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Isolation and Initial Characterization of Thermoresistant RIF Tumor Cell Strains

George M. Hahn[1] and Ine van Kersen

Department of Therapeutic Radiology, Stanford University School of Medicine, Stanford, California 94305

ABSTRACT

Heat-resistant cell strains were obtained from RIF-1 mouse tumor cells by repeated heatings of cells derived from survivors of previous heating cycles (60 min; 45°C). Twenty thermally resistant (TR) strains were derived from single cells that had survived 11 heating and regrowth cycles. These were then analyzed for appropriate characteristics *in vitro* and *in vivo*. *In vitro* we looked for: marked heat resistance; high plating efficiency; growth rate similar to that of RIF-1 cells; and no obvious morphological abnormalities. In syngeneic hosts, we looked for: ability of the cells to form tumors whose growth rates were similar to that of RIF-1 tumors; high cellular heat resistance; good plating efficiency of tumor-derived cells; and low immunogenicity. Five strains having these desired characteristics were analyzed for survival kinetics. The heat-resistant phenotype was found to be stable *in vitro*, although partial reversion *in vivo* was seen occasionally. The "break" in the Arrhenius plot was found to occur at 45°C in TR strains *versus* 43°C in RIF-1. All TR strains and the RIF-1 line developed similar levels of thermotolerance (as defined by slope ratios) when given isosurvival heat exposures. X-ray responses of TR and RIF-1 cells were indistinguishable both with respect to survival and to heat-induced radiosensitization. While the number of live cells required to give tumor takes in 50% of the recipients for TR strains was appreciably higher than that for RIF-1 cells, radiation-killed cells from none of the strains were able to immunize efficiently against subsequent challenges by live cells.

INTRODUCTION

In spite of a large amount of effort by many laboratories, several important questions regarding the way hyperthermia is efficacious in the clinic have remained unanswered. Considerable data implicate endothelial systems as being the "target" for tumor inactivation by mild (42–45°C) temperatures (1–3), but the role that tumor cell killing *per se* plays has not been established with any degree of confidence. Similarly, in spite of considerable study and sophisticated investigations, the precise way that heat inactivates cells, either endothelial or tumor, is also not known. Finally, on the molecular level, there still remain many unanswered questions regarding the events that follow a nonlethal or lethal heat shock. For example, the functional role of the set of proteins that are preferentially synthesized following heating, the heat shock proteins, is not understood. *A fortiore*, the genes responsible for heat resistance have not been identified.

One of the major reasons for the paucity of precise information in the several areas indicated is the lack of an appropriate test system. Traditionally, in areas such as chemotherapy and to a lesser extent radiotherapy, the role of treatment-resistant mutants and revertants has been very useful, perhaps essential, for the study of modes of action of the treatment itself. In the case of hyperthermia, however, only very few heat-resistant mutants have been reported (4–7), and these have only been exploited minimally for experiments designed to answer many of the questions just raised. We report here on the isolation and initial characterization of a set of heat-resistant mouse tumor cell strains. Our objective was to obtain cell strains with the following properties: (*a*) the cells all derive from a single parent line; (*b*) the cells show a large difference in heat resistance from the parent cells; (*c*) *in vitro*, growth rates of all the cell strains do not vary appreciably from that of the parent line (*d*) when injected into syngeneic hosts, cells from the strains give rise to tumors; (*e*) tumor growth rates of wild type and thermally resistant strains are similar; (*f*) there are no unusual morphological identifying characteristics among the cell strains; (*g*) cells from each of the cell strains grown *in vitro* give rise to colonies with good plating efficiencies; (*h*) cells obtained directly from tumors, when dissociated and plated *in vitro*, also give rise to colonies, thus permitting precise assays of cellular responses *in vivo*; and (*i*) at least some of the heat-induced cell strains should evoke only a minimal immune response when injected into syngeneic hosts. As the data will demonstrate, we have been quite successful in obtaining such cell strains, except perhaps for the last requirement. We believe that the system of heat-resistant strains should have considerable usefulness, both for investigations *in vivo* as well as *in vitro*.

MATERIALS AND METHODS

Isolation of TR[2] Cells

Parent Cell Line. We started with cells from the RIF-1 tumor system. The cells were originally derived from a radiation-induced fibrosarcoma by Twentyman *et al.* (8). This well-characterized tumor system has several advantages. The cells are minimally antigenic, in the sense that it is difficult if not impossible to preimmunize hosts against the tumor cells. Cells derived from this tumor form colonies when plated *in vitro*. The system has been used in many tumor studies, and it is generally accepted as one of the tumor reference systems for radiation and drug and for heat and drug studies.

Isolation Procedure for RIF-TR Cell Strains. RIF-1 cells *in vitro* were maintained in RPMI medium supplemented with 15% fetal calf serum. The cells were not mutagenized. They were grown to confluence and then heated at 45°C for 60 min. This exposure of RIF-1 cells results in a surviving fraction of approximately 10^{-5} (Fig. 1). The surviving cells were allowed to proliferate and were then divided into two parts. A heat survival curve (45°C) was constructed from the progeny of one group of cells; the others were grown to confluence again, and those cells were once more exposed to 45°C for 60 min. After four cycles of such heatings and regrowth, a clear shift in the survival curve of the progeny towards increased heat resistance was observed (Fig. 1). With additional selection cycles, heat resistance increased, until the heating and subsequent growth to confluence procedure had been repeated 7 times. In these and all subsequent experiments that measure survival, the assay used was clonogenicity. We then exposed survivors of the 7 selection cycles to 3 cycles at 45°C for 90 min. There was, however, little difference between the heat responses after Cycle 7 or Cycle 10 (Fig. 1). Cells from passage 10 were once more exposed to the high temperature, and the surviving cells were allowed to form colonies of approximately 200 to 400 cells each. Twenty of these colonies were then isolated, and cell strains grew up. These are termed RIF-TR-1 through RIF-TR-20. Survival after exposure to 45°C of one of these,

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[1] To whom requests for reprints should be addressed.

[2] The abbreviations used are: TR, thermally resistant; RIF-TR, thermally resistant RIF; TD_{50}, number of live cells required to give tumor takes in 50% of the recipients.

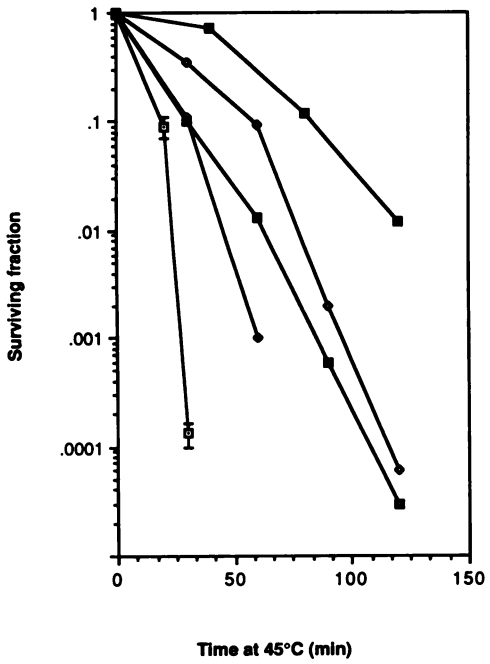

Fig. 1. Development of heat resistance during the selection process. Shown are survival curves for RIF-1 cells, cells obtained after 4, 7, and 10 selection cycles as described in the text, and finally the heat response of one of the selected clones (TR-1). ⊡, RIF-1; ◆, 4 selection cycles; ■, 7 selection cycles; ◆, 10 selection cycles; ■, RIF-TR-1.

designated RIF-TR-1, is also shown in Fig. 1. That figure, in addition, depicts survival curves constructed from the cell populations obtained at isolation (Cycles 4, 7, and 10).

Selection of RIF-TR Cells for Characterization

There did not seem to be any need for 20 strains. We proceeded to narrow down that number to 4 or 5 strains for detailed study. Our initial two criteria for cell strain selection were thermoresistance and plating efficiency *in vitro*. Based on these criteria, 8 cell strains were chosen for further characterization. Complete 45°C survival curves were now obtained from these strains as well as growth rates *in vitro*. Our next step was to test for tumor formation *in vivo*. Up to 2×10^5 cells from each of the remaining cell strains were injected into the flanks of syngeneic hosts (C3H mice from the Stanford colony), and the rate of tumor induction and the rate of tumor growth were determined for each cell strain. We also examined the *in vivo* response by immersing RIF-TR tumors in a temperature-controlled water bath, excising the tumors immediately after heating. Single cell suspensions were then obtained, and cells were plated *in vitro* for colony formation. Data for the 8 cell strains are summarized in Table 1. This table also presents, for comparison, results obtained in the wild type (RIF-1). Based on all these results, we selected four strains to be studied in detail. Examination of these cells by light microscopy showed that they had an appearance not noticeably different from the parent line. The clones chosen were RIF-TR-4, 5, 10, and 20. In the remainder of this paper only data on these strains and on the wild type, RIF-1, will be presented.

RESULTS

Heat Responses of the Selected TR Cells

Survival. Figs. 2 and 3 compare the survival of heated RIF-1 and RIF-TR-5 cells in the temperature range of 41–46°C. The major point of interest is the "break" (so called because at that temperature a marked change in an Arrhenius plot is observed) in the survival characteristics. In the RIF-1 cells, this break appears between 42°C and 43°C, consistent with the behavior of almost all the other mouse cell lines described in the literature. In the RIF-TR-5 cells, however, this break has been shifted and now occurs between 4°C and 45°C.

Thermotolerance. We next examined the question of whether the heat-resistant cells can develop thermotolerance, thus becoming temporarily even more heat resistant. When both RIF-1 and RIF-TR-5 cells were given a similar induction dose, *i.e.*, 45°C for 10 min, only the RIF-1 cells developed appreciable thermotolerance. On the other hand, if the two cell lines were given isosurvival doses, 45°C/10 min for RIF-1, 46°C/20 min for RIF-TR-5, both groups of cells developed considerable thermotolerance. The amount of tolerance, if measured as the change in the slope of the survival curve, was similar for the two lines. This is shown in Fig. 4. All the other TR strains examined also were able to develop tolerance.

Stability of the Heat-resistant Phenotype. Cell strains have now been maintained for the full maintenance schedule that was originally developed for RIF-1 by Twentyman *et al.* (5). The schedule is reproduced in Fig. 5. Usually cells at the end of the passage protocol respond to heat in the same way as do cells from the early passages, although occasionally partial reversions occur. These seem to be associated with passage of the cells in the mouse. Results from an experiment in which we observed such a partial reversion are shown in Fig. 6. In these cells the "break" of the survival curves has been shifted to a value between that seen in RIF-TR-5 and RIF-1 and occurs between 43°C and 44°C.

We have also maintained some of the RIF-TR strains *in vitro* without any passages in mice. Under such conditions, we saw no change in heat sensitivities for a period in excess of 50 doubling times of TR-5 and TR-10 cells.

Radiation Response

Survival without Heat. There was no discernible difference between the responses of any of the TR cells and the RIF-1 cell

Table 1 *Characterization of eight cell strains and the wild type (RIF-1)*

	RIF-1	RIF-TR-1	RIF-TR-3	RIF-TR-4	RIF-TR-5	RIF-TR-6	RIF-TR-8	RIF-TR-10	RIF-TR-20
Plating efficiency *in vitro* (%)	79	75	79	79	100	100	72	84	72
Cells surviving 90 min at 45°C *in vitro* (%)	10^{-5}	1	4	76	0.6	2	0.5	0.2	2.8
Doubling time of cells *in vitro* (h)	9.6	14.4	12	14.4	4.8	NM[a]	14.4	16.8	19.2
Plating efficiency *in vivo-in vitro* (%)	48	43	6	57	19	82	6	38	51
Cells surviving 60 min at 45°C *in vivo* (%)	10^{-5}	4	NM	10	32	4[b]	NM	40	25
Tumor doubling time (days)	3	>10	>10	3	2.5	NM	4.5	3	8

[a] NM, not measured.
[b] After exposure to 45°C for 45 min.

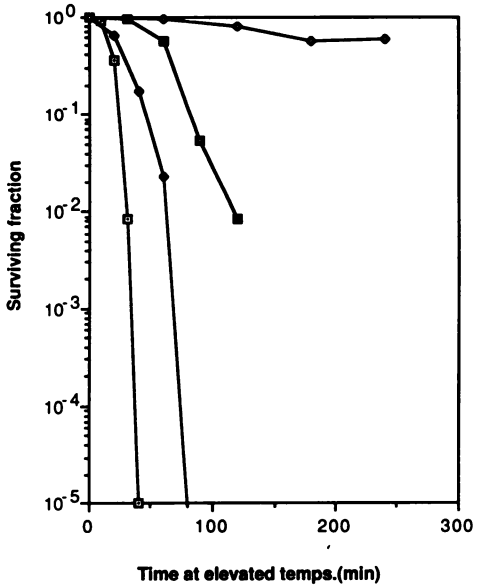

Fig. 2. Survival kinetics of RIF-1 cells at the indicated temperatures. Note the "break" between 42°C and 43°C. ⊡, 45°C; ◆, 44°C; ■, 43°C; ◆, 42°C.

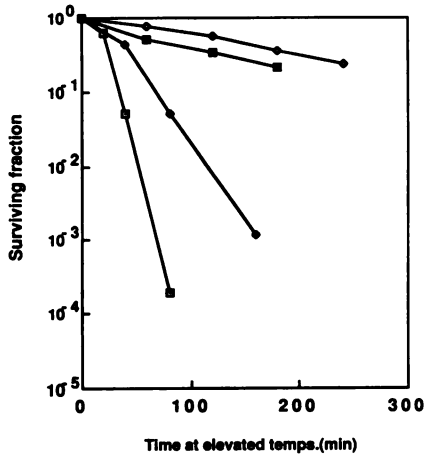

Fig. 3. Survival kinetics of typical RIF-TR strain at the indicated temperatures. Note that, in contrast to the data in Fig. 2, the "break" occurs between 44°C and 45°C. ⊡, 46°C; ◆, 45°C; ■, 44°C; ◆, 43°C.

line to single doses of X-irradiation. This is shown in Fig. 7, where survival data for cells exposed to doses between 0 and 12 Gy are shown.

Heat-induced Sensitization to X-Irradiation. Fig. 7 also shows the sensitization to X-irradiation provided by a heat exposure given just before X-irradiation. We compared the effects on RIF-1 and TR-4 given either an equal heat exposure or an isosurvival exposure. When the two cell lines were exposed to isosurvival doses, the amount of sensitization was far greater for the TR cells than for the parent line. When the two cell lines were exposed to similar heat doses, however, similar sensitization to X-irradiation was seen.

Responses *in Vivo*

Heat Survival. Fig. 8 shows survival curves of TR-5 cells obtained after exposing tumors *in vivo* to 45°C exposures for various lengths of time. Shown on that graph are also RIF-1 cells treated similarly. It is clear that the TR-5 cells maintained heat resistance *in vivo*. Similar data have also been obtained for the other TR cell strains.

Antigenicity. To assess the relative antigenicity and immu-

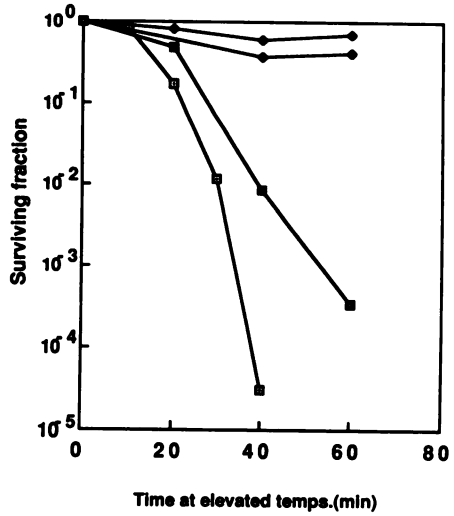

Fig. 4. Comparison of thermotolerance of TR and RIF-1 cells. Cells of both the parent line and strain TR-5 were given isosurvival doses (RIF-1, 10 min/45°C; RIF-TR-5, 20 min/46°C). The response of cells not made tolerant is shown for comparison. Induction of tolerance is obvious for both cell lines. Note also that the slope ratio is not too different for the two cell strains. ⊡, RIF-1, 45°C; ◆, RIF-1, TT; ■, RIF-TR-5, 46°C; ◆, RIF-TR-5, TT.

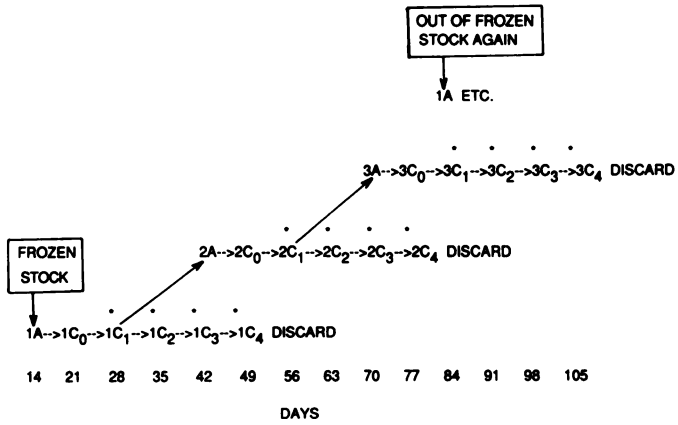

Fig. 5. Protocol for maintenance of RIF-1 and RIF-TR cells. Cells are frozen in large quantities at a fixed passage. Vials are thawed, and the cells are passaged *in vivo* and then 5 times *in vitro*. The C_1 culture is passaged *in vitro* as well as *in vivo*. The passage sequence is repeated no more than 3 times, then the cells are discarded, and a new vial is thawed. This is the procedure of Twentyman *et al.* (8). →, passage in animal; - - -, passage in culture; ★, cells to be used for inoculating C3H mice and *in vitro* experiments.

nogenicity of RIF-TR strains 1, 4, 5, 10, and 20 and the wild line, the TD_{50} was determined in both control and preimmunized mice. Strains 1 and 20 were included because we wanted to see if their slow growth rates *in vivo* reflected antigenicity.

Mice were preimmunized by i.p. inoculation of 10^6 radiation-killed cells (100 Gy) at 20 and 8 days before inoculation of live cells. Immunization was always with cells from the same strain as the cells used to determine the TD_{50}. Live cells were taken from the C_1 culture passage (Fig. 5), and these were administered intradermally in a volume of 0.05 ml of saline. Cell numbers of 10, 10^2, 10^3, 10^4, and 10^5 cells were inoculated. From the number of tumors that developed, the TD_{50}s were estimated. Results are shown in Table 2.

The TD_{50}s for all the TR strains were substantially higher than for the RIF-1. This was true for control mice as well as for the preimmunized mice (Columns 1 and 2). Immunogenicity, however, depends on the ratio of the TD_{50}s (*i.e.*, TD_{50} preimmunized/TD_{50} control). This quantity (Column 3) was quite similar for all the lines examined here. Thus, the TR strains, by this criterion at least, are no more antigenic than the

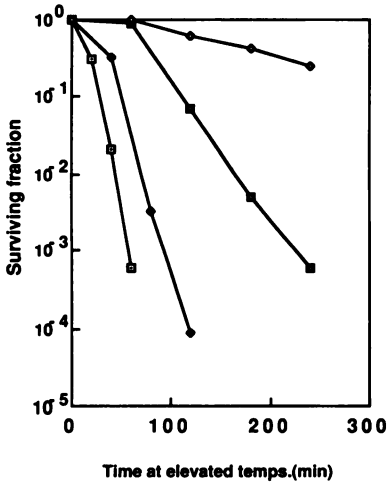

Fig. 6. Survival kinetics of partial revertants. Cells from RIF-TR-5 were passed once in syngeneic hosts, and the cells from resulting tumors were then heated *in vitro*. In these cells the "break" is now at a temperature intermediate to that of the cells whose survival data are shown in Figs. 1 and 3; *i.e.*, between 43°C and 44°C. ⊡, 46°C; ◆, 45°C; ■, 44°C; ◈, 43°C.

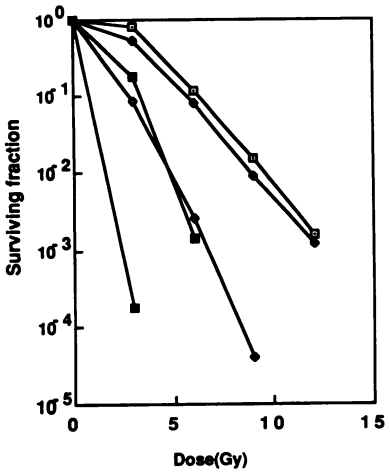

Fig. 7. Radiation responses and thermal sensitization of RIF cells. RIF-1 and RIF-TR-4 cells were given graded doses of X-ray (80 kV, 10 mm aluminum filtration, 1 Gy/min). The survival curves were indistinguishable. The cells were also given isosurvival heat treatments and then irradiated (RIF-TR-4, 30 min/45°C; RIF-1, 10 min/45°C). RIF-TR-4 cells were sensitized appreciably more than RIF-1 cells. When the cells were given identical heat exposures (10 min/45°C) followed by X-irradiation, the responses were again indistinguishable. ⊡, RIF-1; ◆, RIF-TR-4; ■, RIF-1, 10 min, 45°C; ◆, RIF-TR-4, 10 min, 45°C; ■, RIF-TR-4, 30 min, 45°C.

RIF-1 parent line. Furthermore, antigenicity did not explain the slow growth rates of the TR-1 and TR-20 strains. We can offer no explanation as to why the individual TD_{50}s are so much higher for TR cells than they are for RIF-1. Perhaps the TR cells have altered membrane characteristics that impair their ability to form tumors in the hosts.

DISCUSSION

The TR cells that we have isolated clearly show that some cells obtained from the RIF-1 tumor show great heat resistance. This is true in spite of the fact that we did not expose the cells to any mutagens, unless heat itself is a mutagen, a question of some controversy (reviewed in Ref. 9). It is not clear from our results whether these heat-resistant cells were necessarily part of the original cell population. We need to distinguish here between moderately heat-resistant cells, such as those isolated

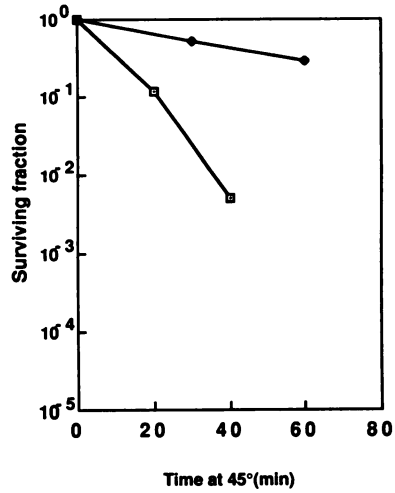

Fig. 8. Heat response of cells *in vivo*. Tumors arising from either RIF-1 cells or RIF-TR-5 cells were heated *in vivo* and then harvested. Single cell suspensions obtained from these tumors were then plated for colony formation *in vitro*. ⊡, RIF-1; ◆, RIF-TR-5.

Table 2 *TD_{50}s of RIF-1 and RIF-TR cells*

Cell	Controls	Preimmunized	Ratios[a]
RIF-1	14	120	7.5
RIF-TR-1	5×10^4	1×10^5	2
RIF-TR-4	3×10^3	1.5×10^4	5
RIF-TR-5	5×10^2	1×10^4	20
RIF-TR-10	5×10^2	1.2×10^3	2.5
RIF-TR-20	1×10^4	1×10^5	10

[a] None of the values is statistically different from either each other or 1 ($P > 0.1$) (except for RIF-TR-5 where $P \cong 0.05$).

at the fourth heating cycle, *versus* the very heat-resistant TR cells. Assuming that each flask at the beginning of the first cycle contained approximately 10^6 cells, and that each heating reduced the population by a conservative 10^2, then after four cycles of heating the total number of cells at risk was $10^6 \times (10^2)^4$, *i.e.*, approximately 10^{14} cells. Since only a few hundred of these cells, at most 10^3, were as heat resistant as the cells from TR lines, this suggests one of two possibilities: either occurrence of extreme heat resistance is a very rare event (frequency, less than 10^{-11}); or the repeated heatings induced a permanent state of resistance in a small fraction of the cells. This latter possibility, particularly in view of the fact that we did not use any mutagens, seems unlikely, suggesting indeed that resistance at the TR level is very rare indeed. On the other hand, moderate heat resistance occurs at a much higher frequency. Since the fourth heating cycle already demonstrated partial heat resistance (Fig. 1), the probability of occurrence of partial heat resistance appears to be on the order of 10^{-8} per cell. For a tumor with 10^{12} cells at the initiation of treatment, this would mean that at that time the tumor contains about 10^4 moderately heat resistant cells.

Our finding that heat resistance can involve a change in the position of the "break" in the survival curves has implications regarding the definition of heat dose. Sapareto and Dewey (10) have suggested that "43°C equivalent minutes" are an appropriate measure of heat dose. They based their attractive concept on measurements of thermal behavior of most mouse cell lines. These show the characteristic break of the Arrhenius plot at 43°C; hence, the term, 43°C equivalent minutes. But for the TR strains, where the break is at 45°C, the 43°C equivalent minutes would not be an appropriate dose. Little is known about the detailed thermal behavior of cells from human tumors. These are known, however, to be appreciably more re-

sistant than equivalent mouse cells. If many human tumors had cells whose thermal responses are similar to those of the TR strains, 43°C equivalent minutes could lead to gross undertreatment of these tumors if the dose is calculated from treatments below 45°C.

Although our data were obtained primarily for initial characterization of the cell strains, nevertheless they already yield useful information on two biological points. (*a*) The results indicate that the induction of thermotolerance and heat-induced cell death may be consequences of similar events. Induction of thermotolerance in both the RIF-1 line and the RIF-TR strains requires thermal exposures that are nearly lethal to the surviving cells. This is in spite of the fact that the thermal exposures so required differ appreciably for the two cells. This suggests that heat death and induction of tolerance involve similar pathways, or that the degree of cell damage determines the level of tolerance induced. This view has been expressed before (reviewed in Ref. 9), but was not tested under the stringent conditions described here.

The second point pertains to the heat-induced radiosensitization. The data of Fig. 7 indicate that heat resistance is not related to radiosensitization. When RIF-1 and RIF-TR cells were exposed to similar heat treatments (not isosurvival treatments) and then irradiated, the amount of heat-induced sensitization was quite similar. While several authors have pointed out that no correlation exists between heat resistance and thermal radiation sensitization, this is the first such demonstration in closely related cells of stable phenotype. This finding obviously implies that the molecular events leading to radiosensitization are not closely linked to those events that lead to thermal death or to thermotolerance.

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