A Multicenter Study of 4-Chloro-m-cresol for Diagnosing Malignant Hyperthermia Susceptibility

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Standardization of the *in vitro* contracture test (IVCT) for malignant hyperthermia (MH) susceptibility has resulted in very rare false negative tests. However, false positive results stigmatizing the patient seem to be more frequent than false negative results and make supplementary tests desirable. This multicenter approach studied the usefulness of an IVCT with 4-chloro-m-cresol (4-CmC), a ryanodine receptor-specific agonist for a better definition of MH susceptibility. Diagnosis made by the standard IVCT was compared with the results of this 4-CmC test on muscle specimens of 202 individuals from 6 European MH centers. In the 4-CmC test, the results of the MH susceptible group differed significantly from both the MH normal and the MH equivocal group. 4-CmC revealed a qualitatively dose response-curve similar to caffeine. A correlation index of $r = 0.79$ for the concentration thresholds underlined the strong concordance of the caffeine and the 4-CmC effects. The optimal threshold concentration was determined to be 75 μM in the pooled data of all centers and is much lower than that of caffeine (2 mM), suggesting a more than 25-fold higher affinity of 4-CmC. The predictive value of 4-CmC is as high as that of caffeine and consequently higher than that of halothane. 4-CmC seems to be a suitable drug to refine diagnosis of MH susceptibility and could be used as an additional test substance. **Implications:** Although *in vitro* contracture testing for malignant hyperthermia diagnosis is well standardized, with a relatively high sensitivity and specificity, false test results cannot be excluded and may be associated with serious disabilities for the concerned individuals. In this multicenter study, 4-chloro-m-cresol was evaluated as a new test substance for the *in vitro* contracture testing. Its use improves the accuracy of *in vitro* diagnosis of malignant hyperthermia susceptibility.

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Malignant hyperthermia (MH) is a pharmacogenetic disorder of skeletal muscle (1,2). Triggered by volatile anesthetics and succinylcholine, MH may lead to a life-threatening crisis. The introduction of dantrolene in 1979 made a potent therapeutic drug available (3) and decreased the mortality rate from 70%–80% to <10%. The search for a test that allows diagnosis of the MH trait before a possible anesthetic reaction led to the introduction of the *in vitro* contracture test (IVCT) in a standardized form in 1984 in Europe (4) and in North America in 1989 (5). These tests are broadly accepted and are still the gold standard for the laboratory diagnosis of MH susceptibility (6). They proved to be highly reliable, with a sensitivity of 97%–99% and a specificity of up to 93.6% (7,8), remarkably good for a biological test. The risk of a false negative result is relatively low (<1% for the European and 3% for the North American protocol), but the occurrence of a false positive result is still between 7% (European protocol) and 22% (North American protocol).

The revolutionary progress of molecular biological techniques, especially after the identification of the first mutation causative for MH susceptibility in the skeletal muscle ryanodine receptor (RYR1) gene, increased hope of a less invasive NA-based diagnosis of

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MH susceptibility. There has been significant progress toward this aim; however, the enormous size of the RYR1 gene and the genetic heterogeneity of MH do not allow replacement of the IVCTs. As these tests have a specificity and sensitivity of <100%, they may produce false positive and false negative results. To further increase sensitivity and specificity and therefore reduce diagnostic errors, additional tests are necessary. Moreover, the group of patients showing an equivocal result (MHE) needs clarification. Clinically, for safety reasons, these patients are regarded as MH susceptible (MHS), although some of them do not carry the mutation found in their family and do not carry the genetic predisposition to MH. Because more than half of the MH families are genetically linked to the RYR1 gene and almost all of the known mutations are located in this gene (9), the interest focused on agents that specifically interact with the protein coded by this gene (e.g., ryanodine, 4-Chloro-m-cresol [4-CmC]).

A ryanodine contracture test has been evaluated in some laboratories, but an overlap between the MHS and malignant hyperthermia normal (MHN) groups has been observed (10–12). Additionally, the intercenter variability of the ryanodine contracture test results did not allow for a definition of common cutoff values for all laboratories, which makes the introduction and evaluation of the test difficult. Moreover, the problem of the MHE group could not be clarified, because the values were borderline and the sensitivity and specificity of this test were not increased.

Present tests have been designed to reach a very high sensitivity to prevent false negative results, which could induce the administration of triggering agents and a fatal crisis. Indeed, false negative results are extremely rare (7,8,13). This design leads to a rate of false positive results of up to more than 20% and many disadvantages for those labeled as MHS. They are not only discouraged from participating in sports on a high performance level, but also prevented from enlisting in the military, may not be allowed to serve in police or fire departments, and may be denied operations in smaller hospitals not prepared for anesthetic crises. In some countries, they will experience serious difficulties obtaining adequate health, disability, and life insurance.

Recently, 4-CmC, a potent and specific activator of the RYR1, has been suggested as a new test substance for *in vitro* testing of susceptibility to MH (14). Similar to caffeine (14–17), 4-CmC releases calcium from the sarcoplasmic reticulum of skeletal muscle by interaction with the RYR1; however, the binding sites seem to be different, as 4-CmC seems to exert its effect from the luminal side of the sarcoplasmic reticulum (16). The substance has no other known effects on skeletal muscle and is more specific, as 15-fold lower concentrations than those for caffeine increase the open probability of the isolated Ca^{2+} channel. In reports of single MH centers, the addition of a 4-CmC contracture test seems to improve phenotypic classification of patients indicative of MH (18–20). This study collates the data of more than 200 patients from five European countries.

Methods

Two hundred two patients from six European MH centers performing the IVCT participated in the study. The investigation was indicated by clinically-suspected MH in the patient, a relative, or in individuals revealing familial chronic creatine kinase elevation with no known neuromuscular disease. The study protocol complies with the standards described in the declaration of Helsinki and was approved by the ethics committees of the participating centers; informed consent was obtained by the patients. In all individuals participating in this investigation, a single or double test with 4-chloro-m-cresol, in addition to the routinely performed duplicate, cumulative halothane and caffeine tests, was performed.

IVCT

Muscle bundles from the vastus lateralis muscle were excised under regional anesthesia (three-in-one block or spinal anesthesia) or general anesthesia without triggering agents. Diagnosis of MH susceptibility was made with the halothane and caffeine test according to the protocol of the European Malignant Hyperthermia Group (4). Diagnostic criteria were applied as follows: MHS, caffeine threshold at 2 mM or less *and* halothane threshold at 0.44 mM or less; MHN, caffeine threshold at 3 mM or more *and* halothane threshold at 0.66 mM or more; MHEc, caffeine threshold at 2 mM or less *and* halothane threshold at 0.66 mM or more; MHEh, halothane threshold at 0.44 mM or less *and* caffeine threshold at 3 mM or more.

4-CmC Test

For the 4-CmC test, muscle bundles were immersed in Krebs-Ringer solution, stretched to 150% of their resting length and electrically stimulated (frequency 0.2Hz, pulse duration 1 ms, constant current 80–100mA). The tissue bath temperature was maintained at 37°C, and the bath was continuously bubbled with carboxygen (95% O_2, 5% CO_2). Baseline force and twitch amplitudes were recorded on-line. If viability criteria were achieved (twitch amplitude = 10 mN) and the baseline was stable (change of baseline = 2 mN during the last 10 min), 4-CmC was added to the tissue bath in increasing concentrations (25,50,75,100,150, and 200 μM; some centers used additional concentration steps [10 μM and 125 μM, respectively]) in some tests. By adding the appropriate amount of stock solution to the bath, exposure to the next concentration step was undertaken after a time

frame specific for each laboratory (3–6 min), when muscle contracture had reached the maximal strength. According to the European protocol, the threshold of a muscle bundle was defined as the 4-CmC concentration at which baseline force increased by ≥2.0 mN. When two or more muscle bundles were examined, the IVCT results of the muscle bundle with the highest sensitivity were taken, identical to the procedure proposed for the responses to caffeine and halothane. To define the diagnostic threshold indicative of MH susceptibility for the 4-CmC test, a receiver operating characteristic curve was generated, varying the diagnostic threshold: true positive values (sensitivity) were plotted against false positive values (1 − specificity), whereby the area under the curve is a measure for the accuracy of the test (21). The optimal threshold was selected based on the maximal Youden index (sensitivity + specificity − 1) (22). 4-CmC was purchased from Fluka (Neu-Ulm, Germany) or Sigma-Aldrich (Steinheim, Germany). Both were of analytical grade or higher purity (>98%). Additionally, the purity was assessed by high-performance liquid chromatography and mass spectroscopy by one laboratory. The concentration of the 4-CmC solutions has been evaluated by spectrophotometrical methods at a wavelength of 280 nm by some centers.

Linear regression analysis and the unpaired Student's *t*-test were performed by using ORIGIN 5.0© software (Microcal Software, Inc., Northampton, MA). A value of $P < 0.01$ was taken to indicate statistical significance. Data were presented as mean values ± SEM.

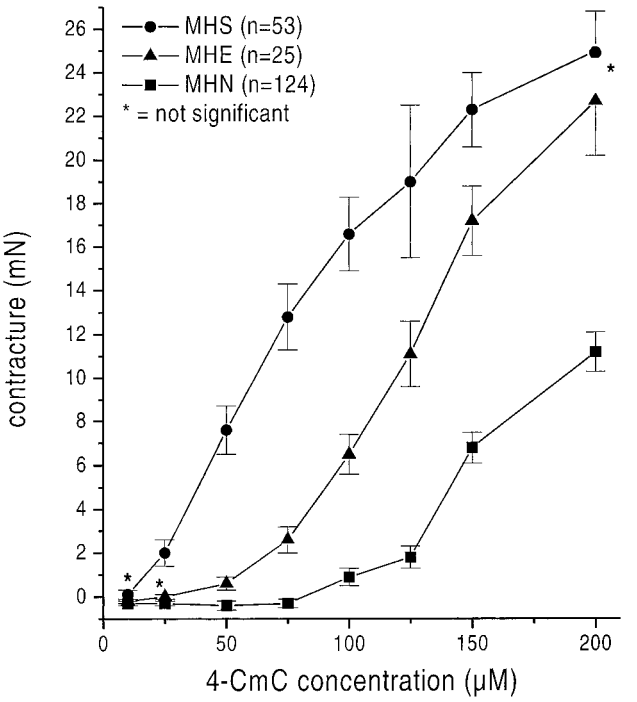

Figure 1. Dose response curve to cumulative application of 4-Chloro-m-cresol (4-CmC) to the (most sensitive) muscle bundle of each subject ($n = 202$) separated in malignant hyperthermia susceptible (MHS), malignant hyperthermia equivocal (MHE), and malignant hyperthermia normal (MHN) groups according to the caffeine halothane *in vitro* contracture test result. Differences are significant ($P < 0.01$) with exception at 10 μM (all groups), at 25 μM (MHE group versus MHN group) and at 200 μM (MHS group versus MHE group). At 10 μM and 125 μM 4-CmC, only 37 and 35 subjects, respectively, have been tested. At all other concentration steps, all individuals have been tested. Error bars indicate SEM.

Results

Purity analysis of 4-CmC by high-performance liquid chromatography and mass spectroscopy detected neither other organic components nor other contaminations. Evaluation of the applied bath solutions (10–200 μM 4-CmC) was performed by spectrophotometrical analysis. This revealed a deviation of the 4-CmC concentration of <10% from the nominal value, whereby baseline value (absorption of Krebs-Ringer solution alone) was zero for the employed wavelength of 280 nm.

Six MH centers from five countries in Europe participated in this study. Of the 202 patients, 53 patients (26.2%) were classified as MHS, 124 (61.4%) as MHN, 23 (11.4%) as MHEh and 2 (1.0%) as MHEc. After exposing the muscle bundles to 4-CmC, contractures were found to be significantly greater at all concentrations from 25 μM to 150 μM in the MHS group compared with both the MHE and the MHN group ($P < 0.01$). Each group (MHS, MHE, and MHN) differed significantly ($P < 0.01$) at most concentrations (Figure 1). By comparing the contracture forces at the threshold concentration of 4-CmC (75 μM) versus caffeine (2 mM), 4-CmC shows a somewhat stronger contracture for 4-CmC in the MHS (12.8 ± 1.5 mN versus 9.8 ± 1.2 mN) and the MHE group (2.6 ± 0.6 mN versus 0.5 ± 0.1 mN) whereas 2% halothane evokes the strongest contracture (MHS: 20.3 ± 2.0 mN, MHE: 5.2 ± 0.7 mN). The specimens of MHE patients at all concentrations exhibited baseline contractures intermediate to those of the specimens from patients classified as MHS and MHN, respectively. No contracture was seen in the MHN group at these concentrations.

Figure 2 shows the receiver operating characteristic curve generated for the 4-CmC test, varying the diagnostic thresholds. For diagnostic testing of a potentially fatal disposition, a high degree of sensitivity (no false negatives) and an acceptable level of specificity (few false positives) are desired. For the 4-CmC test, the above-mentioned premise was fulfilled at a cutoff point of 75 μM, at which sensitivity was 94% and specificity 95% (compared with the standard IVCT) and the Youden index (sensitivity + specificity − 1) was maximal (0.89). The optimal threshold concentration responsible for a contracture of ≥2mN (defining MH susceptibility by the 4-CmC test) was therefore 75 μM, leading to a classification as MHS at thresholds

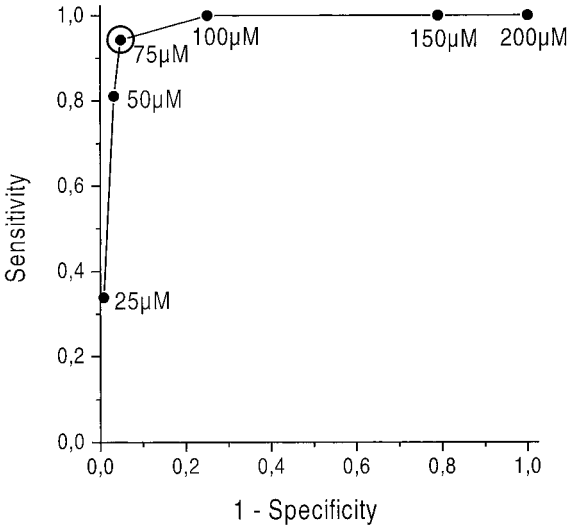

Figure 2. Receiver operating characteristic curve for the cumulative 4-Chloro-m-cresol (4-CmC) test (25–200 µM) based on 124 malignant hyperthermia normal and 53 malignant hyperthermia susceptible subjects. The circled point on the curve is the cutoff point determined by calculation of the maximal Youden index.

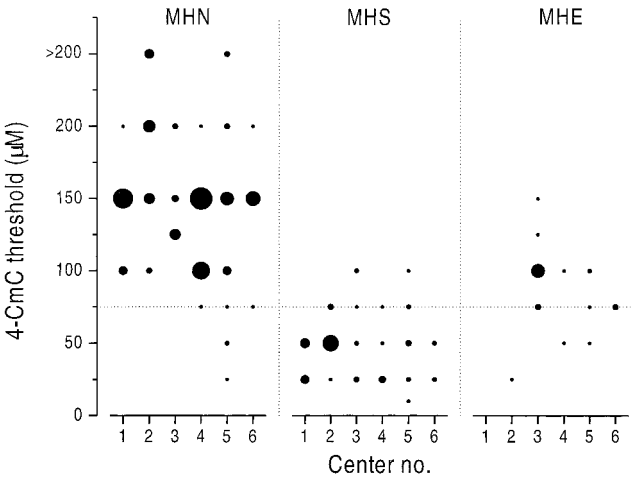

Figure 3. Threshold of muscle specimens from subjects classified by the caffeine-halothane contracture test subdivided into centers. The size of the dots is proportional to the number of patients. The dashed line symbolizes the 4-Chloro-m-cresol (4-CmC) threshold concentration (75 µM). MHN = malignant hyperthermia normal, MHS = malignant hyperthermia susceptible, MHE = malignant hyperthermia equivocal.

≤75 µM 4-CmC and as MHN for thresholds ≥100 µM 4-CmC.

When classifying the patients according to this definition, 50 of 53 the patients, classified as MHS by the caffeine halothane contracture test, also belong to the MHS group of the 4-CmC test. Three of the MHS patients (according to the standard IVCT) achieved the postulated contracture level (2 mN) at a 4-CmC concentration of 100 µM, which would lead to a classification as MHN in the 4-CmC test at a given threshold of 75 µM (Figure 3). Only one patient classified MHS by the standard IVCT was classified MHN by the 4-CmC test. Muscle bundles from 118 of 124 MHN patients (standard IVCT) developed no significant contracture (<2 mN) up to 75 µM 4-CmC, which leads to an MHN classification in the 4-CmC test, too. Six of the 124 MHN patients, however, showed a significant increase in tension (≥2 mN) at contracture concentrations typically observed in MHS patients (25–75 µM 4-CmC). In only two centers can an underlying individual threshold (100 µM for center Number 3 and 50 µM for center Number 6) be found. The reason for the difference between laboratories is still unclear, but to exclude systematic errors, such as deviations in the concentration of the test agents, regular determinations of the applied bath concentrations would be necessary.

The sensitivities of the muscle specimens in reaction to 4-CmC and caffeine were compared for each patient. Most of the patients with a low caffeine threshold also exhibited a low 4-CmC threshold and vice versa, which indicates a high correlation of the effects of these two agents. The regression analysis of the 4-CmC threshold versus the caffeine threshold revealed a correlation index of $r = 0.79$. This value underlines the strong concordance of the caffeine and the 4-CmC results. Analysis of the distribution of the threshold concentration of the 4-CmC test in the four different patient groups (MHS, MHEc, MHEh, MHN) is shown in Table 1. Analysis of the 4-CmC thresholds of the patients tested by the standard IVCT revealed that muscle bundles of 81.2% of the MHS patients exceeded the postulated baseline contracture (2 mN) at a concentration = 50 µM and 94.3% achieved the 2-mN increase at a 4-CmC concentration of ≤75 µM. No MHS patient required a 4-CmC concentration of >100 µM. 95.2% of all MHN the patients revealed a 4-CmC threshold of ≥100 µM, 4.8% had a threshold of ≤75 µM.

Discussion

Cresols are commonly used in pharmacology as disinfectants and preservatives and are used in many preparations. Interest in the effects of 4-CmC on MHS individuals and their skeletal muscles has been concentrated because this agent, similar to ryanodine (23), has been found to be a specific *in vitro* activator of the RYR1 (15,16) that is mutated in many of these individuals.

4-CmC is used as a preservative not only with succinylcholine but also with many other drugs such as insulin and heparin. However, MH crises potentially induced by these drugs have been scarcely reported (24). This can be adequately explained by the low serum levels usually achieved when administering these drugs (19) and by rapid metabolism of this agent in the liver (25). Nevertheless, it could be shown that the administration of very high doses of 4-CmC can trigger MH in susceptible swine (25,26).

Table 1. Contracture Thresholds for 4-Chloro-m-cresol

MH status	10 μM	25 μM	50 μM	75 μM	100 μM	150 μM	200 μM	>200 μM	Total
MHS									
n	1	17	25	7	3				53
%	1.9	32.1	47.2	13.2	5.7				100
MHEc									
n				1	1				2
%				50	50				100
MHEh									
n			3	7	11	2			23
%			13.0	30.4	47.8	8.7			100
MHN									
n		1	2	3	25	67	17	9	124
%		0.8	1.6	2.4	20.2	54.0	13.7	7.3	100

Values are *n* or % of patients.
MH = malignant hyperthermia, MHS = malignant hyperthermia susceptible, MHEc = malignant hyperthermia equivocal (pathological result with caffeine), MHEh = malignant hyperthermia equivocal (pathological result with halothane), MHN = malignant hyperthermia normal.

Table 2. Suggested IVCT Classification Using Two Agents

Classification (*n*)	Halothane and caffeine	Halothane and 4-CmC	Caffeine and 4-CmC
MHS	53	60	51
MHN	124	119	131
MHEc	2	—	4
MHEh	23	16	—
MHEcmc	—	7	16

IVCT = *in vitro* contracture test, 4-CmC = 4-chloro-m-cresol, MHS = malignant hyperthermia susceptible, MHN = malignant hyperthermia normal, MHEc = malignant hyperthermia equivocal (pathological result with caffeine), MHEh = malignant hyperthermia equivocal (pathological result with halothane), MHEcmc = malignant hyperthermia equivocal (pathological result with 4-CmC).

Table 3. Suggested IVCT Classification Using Three Agents

Responses	Used agents:	Number of patients	Suggested classification
Abnormal (only) to	Halothane, Caffeine and 4-CmC	50	MHS
	Caffeine and 4-CmC	1	MHE
	Caffeine and Halothane	3	MHE
	Halothane and 4-CmC	10	MHE
	Caffeine	1	MHN
	Halothane	13	MHN
	4-CmC	6	MHN
Normal to	Halothane, Caffeine and 4-CmC	118	MHN

ICVT = *in vitro* contracture test, 4-CmC = 4-Chloro-m-cresol, MHS = malignant hyperthermia susceptible (redefined as pathological result with all three test agents), MHE = malignant hyperthermia equivocal) (redefined as pathological result with two of the three test agents, MHN = malignant hyperthermia normal (redefined as normal results for at least two of the three test agents, one pathological result is tolerated).

In vitro 4-CmC in the μM range regularly causes muscle contractures in MHS humans and pigs at significantly lower concentrations than in normal individuals. As in previous studies from single centers, this multicenter study identified a common threshold concentration (75 μM) that differentiated between normal and pathologic contractures (18–20). This concordance may be the consequence of the standardization of the 4-CmC test regarding manufacturing, purity, dissolution, etc., of the drug. Moreover, the results of the 4-CmC test are highly consistent with those of the standard IVCT test: discrimination between MHS and MHN as diagnosed by standard *in vitro* contracture testing with halothane and caffeine is possible in 95% of the patients.

As the reliability of the standard IVCT test is <100%, some results are expected to differ if a new test is better than the current one. Possibilities for a test classification using only two agents are displayed in Table 2. For example a halothane-4-CmC test defines seven more individuals as MHS than the standard caffeine-halothane test. Taking the sensitivity of 99% of the standard test into account, these seven additional MHS results are most likely the result of false positive tests. In contrast, the caffeine-4-CmC test

dis characterized by both a similar number of MHS ($n = 51$) as in the standard test and the lowest number of MHE ($n = 20$ compared with 25 and 23 in the other test combinations). Thus a replacement of the standard test using caffeine and halothane by the caffeine and 4-CmC test could be considered.

An alternative is the use of all three drugs for a safe diagnosis. Unfortunately, additional test agents will increase the percentage of patients whose muscle samples will abnormally react to a single substance and thus increase the percentage of MHE results. We therefore suggest a way of classification shown in Table 3. MHE would then be defined as pathologic responses to two of three agents, whereas MHN would allow none or a maximum of one agent to cause an abnormal reaction, i.e., in the sense of an outlier. This classification would lead to 50 MHS, 14 MHE (7%) only and 138 MHN, the latter including 14 individuals (13 abnormal halothane and 1 abnormal caffeine response) who originally would have been classified as MHE. Because the predictive value of an abnormal halothane response seems to be small [16% (27) to 22% (28)], the suggested classification may be acceptable.

Because of the limited possibilities of genetic diagnosis (for review see Reference 2) improvement of the functional tests by additional procedures is desired. This multicenter study demonstrates the high predictive value of the 4-CmC test for the susceptibility to MH, and the European Malignant Hyperthermia Group has recently recommended the use of 4-CmC as an optional test. Further advantages of this agent are its high specificity for the RYR1 and the absence of other effects on skeletal muscle.

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