Growth of Tumor Fragments X-radiated in Vitro Following Pretreatment with Cysteine

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Since the discovery of glutathione (16), many workers have demonstrated its occurrence and the significant role of sulfhydryl groups in growing tissues and organisms (13, 17, 24). More recently, the studies of Hellerman et al. (14), Rapkine (25), and Barron et al. (1, 8) have emphasized the importance of -SH groups in enzyme systems. Barron et al. (1) found that small amounts of x-radiation readily inhibited the action in vitro of enzymes whose activity was dependent upon the presence of -SH groups, and, after low doses of radiation, the enzymatic activity could be restored with sulfhydryl compounds such as glutathione. Ephrati (8) reported that cysteine and other reducing compounds added to culture media lessened the destruction in vitro of x-radiated bacterial toxins. Similarly, it has been shown that sulfhydryl compounds, such as thioglycollic acid and cysteine, as well as other amino acids, added to culture media will protect bacteria from the lethal action of x-rays (10, 15).

Patt et al. (21) and Chapman et al. (6) found that intravenous injection of cysteine and subcutaneous injection of glutathione, administered prior to total-body x-radiation, significantly increased survival of rats and mice. Rats bearing well implanted Walker 256 mammary carcinomas were given intravenous injections of cysteine immediately before exposure to 800 r x-radiation (25). The protection afforded the cysteine-injected rats was concomitant with the partial protection that was observed in the tumors. Protection of hair follicles in a localized area was demonstrated by Forsberg (10) following intracutaneous injection of a cysteine solution into a limited region of the dorsal skin of the guinea pig.

The experiments presented here were undertaken (11) to investigate the influence of cysteine upon the radiosensitivity of tumor fragments irradiated in vitro as determined by their survival and growth after implantation in nonirradiated hosts.

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In some experiments measured dose rates as much as 2 per cent higher or lower were employed. The tumor fragments were x-radiated while they lay in a single layer on the bottom of a culture dish, slightly covered with about 0.25 cc. of the solution with which they had been treated. Subcutaneous, bilateral axillary implants were made soon after irradiation, although occasionally fragments were kept in an icebox up to 1½ hours before implantation. Usually, the cysteine-treated tumors were the last to be implanted, but control implants clearly indicated that the time involved in making the inoculations did not influence the results appreciably. Over 400 NaCl and cystine-treated and 100 cysteine-treated, nonirradiated implants were used as controls. In no case did a nonirradiated control implant fail to grow, nor did any host survive the effects of the control tumors.

RESULTS

The survival and growth of the tumor fragments of the "J" series, after a 15-minute treatment with 0.008 M cysteine or saline solution, and with and without radiation treatment, are summarized in Chart 1. After exposure to 4,000 r, 55 per cent of the cysteine-treated implants survived, but none of the similarly irradiated, untreated implants grew. After exposure to 8,000 r, only 70 per cent of the untreated implants grew, in contrast to 100 per cent of the cysteine-treated ones. All the untreated and cysteine-treated nonirradiated implants survived. This was also true of those irradiated with 1,000 r, but, after 2,000 r x-radiation, one implant out of twenty of both the cysteine- and untreated implants failed to grow.

Since the maximum number of growing tumors was present in the first weeks following implantation, the growth curves during this period have greater significance than those for the later period. When the ratio of tumor mass to that of the host becomes large, the host sickens, the growth of a tumor may be retarded, and is eventually terminated with the death of the host. This results in large fluctuations of the growth curves, especially prominent after the tumors reach an average diameter of approximately 10 mm.

It is apparent from the data of Chart 1 that, in addition to its obvious lethal action on tumor fragments, radiation notably increased the time required for an implant to initiate growth, although after growth was initiated the irradiated and nonirradiated tumor fragments grew at similar rates.

![Chart 2. Effect of x-radiation upon delay in initiation of growth of tumor implants with and without pretreatment with cysteine (semilogarithmic graph).](chart2.png)

The larger doses of radiation increased significantly the time required for an implant to resume growth in a new host. When this delay, or "latent period," was measured as the additional time required for irradiated implants to reach the average diameter of the nonirradiated control implants at one week, it was found to be closely proportional to an exponential function of the radiation dose as shown in Chart 2. It is noteworthy that cysteine pretreatment significantly reduced by the effectiveness of each radiation dose as determined by the duration of the latent period.

Inhibition and delay of cell division are known to follow exposure of cells to moderate doses of radiation and must account to some extent for the increase in time required for an irradiated implant to resume growth. It is also known that implant size is an important factor in determining the apparent latent period; usually the smallest implants show the longest "latency." For example, a slice of tumor was thoroughly triturated in saline solution, and 0.1 ml. of the brei containing an estimated 24,000 mostly isolated cells was inoculated subcutaneously into young mice. Only a few of these implants grew, and the appearance of detectable growth was delayed 7–10 days beyond...
that of the 8-12-mg. control implants. From the data of Chart 1, it is evident that the lethal action of the radiation and, by inference, a concomitant effective reduction of implant size, as measured by the number of potentially viable cells, increased with dosage. Thus, the observed extension of the latent period by irradiation of tumor implants can be related to a temporary retardation of cellular processes associated with cell division and growth and to the death of sufficient cells to cause an effective reduction in implant size.

The data of all the experiments on survival of tumor fragments after irradiation with and without cysteine are summarized in the curves plotted in Chart 3. The curves were fitted from points calculated from probit transformations of the data. The mean survival percentages for each radiation dose fall very close to the calculated curves. At radiation doses of 2,000 r or less, treatment with cysteine had little effect upon the number of “takes.” At doses above 2,000 r, protection against the effects of x-radiation was clearly demonstrated by the significantly greater survival of the cysteine-treated implants as compared to those not treated with cysteine. Of 160 noncysteine-treated implants, none grew after irradiation with 4,000 r, although 32 of 140, or 23 per cent, of the cysteine-treated implants survived the effects of the same amount of radiation. After 3,000 r, 39 per cent and after 5,000 r, 38 per cent more of the cysteine-treated implants survived than did their respective control implants.

Analysis of the tumor-survival curves yields the following statistics: LD50 for controls 2,975 ± 49 r; with cysteine, 3,544 ± 55 r. The difference is 569 r, or about 8 times its standard deviation of ± 74 r, and therefore highly significant. Analysis by Tippett’s method (96) yielded the information that the apparent difference in the slope of the two curves is not significant (0.3 > P > 0.2). It appears then that cysteine treatment of the tumor fragments has an effect on the radiation response which is equivalent to a reduction of the dose by about 18 per cent.

Protection of tumor fragments against radiation injury by cysteine is also clearly demonstrated by the average diameters and weights of the tumors developed from the implants. Examination of the growth curves of Chart 1 reveals that, after x-radiation with 2,000 and 3,000 r, the difference in the mean diameters of the tumors developing from cysteine-treated and control implants at 2 weeks was 1.4 and 5.4 mm. These differences when analyzed by the method given by Fisher (9) were found to have a statistical significance (P values) of < 0.05 and < 0.001, respectively. In Table 1 are listed the average weights of the tumors that developed from irradiated and nonirradiated implants 18–24 days following implantation. After 22–23 days, the cysteine-treated, x-radiated implants (3,000 r) had grown into tumors whose average weight was nearly three times that of the untreated ones irradiated with the same dose. The ratios of the average weights of the cysteine-treated to the noncysteine-treated tumors were consistently higher in both the irradiated and nonirradiated series. The average ratio for the irradiated series was 2.91 and for the nonirradiated series, 1.33.

Without cysteine pretreatment, only 44 per cent of 140 tumor fragments survived 3,000 r radiation and in 22–24 days they grew to an average weight of only 245 mg. With cysteine pretreatment, 85 per cent of 120 implants survived the same radiation and attained an average weight during the same time of 718 mg. There is, then, positive correlation between survival after exposure to radiation and tumor size. Since the slopes of the growth curves in Chart 1 are much alike, the primary factor determining differences in tumor average sizes cannot be attributed to depression of the fundamental growth rate of the tumor. However, there does appear to be a significant relationship between the effect of cysteine-pretreatment and irradiation upon the delay in initiation of growth following implantation, and this delay very significantly affects the size of tumors measured in the early stages of growth.

The larger size of the cysteine-treated, as compared to the noncysteine-treated, nonirradiated...
implants probably was the result of their implantation into more favorable hosts whose average age was about 3 weeks younger and whose average weight was 3 gm. less than the hosts bearing the untreated control implants. Further support for the belief that cysteine treatment alone did not influence the growth of nonirradiated implants is given in Chart 1 and by the absence of a significant difference in the average diameters of tumors that developed from an additional 60 cysteine-treated, and 100 untreated, nonirradiated implants.

Since in the experiments described above, the tumor fragments were irradiated immersed in the solution with which they were pretreated, additional experiments were undertaken to evaluate the importance of the cysteine in the ambient fluid during irradiation. Tumor fragments were prepared and placed in cystine or cysteine solutions, as described previously, for 20 minutes. Immediately before irradiation, the cysteine-treated implants were thoroughly washed for 2 minutes, by vigorous shaking, in two separate washings of 3 ml. of cysteine-free physiological salt solution. The implants were then exposed directly to 3,000 r x-radiation but with only a minimum of fluid (0.85 per cent NaCl) clinging to the tumor fragments. For comparison, two separate series with cystine- and cysteine-treated, nonwashed implants were irradiated, with the results shown in Table 2. These results demonstrate that cysteine in the solution

**TABLE 1**

**THE INFLUENCE OF 5,000 r X-RADIATION UPON THE SURVIVAL AND GROWTH OF MOUSE TUMOR IMPLANTS WITH AND WITHOUT PRETREATMENT WITH CYSTEINE**

<table>
<thead>
<tr>
<th>Without cystine Cysteine-treated</th>
<th>Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Per cent Av. wt.</td>
<td>Per cent Av. wt.</td>
</tr>
<tr>
<td>takes (gm.)</td>
<td>takes (gm.)</td>
</tr>
<tr>
<td>55</td>
<td>0.216 (11)</td>
</tr>
<tr>
<td>50</td>
<td>0.195 (10)</td>
</tr>
<tr>
<td>20</td>
<td>0.379 (4)</td>
</tr>
<tr>
<td>5</td>
<td>0.308 (1)</td>
</tr>
<tr>
<td>45</td>
<td>0.085 (9)</td>
</tr>
<tr>
<td>95</td>
<td>0.494 (19)</td>
</tr>
<tr>
<td>40</td>
<td>0.185 (8)</td>
</tr>
<tr>
<td>Summary</td>
<td>44</td>
</tr>
</tbody>
</table>

Results with nonirradiated but similarly treated control implants:

| 100 | 0.876 (14) | 100 | 1.598 (18) | 1.85 | <0.01 |
| 100 | 1.537 (7) | 1.08 | <0.01 |
| 100 | 0.929 (16) | 1.12 | <0.01 |
| 100 | 0.917 (20) | 1.146 (18) | 1.24 | <0.05 |
| Summary | 100 | 0.904 (57) | 100 | 1.279 (30) | 1.35 | <0.001 |

*The statistical significance of the difference in per cent survival of the cysteine- and noncysteine-treated control tumor fragments for individual experiments and the summaries in this table and elsewhere are indicated by the "P" values which were determined graphically by the use of Biomial Probability Paper (Mosteller and Tukey [19]).

† Per cent of 80 tumor implants that grew to a diameter of 8 mm. or more in 18–16 days (irradiated implants), and in 15–14 days (nonirradiated implants).

‡ Average weight is followed by number of tumors weighed.

§ Ratio of average weight of tumors from cysteine-treated to average weight of tumors from implants not treated with cysteine.

‖ Probability of difference in per cent "takes."

¶ Probability of difference in average weights (Fisher [9]) of tumors developed from implants which were treated with and without cysteine.

**TABLE 2**

**THE FAILURE OF CYSTEINE IN THE AMBIENT MEDIUM TO AFFECT THE RADIOSENSITIVITY OF TUMOR FRAGMENTS EXPOSED TO 3,000 r X-RADIATION**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Irradiated in:</th>
<th>Exp. No. implants</th>
<th>No.</th>
<th>Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 min. in 0.85 per cent NaCl plus 0.008 M cysteine then washed in 0.85 per cent NaCl</td>
<td>0.85 per cent NaCl</td>
<td>Qk</td>
<td>20</td>
<td>18</td>
</tr>
<tr>
<td>Summary</td>
<td></td>
<td></td>
<td>40</td>
<td>33</td>
</tr>
<tr>
<td>0.008 M cysteine</td>
<td>0.008 M cysteine</td>
<td>Qk</td>
<td>20</td>
<td>18</td>
</tr>
<tr>
<td>Summary</td>
<td></td>
<td></td>
<td>40</td>
<td>31</td>
</tr>
<tr>
<td>Cystine (sat.)</td>
<td>Cystine (sat.)</td>
<td>Qk</td>
<td>20</td>
<td>9</td>
</tr>
<tr>
<td>Summary</td>
<td></td>
<td></td>
<td>40</td>
<td>19</td>
</tr>
</tbody>
</table>
bathing the tumor fragments was not essential during the period of irradiation for protection of the implants from the effects of ionizing radiation; for the twice-washed, cysteine-treated tumor fragments survived exposure to 3,000 r in even slightly greater numbers than those irradiated in the cysteine solution (85/40 as compared to 81/40). Of the 40 implants treated with and x-radiated in cysteine, only nineteen survived exposure to 3,000 r. The results of these experiments clearly indicate that cysteine in the ambient medium had no active part in protection of the tumor fragments other than to supply the cells with additional sulfhydryl compound.

These experiments lead to a brief discussion of the mechanism of the protective action of -SH compounds and of the relative effectiveness of intracellular, as opposed to extracellular cysteine or related sulfhydryl compounds, in protecting cells from ionizing radiations. The available evidence supports the view that cysteine, glutathione, BAL, and related sulfhydryl compounds readily enter cells both in vivo and in vitro. For example, Braunstein and co-workers (4) have demonstrated the rapid entry of cysteine from the culture medium into intact tissue slices in vitro, by determining its synthesis into glutathione. In addition, Barron et al. (2) and Voegtlín et al. (28) have presented other evidence which can be interpreted to signify that BAL and glutathione (and presumably cysteine) readily diffuse into tissue slices in vitro.

The action of cysteine in protecting cells from ionizing radiations must be a chemical one, perhaps by the formation of protective combinations with essential enzymes or genes, or by the induction of other more general cellular effects (Fatt et al. [20]). However, the evidence of the work in vitro with enzyme inhibitors and the well known facility of sulfhydryl compounds to react rapidly with oxidizing agents suggest that the protective action of cysteine and related sulfhydryl compounds to a considerable degree may result from the direct and immediate neutralization of peroxides and oxidizing radicals formed from ionized water molecules (Weiss [29]). It is possible that some protection may also result from the immediate and rapid reactivation of inactivated enzymes by excess -SH groups present at the time that critical intracellular enzymes are oxidized. The possibility exists also that compounds with free -SH groups may partially protect biological systems from ionizing radiations by interfering indirectly with the formation of peroxides and derived oxidizing hydroperoxyl radicals, HO₂⁻, perhaps by reducing the concentration of dissolved oxygen available for formation of these active entities, which Burton (5) considers to be responsible for much of the effect of ionizing radiation upon biological systems.

Since the radius of diffusion of the free radicals produced by x-rays in an aqueous medium is very restricted in relation to the gross dimensions of a cell (Lea [18]) and since free radicals are very short-lived, any theory of protection by direct and immediate neutralization of oxidizing radicals requires that the compounds supplying the active -SH groups be present within the immediate region where oxidizing radicals and injurious molecular changes occur. The results of several investigations of the relative radiosensitivity of specific regions of the cell, as determined by survival and other criteria, fully support the view that the nucleus and cytoplasm are very much more sensitive and more seriously damaged by exposure to minimum effective doses of radiation than is the cell membrane (Zirkle [30], Petrová [22], and Durьеe [7]). Protection of the cytoplasm and nucleus from ionizing radiations must be then of maximum importance to the viability of a cell. These considerations lead to the suggestion that chemical protection of cells from ionizing radiations afforded by cysteine and other sulfhydryl compounds is primarily the result of their intracellular action.

SUMMARY AND CONCLUSIONS

The data presented in the survival and growth of mouse tumor fragments following x-radiation in vitro with and without prior treatment with 0.008 M cysteine clearly demonstrate the prevention and alleviation of radiation injury of mammalian tumor cells by cysteine. The radiation dose had to be increased by about 18 per cent to effect equivalent injury of cysteine-treated implants. Delay in initiation of growth by tumor fragments following implantation was increased by radiation effects, and the increase in latency was found to be determined by an exponential function of the radiation dose. Cysteine in the ambient medium was not found to be an effective agent in reducing the radio-sensitivity of tumor fragments. The results indicate that the prevention and alleviation of the effects of ionizing radiations upon cells by pretreatment with cysteine and related compounds is due to the intracellular action of their sulfhydryl groups.

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REFERENCES


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