INTRODUCTION

Glioblastoma multiforme is among the most resistant tumors to radiation. This resistance may be due to several different factors, such as a low intrinsic radiation sensitivity, a high recovery capacity, an increased number of clonogens, and a high hypoxic fraction. Previously, we have demonstrated a very wide range of intrinsic radiation sensitivities of cells of glioblastoma multiforme cell lines in vitro after single-dose irradiation. That is, the cells of some glioblastoma multiforme cell lines were quite sensitive, while for others the sensitivity of glioblastoma multiforme was among the lower range of sensitivities reported in the literature. This finding indicates that inherent cellular radiation sensitivity is not the sole determinant of the in vitro response of glioblastoma multiforme tumors. In this report, we evaluate the role of split-dose recovery determined in vitro in relation to the poor clinical outcome of glioblastoma multiforme. Cells of seven early-passage glioblastoma multiforme cell lines and six cell lines derived from tumors of a type frequently treated successfully (two squamous cell carcinomas of head and neck, three breast cancers, and one low-grade astrocytoma cell line) were studied. The in vitro split-dose recovery has been measured using colony formation as an end point. The cells were maintained at 37°C for a period of 6 h between the doses of radiation. Results are presented in terms of a recovery ratio: the ratio of the mean inactivation dose of split-dose radiation to that of single-dose radiation. The data show significantly higher recovery ratios for glioblastoma multiforme than for the other types of histology; however, glioblastoma multiforme showed a wide range of recovery ratios, varying from 1.12 to 2.02. This indicates that cells of some glioblastoma multiforme cell lines exhibit minimal split-dose recovery. No correlation was found between the recovery ratio and the intrinsic radiation sensitivity of the cell lines studied. From these data, we conclude that the recovery capacity may not be the major determinant of the clinical radiation resistance of some glioblastoma multiforme.
was negative for Vimentin. All glioblastoma cell lines were tumorigenic in immunodeficient nude mice. Survival curves were determined for early passage cells (2 to 12). The two squamous cell carcinoma (SCC) cell lines (SQ2OB and JSQ3) were established in the laboratory of Dr. Little (Harvard School of Public Health) and kindly provided by W. Dahlberg, and the three breast cancer cell lines were obtained from the ATCC. All cell lines were studied concurrently in regular single-dose and split-dose survival assays.

The cells were maintained at 37°C in an atmosphere of 5% CO₂ in air, in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum and 1% antibiotics (0.05 mg penicillin/ml, 0.05 mg streptomycin/ml, and 0.1 mg neomycin sulfate/ml).

**Radiation Cell Survival Assays**

Tumor cells in exponential phase were plated on 25-cm² tissue culture flasks in cell numbers appropriate for colony counting. Heavily irradiated feeder cells were added to HGL9, HGL16, SQ2OB, JSQ3, MDA-MB-134, MDA-MB-157, and MDA-MB-231 cells to bring the total number of cells to 40,000 per flask. For the other cell lines (HGL4, HGL5, HGL11, HGL12, and HGL13) feeder cells were not used because preliminary studies showed no gain in plating efficiency or change in radiation sensitivity parameters when feeder cells were added. In all experiments, the flasks were seeded at two or three different cell densities for each of six or seven radiation doses. The cells were irradiated at 18–24 h after plating.

The irradiation was performed using a 250-kVp X-ray machine; the beam was characterized by an HVL of 0.4 mm Cu and a dose rate of 1.54 Gy/min. The cells were maintained at room temperature (20–22°C) under aerobic conditions, with the flasks placed on a rotating Lucite block. For the split-dose experiments, the cells were maintained at 37°C for 6 h between the first and second dose: the period required to observe complete recovery (unpublished data). Split-dose experiments were performed in parallel with single-dose survival experiments. After 2 to 3 weeks of incubation at 37°C in an atmosphere of 5% CO₂ in air, the cells were fixed with methanol and then stained with 1% crystal violet and counted. The number of colonies was determined by counting circular aggregates containing more than 50 cells. Six to eight replicate flasks were plated for each dose level. Two to five survival curves were determined for each cell line. For MDA-MB-134 and MDA-MB-157, only one experiment was performed.

**Statistical Analysis**

The analysis of variance was performed on the pooled data from all experiments for each cell line, using a software package (StatWorks, Cricket Software, Philadelphia, PA). For the calculation of α and β, In SF of all data versus dose was fitted by polynomial regression. For the calculation of D₀ and n, In SF (the linear part of the curve <0.1 SF) versus dose was fitted by simple linear regression. All data points were weighted equally. The mean inactivation dose was calculated from the α and β using the published method of Fertil et al. (6).

The survival curve is conveniently summarized by a global parameter such as D (0–8); we have selected the D to assess split-dose recovery. The recovery capacity is defined by the difference between D calculated from the split-dose survival curve and the D calculated from the single-dose survival curve. This quantifies the amount of repaired damage and can be described in units of radiation dose. The recovery ratio (RR) is defined as the D calculated from the split-dose curve divided by the D calculated from the single-dose curve (7, 8).

3 The cell lines SQ2OB and JSQ3 as well as the breast cancer cell line MDA-MB-231 were tumorigenic in nude mice. AST-2 was not tumorigenic. MDA-MB-134 and MDA-MB-157 were not tested.

**RESULTS**

**Survival Curve Parameters**

Table I shows the SF2 and D̄ computed from the single-dose and split-dose survival curves, as well as the RR and the recovery capacity calculated from the D̄. The RR for cells of the seven glioblastoma multiforme cell lines varied between 1.12 and 2.02, and the recovery capacity between 0.30 and 1.67 Gy.

Figures 1 and 2 show survival curves for single- and split-dose irradiation for HGL9, HGL11, HGL5, and HGL12 cells with RRs of 1.12, 1.20, 1.40, and 1.57, respectively. HGL4 cells showed the highest recovery ratio (2.02) and HGL9 cells showed the lowest (1.12). The three breast cancer cell lines showed a relatively low recovery ratio (1.09 to 1.12), and the low-grade astrocytoma showed almost no recovery (RR, 1.09).

Figure 3 shows the recovery ratio for cells of the seven glioblastoma multiforme cell lines and that of cells of the cell lines derived from the other types of histology (one low-grade astrocytoma, two SCC, and three breast cancer cell lines). The figure shows a wide range of RRs for cells of the glioblastoma multiforme cell lines, but a very narrow range (1.09 to 1.12) for cells of the other cell lines. The RR and recovery capacities of glioblastoma multiforme cell lines were significantly higher than those of these tumors (P < 0.05, two-tailed t test).

**Relationship between Recovery Ratio and Radiation Sensitivity**

Figures 4a and 4b present RR plotted against intrinsic radiation sensitivity expressed as D̄ (a) or SF2 (b) for cells of the 13 cell lines. No correlation was found (r² = 0.036 and 0.287 for D̄ and SF2, respectively).

Figure 5 shows a significant correlation between the D̄ and the SF2 for single-dose and for split-dose radiation (P = 0.0005, r² = 0.69) (P = 0.0008, r² = 0.74). A significant correlation was also found for α (P = 0.0009, r² = 0.675) and for β (P = 0.0016, r² = 0.647) for single-dose and split-dose radiation. On the other hand, no correlation was found between the D₀ of the single-dose radiation and that of the split-dose radiation (P = 0.15, r² = 0.41). This suggests that the intrinsic radiation sensitivity of the cell lines did not change when the cells were given single- or split-dose of radiation. These data also demonstrate that the recovery from SLD might affect the initial slope of the survival curve, but not the slope of the distal part, in contrast to PLD, which affects the proximal and the distal part of the curve (α, β, D̄, and D₀) as was demonstrated by Deschavanne et al. (8).

**DISCUSSION**

We have shown previously (1, 2) that the average intrinsic radiation sensitivity of cells of glioblastoma multiforme...
TABLE I
Radiation Response of Cells of Tumor Cell Lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>SF2</th>
<th>SF2</th>
<th>$\bar{D}$ single dose (Gy)</th>
<th>$\bar{D}$ split dose (Gy)</th>
<th>Recovery ratio$^e$</th>
<th>Repair capacity (Gy)$^f$</th>
</tr>
</thead>
<tbody>
<tr>
<td>HGL4$^a$</td>
<td>0.30</td>
<td>0.60</td>
<td>1.65</td>
<td>3.32</td>
<td>2.02</td>
<td>1.67</td>
</tr>
<tr>
<td>HGL5$^a$</td>
<td>0.60</td>
<td>0.69</td>
<td>2.96</td>
<td>4.14</td>
<td>1.40</td>
<td>1.18</td>
</tr>
<tr>
<td>HGL9$^a$</td>
<td>0.50</td>
<td>0.53</td>
<td>2.56</td>
<td>2.86</td>
<td>1.12</td>
<td>0.30</td>
</tr>
<tr>
<td>HGL11$^a$</td>
<td>0.66</td>
<td>0.72</td>
<td>3.36</td>
<td>4.04</td>
<td>1.20</td>
<td>0.68</td>
</tr>
<tr>
<td>HGL12$^a$</td>
<td>0.73</td>
<td>0.90</td>
<td>3.58</td>
<td>5.63</td>
<td>1.57</td>
<td>2.05</td>
</tr>
<tr>
<td>HGL13$^a$</td>
<td>0.75</td>
<td>0.78</td>
<td>3.77</td>
<td>4.44</td>
<td>1.18</td>
<td>0.67</td>
</tr>
<tr>
<td>HGL16$^a$</td>
<td>0.39</td>
<td>0.54</td>
<td>1.95</td>
<td>2.61</td>
<td>1.34</td>
<td>0.66</td>
</tr>
<tr>
<td>AST-2$^b$</td>
<td>0.31</td>
<td>0.36</td>
<td>1.73</td>
<td>1.88</td>
<td>1.09</td>
<td>0.15</td>
</tr>
<tr>
<td>SQ200$^b$</td>
<td>0.51</td>
<td>0.53</td>
<td>2.49</td>
<td>2.77</td>
<td>1.11</td>
<td>0.28</td>
</tr>
<tr>
<td>JSQ3$^b$</td>
<td>0.68</td>
<td>0.71</td>
<td>3.54</td>
<td>3.91</td>
<td>1.10</td>
<td>0.37</td>
</tr>
<tr>
<td>MDA-MB-231$^d$</td>
<td>0.43</td>
<td>0.49</td>
<td>2.01</td>
<td>2.25</td>
<td>1.12</td>
<td>0.24</td>
</tr>
<tr>
<td>MDA-MB-134$^d$</td>
<td>0.63</td>
<td>0.68</td>
<td>3.05</td>
<td>3.34</td>
<td>1.10</td>
<td>0.29</td>
</tr>
<tr>
<td>MDA-MB-157$^d$</td>
<td>0.47</td>
<td>0.49</td>
<td>2.16</td>
<td>2.36</td>
<td>1.09</td>
<td>0.20</td>
</tr>
</tbody>
</table>

$^a$ Glioblastoma multiforme.
$^b$ Low-grade astrocytoma.
$^c$ Squamous cell carcinoma.
$^d$ Breast cancer.
$^e$ The recovery ratio is defined as the ratio of the $\bar{D}$ of the split-dose curve over that of the single-dose curve.
$^f$ The recovery capacity is defined as the difference between $\bar{D}$ for the split dose curves and that of single-dose radiation curves.

Cell lines in vitro is among the lowest studied in the literature; however, there was a broad distribution of sensitivities with extensive overlap of the radiation sensitivities of cell lines derived from epithelial tumors. The latter are often treated effectively by radiation, in contrast to glioblastoma multiforme, which is almost never eradicated by radiation. We concluded that intrinsic radiation sensitivity may not be the dominant factor in the determination of the poor clinical outcome of radiation treatment of patients with glioblastoma multiforme.

Several reports (9–16) have mentioned that the measurement of recovery from radiation damage might serve as a predictor of the response of tumors to radiation. To the extent that this applied to glioblastoma multiforme, we

![Graphs](image-url)
FIG. 2. Radiation survival curves for single-dose (●) and split-dose (▲) irradiation of cells of glioblastoma multiforme cell lines HGL5 (left panel) and HGL12 (right panel). HGL5 cells showed a recovery ratio of 1:40, and HGL12 cells, 1:57.

would expect that cells of most of the glioblastoma multiforme cell lines express a proficient recovery from radiation damage. This could account for part of the radiation resistance observed in the clinic. In the present study, the average recovery ratio for the cells of the seven glioblastoma multiforme cell lines showed a suggestive trend to be higher than that of cells of the cell lines from other types of tumors often treated successfully; however, they showed a wide range of RRs varying between a very low recovery (HGL9) to a relatively high one (HGL4).

This wide range of RRs suggests that recovery cannot be a major determinant of clinical failure of glioblastoma multiforme. These findings indicate that in some glioblastoma multiforme tumors, factors other than the intrinsic radiation sensitivity (1, 2) and the recovery capacity play an important role in the determination of the poor survival of glioblastoma multiforme patients.

In several studies, Weischelbaum et al. (11–16) measured the repair of PLD in cells of cell lines derived from head and neck tumors. They demonstrated a correlation between the presence of radiation-resistant and repair-proficient tumor cells with clinical failure. They suggested that the presence of repair-proficient cells may be responsible for local failure following radiation therapy. In another study, Guichard et al. (9) found that the amount of PLD repair was higher for cells derived from tumors with a low probability of tumor control by radiation treatment (glioblastoma multiforme, hypernephroma, osteosarcoma, melanoma) than that for cells derived from tumors of high curability (breast carcinoma, neuroblastoma). They stated that the magnitude of this repair, in certain cases, is sufficient to explain the incurability of a tumor by radiation therapy. However, in their experiments, only one radiation dose was used to study PLD repair. They assumed an underlying multitarget model of radiation effect, which predicts that the RR should reach a plateau when the dose used per fraction is beyond the shoulder of the acute survival curve.

Peacock et al. (17) studied the split-dose recovery of cells of several cell lines. They found that cellular recovery was the greatest in cells of the most radiosensitive cell lines, suggesting that increased radiation sensitivity does not result from low recovery capacity. Hall et al. (18) studied the repair of PLD and SLD in cells of six human tumor cell

FIG. 3. The recovery ratio (the ratio between the \(\hat{D}\) calculated from the split-dose curve and the \(\hat{D}\) calculated from the single-dose curve) of all glioblastoma multiforme cell lines and tumors with other types of histology (two squamous cell carcinoma, three breast cancer, and one low-grade astrocytoma cell lines).
lines and three diploid fibroblast lines derived from normal lungs and skin. They found no correlation between the extent of repair and the radiation responsiveness of a particular tumor type. In fact, they found an inverse correlation, with the most resistant tumor cells showing the smallest amount of repair, which is in agreement with Peacock et al. (17).

Deschavanne et al. (8) studied reports on cells of 22 human tumor cell lines collected from the literature. They found no correlation between intrinsic radiation sensitivity and PLD repair, in agreement with Hall et al. (18) and Peacock et al. (17). However, for cells of fibroblast cell lines, such a correlation existed; i.e., the PLD repair was proportional to the intrinsic radiation sensitivity of the fibroblast cell line. In their review, the authors found a significant correlation between the parameters $\alpha$, $\beta$, $\bar{D}$, and $D_0$ for cells plated immediately after irradiation and those for delayed-plating cells. This shows that PLD repair affects the whole curve. However, the SLD repair in our data affected only the proximal part ($\alpha$ and SF2) and the shoulder ($\beta$) and did not affect the slope of the distal part of the curve ($D_0$).

On the other hand, Malaise et al. (7) reviewed data from assays on cells of 27 fibroblasts and 33 tumor cell lines in exponential and plateau phase using immediate and delayed plating. The recovery ratio was calculated using the $\bar{D}$ parameter. The authors found a bell-shaped correlation between the repair capacity and the intrinsic radiation sensitivity with a peak at a $\bar{D}$ of 2.2 Gy. The authors concluded that PLD repair is a reasonable reflection of intrinsic radiation sensitivity up to 2.2 Gy, and above that, the relationship is reversed: the greater the radiation resistance, the lower the PLD repair. However, Malaise et al. (7) analyzed both the fibroblast and the tumor cell lines in one plot. Since the data for most of the fibroblast lines were in the part of the plot indicating radiosensitivity, the correlation between PLD repair and intrinsic radiation sensitivity corresponded to that of the fibroblast lines, which is in agreement with the finding of Deschavanne et al. (8). However, for the tumor cell lines, a reverse correlation was found, and this is in agreement with the other studies (17, 18).

In our study, using the split-dose radiation assay, no correlation was found between the recovery ratio or capac-

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**FIG. 4.** The relationship between the recovery ratio and the intrinsic radiation sensitivity of all cell lines. (a) The recovery ratio (calculated from $\bar{D}$ of the 13 cell lines plotted versus the $\bar{D}$ of each cell line). (b) The recovery ratio calculated from the surviving fraction at 2 Gy (SF2) of the 13 cell lines plotted versus the SF2 of each cell line.

**FIG. 5.** (a) The relationship between the SF2 of the split-dose curve and the SF2 of the single-dose radiation curve. (b) The relationship between the $\bar{D}$ of the split-dose curve and the $\bar{D}$ of the single-dose radiation curve.
ity and the intrinsic radiation sensitivity (Figs. 4a and 4b), which is in agreement with other investigators (7, 8, 17, 18).

In conclusion, our data for cells of glioblastoma multiforme cell lines demonstrate a capacity for split-dose recovery which is higher than that of cells of cell lines derived from tumors often treated successfully. However, not all glioblastoma multiforme cell lines expressed this high recovery ratio, and in fact, they showed a wide range of recovery. The low RR for some glioblastoma multiforme cell lines suggests that the invariably fatal outcome is not exclusively a consequence of a great recovery capability. No correlation was found between the intrinsic radiation sensitivity and the recovery capacity of the cell lines studied. From these data, we conclude that the recovery capacity of glioblastoma multiforme may not be the major determinant of the clinical radiation resistance of some of these tumors, and factors other than the intrinsic radiation sensitivity and the recovery capacity play an important role in the determination of the poor survival of glioblastoma multiforme patients.

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