*m*C.[18] Cells were stimulated with increasing concentrations of 4-C*m*C up to 1,000 μM, and the amount of $^{45}Ca^{2+}$ released was calculated as a percentage relative to that which could be released by 800 nM thapsigargin. The amount of $^{45}Ca^{2+}$ released from MHS cells was significantly greater than that from MHN cells at all concentrations of 4-C*m*C used (fig. 5). The EC_{50} of $^{45}Ca^{2+}$ was lower for MHS cells (370 μM) than for MHN cells (670 μM), indicating a higher sensitivity to 4-C*m*C (fig. 5).

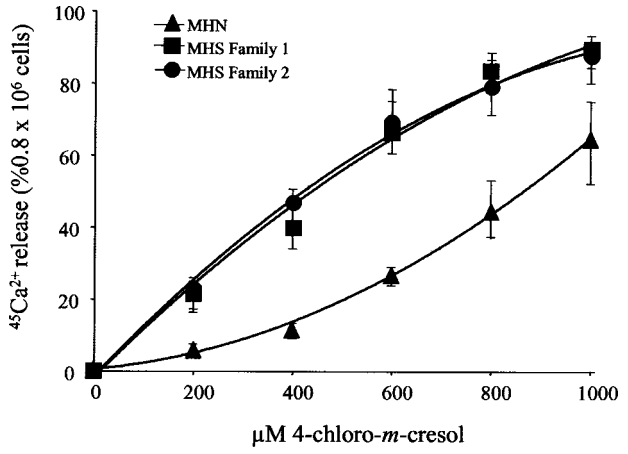

Fig. 5. 4-Chloro-*m*-cresol (4-C*m*C) concentration–response stimulation of B lymphocytes. Concentration–response curves to 4-C*m*C and regression analysis of mean values (± SD; where n = 6 for each cell line used) of $^{45}Ca^{2+}$ released induced by the indicated concentrations of 4-C*m*C. R = 0.94 (P = 0.0064) for malignant hyperthermia–susceptible (MHS) family 1 (fig. 1, IV:2 and V:13); R = 0.94 (P = 0.0062) for MHS family 2 (fig. 3, II:4), and R = 0.97 (P = 0.0097) for malignant hyperthermia–negative (MHN) individuals from both families (fig. 1, V:10 and V:15, and fig. 3, III:4). The confidence interval was set at 95%. Half-maximal release of $^{45}Ca^{2+}$ induced by 4-C*m*C occurred at 370 μM for lymphocytes from both MHS individuals and at 610 μM for MHN lymphocytes. Observed counts-per-minute values in all experiments were normalized to the intensities obtained with 800 nM thapsigargin considered to be 100%, and the amount of $^{45}Ca^{2+}$ released by the different concentrations of 4-C*m*C was calculated as a percentage relative to the amount released by 800 nM thapsigargin.

Discussion

A novel *RYR1* C14997T mutation has been identified from postmortem tissue of two individuals who had fatal MH episodes, as well as from genomic DNA of relatives. MH susceptibility was confirmed by positive IVCT results of relatives. In addition, we have shown unequivocal evidence of cosegregation of the C14997T mutation with clinical diagnosis of MHS in two patients with fulminant MH episodes and in four patients diagnosed as MHS by IVCT, and absence of this mutation in 15 relatives diagnosed as MHN or MH-equivocal by IVCT. Taken together, these observations suggest a causative role in MH for the C14997T mutation.

We have shown that H4833Y is a potential candidate for MHS rather than a rare polymorphic codon by performing biochemical assays with 4-C*m*C in immortalized B lymphocytes from MHS individuals carrying the mutation and MHN individuals from two families. The amount of actively loaded $^{45}Ca^{2+}$ released by 4-C*m*C from MHS cells was significantly greater than that released from MHN cells. Compared with MHN cells, 1.8-fold lower concentrations of 4-C*m*C were required to activate the release of 50% of the releasable $^{45}Ca^{2+}$ from lymphocytes of MHS individuals harboring the skeletal muscle ryanodine receptor H4833Y mutation. This suggests that the increased $^{45}Ca^{2+}$ response by B cells of MHS individuals to lower concentrations of 4-C*m*C is associated with this *RYR1* mutation. These results are similar to those of previous studies on other *RYR1* mutations that showed a 1.8- to 2.0-fold decrease in EC_{50} for 4-C*m*C–induced Ca^{2+} release from MHS compared with MHN cells.[14,34–36] The H4833Y mutation does not seem to affect the thapsigargin-sensitive calcium pool, indicating that it is likely to be an MH-specific mutation rather than causative of CCD, because many CCD mutations in this region of the gene cause the channel to be leaky.[1]

We have developed a robust DNA-based diagnostic test for MH susceptibility in these two families so that other family members can be tested without the need for the IVCT. According to the European Malignant Hyperthermia Group guidelines,[37] a negative result for the DNA test does not necessarily confer nonsusceptibility; however, a negative mutation analysis coupled with a negative B-lymphocyte test should be sufficient to make an

MHN diagnosis. Of course, this does not rule out spontaneous mutations in the dihydropyridine receptor or other genes yet to be identified with MH. Some caution must also be exercised with an MHN diagnosis by DNA analysis, in families where discordance has been reported.[21,38–40]

This method of identifying MH susceptibility from postmortem tissue has yielded positive results in two other individuals. Both were sudden deaths unrelated to anesthesia, and both were from a family with a known *RYR1* mutation. DNA was extracted from archival postmortem tissue from a female child who died at 9 months of age in 1969 and from a 2-yr-old girl several months after death following a 3-day viral illness. In both of these cases, DNA was extracted from postmortem tissue, and the familial mutation was demonstrated in both specimens.[20]

Patients may present for anesthesia with a family history of a fatality associated with anesthesia in a family member, which has not been previously investigated. In some cases, it may be possible to investigate the index case by postmortem analysis.[21] Such a practice will assist in confirming MH susceptibility in families where little historic clinical information is available.

In conclusion, we suggest that the H4833Y substitution is causative of MH because Y4833 is associated with the MHS phenotype in two families and is absent in control individuals, H4833 is conserved across species, and it is located in an MH/CCD hot spot region of the *RYR1* gene. Moreover, functional assays using immortalized B lymphocytes from patients harboring this mutation showed a significant increase in the amount of $^{45}Ca^{2+}$ released compared with MHN cells.

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