Europ. J. Cancer Vol. 15, pp. 1449–1457
© Pergamon Press Ltd. 1979. Printed in Great Britain

0014-2964/79/1201-1449 $02.00/0

Effect of Lysosome Modification on the Heat Potentiation of Radiation Damage and Direct Heat Death of BP-8 Sarcoma Cells*

KURT G. HOFER, BILLY BRIZZARD and MARIA G. HOFER

Institute of Molecular Biophysics, Florida State University, Tallahasse, Florida 32306, *U.S.A.*

Abstract—*It has been suggested that lysosomes represent the intracellular target responsible for heat potentiation of radiation damage and direct heat death in mammalian cells. To investigate the hypothesis that heat damages cells by lysosmal activation, BP-8 murine sarcoma celle were treated with trypan blue. This agent accumulates within lysosomes and causes pronounced inhibition of lysosmal enzymes. To evaluate the possibility that heat damage is mediated by lysosmal membrane instability and leakage of hydrolases into the cytoplasm, BP-8 cells were treated with a membrane labilizer (retinol) or stabilizer (hydrocortisone). The pre-treated cells were then subjected to hyperthermia* (1 hr *incubation at* 37°–43°C), *or to X-irradiation at normal* (37°C) *vs elevated* (41.5°C) *temperatures. The results indicate that pretreatment with trypan blue, retinol or hydrocortisone does not exert any effect on the heat response of BP-8 sarcoma cells. Therefore, it would seem unlikely that lysosomes are responsible for either thermal enhancement of radiation damage or direct heat-induced cell death.*

INTRODUCTION

THERE is ample evidence that hyperthermia (41°C or higher) potentiates radiation killing of mammalian cells [1–6]. At somewhat higher temperatures, hyperthermia causes direct heat induced cell death [7–11]. The mechanisms responsible for these effects are still poorly understood, but in recent years a number of authors have suggested that lysosomes may constitute the primary target site for both heat potentiation of radiation damage [12] and direct heat death [8, 9, 11, 13].

Several different types of lysosomal damage have been postulated. For example, heat could damage cellular membranes, including lysosomal membranes [13, 14]. This could lead to enhanced membrane permeability or perhaps complete lysosomal rupture resulting in leakage of lysosomal enzymes into the cytoplasm. Such discharge of hydrolases into extralysosomal cell compartments could cause severe cell damage or outright cell death [15].

Alternately, heat treatment may induce lysosomal activation, that is, increased lysosomal enzyme activity [9, 11, 12]. In addition, heat may also induce an increase in the number of lysosomes per cell [9, 11]. Thus, even if hyperthermia does not impair the structural integrity of lysosomes, the increase in lysosomal activity could still prove damaging to cells.

In this investigation an attempt was made to evaluate the lysosome concept of heat damage and to determine whether the observed lysosomal changes are causally related to heat potentiation of radiation damage and/or direct heat death in mammalian cells.

MATERIALS AND METHODS

Tumor system and viability assay

The experiments reported in this paper were carried out on BP-8 murine sarcoma cells. The BP-8 cells were maintained in ascites form in the peritoneal cavity of 10–12 week old C3H or ICR female mice by weekly passage of 10^6 cells.

Accepted 15 May 1979.

*This investigation was supported by Grant No. RD-21 from the American Cancer Society and by Grant No. F 78FS-1 from the Florida Division of the American Cancer Society.

The fractional survival of BP-8 cells subjected to hyperthermia and/or irradiation was evaluated with the ^{125}I-iododeoxyuridine (^{125}IUdR) prelabeling technique. This assay system has been described in detail elsewhere [16]. Briefly, peritoneal BP-8 cells were labeled with 1.5 μCi of ^{125} IUdR/mouse, administered i.p. in three injections of 0.5 μCi/mouse on day 4 of tumor growth. The three injections were given at successive intervals of 4 hr, i.e. the total labeling time was 8 hr. Using this procedure it was found that about 10–15% of the injected ^{125}IUdR was incorporated into the DNA of BP-8 ascites cells. Autoradiographic examination of cell samples revealed that the labeling index was 90% or higher. This labeling schedule was closely followed in all experiments to avoid the radiation damage and increased cell death observed after incorporation of higher doses of ^{125}IUdR [17].*

Two hours after completion of the ^{125}IUdR labeling course, the tumor cells were aseptically removed from the peritoneal cavity by repeatedly rinsing the cavity with cold, heparinized (2 U/ml) Earle's balanced salt solution (EBSS). The cells were pooled, pelleted by centrifugation at 600 rev/min, and diluted in fresh EBSS. All procedures were carried out at 4°C to avoid damage to the cells. When aliquots of the final cell suspension were precipitated in ice cold 10% trichloroacetic acid, less than 3% of the total ^{125}I activity was found in the supernatant, i.e. more than 97% was associated with the DNA of the tumor cells [23].

To produce a logarithmically growing cell population and to eliminate the erythrocyte contamination frequently observed during advanced stages of tumor growth, the prelabeled tumor cells were inoculated i.p. into groups of 'intermediate' host mice. The size of the intermediate inoculum was approximately 50 $\times 10^6$ cells/mouse. About 15–18 hr later the cells were harvested from the intermediate hosts, subjected *in vitro* to hyperthermia and/or X-irradiation as described below, and reinjected into groups of 5 final test mice (10 $\times 10^6$ cells/mouse).

One or two days before the final test mice were inoculated with ^{125}IUdR labeled tumor cells, their drinking water was supplemented with 0.1% sodium iodide to depress subsequent accumulation of ^{125}I in the thyroid. Immediately after tumor inoculation and at daily intervals thereafter, the whole body ^{125}I activity of the final test mice was monitored by counting individual live mice in a well-type crystal scintillation counter. All counts were corrected for radioactive decay and small day-to-day fluctuations in the counting efficiency by monitoring a standard sample of ^{125}I. The results were calculated to provide the percentage of the inoculated radioactivity retained in each animal at the time of counting. Previous studies have shown that any loss of ^{125}I from such mice can be taken as an index of natural and treatment induced cell death *in vivo* [6, 16, 20,–22].

To convert the ^{125}I retention values into standard dose survival curves, the surviving fraction (S) of cells was calculated by interpolation between live and dead control values according to the formula:

$$S=\frac{T_x-D}{T_o-D} \qquad (1)$$

where T_o is the percent retention of ^{125}I activity in the untreated live control group, T_x means retention of radioactivity in the experimental treatment group, and D stands for retention of ^{125}I activity in mice inoculated with labeled BP-8 cells which had been pre-killed before implantation into final test mice. The pre-killing was achieved by incubation at 65°C for 15 min in heat experiments and by exposure to 5000 rad in radiation experiments. If the T_o, T_x and D values are obtained after heat-induced or radiation-induced cell death has become fully reflected in reduced ^{125}I retention (8–10 days after implantation), equation (1) appears to give accurate data for survival values greater than 0.05.

Heating and irradiation procedures

To study the heat response of cells, ^{125}I labeled BP-8 cells harvested from intermediate hosts were suspended in EBSS (10×10^6 cells/ml), aerated on ice for 15 min, and placed for 1 hr in a precision controlled circulating water bath (accuracy greater than ± 0.1°C) at temperatures ranging from 37–43°C. During the 1 hr incubation period the suspensions

*Previous experiments had shown that high doses of DNA-associated ^{125}I (0.02 μCi/10^6 cells or higher) cause pronounced division delay [18] and all lethality [17, 19] in a variety of mammalian cell lines. In the experiments reported here the ^{125}I content of the labeled cells did not exceed 0.002 μCi/10^6 cells. Such low ^{125}I doses do not cause any detectable changes in tumor proliferation kinetics [20, 21] or in tumor cell death [18] even if the labeled cells are subsequently subjected to incubation at elevated temperatures [6, 16, 22].

were agitated every 5 min to prevent settling of the suspended BP-8 cells. Also, the pH of the cell suspension was monitored to ascertain that it did not drop by more than 0.2 pH units from the initial pH of 7.2.

For irradiation two circulating water baths (37°vs 41.5°C) were placed on a rotating table in the center of the X-ray beam. Cells were immersed in the water bath at min 0, X-irradiation was performed between min 10 and 20 and the irradiated cells were removed from the water bath at min 60. All irradiations were performed on a GE maxitron 300 X-ray machine. The X-ray machine was operated at 250 kVp, 20 mA, and the beam was filtered with 2.4 mm Al (HVL 11 mm Al). The dose rate employed was 250 rad/min as measured with a Victoreen ionization chamber. During irradiation the turntable with the circulating water baths was rotated at 18 rev/min.

Modification of lysosomes

To evaluate the validity of the hypothesis that lysosomal activation is the primary mechanism of heat damage, the heat response of control BP-8 sarcoma cells was compared to that of cells subjected to trypan blue. This dye is known to accumulate in lysosomes and to inhibit a variety of lysosomal enzymes [24]. The experimental procedure was as follows: BP-8 cells harvested from intermediate hosts were suspended in EBSS supplemented with 1% heat-inactivated calf serum (10×10^6 cells/ml) and trypan blue was added to yield a final concentration of 100 μg/ml. The cells were then incubated at 37°C for 1 hr to permit accumulation of the dye in the lysosomes. At the end of the 1 hr incubation the cells were pelleted by centrifugation and resuspended in fresh EBSS for heat and/or radiation treatment as outlined above.

To investigate the possibility that hyperthermia acts via lysosomal membrane changes, BP-8 cells were subjected to retinol (vitamin A alcohol) or hydrocortisone. It is known that administration of retinol causes increased permeability of lysosomal membranes [25, 26], whereas hydrocortisone appears to stabilize lysosomal membranes [27, 28]. Thus, experiments with these two agents should reveal whether heat acts by impairing or destroying the physical integrity of lysosomes.

Retinol was usually administered *in vivo* in doses of up to 15,000 i.u./mouse. In most experiments retinol was injected i.p. into prospective intermediate host mice. The tumor cells (50×10^6 cells/mouse) were implanted i.p. 15 min after retinol injection. This treatment regimen was chosen to avoid exposure of the BP-8 cells to the solvent (30 μl of absolute alcohol) in which the retinol was administered. Since retinol precipitated within the peritoneal cavity immediately after injection, this regimen provided optimal conditions for exposure of BP-8 cells to high concentrations of the vitamin. Eighteen hours later the cells were removed from the peritoneal cavity and subjected to heat and radiation treatment as described above. In some experiments retinol was administered *in vitro* for 1 hr before heat treatment. In two experiments retinol was administered both *in vivo* and *in vitro*.

Hydrocortisone was administered *in vitro* in concentrations ranging from 20 to 500 μg/ml to BP-8 cells suspended in EBSS (10×10^6 cell/ml). The cells were incubated in this solution at 37°C for 1 hr before heat treatment. In most experiments the cells were washed free of hydrocortisone before heat treatment, but in three experiments hydrocortisone treatment was continued throughout the subsequent 1 hr exposure to elevated temperatures. In addition, experiments were performed where hydrocortisone was administered 6, 24, 48 or 72 hr before heating.

Cell homogenization and lysosome isolation

To obtain lysosomes for *in vitro* examination, aliquots of BP-8 cells were suspended (10×10^6 cells/ml) in 0.25 M sucrose buffered with 30 mM Tris–HCl (pH 7.4). The cells were then broken by sonication with a Bronwill Biosonic III sonifier. Optimal breakage of cells was achieved when 10 ml of the cell suspension was sonicated for 2–6 sec at position 40 on the Bronwill sonifier. The degree of breakage of the cells was checked by microscopic examination of the homogenate. To avoid excessive damage to the subcellular particles, sonication was discontinued when about 2/3 of the cells had been disrupted. After sonication the cell suspension was homogenized in a glass homogenizer with 10 strokes with a loosely fitting pestle.

Lysosomes were recovered from the homogenate by a modification of the method described by Wang and Touster [29]. Briefly, the cell homogenate was centrifuged at 5000 ***g*** for 10 min in a Spinco Model L ultracentrifuge to pellet whole cells, nuclei and larger cell fragments. The supernatant fraction was further centrifuged at 20,000 ***g*** for 30 min to

pellet lysosomes and mitochondria. The L and M pellet was a brownish layer (intensely blue in the case of trypan blue containing lysosomes) with a thin loose white layer on top. The white layer was scraped off and removed with a pipette.

The remaining lysosomal pellet was resuspended in buffered 0.25 M sucrose and subjected to density gradient centrifugation on a continuous sucrose gradient (20–60% w/v). Two milliliters of the lysosomal suspension was layered onto the gradient. Centrifugation was performed in the SW 25.1 rotor of a Spinco Model L ultracentrifuge at 15,000 rev/min for $5\frac{1}{2}$ hr. Each tube was divided into approximately 15 2-ml fractions (starting from the top of the gradient) by aspiration with a syringe.

The fraction containing the lysosomes was determined by assaying the acid phosphatase activity of each fraction. Moreover, with lysosomes obtained from trypan blue treated cells the lysosomal fraction was easily visible on the gradient as a blue band. To determine the trypan blue concentration within lysosomes, the lysosomal fraction was pelleted by centrifugation at 20,000 ***g*** for 30 min, resuspended in 1 ml of 0.25 M sucrose and again pelleted in a thin (i.d. 2 mm) centrifuge tube. After this number of centrifugations the supernatant remained completely clear, indicating that all the remaining trypan blue was associated with the lysosomes.

To determine the intralysosomal trypan blue concentration, the final lysosome pellet was resuspended in 0.25 M sucrose at a volume ratio 1:100 and the absorbance of the suspension was determined at 590 nm, the wavelength of maximal absorbance for trypan blue. To obtain the net absorbance value, the trypan blue containing lysosomes were read against a reference sample of equally prepared lysosomes derived from control BP-8 cells. The concentration of the dye in the diluted lysosome sample was then determined from a calibration graph generated by plotting the A_{590} against known concentrations of trypan blue (the resulting plot yielded a straight line indicating conformity to Beer's law). The concentration value obtained in this manner was then multiplied by 100 to derive the nominal concentration of the dye within the original lysosome pellet. Since the final pellet still contained appreciable quantities of mitochondria whose volume was included in the volume of the pellet, this procedure provided a minimum estimate of the trypan blue concentration within lysosomes.

Acid phosphatase assay

The presence of acid phosphatase was used as a marker for lysosomes. For enzyme assay the lysosomes were diluted in 0.25 M sucrose containing 0.1% Triton X-100 to facilitate release of lysosomal enzymes. The assay procedure [30] was as follows: 0.3 ml of the enzyme sample was mixed with 1.2 ml of 0.1 M sodium acetate–acetic acid buffer (pH 5.0). The mixture was incubated for 10 min at 37°C to destroy glucose-6-phosphatase. The reaction was then started by the addition of 0.5 ml of substrate solution (32 mM 4-nitrophenyl phosphate, disodium salt, dissolved in water), giving a final substrate concentration of 8 mM. After 30 min incubation at 37°C, the reaction was stopped with 2 ml of ice-cold Tris–phosphate reagent and the absorbance of the solution at 420 nm (against appropriate blanks) was determined on a Beckman Model 25 spectrophotometer. To evaluate the effect of trypan blue on the activity of acid phosphatase, the same assay procedure was carried out at trypan blue concentrations ranging from 0–150 μg/ml.

RESULTS

Effect of trypan blue on cell viability

Figure one (solid lines) shows the rate of ^{125}I loss from C3H mice inoculated i.p. with

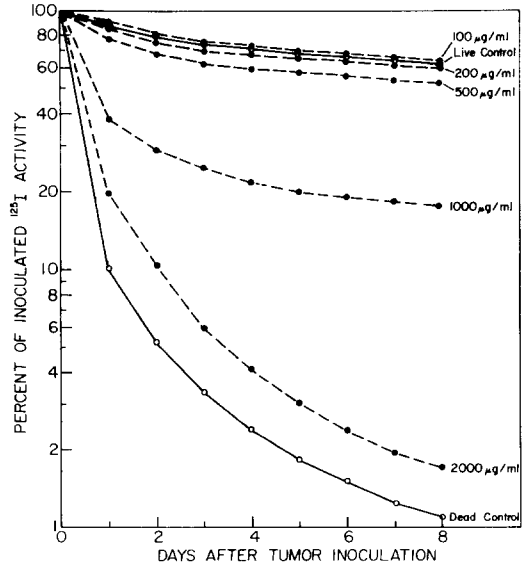

Fig. 1. Loss of ^{125}I from mice inoculated i.p. with ^{125}IUdR labeled BP-8 sarcoma cells. The solid lines represent ^{125}I retention in mice bearing live control or heat killed BP-8 cells, the dashed lines show ^{125}I retention in mice inoculated with trypan blue (100–2000 μg/ml) treated cells. Each data point represents the average retention value of 5 mice, expressed as a percentage of the initial ^{125}I activity. The variation within each treatment group was less than ±8%.

living or heat killed ^{125}IUdR labeled BP-8 cells. Following inoculation of living BP-8 cells, about 10–12% of the ^{125}I activity was lost from the tumorous mice each day during the initial phases of tumor growth. Between day 2 and day 8, the rate of ^{125}I loss declined to 6–8% per day, but during advanced stages of tumor development ^{125}I loss once again increased to about 12–15% per day. Implantation of dead labeled tumor cells (killed by incubation at 65°C for 15 min) was followed by rapid excretion of most of the inoculated ^{125}I activity, indicating very low rates of ^{125}I reutilization after cell death.

The dashed lines in Fig. 1 indicate the rate of ^{125}I loss from mice bearing tumor cells which had been exposed to various concentrations of trypan blue (up to 2000 μg/ml). Trypan blue was administered *in vitro* to BP-8 cells suspended in EBSS (10×10^6 cells/ml). The cells were incubated in the presence of the dye for 1 hr at 37°C, followed by centrifugation and inoculation into final test mice. From the data shown in Fig. 1 it appears that trypan blue doses of up to 200 μg/ml did not produce any adverse effects on tumor cell viability. At higher concentrations trypan blue induced a marked decrease in tumor cell survival. Using equation (1) described under Materials and Methods, it can be shown by interpolation between live and dead control values (day 8) that the survival of trypan blue treated cells was 84% at 500 μg/ml, 27% at 1000 μg/ml and less than 1% at 2000 μg/ml.

Accumulation of trypan blue in lysosomes

Based on the data presented in Fig. 1, most subsequent experiments were carried out at a trypan blue concentration of 100 μg/ml. No adverse effects on tumor viability were ever noted at this dose level.

To determine the degree of dye accumulation within lysosomes, aliquots of trypan blue treated cells were disrupted by sonication and the lysosomal fraction was isolated as outlined under Materials and Methods. The results of these studies showed that BP-8 cells exposed to 100 μg/ml of trypan blue for 1 hr exhibited an intralysosomal dye concentration equivalent to about 700–1200 μg/ml.

Even these high values probably represent minimum estimates because the final lysosome pellet was invariably contaminated with mitochondria which banded at the same density as lysosomes. The presence of the mitochondria (which did not incorporate trypan blue but did contribute to the volume of the pellet), caused a corresponding underestimate in the calculated concentration of trypan blue within lysosomes.

Effect of trypan blue on acid phosphatase activity

Trypan blue is known to cause pronounced inhibition of a variety of lysosmal enzymes such as deoxyribonuclease, ribonuclease, β-glucuronidase and acid phosphatase [24]. Figure two demonstrates this effect on acid phosphatase. From the data it is apparent that trypan blue causes a significant reduction in acid phosphatase activity even at concentrations as low as 25 μg/ml. At 100 μg/ml almost 90% inhibition of enzyme activity was observed. Thus, the 700–1200 μg/ml concentration of the dye observed in lysosomes isolated from trypan blue treated cells should be more than sufficient to cause pronounced inhibition of lysosomal enzymes.

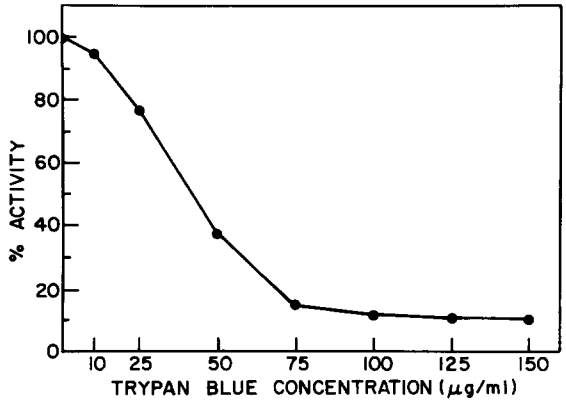

Fig. 2. Inhibition of acid phosphatase activity by trypan blue.

Heat potentiation of radiation damage in trypan blue treated cells

To determine whether heat potentiation of radiation death is indeed related to lysosomal enzyme activity, control and trypan blue treated cells were subjected to X-irradiation at 37° vs 41.5°C. The heating time was 1 hr and the irradiation was performed between min 10 and min 20 of the 1 hr incubation period. After completion of this treatment regimen the cells were transplanted into new host mice and the fractional survival of the cells was evaluated with the ^{125}IUdR prelabeling assay.

From the survival data shown in Fig. 3 it is clear that heating at 41.5°C caused significant potentiation of radiation damage both in control cells and in trypan blue treated cells. With both types of cells the D_o was 260 rad for cells irradiated at 37°C and 170 rad for cells irradiated at 41.5°C, i.e., the degree of radiosensitization due to hyperthermia was

identical in treated and untreated BP-8 cells. It can be concluded, therefore, that inhibition of lysosomal hydrolases does not protect cells from the radiosensitizing effects of elevated temperatures.

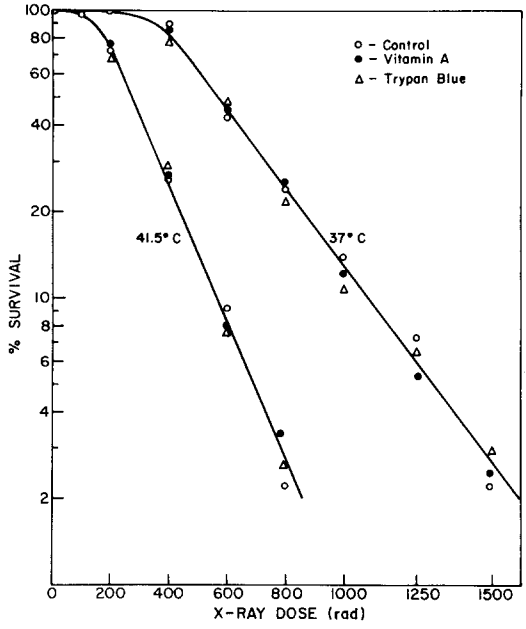

Fig. 3. Effect of trypan blue (100 μg/ml) *and vitamin A* (10,000 *i.u./mouse*) *on heat potentiation of radiation damage in BP-8 sarcoma cells.*

Direct heat death of trypan blue treated cells

Additional experiments were performed to determine whether treatment with trypan blue conferred enhanced resistance to direct heat induced cell death. In these experiments BP-8 cells were heated in EBSS* for 1 hr at temperatures ranging from 37° to 43°C. Once again trypan blue treated cells did not demonstrate any greater heat resistance than untreated control cells (Fig. 4).

Effect of retinol and hydrocortisone on the heat response of BP-8 cells

Since inhibition of lysosomal enzymes did not cause any change in the heat response of

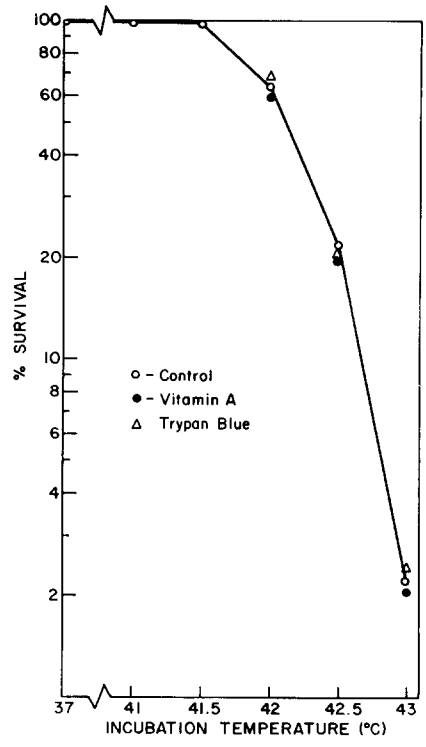

Fig. 4. Effect of trypan blue (100 μg/ml) *and vitamin A* (10,000 i.u./*mouse*) *on direct heat death of BP-8 sarcoma cells.*

BP-8 cells, an attempt was made to investigate the possibility that heat damage might be mediated by changes in the stability of lysosomal membranes. The possibility of outright lysosomal rupture was ruled out by microscopic examination. Control BP-8 cells and BP-8 cells which had previously been subjected to 1 hr incubation at 42° or 43°C were stained with acridine orange and examined on a fluorescence microscope. No change in the number, size or staining properties of lysosomes was noted in heated cells.

However, since the heat response of mammalian cells might involve more subtle changes in the integrity of lysosomal membranes, additional experiments with membrane labilizers (retinol) and stabilizers (hydrocortisone) were performed. It has been known for many years that administration of retinol (vitamin A alcohol) causes increased permeability of lysosomal membranes both in isolated lysosomes and in whole cells [25, 26]. From the data shown in Figs. 3 and 4, it is apparent that such lysosomal labilization did not enhance the heat response of BP-8 cells.

In the experiments illustrated in Figs. 3 and 4, retinol (10,000 i.u.) was administered 18 hr before heating by i.p. injection into intermediate hosts. Eight additional studies performed with different retinol doses (500–15,000

*Previous experiments in our laboratory demonstrate that incubation in EBSS does not provide optimal conditions for maximizing the heat resistance of mammalian cells. For example, L1210 leukemia cells growing in the peritoneal cavity of mice do not show any decline in cell viability if the leukemic mice are subjected to whole body heating at 42°C for 1 hr [6]. In contrast, L1210 cells suspended in EBSS and subjected to the same heating schedule exhibit death rates in excess of 80% (unpublished observation). Thus, the comparatively low survival values shown in Fig. 4 do not indicate that BP-8 cells are intrinsically more sensitive to heat damage than other types of tumor cells.

i.u.) and different treatment schedules (*in vivo* vs *in vitro* administration, or combined administration from 1 to 24 hr before heating) yielded identical results. In all experiments retinol treated cells were no more sensitive to heat effects than control cells.

Similarly, administration of hydrocortisone, a lysosomal stabilizer [27, 28], did not alter the heat response of BP-8 cells (data not shown). In these experiments the hydrocortisone dose was varied between 20 and 500 μg/ml, and the time of administration was between 1 and 72 hr before heating. In three experiments hydrocortisone treatment was continued throughout the 1 hr heating period, also with negative results. It therefore appears that neither lysosomal labilization by retinol or stabilization by hydrocortisone or inhibition of lysosomal enzymes by trypan blue exerted any effect on the heat response of BP-8 sarcoma cells.

DISCUSSION

Lysosomes have been implicated in a large variety of cellular processes such as phagocytosis, pinocytosis, cellular digestion, control of cell division, cell renewal by autophagy and autolysis after cell death [31]. In addition, exposure to adverse stimuli such as streptolysin S may cause discharge of lysosomal enzymes into the cell cytoplasm resulting in cell death [15]. Exposure to ionizing radiations or heat seems to be associated with pronounced changes in lysosome morphology and distribution, as well as with changes in the activation state of lysosomal enzymes. Based on such findings a number of authors have suggested that damage to lysosomes may be the primary cause of radiation death, heat death, or heat potentiation of radiation damage in mammalian cells [8, 9, 11–14, 32].

Previous studies in our laboratory have shown that lysosomes are not the primary target for radiation induced death [20, 33]. These findings, however, do not exclude the possibility that lysosomes may be involved in heat potentiation of radiation damage or direct heat death. In fact, recent literature reports provide strong evidence that there is a close correlation between the degree of lysosomal activation and thermal enhancement of X-ray damage in mammalian cells [12]. It is not clear, however, whether the observed lysosomal changes are the cause of the heat response, or whether the lysosomes are only incidentally damaged [14].

This investigation represents an attempt to evaluate the lysosome concept of heat damage by studying the heat response of cells subjected to lysosomal modification by means other than heat or radiation. From the data shown in Figs. 3 and 4, it appears that neither inhibition of lysosomal enzymes nor labiliztion or stabilization of lysosomal membranes causes any change in the heat response of cells. These findings suggest that lysosomes may not be causally involved in thermal enhancement of radiation damage or direct heat death in mammalian cells.

However, several objections could be raised against this interpretation. For example, it could be argued that heat treatment causes a discharge of lysosomal enzymes into the cell cytoplasm. Since trypan blue is retained within the lysosomes, the released hydrolases would no longer be subject to inhibition by the dye. Thus, if the heat damage is mediated entirely by those hydrolases which are active in extralysosomal cell compartments, inhibition of intralysosomal hydrolases would not cause any enhancement in the heat resistance of trypan blue treated cells.

However, the studies with retinol and hydrocortisone treated cells argue against this explanation. If heat effects were indeed caused by increased release of hydrolases, retinol treated cells should be more sensitive and hydrocortisone treated cells more resistant to heat than control cells. In fact, trypan blue itself also seems to inhibit the release of lysosomal enzymes [34] and should therefore confer some protection as a lysosomal membrane stabilizer. Since neither of the three agents causes any change in the heat response of cells, it appears unlikely that the primary effect of heat is damage to lysosomal membranes. This conclusion is supported by recent data from Hume and Field [12] who observed no change in lysosomal membrane stability in heated mouse spleens after 1 hr exposures to 41.8°C.

However, even if the physical integrity of lysosomes is not affected by hyperthermia, our data do not completely rule out the possibility that the heat response of cells may in some way be related to lysosomal activation. Although trypan blue causes pronounced inhibition of several important lysosomal enzymes (deoxyribonuclease, ribonuclease, β-glucuronidase, acid phosphatase), other hydrolases may be less sensitive to the dye. For example, acid protease is inhibited only at trypan blue concentrations of 1 mg/ml or higher [24]. It is conceivable, therefore, that

heat damage is mediated by activation of one or more hydrolases which are insensitive to inhibition by trypan blue. Further studies are needed to prove or disprove this possibility.

In this context it may be important to note that heat potentiation of radiation damage can also be demonstrated on lambda bacteriophages (unpublished observations). Except for the higher D_o and the absence of a shoulder in the radiation survival curve of phages, the pattern of thermal radiosensitization in bacteriophages appears to be similar to that observed in mammalian cells. Direct heat inactivation of bacteriophages (heated in physiological saline) also follows a pattern similar to that seen in mammalian cells. While these findings do not prove that the mechanism of thermal damage is the same in phages and cells, the data do suggest that marked thermal responses can be obtained under conditions which clearly exclude the possibility of lysosomal involvement.

In conclusion, the findings reported in this communication suggest that lysosomes are probably not the primary target site for either heat potentiation of radiation damage or direct heat-death in mammalian cells.

REFERENCES

1. O. S. Selawry, J. C. Carlson and G. E. Moore, Tumor response to ionizing rays at elevated temperatures. A review and discussion. *Amer. J. Roentgenol.* **80,** 833 (1958).
2. E. Ben-Hur, M. M. Elkind and B. V. Bronk, Thermally enhanced radioresponse of cultured Chinese hamster cells: inhibition of repair of sublethal damage and enhancement of lethal damage. *Radiat. Res.* **58,** 38 (1974).
3. J. E. Robinson, M. J. Wizenberg and W. A. McCready, Combined hyperthermia and radiation suggest an alternative to heavy particle therapy for reduced oxygen enhancement ratios. *Nature (Lond.)* **151,** 521 (1974).
4. A. Yerushalmi and I. Har-Kedar, Enhancement of radiation effects by heating of the tumor. *Israel J. med. Sci.* **10,** 772 (1974).
5. D. E. Thrall, E. L. Gillette and W. C. Dewey, Effect of heat and ionizing radiation on normal and neoplastic tissue of the C3H mouse. *Radiat. Res.* **63,** 363 (1975).
6. K. G. Hofer, D. A. Choppin and M. G. Hofer, Effect of hyperthermia on the radiosensitivity of normal and malignant cells in mice. *Cancer (Philad.)* **38,** 279 (1976).
7. R. Cavaliere, E. C. Ciocatto, B. C. Giovanella, C. Heidelberger, R. O. Johnson, M. Margottini, B. Mondovi, G. Morrica and A. Rossi-Fanelli, Selective heat sensitivity of cancer cells. *Cancer (Philad.)* **20,** 1351 (1967).
8. M. Von Ardenne, Selective multiphase cancer therapy: conceptual aspects and experimental basis. *Advanc. Pharmacol. Chemother.* **10,** 339 (1972).
9. K. Overgaard and J. Overgaard, Investigations on the possibility of thermic tumor therapy I. Short-wave treatment of a transplanted isologous mouse mammary carcinoma. *Europ. J. Cancer* **8,** 65 (1972).
10. H. D. Suit and M. Shwayder, Hyperthermia: potential as an anti-tumor agent. *Cancer (Philad.)* **34,** 122 (1974).
11. J. Overgaard, Ultrastructure of a mouse mammary carcinoma exposed to hyperthermia *in vivo*. *Cancer Res.* **36,** 983 (1976).
12. S. P. Hume and S. B. Field, Acid phosphatase activity following hyperthermia of mouse spleen and its implication in heat potentiation of X-ray damage. *Radiat. Res.* **72,** 145 (1977).
13. B. Mondovi, R. Strom, G. Rotilio, A. F. Agro, R. Cavaliere and A. Rossi-Fanelli, The biochemical mechanism of selective heat sensitivity of cancer cells. I. Studies on cellular respiration. *Europ. J. Cancer* **5,** 129 (1969).
14. C. Turano, A. Ferraro, R. Strom, R. Cavaliere and A. Rossi-Fanelli, The biochemical mechanism of selective heat sensitivity of cancer cells. III. Studies on lysosomes. *Europ. J. Cancer* **6,** 67 (1970).
15. J. G. Hirsch, A. W. Bernheimer and G. Weissmann, A motion picture study of the toxic action of streptolysins on leukocytes. *J. exp, Med.* **118,** 223 (1963).
16. K. G. Hofer, M. G. Hofer, J. Ieracitano and W. H. McLaughlin, Radiosensitization of hypoxic tumor cells by simultaneous administration of hyperthermia and nitroimidazoles. *Radiat Res.* **70,** 362 (1977).

17. K. G. Hofer and W. L. Hughes, Radiotoxicity of intranuclear tritium, iodine-125 and iodine-131. *Radiat. Res.* **47,** 94 (1971).
18. R. L. Warters and K. G. Hofer, Radionuclide toxicity in cultured mammalian cells: elucidation of the primary site for radiation-induced division delay. *Radiat. Res.* **69,** 348 (1977).
19. R. L. Warters, K. G. Hofer, C. R. Harris and J. M. Smith, Radionuclide toxicity in cultured mammalian cells: elucidation of the primary site of radiation damage. *Curr. Top. Radiat. Res.* **12,** 389 (1977).
20. K. G. Hofer, W. Prensky and W. L. Hughes, Death and metastatic distribution of tumor cells in mice monitored with ^{125}I-idodoeoxyuridine. *J. nat. Cancer Inst.* **45,** 763 (1969).
21. K. G. Hofer and M. Hofer, Kinetics of proliferation, migration, and death of L1210 ascites cells. *Cancer Res.* **31,** 402 (1971).
22. K. G. Hofer, Cytotoxic and radiosensitizing effects of Ro 7-0582 in combination with hyperthermia. In *Cancer Therapy by Hyperthermia and Radiation.* (Edited by C. Streffer) p. 264 (1978).
23. K. G. Hofer and W. L. Hughes, Incorporation of ^{125}I-iododeoxyuridine into the DNA of L1210 leukemia cells during tumor development. *Cancer Res.* **30,** 236 (1970).
24. F. Beck, J. B. Lloyd and A. Griffiths, Lysosomal enzyme inhibition by trypan blue: a theory of teratogenesis. *Science* **157,** 1180 (1967).
25. J. A. Lucy and J. T. Dingle, Fat-soluble vitamins and biological membranes. *Nature (Lond.)* **204,** 156 (1964).
26. A. A. Aikman and E. D. Wills, Studies on lysosomes after irradiation. I. A quantitative histochemical method for the study of lysosomal membrane permeability and acid phosphatase activity. *Radiat. Res.* **57,** 403 (1974).
27. G. Weissman and J. Dingle, Release of lysosomal protease by ultraviolet irradiation and inhibition by hydrocortisone. *Exp. Cell Res.* **25,** 207 (1961).
28. H. B. Fell and L. Thomas, The influence of hydrocortisone on the action of excess vitamin A on limb bone rudiments in culture. *J. exp. Med.* **114,** 343 (1961).
29. C. C. Wang and O. Touster, Acid phosphatase of HeLa cells: properties and regulation of lysosomal activity by serum. *Arch. Biochem. Biophys.* **172,** 191 (1976).
30. A. J. Barrett and M. F. Heath, Lysosomal enzymes. In *Lysosomes: A Laboratory Handbook.* Edited by J. T. Dingle) p. 19. North-Holland, Amsterdam (1977).
31. D. Pitt, *Integrated Themes in Biology: Lysosomes and Cell Function.* Longman, New York (1975).
32. D. Brandes, K. W. Sloan, E. Anton and F. Bloedorn, The effect of X-irradiation on the lysosomes of mouse mammary gland carcinomas. *Cancer Res.* **27,** 731 (1967).
33. K. G. Hofer, C. R. Harris and J. M. Smith, Radiotoxicity of intracellular ^{67}Ga, ^{125}I and ^{3}H: nuclear vs cytoplasmic radiation effects in murine L1210 leukemia. *Int. J. Radiat. Biol.* **28,** 225 (1975).
34. J. B. Lloyd, F. Beck, A. Griffith and L. M. Parry, The mechanism of action of acid bisazo dyes. In *The Interaction of Drugs and Subcellular Components in Animal Cells.* (Edited by P. N. Campbell) p. 171. Churchill, London (1968).