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The Effects of Membrane Modification and Hyperthermia on the Survival of P-388 and V-79 Cells*

M. B. YATVIN,†‡ J. W. VORPAHL,† M. N. GOULD† and M. LYTE§

†*Human Oncology Department, University of Wisconsin, Madison, WI 53792, U.S.A. and* §*Department of Membrane Research, Weizmann Institute of Science, Rehovot, Israel*

Abstract—*Cells with greater membrane microviscosities are reportedly less sensitive to being killed by mild hyperthermia. To further study this phenomenon, membrane microviscosity of ascites P-388 tumor cells was increased by adding cholesteryl hemisuccinate (CHS). Unexpectedly, when modified cells were heated for* 60 min *at* 43°C in vitro *and analyzed* in vivo *in* CDF_1 *mice they showed an increased thermal sensitivity. Similar increases in cell membrane microviscosity were obtained with V-79 cells. However, after heat treatment no differences in survival* in vitro *were noted between modified and unmodified cells. Treatment with CHS alone results in a substantial increase in P-388 cells, which take up trypan blue, but not in V-79 cells. When these 'dead' cells are accounted for, the difference in killing between control and CHS-modified P-388 cells is no longer seen. When considered in this light, both P-388 and V-79 cells are similar in their response to heat, which is not influenced by CHS* per se.

INTRODUCTION

AN UNDERSTANDING of the biochemical and molecular events involving heat cytotoxicity would provide a more rational basis for the use of hyperthermia in the treatment of malignant disease. Of particular interest are events involving cell membranes as they reportedly play a primary role in the process of cytotoxicity [1–9]. We have proposed that membrane lipid composition and, concomitantly, microviscosity are correlated with heat sensitivity [3, 10]. These physical properties presumably influence hyperthermic killing of cells by determining the level of interaction of key protein–lipid complexes within the cell membrane matrix [1, 3, 10]. If such were true, then cells in which the membrane lipids were more fluid at the onset of heating would be expected to suffer greater damage. These expectations were shown to be correct in studies with an *E. coli* unsaturated fatty acid-requiring auxotroph. A correlation was found between the fatty acid composition and the membrane fluidity of such bacteria and their thermal sensitivity [3, 6, 10]. Likewise in mammalian cells, an increase of membrane unsaturated fatty acid content brought about either by nutritional manipulation of growth temperature, growth medium or diet resulted in greater heat killing [11–15]. Local anesthetics [3, 6, 7, 12, 16], hypnotics [16] and alcohols [17, 18], all of which acutely perturb membrane structure, also potentiate the effects of hyperthermia.

The addition of cholesterol to an organized membrane decreases the ordering of its fatty acyl chain while the reverse occurs when it is added to a membrane in the liquid–crystalline state [19, 20]. In cholesterol-deficient mutants a correlation has been observed between the level of membrane cholesterol and its microviscosity [21]. The relationship between heat sensitivity and cholesterol content has been studied in a variety of cell lines and a negative correlation was found [22, 23]. The studies described above are consistent with the concept that thermal sensitivity increases as membrane microviscosity decreases.

In vitro modification of membrane lipid microviscosity has been acutely accomplished by

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‡To whom requests for reprints should be sent at: Clinical Science Center, K4/310, 600 Highland Avenue, Madison, WI 53792, U.S.A.

incorporating lecithin, cholesterol or cholesterol analogs into cell membranes [24, 25]. The most efficient method was the use of lipid dispersions in polyvinylpyrrolidone [24]. That method was employed in the current study and the relationship of membrane microviscosity to heat sensitivity of lipid-modified P-388 ascites tumor cells and V-79 Chinese hamster lung cells investigated. Both types of cells were heated *in vitro* and assayed for survival following modification with cholesteryl-hemisuccinate (CHS).

MATERIALS AND METHODS

These studies employed a P-388 ascites tumor system *in vivo* and V-79 Chinese hamster lung fibroblasts *in vitro*.

Membrane modification

To obtain cells for membrane modifications, P-388 ascites cells were withdrawn from the peritoneal cavity of CDF_1 female mice 8–10 days after they were injected i.p. with 10^6 cells. Cells were washed three times in cold phosphate-buffered saline (PBS). V-79 cells, grown in 4-(2 hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered α-modified minimal essential medium (α-MEM) with 10% fetal bovine serum, were harvested from tissue culture dishes by trypsinization and washed by low-speed centrifugation (500 *g*). The cells were modified *in vitro* using the method of Shinitzky *et al.* [24]. All cells were resuspended in PBS containing 3.5% polyvinylpyrrolidone, 0.2% glucose, 1% bovine serum albumin and 1% ethanol. For modification, CHS in ethanol was added to the medium (100 μg/ml of medium). In initial studies incubation time ranged from 15 to 180 min. As a maximum response was attained by 30 min, in the experiments reported here modification of the cells was accomplished by incubating at 37°C for 30 min. Cells were pelleted by low-speed centrifugation (500 *g*) and washed three times in α-MEM.

Measurement of fluorescence depolarization (P)

Steady-state fluorescence depolarization measurements were performed on PBS-washed cells or cell fragments. An aliquot (1 μl) of a stock solution of 1-6-diphenylhexatriene (DPH) in tetrahydrofuran was added to 1 ml of the suspensions while gently vortexing. The final concentration of the DPH in the cell or membrane fraction suspensions was 10^{-6}M. Measurement of P values was performed directly in an Elscint MV-1A microviscosimeter.

Hyperthermia and cell survival

The P-388 cells were pipetted into 125 ml Erlenmeyer flasks equilibrated at 43 ± 0.1°C (5×10^5 cells/ml) and heated in a gyratory shaking water bath for 60 min. After heating, the cells were pelleted, resuspended in α-MEM (10^7 total cells/ml) and 0.1 ml of the cell suspension injected i.p. Animal survival was monitored daily. Immune-depressed animals were produced by giving 525 rad of whole-body X-irradiation 24 hr prior to injection of cells.

The V-79 cells were resuspended in α-MEM with 10% fetal bovine serum (FBS) and appropriate numbers (always less than 10^5/ml) were heated in 50-ml tubes. The cell suspensions were rapidly equilibrated to a temperature of 43 ± 0.1°C and then heated for varying periods of time from 0 to 4 hr. Appropriate cell dilutions were plated in triplicate in 100-mm diameter tissue culture dishes in α-MEM + 10% FBS. They were then incubated at 37°C in a 5% CO_2 incubator for 8 days. The dishes were then stained and cells that grew into colonies that contained 50 cells or more were considered to have survived the hyperthermia. Survival was based on these observations and was corrected for plating efficiency and multiplicity.

Cells were washed and resuspended (10^7/ml) in homogenizing buffer (0.01 M HEPES, pH 7.4, containing 0.25 M sucrose, 0.065 M NaCl and 0.075 M KCl). The cells were disrupted in a French Pressure Cell under a pressure of 3000 lb/in^2. The homogenate was diluted 10-fold with homogenizing buffer. Any undisrupted cells, cell debris and nuclei were removed by centrifugation at 750 *g* for 10 min in a Sorvall HG-4 rotor. Mitochondria were pelleted from the low-speed supernatant by centrifugation at 8300 *g* for 10 min in a Spinco No. 30 rotor. A crude microsome fraction was pelleted from the mitochondrial supernatant by centrifugation at 104,000 *g* for 60 min. The plasma membrane was separated from the microsomal membrane in a continuous Ficoll gradient with a density range of 0–1.09 using 0.001-M HEPES buffer at pH 8.2 as the gradient buffer and underlaid with a Ficoll cushion. Prior to centrifugation on the Ficoll gradient the pellet was resuspended in 3.0 ml of the gradient solution. The final gradient consisted of 5 ml of 1.09 gradient solution on the bottom, followed by 7.0 ml of the continuous gradient and 3.0 ml of sample. The gradient tube was placed into a SW 27 Spinco rotor and centrifuged at 75,000 *g* for 18 hr. Plasma membrane concentrated in the gradient. These fragments were carefully removed with a Pasteur pipet, diluted with homogenization buffer and centrifuged at 104,000 *g* for 60 min at 4°C. The

microsomes which pelleted through the gradient were collected and washed in homogenization buffer. All cell fractions were resuspended in PBS for fluorescence depolarization measurements.

Trypan blue exclusion by cells

For estimates of cell viability, cells (10^6/ml) were mixed with an equal volume of 0.4% trypan blue in PBS at 37°C. Total cells and cells unable to exclude dye were counted.

RESULTS

Radioactive cholesterol is incorporated into P-388 cells during a 30-min incubation at 37°C; however, this addition did not alter the P value of whole cells when measured with DPH (Table 1). Likewise, no significant change of P values was observed in cholesterol-modified V-79 cells. In contrast, enrichment of P-388 cells with the combination of cholesterol and CHS or CHS alone significantly increased whole-cell P values (Table 1 and 2). In addition, CHS greatly facilitated the uptake of labeled cholesterol by P-388 cells. Modification of P-388 cells with cholesterol alone had no effect on their estimated survival following hyperthermia.

The consequences of altering membranes with CHS only was studied and the results are shown in Table 2 and Figs 1–4. Both P-388 and V-79 cells show significant increases in their P values after incubation in the CHS-containing medium (Table 2). Furthermore, the alteration produced by CHS occurs to various degrees in all membrane fractions studied, the effects being relatively greater for P-388 cells than for V-79 cells.

From Fig. 1, P-388 cell survival after heating can be inferred from the animal survival data, as cell survival and/or growth potential is inversely related to survival of recipient animals. Animals injected with CHS-modified-only or heated-only cells survive longer than animals injected with control cells. Although the increase in survival time was small, the same trend was observed in three separate experiments. Animals receiving cells that were both CHS-modified and heated survived longer than animals in all other treatment groups. Each group consisted of at least ten animals.

Table 1. Uptake of [^{3}H]-cholesterol and fluorescence polarization of P-388 cells

	dpm/10^6 Cells	Whole cell fluorescence depolarization (P)
Control	—	0.195
Cholesterol I	17,000	0.195
II	3500	0.195
Cholesterol + CHS III	50,000	0.245
IV	13,500	0.261

Incorporation of [^{3}H]-cholesterol was followed in media containing either: (I) [^{3}H]-cholesterol (1 μCi/ml); (II) [^{3}H]-cholesterol (1 μCi/ml) and cholesterol (10 μg/ml); (III) [^{3}H]-cholesterol (1 μCi/ml) and CHS (10 μg/ml); or (IV) [^{3}H]-cholesterol (1 μCi/ml), cholesterol (10 μg/ml) and CHS (10 μg/ml).

Table 2. Fluorescence polarization of whole cells and membrane fractions of control and CHS modified P-388 and V-79 cells

	Control	CHS	ΔP
V-79			
Whole cells	0.247 ± 0.027 (8)	0.285 ± 0.027 (6)	0.038
Mitochondria	0.231 ± 0.007 (2)	0.246 ± 0.014 (2)	0.015
Microsomes	0.278 ± 0.005 (2)	0.294 ± 0.008 (2)	0.016
Plasma membrane	0.314 ± 0.006 (2)	0.326 ± 0.010 (2)	0.012
P-388			
Whole cells	0.199 ± 0.016 (14)	0.256 ± 0.210 (7)	0.057
Mitochondria	0.180 ± 0.015 (3)	0.225 ± 0.026 (3)	0.045
Microsomes	0.232 ± 0.004 (3)	0.259 ± 0.007 (3)	0.027
Plasma membrane	0.277 ± 0.011 (3)	0.302 ± 0.012 (3)	0.025

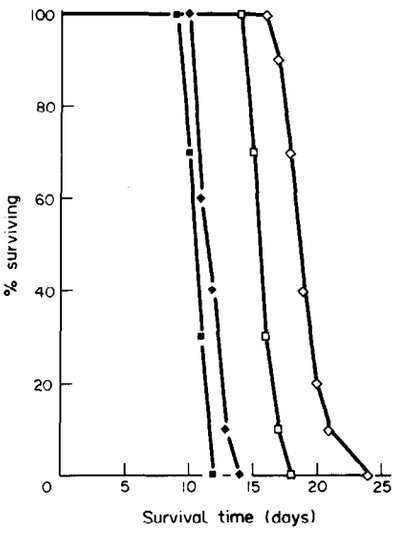

Fig. 1. Survival of female CDF$_1$ mice injected with ascites P-388 cells that were: unmodified and unheated (■——■); CHS-modified and unheated (◆——◆); unmodified and heated at 43°C for 60 min (□——□); and CHS-modified and heated (◇——◇). Results shown represent 1 of 2 replicate experiments. Each experiment had qualitatively similar results. In each replicate experiment the individual groups were composed of 10 animals.

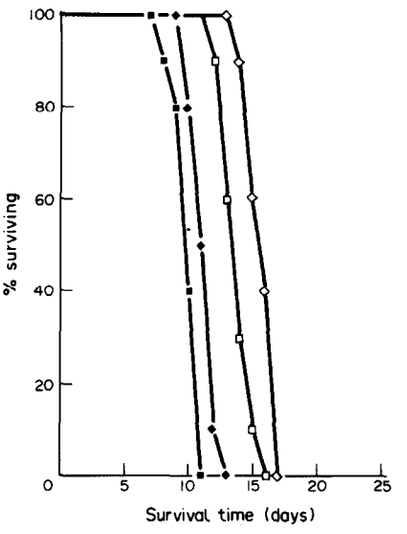

Fig. 3. Survival of irradiated (525 rad) female CDF$_1$ mice injected with ascites P-388 cells that were: unmodified and unheated (■——■); CHS-modified and unheated (◆——◆); unmodified and heated at 43°C for 60 min (□——□); and CHS-modified and heated (◇——◇). Results represent 1 of 2 replicate experiments with 10 animals in each of the groups in both experiments.

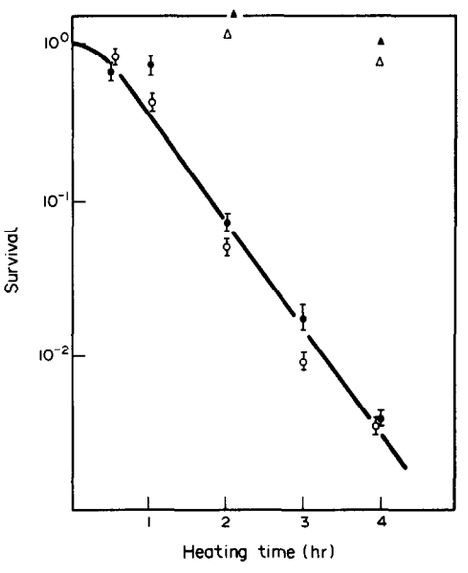

Fig. 2. In vitro survival of V-79 cells that were: incubated at 37°C when either unmodified (▲) or CHS-modified (△); or heated at 43°C when either unmodified (○) or CHS-modified (●).

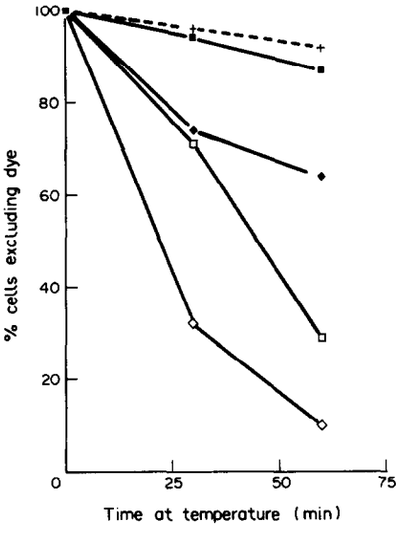

Fig. 4. Percentage of P-388 and V-79 cells excluding trypan blue following treatment with CHS alone or in combination with 43°C for up to 60 min. For P-388 cells: unmodified and unheated (■——■); CHS-modified and unheated (◆——◆); unmodified and heated at 43°C (◇——◇); CHS-modified and heated at 43°C (□——□). The exclusion of trypan blue by V-79 cells was not influenced by any of the treatments over 60 min; all treatment groups are represented by the same line (+).

The survival of V-79 cells after CHS modification and heating is shown in Fig. 2. There is no significant difference in the survival of modified and unmodified cells heated in suspension at 43°C. Maintaining modified or unmodified cells in suspension at 37°C for up to 4 hr did not alter plating efficiency relative to zero time controls. Similar survival results were obtained when cells were heated while attached to the surface in tissue culture flasks.

Survival studies were performed with P-388 cells in irradiated animals, which were shown to

be immune-suppressed using heterograft time to rejection techniques. The relative pattern of survival for the various treatment groups assayed in irradiated animals was the same as that observed in unirradiated mice (Fig. 3). The only difference was that survival time in all treatment groups was reduced.

The influence of CHS and heat on the ability of P-388 and V-79 cells to exclude trypan blue dye was studied (Fig. 4). None of these treatments affected exclusion of trypan blue by V-79 cells, whereas P-388 cells were differentially affected. The percentage of CHS-modified cells that excluded trypan blue decreased from 75% after 30 min to 63% after 60 min of incubation at 37°C. Heating alone for 60 min decreased the number of cells excluding the dye to 28%. The combination of CHS modification and heat treatment produced the greatest effect. Only 10% of cells so treated exclude trypan blue at the end of 60 min of heating. In contrast, cholesterol, when added to the membrane of P-388 cells, did not influence dye exclusion at either 38 or 43°C.

DISCUSSION

We have previously suggested that *Escherichia coli* thermal sensitivity is correlated with the physical state and lipid composition of their membranes [3, 10]. Furthermore, cholesterol content in a variety of cell lines is inversely correlated to heat sensitivity [22, 23]. Cholesterol content can also be varied in cells and those grown at 41°C had a higher cholesterol/phospholipid ratio and survived hyperthermia better than those grown at lower temperatures [14]. Fluorescence polarization values indicated that growth at higher temperatures increased microviscosity [14].

We have used the *in vitro* method of Shinitzky *et al.* [24] to acutely enrich cell membranes with cholesterol and/or CHS. DPH monitors the average microviscosity of the plasma membrane and of all the intracellular membranes; therefore the P value is the average of all the membranes. Cholesterol alone, which presumably only enters the plasma membrane of whole cells, did not markedly alter whole-cell P values. In contrast, CHS markedly increases the P values of both whole cells and 'subcellular organelles'. We interpret our data as indicating that there is a relative increase in membrane microviscosity (decrease in fluidity) in membrane fractions that have been enriched with CHS. Although there is controversy over the interpretation of steady-state P measurements, we do not feel that it influences our conclusions. They are based on average membrane microviscosity values and are not contingent on detailed knowledge of the physical state of microdomains within the membranes. Despite the evidence of others, cited above, which indicates that increased microviscosity is protective against the adverse effects of heat, neither cholesterol nor CHS increased survival in cells heated at 43°C. In fact, the reverse was true for P-388 cells heated and assayed *in vivo*. Furthermore, the CHS-treated group incubated at 37°C for 60 min was more sensitive than its untreated 37°C control group.

One possible explanation for the greater killing of CHS-modified cells *in vivo* could be increased immunogenicity, which reportedly occurs [24]. However, our results with animals immunosuppressed by radiation make this explanation unlikely.

The data from the trypan blue exclusion studies provide a more likely explanation. Treatment with CHS alone results in a substantial increase in cells which take up trypan blue (32% after 60 min at 37°C), suggesting that membrane integrity is altered by the addition of CHS. Consistent with a CHS-induced change in the plasma membrane (permeability) is the 7-fold increase in [^{3}H]-cholesterol uptake. When the cells that are made permeable to trypan blue by CHS addition are corrected for, the difference in killing between control and CHS-modified cells after 1 hr of heating is no longer seen. Thus the heat killing of CHS-modified P-388 cells seem to be more apparent than real. When considered in this light, both P-388 and V-79 cells are similar in their response to heat, which is not influenced by CHS *per se*.

The results from the current study are consistent with observations which conclude that probe measurements of lipid fluidity did not always correlate with hyperthermic sensitivity. In mammalian cells, Yau found no change in P values of whole V-79 cells measured by DPH in the presence of 10 mM procaine, although he did find evidence of fluidization using a perylene probe [7]. Lepock *et al.* [26] found that butylated hydroxytoluene (BHT), which markedly fluidized V-79 cell membranes when measured by EST techniques, did not potentiate cell killing by hyperthermia.

How then can these findings be rationalized to fit with our hypothesis of cell killing by hyperthermia? Recent evidence suggests that lipids are not homogeneously distributed in membranes but, rather, populations are organized into domains. Asymmetric distribution of phospholipids in bilayer leaflets, first demonstrated to exist in erythrocytes [27], is now accepted for a variety of biological membranes

[28] and a number of techniques [29–31] suggest that gel and liquid-crystalline phases coexist. However, more speculative, lateral domains of lipids are thought to occur as a result of preferential interaction of headgroups [32] and through association with either cholesterol [33, 34] or proteins [35–37]. As membrane function seems to reflect the needs of a specific lipid-protein compartment (gel or liquid-crystalline) rather than a generalized lipid fluidity [38, 39], it should come as no surprise that membrane function and thermal sensitivity cannot always be correlated with the average value of membrane microviscosity measured by various probes. This is consistent with the conclusions of Poon and Clark [40] that interpretation of results using ESR and DPH to measure the physical state of biological membranes should be approached cautiously. Thus our data may in part be explained by intrinsic differences in membrane lipid components of P-388 and V-79 cells. These differences could explain the differential permeability of these cells to trypan blue following the addition of CHS to their membranes since their lipid domains would most likely have varying defects in packing and boundary mix matches. Thus it is not unexpected that they respond differently when CHS is inserted into their respective bilayers.

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