Effects of anaesthetic agents on erythrocyte fragility: comparison of normal and malignant hyperthermia susceptible patients

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The effects on erythrocyte fragility of two general anaesthetic agents (halothane and ethanol) and succinylcholine were examined using preparations from 13 normal and four malignant hyperthermia susceptible patients. Erythrocyte fragility was determined by the degree of haemolysis induced in solutions of decreasing osmolarity of NaCl. Halothane caused haemolysis of erythrocytes in an isoosmolar solution, being more potent at 42° C than at 32° C. Haemolysis produced by an hypoosmolar medium or halothane was potentiated by exogenously added phospholipase A_2. Ethanol did not markedly alter the haemolysis of erythrocytes under conditions of decreasing osmolarity. Succinylcholine 10 mM did not significantly alter the susceptibility of erythrocytes to lysis by halothane. No differences in erythrocyte fragility were observed between preparations from normal and malignant hyperthermia susceptible patients under any of the conditions tested, despite the inclusion of malignant hyperthermia triggering agents in some instances. Although sampling a larger patient population might reveal slight differences between the groups, erythrocyte fragility tests do not appear to be useful in differentiating malignant hyperthermia susceptible from normal patients.

Key words

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Malignant hyperthermia (MH) susceptibility is identified either by a proven personal or family history of MH, or the contracture response of biopsied muscle to halothane or caffeine *in vitro*.[1,2] Due to its invasive nature and the equipment and manpower requirements, the *in vitro* contracture test is not useful for screening the large population that undergoes anaesthesia each year. A test to detect susceptibility to MH based on the analysis of blood components is, therefore, desirable. Biochemical tests using platelets have been investigated as a means of discriminating MH susceptible from normal individuals,[3] but have not proven to be satisfactory.[4,5]

Erythrocytes from MH susceptible pigs are more fragile than those from normal pigs when incubated in hypoosmolar solutions of sodium chloride.[6-8] In contrast to the consistent findings in pigs, investigations on erythrocytes from humans with MH show increased, decreased, or no change in fragility when compared to control patients.[9-11]

In the present study we attempted to optimize the conditions for discrimination of MH susceptible from normal patients based on our current understanding of the mechanisms underlying the disorder (i.e., an enhanced phospholipase A_2 activity). We used a buffer that would enhance the activity of phospholipase A_2, which is believed to be the biochemical defect in pigs[12] and humans[13] susceptible to MH. Halothane and succinylcholine are the major triggering agents for MH. Therefore, the effects of halothane with and without succinylcholine on erythrocyte fragility were also examined. In addition the effect of ethanol on osmotic fragility was tested, as this weak general anaesthetic agent is known to affect membrane characteristics[14] and to decrease sarcolemmal calcium uptake.[15]

Methods

Erythrocyte preparation

Blood (4.5 ml) was collected in tubes containing 0.5 ml

buffered citrate (16 mg sodium citrate and 2.1 mg citric acid) and maintained at 4°C until used. The donors included 13 normal patients and four with susceptibility to MH as ascertained by *in vitro* halothane contracture testing using previously reported techniques.[16] In one case the patient also had previously experienced a fulminant MH episode. Approval for this study was obtained from the Hahnemann University Human Studies Committee. Patients with chronic anaemia were excluded from the study. After centrifugation of the blood samples at 1000 × g for eight minutes, the supernatant was drawn off and the erythrocyte pellet resuspended in an equal volume of HEPES (N-2-hydroxyethyl piperazine-N^1-2-ethane sulfonic acid) buffer (NaCl 130 mM, HEPES 20 mM, $CaCl_2$ 2 mM, pH 7.40). The centrifugation and resuspension procedure was then repeated twice. The erythrocytes were centrifuged a final time and the supernatant discarded, leaving packed erythrocytes. Citrate was presumed to be removed by these washings. Studies were initiated within three hours of venipuncture.

Osmolarity studies
Ten stock solutions were prepared containing $CaCl_2$ 2mM, HEPES 20 mM at pH 7.40, and NaCl at 13, 26, 32.5, 39, 45.5, 52, 58.5, 71.5, 84.5, or 130 mM concentration. Each solution was designated as a fraction of the concentration of the NaCl divided by 130; specifically those mentioned correspond to 0.1, 0.2, 0.25, 0.30, 0.35, 0.40, 0.45, 0.55, 0.65, and 1.0 fractional concentration of NaCl 130 mM. For osmotic fragility studies, 5 ml of stock solution was delivered into each tube (16 × 100 mm). For studies involving ethanol, a volume of 95 per cent ethanol equal to 1, 5, or 10 per cent of 5 ml was added to each tube. When phospholipase A_2 was used, it was added in a volume of 50 μl. The tubes containing ethanol or phospholipase A_2 were thoroughly mixed. Fifty μl of packed erythrocytes was added to each tube. The tubes were again thoroughly mixed and the samples incubated at 32°C or 42°C for one hour in uncapped test tubes. The ethanol experiments were done in capped tubes.

After incubation, samples were centrifuged at 1000 × g for 8 min. The absorbance of the supernatant was read at 540 nm against a distilled water blank on a Shimadzu UV-120-01 spectrophotometer. The absorbance of any sample was compared to the value representing 100 per cent haemolysis (50 μl of erythrocytes in 5 ml distilled water or 100 per cent halothane saturated solution, respectively) to yield per cent haemolysis.

Halothane studies
Into a glass bottle was added 500 ml of buffer containing NaCl 130 mM, $CaCl_2$ 2 mM, HEPES 20 mM at pH 7.40, along with 25 ml of liquid halothane and a magnetic

stirbar. The glass stoppered bottle was stored at 32°C with continuous stirring for several hours. The aqueous phase of this two phase (buffer/halothane) solution was designated the halothane saturated solution.

The halothane and buffer solution was left at room temperature for 5 min before use. To test tubes (16 × 100 mm; size is critical) containing 50 μl of erythrocytes in precalculated volumes of NaCl 130 mM, HEPES 20 mM, $CaCl_2$ 2.0 mM buffered solution were added the appropriate volume of the halothane saturated solution to produce a final volume of 5 ml and final concentrations of 100, 90, 80, 70, 60, 50, 40, 30, and 20 per cent halothane saturated solutions. For example, a final 60 per cent halothane saturated solution would consist of 2 ml NaCl/HEPES/$CaCl_2$ buffer and 3 ml of halothane saturated solution. The tubes were immediately capped with rubber stoppers and thoroughly mixed. If succinylcholine was included, it was added to the erythrocyte-buffer suspension at a final concentration of 0.01 M. Likewise, if phospholipase A_2 from bee venom (Sigma Chemical Co., St. Louis, MO) was used it was added to a final concentration of 1 μM. The tubes were then incubated at 32°C or 42°C for one hour and the absorbance determined as described above.

Results
We first tested the hypothesis that elevated phospholipase A_2 activity can potentiate the effects of halothane on erythrocytes in the same manner as observed in skeletal muscle.[13] Bee venom phospholipase A_2 1 μM in NaCl 130 mM, HEPES 20 mM, $CaCl_2$ 2 mM at pH 7.4, did not induce haemolysis of red blood cells (Figure 1). A 40 per cent halothane saturated solution also did not induce more than five per cent haemolysis under the same conditions (Figure 1). The combination of a 40 per cent halothane saturated solution and phospholipase A_2 1 μM resulted in levels of haemolysis, which were significantly greater ($p < 0.001$; two-tailed grouped Student's t test) than in the absence of phospholipase A_2 (Figure 1). Thus, phospholipase A_2 activity does potentiate the action of halothane on red blood cells in a manner similar to that observed in skeletal muscle. Therefore, all subsequent studies were done in Ca^{2+}-containing buffer to elevate the activity of any calcium-dependent endogenous phospholipase A_2 that may be present in human erythrocytes. Phospholipase A_2 addition also enhanced the fragility of red blood cells as determined by osmotic lysis. Incubation of erythrocytes in an 0.50 fraction of initial NaCl solution resulted in very little haemolysis; whereas, incubation of the cells in the same medium containing phospholipase A_2 1 μM resulted in significantly greater ($p < 0.001$; two-tailed grouped Student's t test) haemolysis than in the absence of phospholipase A_2 (Figure 1).

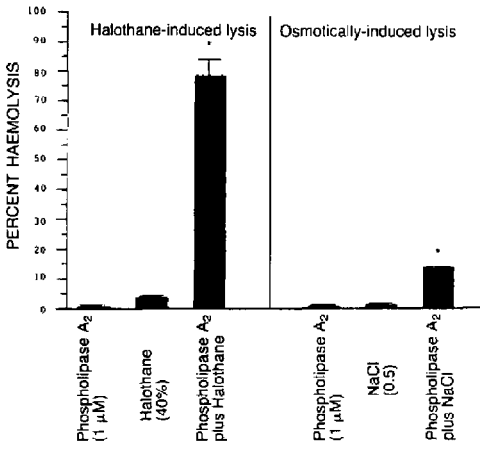The per cent haemolysis as a function of changing

FIGURE 1   Effects of phospholipase A$_2$ on halothane- and osmotically-induced lysis of red blood cells. In the left panel a solution of halothane equivalent to 40 per cent of the saturated halothane solution was incubated in the presence or absence of phospholipase A$_2$ 1 μM. In the right panel a solution containing 50 per cent fraction of the NaCl concentration was incubated in the presence or absence of phospholipase A$_2$ 1 μM. Shown are the mean ± SD for three determinations. The asterisk denotes significantly (p < 0.001) greater haemolysis than in the absence of phospholipase A$_2$.

FIGURE 3   Scatter plots of 50 per cent haemolysis points based on varying osmolarity of NaCl solutions for normal and MH susceptible patients. Each solution contains HEPES 20 mM, CaCl$_2$ 2 mM, and NaCl at various concentrations.
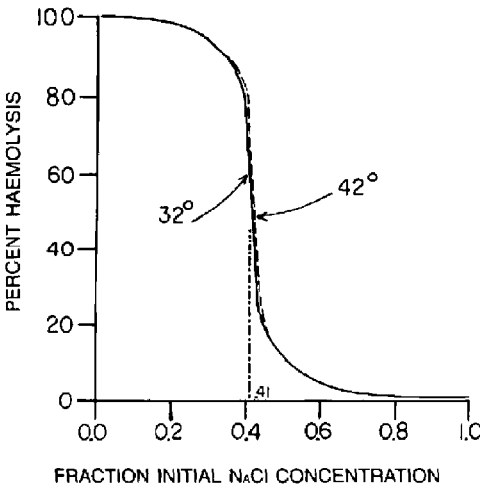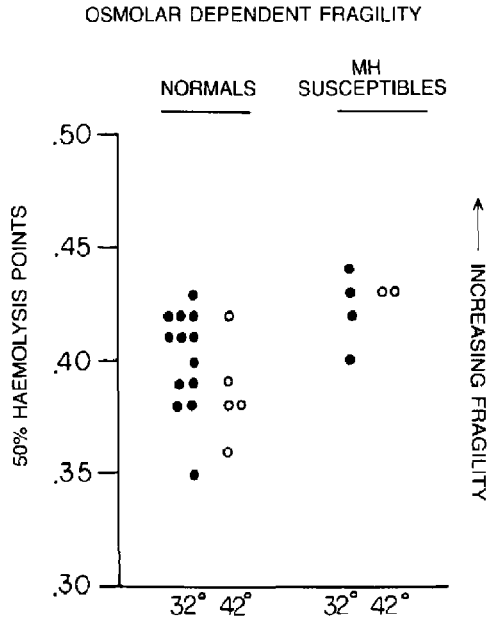

FIGURE 2   Representative NaCl osmolarity dependent haemolysis curves of erythrocytes incubated at 32°C and 42°C for a single non-MH susceptible patient. The NaCl fraction is expressed as a fraction of NaCl 130 mM. The 50 per cent haemolysis points for both temperatures are equivalent to NaCl 53 mM.

osmolarity is shown in Figure 2 for one patient not susceptible to MH. Samples from each patient were run in duplicate and were usually within one per cent of each other. The 50 per cent haemolysis point is equivalent to a solution containing NaCl 53 mM, HEPES 20 mM, and CaCl$_2$ 2 mM.

Lysis of red blood cells from MH susceptible patients was then compared to those from normal patients in an hypoosmolar solution. The 50 per cent haemolysis points based on varying osmolarity are shown in Figure 3. Although tending toward the expected decreased fragility at higher temperatures,[17] there was no statistically significant difference (p > 0.05) between 50 per cent haemolysis points for normal patients at 32°C (0.401 ± 0.006; mean ± SEM) or 42°C (0.386 ± 0.010) as determined by the two-tailed grouped Student's t test. The values of 50 per cent haemolysis for normal patients at 32°C were not statistically different (p > 0.05; two-tailed grouped Student's t test) and, more importantly, overlap considerably with those from MH susceptible patients (0.423 ± 0.008).

Figure 4 demonstrates representative haemolysis curves for one patient not susceptible to MH as a function of per cent halothane saturation. Fifty per cent haemolysis
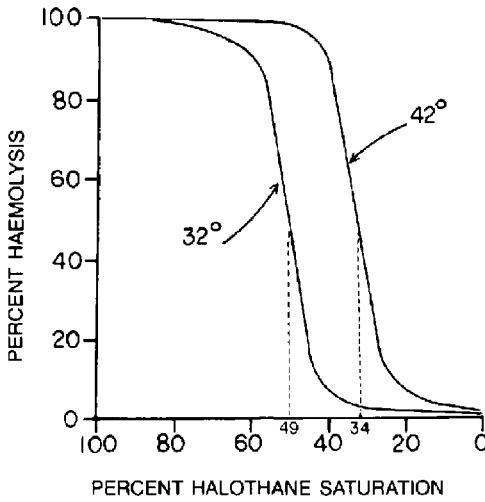
FIGURE 4   Representative haemolysis curves for erythrocytes incubated at 32°C and 42°C as a function of per cent halothane saturation for a single non-MH susceptible patient. The suspension medium contains NaCl 130 mM, HEPES 20 mM and $CaCl_2$ 2 mM at pH 7.4. Fifty per cent haemolysis points occur at 49 and 34 per cent saturation for preparations incubated at 32°C and 42°C, respectively.

points at 32°C and 42°C are 49 and 34 per cent halothane saturation, respectively, suggesting an increase in susceptibility to halothane lysis at 42°C. Curves identical to those for nonsusceptible patients were observed with erythrocytes from MH susceptible patients.

The effect of succinylcholine on halothane-induced lysis was compared for normal and MH susceptible patients. Figure 5 shows results based on halothane enhanced fragility. A one-way ANOVA (completely randomized design) and Duncan's multiple-range test (at

TABLE   Fraction of NaCl concentration to produce 50% haemolysis

	Volume per cent ethanol solution			
	0%	1%	5%	10%
MH negative	0.35–0.43 (13)	0.41–0.43 (3)	0.41–0.44 (3)	0.44–0.48 (3)
MH positive	0.40–0.44 (4)	0.40–0.43 (2)	0.42–0.43 (2)	0.42–0.47 (2)

The range of 50% haemolysis points for preparations from MH negative and MH positive patients at various concentrations of ethanol in solutions containing NaCl 13–130 mM, HEPES 20 mM, $CaCl_2$ 2 mM at 37°C and pH 7.4. Values are expressed as the fractional concentration of NaCl causing 50% hemolysis (see Methods). Numbers in parentheses represent the number of patients studied.
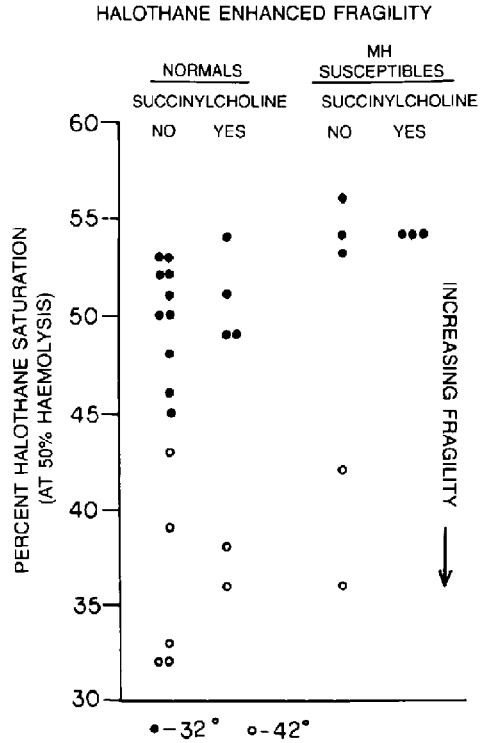

FIGURE 5   Scatter plots of 50 per cent haemolysis points based on varying halothane solutions. The suspension medium contained NaCl 130 mM, HEPES 20 mM and $CaCl_2$ 2 mM at pH 7.4, with or without succinylcholine 0.01 M.

a level of $p < 0.05$) were used to analyze the data in Figure 5, excluding conditions in which there were only two data points. Again the values of the 50 per cent haemolysis points between normal (50.0 ± 0.9) and MH susceptible (54.3 ± 0.9) patients were not significantly different and overlap at 32°C. For both the normal and MH susceptible patients, addition of succinylcholine did not change the values of 50 per cent haemolysis at 32°C. The only statistically significant finding ($p < 0.05$) was the increased fragility to halothane at 42°C (35.8 ± 2.2) compared to 32°C (50.0 ± 0.09) for erythrocytes from normal patients.

The effects of ethanol on hypoosmolar lysis were tested at three concentrations of ethanol. The ranges for 50 per cent haemolysis of solutions containing ethanol at varying osmolar concentrations are shown in the Table. Preparations from nonsusceptible patients were slightly more fragile in the presence of ethanol ten per cent when

compared to the absence of ethanol ($p < 0.05$; two-tailed grouped Student's t test). No differences were found between the response of erythrocytes from normal and MH susceptible patients.

In order to determine if the halothane concentrations were influenced by the number of erythrocytes in a sample, the halothane haemolysis studies without succinylcholine were repeated for preparations from four patients using volumes of erythrocytes from 25 to 150 μl. No change in the haemolysis curves were noted (results not shown).

Discussion

The standard method of determining osmotic fragility involves serial dilutions of a sodium chloride solution in a phosphate buffer.[17,18] The goal of the present study was to alter the normal incubation conditions in such a manner as to increase the probability of distinguishing MH susceptible from normal patients. Our choice of incubation buffer was based on the current understanding of mechanisms underlying MH.

Elevated phospholipase A_2 activity is believed to be the defect in porcine[12] and human[13] MH. In the present study we found that enhanced phospholipase A_2 activity can potentiate halothane-induced haemolysis of erythrocytes similar to the synergism observed with these agents in skeletal muscle.[13] In addition, increased phospholipase A_2 activity results in red blood cells with greater fragility when tested for hypoosmolar lysis. Since in most tissues phospholipase A_2 activity appears in virtually all cell fractions,[19] it should be present in all membranes, including the plasma membrane of red blood cells. The presence of phospholipase A_2 in the red blood cell of pigs could explain the ability to distinguish MH susceptible from nonsusceptible pigs. While phospholipase A_2 activity is abundant in red blood cells from some species, it is not present to a great extent in red blood cells from all species.[19] Human red blood cells do not contain detectable levels of a major product of phospholipase A_2 activity, free arachidonic acid, suggesting these cells do not have much, if any, phospholipase A_2 (unpublished observations). In contrast porcine red blood cells have detectable amounts of arachidonic acid (about 4 pmol·mg^{-1} protein; unpublished observations). We added Ca^{2+} to the incubation buffer, to optimize phospholipase A_2 activity,[19] and possibly allow discrimination of MH susceptible and nonsusceptible patients using the red blood cell fragility test. Since calcium ions at a concentration of 2 mM precipitate with the phosphate buffer normally used in studies of erythrocyte fragility, we chose a HEPES buffer for our studies. Since halothane is known to precipitate episodes of MH, we also tested its ability to

discriminate MH susceptible from nonsusceptible patients using the red blood cell model.

The 50 per cent haemolysis points of erythrocytes from the normal patients in the present study range from 0.35 to 0.43 fraction of the NaCl solution. This is equivalent to 127–148 milliosmolarity (mOsmol·L^{-1}). To convert from the fraction of NaCl used in our study to mOsmol·L^{-1}, it is necessary to multiply the fraction of NaCl by 260 (mOsmol·L^{-1} of NaCl) and add 36 (mOsmol·L^{-1} of HEPES 20 mM = 30 at pH 7.4 and the mOsmol·L^{-1} of $CaCl_2$ 2 mM = 6). Normal patients exhibit a range of 50 per cent haemolysis points of 0.400–0.445 per cent NaCl at 20°C.[18] These haemolysis points convert to 0.372–0.417 per cent NaCl at 32°C using the standard temperature correction factor,[17] or 127–143 mOsmol·L^{-1} (342 mOsmol·L^{-1} × fraction of NaCl). Therefore, our modified buffer yields results comparable to buffers traditionally used in studies of red blood cell fragility. In the present study, the range of 50 per cent haemolysis points for MH patients was similar to that of normal patients.

Although lysis of erythrocytes by a saturated halothane solution has been reported,[20] the present study is the first description of dose related haemolysis induced by halothane. This study provides a valuable model for studying the action halothane on membranes, as suggested by the results with phospholipase A_2 and halothane. The relative independence of the value of 50 per cent haemolysis from erythrocyte concentration indicates that haemolysis depends more upon halothane tension than on total halothane concentration. Incubation of erythrocytes in halothane containing solutions at 42°C did markedly increase the fragility of erythrocytes relative to incubation at 32°C. The saturated vapour pressure increases with increasing temperature, whereas the solubility of halothane should decrease. Therefore, the same mass of halothane should have a greater tension at 42°C than at 32°C, further supporting halothane tension rather than total content as the major factor in haemolysis. The temperature-dependent increase in halothane-induced fragility appeared to be the same in MH susceptible and nonsusceptible patients.

In contrast to the marked lytic effects of halothane on erythrocytes, ethanol had only a slight effect on increasing red blood cell fragility. Ethanol did not increase the sensitivity of the osmotic fragility test for distinguishing MH susceptible patients from normal patients. Succinylcholine had no effect on lysis caused by halothane. The effects of ethanol and succinylcholine were similar for preparations from normal or MH susceptible patients.

Although a larger sample size might reveal slight differences between erythrocytes from normal and MH susceptible patients, it is unlikely that fragility tests will

show the sensitivity seen using *in vitro* contracture tests. Addition of calcium, succinylcholine, or halothane did not improve the ability to discriminate MH susceptible from normal patients using erythrocyte fragility tests. If elevated phospholipase A_2 activity in skeletal muscle is the cause of MH, as suggested by some investigators,[12,13] then either human red blood cells have too little phospholipase A_2 activity[19] for detection of MH susceptible patients using erythrocyte fragility tests, or the enzyme in red blood cells differs from that in skeletal muscle.

In summary, the use of erythrocyte fragility tests based on changing osmolarity with or without ethanol and based on changing halothane tensions with or without succinylcholine do not provide enough sensitivity to discriminate MH susceptible patients from the normal population. These results do not exclude the possibility that slight differences in red blood cell membrane structure or function may exist between normal and MH susceptible patients and that such differences may be detected by either a larger sample size or by other assays.

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Résumé

Les effets de la fragilité érythrocytaire produite par deux agents anesthésiques généraux (l'halothane et l'éthanol) et par la succinylcholine ont été examinés à l'aide de préparations provenant de treize patients normaux et de quatre patients susceptibles à l'hyperthermie maligne. La fragilité érythrocytaire a été déterminée par le degré d'hémolyse induit par des solutions d'osmolarité décroissante de NaCl. L'halothane a causé une hémolyse des érythrocytes dans une solution isoosmolaire, qui était plus puissante à 42° C qu'à 32° C. L'hémolyse produite par un milieu hypoosmolaire ou par l'halothane a été potentialisée par l'ajout exogène de phospholipase A_2. L'éthanol n'a pas modifié de façon marquée l'hémolyse des érythrocytes dans des conditions d'osmolarité décroissante. La susceptibilité de lyse des érythrocytes causée par l'halothane n'a pas été modifiée de façon significative par les 10 mM de succinylcholine. On a remarqué aucune différence dans la fragilité érythrocytaire entre les préparations provenant des patients normaux et celles provenant de patients susceptibles à l'hyperthermie maligne, dans aucune des épreuves vérifiées, malgré l'inclusion d'agents déclencheurs d'hyperthermie maligne dans certains cas. Quoique l'échantillonnage d'une population plus grande de patients pourrait révéler des différences légères entre les groupes, les tests de fragilité érythrocytaire ne semblent pas être utiles pour différencier les patients normaux des patients susceptibles à l'hyperthermie maligne.