Electrophoresis of Muscle Proteins Is Not a Method for Diagnosis of Malignant Hyperthermia Susceptibility

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The authors used denaturing polyacrylamide slab gel electrophoresis, employing 7.5–20% and 15–20% polyacrylamide gradients, to compare total skeletal muscle proteins of 12 normal and 19 malignant hyperthermia (MH)-susceptible individuals. The patients' MH status was determined by contracture testing. No consistent qualitative or quantitative differences could be detected. Because MH is believed to be triggered by a loss of control of sarcoplasmic [Ca²⁺], the authors compared: 1) the Ca²⁺-binding proteins of eight normal and ten MH muscles using Ca²⁺-dependent electrophoretic mobility shifts and a transblot/⁴⁰CaCl₂ overlay technique; and 2) the total protein composition of a heavy sarcoplasmic reticulum fraction isolated from five normal and nine MH muscles. Again, no differences were detected. Finally, a similar electrophoretic study was conducted to compare the total protein composition of five normal and six MH-susceptible, central core disease-affected human muscles. No significant differences could be observed. It appears, therefore, that simple electrophoretic techniques cannot be applied in the diagnosis of MH susceptibility. (Key words: Hyperthermia: malignant; Ions: calcium. Muscle, skeletal: diseases. Protein.)

MALIGNANT HYPERTERMIA (MH) is an hereditary disease of skeletal muscle characterized by metabolic acidosis and elevated serum metabolite levels, a rapid increase in body temperature, and often muscle rigidity. This disorder manifests itself during general anesthesia, is most commonly induced by halothane or succinylcholine, and can lead to death if therapy, i.e., administration of dantrolene and symptomatic treatment are not instituted as soon as possible. During the past 15 years considerable advances have been made in MH in areas related to detection of individuals who are at risk, identification of pharmacologic triggering agents, and treatment. Less progress has been made in identifying the basic disordered mechanisms in the disease, although studies with skinned skeletal muscle fibers and isolated sarcoplasmic reticulum have suggested that the Ca²⁺-induced Ca²⁺ release mechanism of the sarcoplasmic reticulum is significantly more sensitive to Ca²⁺ in MH muscle than in normal muscle.

Current methods of diagnosis of MH susceptibility rely on in vitro contracture tests: MH-susceptible muscle developing contracture at significantly less concentrations of caffeine or halothane, for example, than does normal muscle. In this study we investigated the protein composition of skeletal muscle in normal and MH-susceptible persons with two objectives in mind: 1) possible identification of a missing or different protein in MH-susceptible muscle compared with normal muscle, which could represent the fundamental defect in this genetic disorder; and 2) development of a simple biochemical diagnostic method for detecting MH susceptibility that would necessitate biopsy of only small tissue samples. Patients were classified as normal or MH-susceptible by contracture studies. We also applied the same methodology to compare the protein composition of normal human muscle with that of muscle from individuals afflicted with central core disease, a rare congenital myopathy in which MH seems to occur with a very high frequency.

Methods

MUSCLE BIOPSIES

All muscle biopsies were taken from the vastus lateralis muscle. As soon as the muscle was removed it was divided into portions that were used for contracture studies, routine histology and histochemistry, electron microscopy, and biochemical studies. The portion of the biopsy used for these biochemical studies was frozen immediately in isopentane previously chilled in liquid nitrogen and was then placed in liquid N₂ for transport to the laboratory where it was stored at -80°C until used.

In our laboratory, contracture studies are carried out using the methodology described by Britt. In one series of tests, individual muscle strips were exposed to caffeine concentrations ranging from 0.125 to 32 mM, and the contracture response, if there was one, was measured in grams. In another series of tests, the muscle strips were exposed to vaporized halothane in concentrations ranging from 1% to 3% and again contracture responses, if they occurred, were measured in grams. In both test situations up to three strips of muscle could be used, baseline tensions were set at 2 g, and the muscle strips were stimulated throughout the test procedure to ensure their viability.
Halothane concentrations were verified using an infrared analyser. All testing was completed within 2 to 3 h of the time the biopsy was removed.

In our laboratory, two types of contracture responses are considered indicative of MH susceptibility. In one group of patients (A), a contracture response of at least 1 g develops on exposure to both 2 mM caffeine or 3% halothane in the gas phase. In the other group of patients (B), calculation of the caffeine-specific concentration (CSC), as described by Britt, reveals an abnormal response. In our laboratory a CSC of less than 3.5 mM is considered indicative of MH susceptibility.

**SODIUM DODECYL SULFATE (SDS)-POLYACRYLAMIDE GRADIENT SLAB GEL ELECTROPHORESIS**

Muscle samples (approx. 0.1 g) were homogenized while jacketed in an ice bath, using a Brinkmann® Polytron equipped with a PT10ST® probe generator in 20 volumes of 25 mM Tris-HCl (pH 6.8), 0.5% sodium dodecyl sulfate (SDS), 15% glycerol, 1 mM 2-mercaptoethanol, 0.005% bromphenol blue (SDS gel sample buffer), heated in a boiling water bath for 2 min, cooled on ice, and centrifuged at 15,000 × g for 15 min. Supernatants were divided in two, one of which was made 10 mM in CaCl₂ and the other 10 mM in EGTA. Electrophoresis was carried out at 36 mA in 0.1% SDS, 7.5–20% polyacrylamide gradient slab gels or 0.1% SDS, 15–20% polyacrylamide gradient slab gels (1.5 mm thick × 12 cm high × 18 cm wide), with 5% acrylamide stacking gels (2.5 cm high), according to the method of Laemmli. Gels were stained with Coomassie brilliant blue R-250 (0.14%) in 45% ethanol, 10% acetic acid, and diffusion-stained in 10% acetic acid. Gels were scanned with an LKB 2202 Ultrascan® laser densitometer equipped with an HP390A integrator.

**TRANSBLOT®/44CaCl₂ OVERLAY TECHNIQUE**

Proteins separated on 0.1% SDS, 7.5–20% polyacrylamide gradient slab gels were electrophoretically transferred to nitrocellulose membranes (Transblot® transfer medium, BioRad Laboratories, Richmond, CA) and incubated with 44CaCl₂ (1 mCi/l) as described by Maruyama et al. Dried nitrocellulose membranes were exposed to Kodak X-Omat® AR film in a Kodak X-Omat® AR cassette equipped with intensifying screens for 1–3 days.

**SARCOPLASMIC RETICULUM PREPARATION**

Heavy sarcoplasmic reticulum (SR) was isolated from frozen muscle biopsy samples by a modification of the method of Nelson. Muscle samples (approx. 0.1 g each) were homogenized while jacketed in an ice bath, for 3 × 10 s in 5 volumes of 20 mM imidazole-HCl (pH 6.8) with the aid of a Brinkmann® Polytron equipped with a PT10ST® probe generator. The homogenates were centrifuged at 8,000 × g for 20 min, and the pellets were discarded. The supernatants were filtered through glass wool and made 0.6 M in KCl and centrifuged at 12,000 × g for 20 min. The resultant pellets (heavy SR) were suspended in 5 volumes of 20 mM imidazole-HCl (pH 6.8), 0.15 M KCl and centrifuged at 48,000 × g for 30 min. The pellets were suspended in 2.5 volumes of SDS gel sample buffer diluted 1:1 with distilled, deionized water and boiled prior to SDS-polyacrylamide gel electrophoresis.

**RESULTS**

**PATIENT CONTRACTURE RESPONSES AND CLINICAL HISTORY**

A total of 42 patients were studied. Of these, 16 patients were normal, 17 patients demonstrated group A responses, and nine patients showed group B responses. Of the six patients with central core disease, five showed group A responses. In the group A responders (excluding central core disease patients) were three patients who had clinical MH reactions, three first-degree relatives of patients who had died of MH, and a first-degree relative of a patient who survived an episode of MH. In the group B responders, two patients were referred because of a clinical suspicion of MH that was treated before the full clinical syndrome had developed. Two of the central core disease patients were thought to have had clinical MH reactions. Three of the other central core disease patients were first-degree relatives of these patients. Because it was only toward the end of the study that halothane was introduced as one of the test agents in our laboratory, only four of the 17 group-A responders were tested with both caffeine and halothane and produced abnormal responses to both.

**COMPARISON OF NORMAL AND MH-SUSCEPTIBLE HUMAN MUSCLE PROTEINS**

Preliminary experiments involving comparisons of the electrophoretic patterns of total proteins of fresh and frozen rabbit back muscle indicated that no differences were apparent. Consequently, all subsequent studies with human muscle biopsies were carried out using tissue that had been quick-frozen in liquid N₂ and stored at −80°C.

The protein compositions of human muscle samples
Fig. 1. Electrophoretic comparison of normal and MH-susceptible human muscle proteins. Samples (approx. 0.1 g each) of three normal and three MH-susceptible muscles were treated as described under “Methods” prior to electrophoresis in the presence of 0.1% SDS on a 7.5–20% polyacrylamide gradient slab gel (A) and a 15–20% polyacrylamide gradient slab gel (B) in the presence (+) and absence (−) of Ca²⁺ as indicated. Samples from MH-susceptible and normal muscles are indicated “MH” and “N,” respectively. Molecular weights of protein standards electrophoresed on the same gels are indicated on the left in kilodaltons (Kd). Some of the major protein components in the muscle are identified on the right on the basis of subunit molecular weight and relative amount. Protein loads: A—lanes 1 and 2 = 25 μg, lanes 3–5 = 50 μg, lanes 6–10 = 55 μg, lanes 11 and 12 = 50 μg; B—lanes 1 and 2 = 25 μg, lanes 3–5 = 50 μg, lanes 6–8 = 55 μg, lanes 9 and 10 = 59 μg, lanes 11 and 12 = 35 μg.

obtained initially from three normal and three MH-susceptible patients were compared by 0.1% SDS, 7.5–20% polyacrylamide gradient slab gel electrophoresis (fig. 1A) and 0.1% SDS, 15–20% polyacrylamide gradient slab gel electrophoresis (fig. 1B). These techniques also permitted comparison of proteins undergoing a Ca²⁺-dependent electrophoretic mobility shift by electrophoresis in the presence of CaCl₂ (10 mM) or in the absence of Ca²⁺ (presence of 10 mM EGTA). Detailed comparisons of the electrophoretic patterns (fig. 1), including densitometric scanning of the electrophoretograms using a laser densitometer (fig. 2), revealed no consistent qualitative or quantitative differences between normal and MH-susceptible muscle samples. Furthermore, no differences be-
between group A and group B responders were detected. Several Ca$^{2+}$-binding proteins can be identified in the gels of figure 1 on the basis of a Ca$^{2+}$-dependent electrophoretic mobility shift (e.g., proteins of molecular weights approximately 100,000, 65,000, 47,000, and 20,000 daltons; the latter is probably troponin C) (table 1).

Ca$^{2+}$-binding proteins were also examined in total homogenates of normal and MH-susceptible muscle by transblotting the proteins separated on a gel onto a nitrocellulose membrane, which was then incubated with $^{45}$CaCl$_2$. After washing out the excess $^{45}$CaCl$_2$, Ca$^{2+}$-binding proteins were visualized by autoradiography (fig. 3). The major Ca$^{2+}$-binding protein had a molecular weight of approximately 20,000 daltons and can be tentatively identified as troponin C (TnC). It appears as a doublet in some lanes, which may perhaps represent the Ca$^{2+}$-free and Ca$^{2+}$-bound forms of the protein. Other Ca$^{2+}$-binding proteins are apparent of molecular weights approximately 65,000, 47,000, 26,000, and 15,000 daltons. The amount of Ca$^{2+}$-binding proteins varies considerably but there is no correlation between amount and susceptibility to MH; this simply reflects different amounts of total protein loaded on the gel.

We have applied the same methodology (denaturing 7.5–20% and 15–20% polyacrylamide gradient slab gel electrophoresis in the presence and absence of Ca$^{2+}$) to a large number of additional normal (9) and MH-susceptible (16) muscle samples. All these data confirm our conclusion.
that no electrophoretic difference could be detected between normal and MH-susceptible muscle.

Because the Ca\(^{2+}\)-induced Ca\(^{2+}\) release mechanism of the SR has been implicated in MH,\(^5\text{--}^7\) we isolated SR from normal and MH-susceptible muscle and compared their protein compositions by electrophoresis (fig. 4). The major band can be identified on the basis of molecular weight

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\begin{array}{|c|c|c|c|}
\hline
\text{Molecular Weight} & \text{R}_f \text{ Value} & \text{Sodium Lauryl Sulfate Treatment} & \text{Gel Gradient} \\
\hline
100,000 & 0.274 & 0.283 & 7.5--20 \\
65,000 & 0.442 & 0.436 & 7.5--20 \\
47,000 & 0.271 & 0.279 & 15--20 \\
20,000 & 0.798 & 0.750 & 15--20 \\
\hline
\end{array}
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* Molecular weights and \text{R}_f \text{ values of muscle proteins undergoing a Ca}\(^{2+}\)-dependent electrophoretic mobility shift were calculated from the data in figure 1.

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\begin{array}{c}
205- \\
974- \\
66- \\
36- \\
29- \\
20.1- \\
14.2- \\
\end{array}
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\begin{array}{cccccccc}
1 & 2 & 3 & 4 & 5 & 6 & 7 & 8 \\
\end{array}
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\text{FIG. 4.} Electrophoretic comparison of sarcoplasmic reticulum proteins of normal and MH-susceptible human muscle. SR, prepared as described under Methods, was diluted 1:1 with SDS gel sample buffer, heated in a boiling water bath for 2 min, and electrophoresed on a 0.1% SDS, 7.5--20% polyacrylamide gradient slab gel. Key to lanes, with protein loads in parentheses: 1 (10 \(\mu\)g), 2 (20 \(\mu\)g), 3 (2.5 \(\mu\)g), 4 (5 \(\mu\)g), 7 (7.5 \(\mu\)g), 8 (15 \(\mu\)g) = MH-susceptible (caffeine and halothane); 5 (7.5 \(\mu\)g), and 6 (15 \(\mu\)g) = normal. Two samples of each SR preparation (two different loading levels) were analyzed. Therefore, a total of four preparations were examined in this experiment.

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12345678 \\
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\text{FIG. 3.} Identification of Ca\(^{2+}\)-binding proteins in normal and MH-susceptible human muscle by the transblot/\(\text{Na}\)Cl overlay technique. Proteins from normal and MH-susceptible muscles were separated on a 0.1% SDS, 7.5--20% polyacrylamide gradient slab gel and transblotted onto nitrocellulose membranes, which were treated with \(\text{Na}\)Cl. Ca\(^{2+}\)-binding proteins were identified by autoradiography. This figure depicts the resultant autoradiogram. Key to lanes, with protein loads in parentheses: 1 (25 \(\mu\)g), 2 (50 \(\mu\)g), 7 (2.5 \(\mu\)g), 8 (2.5 \(\mu\)g), 11 (10 \(\mu\)g), 12 (15 \(\mu\)g), 17 (40 \(\mu\)g), 18 (40 \(\mu\)g) = MH-susceptible (caffeine and halothane); 3 (5 \(\mu\)g), 4 (25 \(\mu\)g) = MH-susceptible (caffeine alone); 5 (60 \(\mu\)g), 6 (60 \(\mu\)g), 9 (10 \(\mu\)g), 10 (10 \(\mu\)g), 13 (50 \(\mu\)g), 14 (50 \(\mu\)g), 15 (50 \(\mu\)g), 16 (40 \(\mu\)g) = normal.

\[10,000 \text{ daltons}\) as the Ca\(^{2+}\) transport adenosine triphosphatase (ATPase). Whereas the yield of SR protein was very variable from one preparation to another, this bore no relationship to the incidence of MH susceptibility. Furthermore, the protein compositions of the four preparations (1 normal, 2 MH-susceptible) shown in figure 4 were indistinguishable. We have analyzed a total of five normal and nine MH-susceptible muscles in this way. These data fully support the conclusion that the protein compositions of normal and MH-susceptible SR are indistinguishable by this electrophoretic technique.

\text{Comparison of Normal and Central Core Disease Muscle Proteins}

We have also compared the electrophoretic patterns of total homogenates derived from normal and central

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core disease muscle samples, using both 7.5–20% and 15–20% polyacrylamide gradient gels (fig. 5). This study was carried out on muscle from five normal and six MH-susceptible, central core disease individuals. No significant qualitative or quantitative differences between the two groups could be detected.

**Discussion**

Blanch et al.\textsuperscript{14} compared the soluble protein compositions of the vastus lateralis muscle of three MH-susceptible individuals, three MH relatives, and six normal subjects by SDS-15% polyacrylamide gel electrophoresis. They observed large amounts of two low molecular weight (15,000 and 13,500 daltons) proteins in MH muscle that were not found in normal muscle and suggested that this may provide a useful diagnostic procedure for MH, while stressing the need to obtain data on more subjects. We have used a similar approach to attempt to develop a simple diagnostic method for MH susceptibility. Slab gel electrophoresis in the presence of 0.1% SDS in 7.5–20% or 15–20% polyacrylamide gradients enabled a comparison of muscle proteins from 12 normal and 19 MH-susceptible individuals that revealed no consistent or significant differences. We have observed large amounts of two low molecular weight proteins, which are probably identical to those of Blanch et al.\textsuperscript{14} in some muscle extracts but their presence does not correlate with the incidence of MH susceptibility (fig. 1). Furthermore, these proteins are found in all muscle samples we have examined; they merely vary in quantity (fig. 2). Recently, Fletcher et al.\textsuperscript{15} observed similar low molecular weight proteins in muscle of two controls and three MH-susceptible individuals. They suggested that these proteins may, in fact, be the α (15,800 daltons) and β (16,350 daltons) subunits of hemoglobin arising from serum contamination. In two recent letters to the editor, Fletcher and Rosenberg\textsuperscript{16} and Blanch\textsuperscript{17} provided further information in support of this conclusion. Our data strongly support their conclusion that electrophoretic techniques are not suitable diagnostic procedures for identifying MH susceptibility. Similarly, Marjanen and Denborough\textsuperscript{16} found no difference in the one- and two-dimensional electrophoretic patterns of skeletal muscle from normal and MH humans and swine.

Because MH is believed to involve defective control of sarcoplasmic Ca\textsuperscript{2+} concentration, and because the calmodulin content of MH pigs has been reported to be elevated,\textsuperscript{18} we also compared: 1) Ca\textsuperscript{2+}-binding proteins by both a Ca\textsuperscript{2+}-dependent electrophoretic mobility shift and a transblot/\textsuperscript{45}CaCl\textsubscript{2} overlay technique; and 2) SR protein composition between normal and MH-susceptible muscle. In neither case could we detect any significant or consistent differences. The lack of differences in SR protein composition
composition is consistent with the earlier observations of Oku et al. 20

Finally, we have conducted an electrophoretic comparison of total proteins of normal (five samples) and MH-susceptible central core disease (six samples) muscle to determine whether or not we could detect any differences in this group of patients. Again, no differences were detected.

In conclusion, the genetic defect in malignant hyperthermia appears to involve synthesis of a defective protein that, on high-resolution electrophoresis, is indistinguishable from the normal, functional protein, or the defect lies either in a minor protein that is not visible following electrophoresis or a very low molecular weight protein (<6,000 daltons) that is not fixed in the gel. At the moment, therefore, electrophoresis of total muscle proteins or heavy SR proteins does not provide a valuable diagnostic tool for the detection of MH susceptibility. It is possible that analysis of other discrete cell fractions (e.g., T-tubules or sarcolemma) may reveal differences between MH-susceptible and normal muscle protein profiles that were masked in the present study.

References

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