Effect of Cyclophosphamide or X-Rays on Spontaneously Occurring Metastases from Tumors Transplanted into the Tails of Mice

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ABSTRACT

A spontaneous metastases model in mice is being used to test the efficiency of various treatments in eliminating metastases. Solid tumors were transplanted into the tails of mice and removed by tail transection when they had grown to a 4- to 5- or 6- to 7-mm mean diameter. Subsequently, 70 to 95% of mice not given other treatment developed metastases in the lungs or in regional lymph nodes (lumbar sacral region), or in both sites. The present paper reports the effects of whole-body or partial-body treatment on these metastases. The treatments, which started at the time of surgical transection of the tail, included a range of single or fractionated doses of cyclophosphamide (CTX) or X-rays given either to the whole body or locally to the lungs only. CTX reduced the incidence of metastases in both sites although the incidence of lung metastases was reduced by smaller doses of CTX than that of the lumbar sacral metastases. Whole-body irradiation of 6 grays (600 rads) had no effect on the incidence of metastases, whereas local irradiation of the lungs with single doses of 14.5 or 20 grays reduced the number substantially, as did 95 mg or more of CTX per kg. Thus, CTX or radiation reduced the incidence of lung metastases in a system where metastases developed from cells seeded from a primary tumor rather than from a cell suspension injected into the tail vein.

INTRODUCTION

Relatively few studies have been published on the treatment response of metastases occurring naturally from spontaneous or transplanted tumors in animals (6, 8, 11). Most studies have used the technique of injecting malignant cells into the tail vein of mice so as to produce "artificial metastases" in the lungs. Recent work throws doubt on the similarity of the chemotherapeutic response of such artificial metastases and those metastases which seed out naturally from transplanted tumors in mice (2, 13, 17). While chemotherapeutic drugs have been reported to increase the incidence of artificial metastases in the lungs of mice (4, 6, 16, 22), the same drugs also have been reported to kill cells in vivo (10, 11, 15, 20, 21). The promised efficacy of chemotherapeutic agents in eliminating metastases is well known (14), but the animal models contradict this as often as they support it. Thus, there seems to be a place for other animal models of metastases to investigate this apparent contradiction.

This paper presents results obtained during the development of an animal system intended to investigate metastases which seed out spontaneously from tumors transplanted into syngeneic mice. The tumor was selected because it is immunogenic in these mice and because many other investigations have been carried out using this tumor in the same laboratory. Tumors were transplanted into the tails of mice and allowed to metastasize naturally from the primary site. The metastases were found almost exclusively in lumbar sacral lymph nodes and in the lungs. The tails were removed surgically after the tumors had reached a size which allowed for metastases to occur up to 60 days after surgery in most of the mice which received no further treatment.

Results are given of investigations into the effectiveness of CTX (Ward Blenkinsop Pharmaceuticals, Ltd., Bracknell, Berks, United Kingdom) or X-rays in eliminating these spontaneously seeded metastases. This animal system may be considered for use in testing other antimetastatic agents.

MATERIALS AND METHODS

The original differentiated, but now anaplastic, sarcoma F was used. It arose spontaneously in the CBA mouse and was first transplanted by Dr. H. B. Hewitt (12). The sarcoma F tumor was maintained routinely by serial passage s.c. in the flanks of CBA mice by Dr. S. A. Hill. Tumors were transplanted on the tail from these s.c. maintenance tumors.

Five tumor transplants were performed, 2 in 1977 and 3 in 1979. The transplant generation numbers are not known but lie between 180 (in 1977) and 340 (in 1979). A total of 634 mice was used in the experiments reported here.

Small pieces (<1 cu mm) were implanted by trocar about half-way along the top of the tail of male mice aged 10 to 12 weeks. The mice were inspected 3 times weekly until the tumor was palpable in the tail, usually 10 to 30 days after transplant. Three perpendicular diameters were then measured with calipers, either daily or 3 times a week, until the tumors reached an average diameter of either 4 to 5 or 6 to 7 mm, usually 7 to 20 days after becoming palpable. There was a poor correlation, if any, between latent time and growth rate of the tumors in the tails. The selection of the size at which the primary tumor was resected was based on data about the incidence rates of metastases from pilot experiments described in "Results." A metastatic incidence of 70 to 95% after surgical removal of the primary tumor was obtained.

The thickness of the tail was subtracted from one transverse diameter, and the geometric mean was calculated. The tails

Received March 10, 1980; accepted February 4, 1981.

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6 The abbreviations used are: CTX, cyclophosphamide; MTD, maximum tolerated dose.
were cut off, under penthrane anesthesia, 1 cm proximally to the proximal edge of the tumor. The mice were then treated with CTX in one of several schedules, given 6 grays whole-body dose of X-rays (240 kV; half-value-layer, 1.3 mm Cu) or larger doses of 14.5 or 20 grays to the thorax only, or kept as untreated controls. In the first 2 transplants, the first dose or single dose of CTX was given 24 hr before surgical removal, and in the last 3 transplants, it was given a few min after surgical removal.

CTX (Endoxana) obtained from Ward Blenkinsop Pharmaceuticals, Ltd., was dissolved in sterile water. Batches were made up every 5 days and stored at 4°C. Injections were given i.p. in volumes of 0.01 ml/g body weight. The doses were chosen as stated proportions of a nominal MTD assumed to be 250 mg/kg. Table 1 lists the treatment schedules used and the number of mice in each group.

The mice were caged 1 to 5/cage and observed for 120 days (in Experiments 1 and 2) or 60 days after surgical removal of the tumor, or until metastases were clinically evident sooner in either the lumbar sacral region or in the lungs. A 60-day period was chosen because, among several hundred mice in these and pilot experiments, no lumbar sacral metastases were seen in any group at more than 40 days after surgery, and only one mouse was found to have lung metastases when sacrificed as late as 62 days. The mice were sacrificed when they looked sick (i.e., judged by ruffled coat, lethargy), breathed fast and/or irregularly, or became moribund, so as to minimize suffering. No mice died of other diseases during the experiments. At postmortem, the lungs were inflated by intratracheal injection of Bouin’s fixative, then removed, and stored in Bouin’s. Later, all the lungs were inspected for the presence of metastases with the unaided eye or with a x 10 dissecting microscope, and they were scored as positive or negative. In addition, the approximate number and size distribution of metastases in the lung were recorded.

No sickness or death due to bone marrow or gut damage was apparent after CTX treatment. However, both deformity of the incisors and clinical signs of respiratory distress were observed 30 to 40 days after the highest CTX dose used (200 mg/kg). These effects of CTX have been reported by others (1, 5, 9). The mice were unable to eat the standard pellet diet because of the deformed teeth; this was overcome by making the pellet into a soft gruel. Respiratory distress was first observed as an increase in the breathing rate of the mice (19), and later histological examination showed a sclerosing alveolitis.

RESULTS

In the control groups (i.e., tail excision to remove the primary tumor at a size of 6 to 7 mm mean diameter but no other treatment administered), the proportions of mice in which metastases were later found were 78%, 83%, 95%, and 92%, respectively, in the first + second, third, fourth, and fifth transplants. In the first experiment, some of the tails were removed when the tumors were 4 to 5 mm in diameter. The incidence of metastases in this control group was 67%. In pilot experiments, we found that removal of the tumor at 3 mm in diameter or smaller resulted in fewer mice with metastases but that, above 4.5 mm, the proportion of metastases did not increase significantly with size of the primary tumor as shown in Table 2 (the mice recorded in Table 2 are not included in Table 1).

Lumbar sacral lymph node metastases were found from 3 to 40 days after surgical removal of the tail although most occurred within 10 days. Lymph nodes as the metastatic site were confirmed by histological examination. Lung metastases were found in mice sacrificed at times ranging from 3 to 70 days after surgery, mostly within 60 days. Referenced to the time after transplantation of the primary tumor, these times corresponded to 10 to 50 days after transplantation for lumbar sacral metastases and 20 to 100 days for lung metastases.

The metastases in both sites had volume doubling times of about 1 day, although this was measured at a 2- to 4-mm size in the lungs but at a larger size in the lumbar sacral lymph nodes. The doubling times in lungs were determined in a separate experiment in which a suspension of sarcoma F tumor cells was injected into the tail vein. Groups of mice were sacrificed from 5 to 18 days after injection, and the numbers and sizes of the lung colonies were recorded.

The proportions of control mice in the present experiments with lumbar sacral metastases only, with lung metastases only, or with both are listed in Table 3. There are no significant

<table>
<thead>
<tr>
<th>Experiment</th>
<th>No. of mice analyzed</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 and 2</td>
<td>79</td>
<td>Controls, tail excision and no CTX</td>
</tr>
<tr>
<td>38</td>
<td>% CTX, single injection</td>
<td></td>
</tr>
<tr>
<td>43</td>
<td>% CTX, single injection</td>
<td></td>
</tr>
<tr>
<td>39</td>
<td>4 x ¼ CTX, weekly intervals</td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>2 x % CTX, weekly intervals</td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>2 x % CTX, weekly intervals</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>Controls, tail excision and no CTX</td>
</tr>
<tr>
<td>16</td>
<td>% CTX, single injection</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>% CTX, single injection</td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>Whole-body X-rays, 6 grays</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>76</td>
<td>0.15, 0.2, 0.25, 0.3, 0.35</td>
</tr>
<tr>
<td>12–16/group</td>
<td>CTX, single injection</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>Controls, tail excision and no CTX</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Controls, no excision and no CTX</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>12</td>
<td>Controls</td>
</tr>
<tr>
<td>21</td>
<td>14.5 grays X-rays to whole thorax</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>20 grays X-rays to whole thorax</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>529</td>
<td></td>
</tr>
</tbody>
</table>

a All CTX doses are stated as a proportion of a nominal MTD assumed to be 250 mg/kg.
b The first injection was given 1 day before surgical removal of the primary tumor.

c Table 2

<table>
<thead>
<tr>
<th>Size (± 1 mm)</th>
<th>No. of mice</th>
<th>Mice with metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palpable</td>
<td>20</td>
<td>Sacral% Lung% None</td>
</tr>
<tr>
<td>3 mm</td>
<td>27</td>
<td>15 (50) 6 (22) 10 (37)</td>
</tr>
<tr>
<td>5 mm</td>
<td>24</td>
<td>25 (74) 17 (55) 4 (11)</td>
</tr>
<tr>
<td>6.5 mm</td>
<td>24</td>
<td>17 (71) 7 (29) 2 (8)</td>
</tr>
</tbody>
</table>

a Whether or not metastases were also present in the other site.
b Numbers in parentheses, percentage.
c No significant differences between 3- and 6.5-mm results.
differences among the 4 experiments. The largest suspected difference was for lung metastases between Transplants 3 and 4, but $\chi^2$ is only 3.4 ($p = 0.06$). The time distributions of metastases occurring in both sites were recorded. There were no significant changes in the time distributions of the metastases in the treated mice. Metastases in lungs occurred at an average of 10 days later than in the lumbar sacral region. A few mice were found to have metastases in sites other than the lung or the lumbar sacral region, 11 in nodes in the mediastinum but none in neck or axillary nodes. Tail tumors recurred locally in only 4 mice, all from the first 2 transplants.

Chart 1 summarizes the results of the 4 experiments using single doses of CTX. There are no significant differences among the experiments. The upper curve shows the proportion of treated mice with metastases in any site (i.e., lung and/or lumbar sacral). The lower curve shows the proportion of mice with metastases in lung, whether or not these mice also had metastases in the lumbar sacral region. CTX obviously is able to eliminate metastases from both sites when given at a dosage $>150$ mg/kg. It is evident that lung metastases were eliminated by lower doses of CTX than metastases in the lumbar sacral region.

It was observed that, in CTX-treated mice with metastases, the number of nodules in lungs was only one or 2, usually of 1 or 2 mm in diameter, instead of the larger numbers and wider spread of sizes of metastases, including some as large as 5 mm, in untreated controls. CTX was so efficient at eliminating metastases that there were few mice with lung metastases, even at low doses of CTX. Therefore, no detailed analysis of the number and size of lung metastases would yield further information.

Abnormal lungs were found in mice given single doses of 62.5 mg CTX per kg and higher when the mice were sacrificed at 60 days after CTX treatment (Table 4). This effect has been reported before (5, 9). This abnormality was not looked for in the mice given multiple doses of CTX.

Chart 2 shows the results of the multiple-dose CTX schedules used in the first 2 transplants. The effect of 2 or 4 fractions was always less than that of a single fraction of the same size (Table 5). The reduced effectiveness of the fractionated doses could result from the rapid proliferation (doubling time, about 1 day) of any metastatic cells which survived the first fraction.

The proportion of mice with metastases in either the lung or lumbar sacral lymph nodes was not significantly altered after 6 grays of whole-body irradiation. In the lungs, the incidence after whole-body irradiation was 28 of 33 (32%), compared with 25 of 30 (36%) in the control group.

Table 3
Metastases in various sites in control groups

Sites of metastases seeded spontaneously from a tumor transplanted onto the tail of mice. Tumors were removed by tail transection when they had reached 6- to 7-mm mean diameter in size, except for 61 of 79 in Experiments 1 and 2 which were removed at 4 to 5 mm.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Proportion of control mice with metastases</th>
<th>Mice with metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sacral region only</td>
<td>Lungs only</td>
</tr>
<tr>
<td>1 and 2</td>
<td>55/70 (44)</td>
<td>24/55 (22)</td>
</tr>
<tr>
<td>3</td>
<td>25/30</td>
<td>7/25 (28)</td>
</tr>
<tr>
<td>4</td>
<td>17/18</td>
<td>5/17 (29)</td>
</tr>
<tr>
<td>5</td>
<td>12/12</td>
<td>2/12 (17)</td>
</tr>
</tbody>
</table>

* Mice with primary tumors resected and no other treatment.
* Numbers in parentheses, percentage.

Chart 2. Total dose of CTX. The effect of fractionated doses of CTX on metastatic incidence in any site or in only the lungs of mice. The total dose given was kept constant at 187.5 mg/kg (A) or 250 mg/kg (B). Numbers in parentheses, number of mice with tail tumors resected in each group; Bars, S.D.

In the mice given local irradiation to the whole thorax, lung metastases were found in only 3 of 21 mice given 14.5 grays and in 3 of 19 given 20 grays, compared with 9 of 12 in the control group. These proportions are similar to those obtained by single doses of about 90 mg of CTX per kg (Chart 1). As in the CTX-treated mice, there were fewer (less than 4) and

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*Proportion of mice with metastases in various sites.

**S.D.** Standard deviation.

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Table 4
Incidences of grossly abnormal lungs (excluding metastases) in mice at autopsy 60 days after single doses of CTX. None of these mice was irradiated.

<table>
<thead>
<tr>
<th>CTX dose (mg/kg)</th>
<th>Total no. of mice</th>
<th>No. of mice with abnormal lungs</th>
<th>% of mice with abnormal lungs</th>
</tr>
</thead>
<tbody>
<tr>
<td>37.5</td>
<td>16</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>50</td>
<td>12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>62.5</td>
<td>12</td>
<td>3</td>
<td>25</td>
</tr>
<tr>
<td>75</td>
<td>13</td>
<td>5</td>
<td>39</td>
</tr>
<tr>
<td>87.5</td>
<td>11</td>
<td>2</td>
<td>27</td>
</tr>
<tr>
<td>187.5</td>
<td>31</td>
<td>24</td>
<td>77</td>
</tr>
</tbody>
</table>

---

**S.D.** Standard deviation.

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**Chart 1.** Proportion of mice with metastases (MET5) versus the single i.p. dose of CTX. The 3 symbols represent 3 different experiments (EXPTS). Closed symbols, metastases in sacral and/or lung sites; open symbols, metastases in lungs. & CTX dose (mg/kg) mice abnormal lungs abnormal lungs

Effect of CTX on lungs of mice
smaller (1 to 2 mm) metastases in the irradiated lungs than in the lungs of untreated control mice where a wide variation in number (5 to 32) and size (1 to 6 mm) of metastases was found.

DISCUSSION

CTX has been shown to kill cells in solid animal tumors in vivo (6, 15, 21). However, it has been reported that CTX increases the number of metastases from a primary tumor in some circumstances, especially if the tumor is immunogenic (16). In addition, CTX given shortly before injection of tumor cells into the tail vein of mice is a common way of enhancing the plating efficiency of “metastatic” colonies in the lung (4, 6, 16, 22).

The system used in the present studies involved spontaneous metastases from tumors transplanted into the tail, which were removed by transection of the tail when the primary tumors exceeded 6 mm in mean diameter (4 mm in a few early groups). The metastases occurred mainly in lumbar sacral lymph nodes or in the lungs.

The results in this spontaneous metastases system clearly show that the net effect of CTX was a reduction in the proportion of mice in which metastases grew, regardless of whether the drug was given 24 hr before removal of the primary (Experiments 1 and 2) or immediately after removal of the primary (Experiments 3 and 4). Neither was any increase in extrapulmonary metastases observed. The reduction of metastatic incidence by CTX in the present system, as opposed to an enhancement in artificial metastases systems, was not due to drug dose. Drug doses used in the present system were similar to those used in artificial metastases systems.

Using a mammary tumor transplanted s.c., Heppner et al. (11) have reported that CTX, methotrexate, and 5-fluorouracil all enhance the spontaneous production of metastases from the primary tumor. Although the results of Heppner et al. may appear contradictory to those presented here, the many differences between the 2 systems render the results not comparable, i.e., tumor size at time of treatment, removal versus nonremoval of the primary tumor, drug doses, type of mouse, and type of tumor.

Irradiation of only the whole lung with doses similar to those which have been shown to enhance lung metastases in artificial metastases systems (3, 23) also clearly reduced the incidence of lung metastases in the present spontaneous metastases model. Thus, 2 agents commonly used to enhance the plating efficiency of artificially produced lung metastases reduced the incidence of lung nodules in this spontaneous metastasizing system.

This difference, reduction versus enhancement of lung metastases by similar treatments, suggests that there are important biological differences between a system where metastases are allowed to seed out naturally from a transplanted tumor and a system where a bolus of malignant cells is injected rapidly into the tail vein. Perhaps, these differences are related to cell selection in the tail vein technique or to the large numbers of cells entering the bloodstream in a short time as compared to the relatively small numbers seeding out per unit time from a primary tumor. Although, in the present study, systemic irradiation with 8 grays had no effect on the incidence of spontaneous metastases in either site, similar whole-body doses of radiation have been shown to increase the incidence of lung nodules in artificial metastases systems, (3, 23) further suggesting that biological dissimilarities exist between these 2 metastases models.

The elimination of lung metastases by lower doses of CTX rather than metastases in the lumbar region suggests that the colonies of metastatic cells in the lung were smaller when the CTX was given than were the colonies in the lumbar sacral region. The somewhat later time of appearance of lung metastases by an average of about 10 days (although the ranges of time overlap with those of lumbar sacral metastases) is also consistent with this result. Using our CTX dose-response curve (Chart 1) and data on the sensitivity of tumor cells to CTX reported by others, we are able to estimate the possible difference in numbers of cells in the microcolonies in lung and in the lumbar sacral region at the time of treatment. A single dose of 20 and 70 mg of CTX per kg halved the incidence of metastases in the lung and lumbar region, respectively (Chart 1). In vivo, tumor cell kill has been reported to differ by a factor of 3 to 15 as the CTX dose was increased from 20 to 70 mg/kg (6, 14).

For EMT6 cells in vitro, the difference in cell kill was a factor of 20 (10, 21). Thus, it is probable that the ratio of cell numbers in the 2 sites here is in the range of 3 to 20, the smaller metastatic colonies being, of course, in the lung. However, cell kill after CTX has been shown to be greater by 1 log in a small tumor than in a large tumor (6, 15, 20). Thus, the size ratio between metastases in the lumbar region and lung in the present study might be as large as a factor of 100.

At doses which approximated equal whole-body toxicity, the 2 systemic treatments (i.e., CTX or whole-body radiation) did not produce equal effects on metastatic incidence. A single whole-body dose of 6 grays, which is approximately three-fourths of the dose lethal to 50% of animals in 30 days and approaches the radiation MTD for CBA mice (7), was completely ineffective in reducing metastases in either site, whereas three-fourths the MTD of CTX almost completely eliminated metastases in both sites. Thus, at approximately equitoxic whole-body doses, CTX was more effective than was radiation in eliminating metastases in this system.

Radiation therapy, however, most often is given to a localized area of the body. Comparing three-fourths MTD of CTX with a single dose of 14.5 grays to the whole thorax only, both were equally effective in eliminating lung metastases. However, at these dose levels, both treatments have an associated lung toxicity; 77% of the mice given this dose of CTX had abnormal lungs (Table 4), and 50% of the mice given 14.5 grays to the whole lung would have died at 80 to 196 days after radiation (18). Thus, the therapeutic ratio (the ratio of doses of a given treatment which gives a high probability of tumor control with

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**Table 5**

Proportion of mice with metastases in any site after a single dose, 2 doses, or 4 doses of CTX

<table>
<thead>
<tr>
<th>Total CTX dose</th>
<th>Proportion of MTD</th>
<th>Single</th>
<th>2 equal doses</th>
<th>4 equal doses</th>
<th>8 equal doses % Proportion</th>
<th>8 equal doses % Proportion</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>75</td>
<td>%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>62</td>
<td>1/4</td>
<td>50</td>
<td>%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>94</td>
<td>3/8</td>
<td>25</td>
<td>12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>187</td>
<td>3/4</td>
<td>2</td>
<td></td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>250</td>
<td>1 MTD</td>
<td>0</td>
<td>0/34</td>
<td>30</td>
<td></td>
<td>14/39</td>
</tr>
</tbody>
</table>
little normal tissue morbidity) with both of these treatment regimens was less than optimal. Clearly, studies after sublethal doses of radiation to the lung are necessary to determine which treatment results in the best therapeutic ratio. Higher single doses of CTX cannot be tested as they then approach the bone marrow toxicity of the drug. When fractioned doses of CTX were given at 7-day intervals, they were less effective at eliminating metastases than was the same dose given as a single treatment.

The remarkable feature of the present animal metastases system is that either CTX or radiation unequivocally reduces the incidence of metastases instead of increasing it in lungs as has been reported previously (4, 6, 16, 22). The reason for the difference is not known, but it might be due to a smaller number of malignant cells released per unit time from the tumors transplanted into the tail, as here, than from other sites or, of course, from the injection of a bolus of cells into the tail vein.

It is hoped that this experimental metastases system will help to elucidate mechanisms of metastasis and possibly to compare different treatment methods.

ACKNOWLEDGMENTS

We are grateful to Dr. Juliana Denekamp for first suggesting the system. Dr. Adrian Begg commented constructively on the manuscript. We thank Lynda Hall and her colleagues for the breeding and care of the mice, Charles Parkinson for technical assistance, Professor K. H. Kärcher for enabling Dr. G. Reintz to participate, Dr. Richard Maughan for dosimetry, and the late Reg Ransley for technical assistance, Professor K. H. Kärcher for enabling Dr. G. Reintz to participate, Dr. Richard Maughan for dosimetry, and the late Reg Ransley for providing the jigs. Ward Blenkinsop Pharmaceuticals, Ltd., kindly contributed the CTX.

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