Effect of X-Rays and Cyclophosphamide on Solid Tumors and Naturally Occurring Metastases in Mice

Ann M. Chu and Jack F. Fowler

Abstract

Combinations of single doses of X-rays given locally to a transplanted tumor and single i.p. doses of cyclophosphamide (CY) were tested in mice bearing solid transplanted fibrosarcomas. The end points examined were growth delay and local control of the implanted tumor, the incidence of distant metastases, and survival times free of either local recurrence or metastases.

Growth delay and local control increased more steeply with X-ray dose than with dose of CY. From 8 to 10 days of extra regrowth delay required only 10 to 20% extra X-ray dose, but it required a doubling of CY dose from three-eighths to three-fourths of the maximum tolerated dose.

The incidence of metastases and the survival time of the mice also depended more upon the local dose of X-rays than that of CY. This result was not expected and suggests that metastases are eliminated more certainly if the primary (implanted) tumor is locally controlled on a long-term basis.

A significant proportion of mice, over 50%, was cured of both the transplanted tumor and distant metastases only when the highest doses of both X-rays and CY were given simultaneously. Extended intervals between the two agents gave inferior results, intervals of 8 days giving significantly worse results, but 4 days giving not significantly worse results than simultaneous administration, especially when the X-ray treatment was given first. The interval of 4 days would, however, be sufficient to avoid the enhancement of radiation injury in normal tissues in mice.

Introduction

It is axiomatic that local irradiation may provide a reasonable probability of local control of the primary cancer in certain sites of the body but that the treatment of distant and occult metastases requires a systemic method such as chemotherapy (3, 5). A commonly used strategy is to use CY systemically in "spatial cooperation" with radiation, which is aimed at the primary tumor (14, 15, 19).

CY is a bifunctional alkylating agent which is activated by metabolism in vivo. It has been used clinically for many years and shows activity against such human cancers as Hodgkin's disease and acute lymphocytic and myelocytic leukemias, as well as several types of solid tumor including lung, breast, and cervix (4). CY does not have similar cytotoxic effects on all types of tumor, and the reasons for the differential effect are not well understood. Previous studies on the interaction of CY with radiation in the treatment of cancer (2, 9-13, 18, 20, 21, 23-25) have shown no evidence for an interaction other than simply additivity between CY and X-rays (14, 19).

The type of tumor used in the present experiment was relatively resistant both to radiation and to CY, so as to provide a challenge. Single X-ray doses of about 60 grays (1 gray = 100 rads) were necessary in order to control 50% of the locally irradiated tumors.

Growth delays of only about 2 and 8 days, respectively, were caused by 94- and 188-mg/kg doses of CY alone. This tumor is therefore slightly more sensitive to CY than are some experimental tumors (2, 10, 24) but less sensitive than are others (9, 12, 24) in the wide range that has been reported.

Materials and Methods

Two experiments were done using 245 male WHT/Ht mice bearing the fibrosarcoma WH SA FA transplanted onto the anterior chest wall. They were treated with X-ray, CY, or both. This tumor arose spontaneously in 1974 and has been maintained by passage in the strain of origin, WHT/Ht. Its volume-doubling time from 8 to 10 mm was 2.7 days (7, 22). The time between transplantation and reaching the treatment size of 7.5 ± 1 (S.E.) mm was 18 to 25 days. This tumor metastasizes to regional lymph nodes. In the first experiment, 107 mice were used with an i.p. dose of 94 mg/kg (i.e., three-eighths of the MTD of CY) and a localized dose of 0, 35, 50, or 70 grays of X-rays. In the second experiment, in which 138 mice were used, CY (188 mg/kg) was used (three-fourths of the MTD) with the same X-ray doses. The experiments were carried out with 2 consecutive transplants.

The CY was administered i.p. a few min after the X-ray dose (i.e., "simultaneously"), or 4 days before, or 8 days after the X-ray dose (9). Two other groups received the highest dose of CY and X-rays with these intervals reversed. CY (Endoxana), kindly donated by Ward Blenkinsop Pharmaceuticals, Ltd., Bracknell, Berkshire, England, was freshly dissolved in 0.9% (w/v) NaCl solution before each experimental session lasting for several hr. Twenty-one dose groups contained 9 to 17 mice, each bearing one tumor. Four other groups were smaller, containing only 3 to 7 mice (Tables 1 and 2). Tumors were randomly allocated into one of the treatment groups when they reached a geometric mean diameter of 7.5 ± 1 mm; zero time was the time of the first treatment whether CY or X-rays.

X-rays were generated at 240 kV, 15 ma, 1.3-mm half-value layer, and 2.4 grays (240 rads)/min. The mice were anesthetized with sodium pentobarbitone (60 mg/kg) and placed prone in Lucite-lead cradles with the tumor hanging freely through a hole 2 x 2 cm in the lead into a collimated horizontal X-ray beam (2 x 3 cm) which overlapped the bottom of the cradle, as described earlier (6). The dose received by the axillary lymph nodes in the mice was measured as 1 ± 0.2% of the tumor dose using thermoluminescent dosimeters; lithium fluoride rods (1 x 6 mm) were implanted into a cadaver positioned as for the irradiations. The diameter of the tumors was measured with calipers in

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4 The abbreviations used are: CY, cyclophosphamide; MTD, maximal tolerated dose; PM, postmortem.

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were also palpated for metastatic nodules. The geometric mean diameter of the tumors was recorded and processed as described below.

### Methods of Analysis

- **Controls and separate-agent treatments**
  - Controls (no treatment) 6 6(100) 0(0) 0(0) 0(0) 9.1 ± 0.5
  - CY alone (94 mg/kg) 10 10(100) 1(10) 0(0) 0(0) 10.4 ± 1.0
  - Controls (no treatment) 12 12(100) 0(0) 0(0) 0(0) 10.4 ± 0.6
  - CY (188 mg/kg) 11 0(0) 8(73) 0(0) 3(27) 18.1 ± 0.8
  - X-rays alone (50 grays) 169 Previous experiments by Stewart (22) and Denekamp et al. (7) 50 ± 0.2
  - X-rays alone (35 grays) 169 0(0) 0(0) 0(0) 0(0) 33 ± 0.1

### Treatments with 35-gray single dose of X-rays + CY

<table>
<thead>
<tr>
<th>Schedule</th>
<th>Total no. of mice</th>
<th>Local recurrence</th>
<th>Gross metastases</th>
<th>Metastases at PM only</th>
<th>Mice cured at 120 days</th>
<th>Regrowth of tumors</th>
<th>Sacrifice due to gross metastases only</th>
<th>Av. time (days) to</th>
</tr>
</thead>
<tbody>
<tr>
<td>CY (94 mg/kg) + X-rays simultaneously</td>
<td>13 13(100)</td>
<td>0(0)</td>
<td>4(31)</td>
<td>0(0)</td>
<td>46.0 ± 4.8</td>
<td>50.5 ± 5.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CY (94 mg/kg) + 4 days + X-rays (35 grays)</td>
<td>11 8(73)</td>
<td>5(45)</td>
<td>2(18)</td>
<td>0(0)</td>
<td>36.3 ± 4.6</td>
<td>38.9 ± 3.4</td>
<td>46.0 ± 3.8</td>
<td></td>
</tr>
<tr>
<td>X-rays (35 grays) + 8 days + CY (94 mg/kg)</td>
<td>13 11(85)</td>
<td>3(23)</td>
<td>4(31)</td>
<td>0(0)</td>
<td>42.0 ± 1.2</td>
<td>44.2 ± 1.1</td>
<td>45.0 ± 2.0</td>
<td></td>
</tr>
<tr>
<td>CY (188 mg/kg) + X-rays simultaneously</td>
<td>15 15(100)</td>
<td>0(0)</td>
<td>4(27)</td>
<td>0(0)</td>
<td>51.7 ± 1.6</td>
<td>56.1 ± 1.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CY (188 mg/kg) + 4 days + X-rays (35 grays)</td>
<td>11 11(100)</td>
<td>0(0)</td>
<td>3(27)</td>
<td>5(45)</td>
<td>49.5 ± 2.2</td>
<td>52.5 ± 2.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>X-rays (35 grays) + 8 days + CY (188 mg/kg)</td>
<td>11 9(82)</td>
<td>4(36)</td>
<td>3(27)</td>
<td>0(0)</td>
<td>49.7 ± 1.7</td>
<td>52.4 ± 2.3</td>
<td>43.0 ± 0.0</td>
<td></td>
</tr>
</tbody>
</table>

* Gross metastases + metastases found at PM only = total number of mice with metastases.
* Numbers in parentheses, percentage.
* Mean ± S.E.

3 perpendicular directions on 3 days/week, when the axillary nodes were also palpated for metastatic nodules. The geometric mean diameter of the tumors was recorded and processed as described below. When the tumors reached average diameters of 14 mm or when metastases made the mice noticeably ill, they were sacrificed for humane reasons.

All mice were dissected PM to search for metastases which were too small to be detected by palpation in vivo. The pathway of metastatic spread was almost always to the paraxillary (retrobrachial) lymph nodes, either anterior or posterior. Only 2 mice were found to have lung metastases.

The mice were caged 4 or 5 to a box and fed laboratory mouse pellets and water ad libitum. An additional 16 mice were sacrificed during the experiment (not included in the analysis) for the following reasons: radiation damage to intestines, 10; radiation damage to one foreleg, 4; abscess on left eye, 1; worms, 2; mites, 1. No mice died or were sacrificed for any other reason. Soon after this experiment, the colony was rederived by cesarean section, and the proportion of possible.

Normal tissue damage was recorded, but little was observed except for the intestinal radiation injury, which might have been aggravated by worms. It was not related to dose of CY but occurred after 50 or 70 grays of X-rays. Two mice had lung fibrosis, both in the higher CY dose group, one after 50 grays and one after 70 grays of X-rays. Cardiac plaques, representing a possible atherosclerotic complication of CY, occurred at frequencies of 23 of 148 and 14 of 140 in the higher and lower dose groups of CY as determined at PM dissection. The difference was not significant (P = 0.22). There were thus not sufficient data for a "therapeutic ratio" determination of relative effects on tumor and normal tissues of the combined agents.

### Methods of Analysis

- **Tables 1 and 2** give details of the results and the charts which follow present other results and demonstrate the trends. Metastases were observed early in the present experiments (the first being seen at 17 days on PM dissection of mice sacrificed for rapid local growth) and the range of times at which they caused mice to be sacrificed (35 to 92 days) overlapped with the times of sacrifice due to local recurrence (7 to 94 days); therefore, the mean survival times were often similar for the 2 reasons for sacrifice. Most of the metastases became significant between about 45 and 65 days. The results in Tables 1 and 2 were analyzed in 5 ways.

**Growth Delay.** This is one of the most sensitive measures of an improvement in the effectiveness of a treatment on a solid tumor and gives results over a wide range of doses. The time in days at which the geometric mean diameter at the first treatment was exceeded by 4.5 mm (e.g., 7.5 growing to 12 mm) was determined for each mouse (6). This corresponds approximately to a 4-fold volume increase. If a mouse was sacrificed because of metastases before its tumor had reached this size, it was excluded from the analysis of growth delay.

Mice reaching 120 days without a local tumor as large as 2 mm were included as "120 days of growth delay." This allowance for "cured" mice in a growth delay analysis has been used in previous analyses and has been shown to be a reasonable procedure for comparing treatments (8).

**Local Control and Cure.** Local control of the transplanted solid tumors is, logically, the extension of regrowth delay to very long times. It is a more demanding measure of good treatment than is regrowth delay but yields results over a relatively narrow range of doses. The definition of local control used here was less than 2 mm in diameter at 120 days. One tumor was found to be between 2 and 4 mm in diameter at 120 days and was excluded as "ambiguous" for the analysis of local control, although it was included for the other analyses. The latest local recurrence reached the 12-mm datum size at 94 days, preceded by one each at 90, 89, 83, 81, and 80 days, the bulk of the recurrences being much earlier. Thus, a margin of about 30 days was allowed after the latest recurrences before local control was determined.

The latest metastases were found at 96 days (subclinical at autopsy).
or 92 days (gross), with others at 88 to 92 days and the rest earlier than 75 days.

The mice which survived to 120 days without local tumor were also free of metastases and can be regarded as cured, i.e., with no evidence of disease at 120 days, either clinically or at PM dissection. A small number of these "cured" mice were kept to 150 days and showed no evidence of metastases.

Proportion of Mice with Metastases. Mice with grossly visible metastases in the axillary lymph nodes and also mice found at PM to have metastases, even if they had been sacrificed primarily because of a large local recurrence, were both scored as positive. In this part of the analysis, all mice sacrificed for local recurrence without any evidence of metastases were excluded, all other mice being regarded as at risk of metastases. "Cured" mice at 120 days were included in the number of mice at risk.

Mean Survival Time. This is the crudest method of analysis, but it should indicate any optimum in the overall treatment by combining increasing local control with increasing ablation of metastases. Mice were sacrificed for local recurrence (14 mm in diameter), or for definite metastasis (~7 mm diameter), or at the end of the experiment at 120 days. For local recurrence, mice were sacrificed at an average of 2 to 9 days after their tumors reached 12 mm. Survival time for these mice is therefore not as precise an end point as is growth to 4.5 mm larger than the size of tumor at irradiation, although the mouse-to-mouse variation in a given dose group was larger than the variation in the extra days between 12 and 14 mm average diameter; therefore, the method does not give much larger standard errors than does regrowth delay.

If neither local recurrence nor metastasis was observed up to 120 days, these mice were recorded as cured and entered as "120 days survival time," even though they would have lived longer.

Mean Survival Time of Mice Sacrificed Due to Metastases or to Local Recurrence. Mean survival time becomes a more useful measure when broken down into the separate causes of sacrifice. The survival time of those mice sacrificed because of the presence of gross metastases was averaged for each dose group. A progressive increase of this survival time should indicate the degree of success of the systemic CY in eliminating metastases. The mean survival time of mice sacrificed because their locally recurrent tumor had reached 14 mm in diameter was also computed, as a measure of the success of X-rays plus CY in delaying the growth of the transplanted tumor. In each dose group, any cured mice at 120 days were included because this long survival time represented a successful treatment which it would be misleading not to include.

RESULTS

Chart 1 shows the increase in growth delay of the solid transplanted tumors caused by increasing doses of X-rays and CY given simultaneously. Chart 1a shows a steeper increase with X-ray dose than Chart 1b shows with CY dose, as expected for a primarily local agent. However, X-rays have a large threshold dose and then become rapidly effective, whereas CY, when used with the highest dose of X-rays, is almost as effective at the lower dose of 94 mg/kg as at double this dose. The maximum possible delay is 120 days, which was the end
Chart 1. Average time required for transplanted tumors to grow from 7.5 ± 1 mm to a mean diameter of 4.5 mm larger, a, versus X-ray dose, x, with no CY. O, •, with CY (94 or 188 mg/kg, respectively). b, versus CY dose; O, with no X-rays (X), ▲, ▼, ●, with 35, 50, or 70 grays (Gy) of X-rays, respectively. Max, maximum; bars, S.E. Arrows on bars, groups in which some mice were cured at 120 days. Where no bars are shown, the S.E. range is smaller than the size of the symbol.

Chart 2 shows the proportion of mice cured, i.e., mice with neither local tumor nor any metastases present at 120 days, plotted against dose of X-rays (Chart 2a) or CY (Chart 2b) given simultaneously. Again, the response increases more steeply with increasing dose of X-rays than of CY, as shown by the steep but crowded curves in Chart 2a contrasted with Chart 2b. This result is not so intuitively obvious, because cure includes the elimination of all metastases. The error bars represent one-half the range of the 95% confidence limits.

Chart 3 shows the decreasing incidence of metastases with increasing doses of X-rays and CY. When they are given simultaneously, the same tendency towards a steeper dependence of response on dose of X-rays than of the systemic drug is apparent. This result is even more unexpected than that in Chart 2.

DISCUSSION

As higher doses of CY and radiation were given, the nature
of the responses in mice changed in a logical way; although
the steeper rates of change for all the factors measured with
increasing dose of X-rays than of CY were not expected.

CY alone at the lower dose used (three-eighths of the MTD)
caused a negligible increase in growth delay of the tumors, but
three-fourths MTD increased the regrowth time from 10 days
in untreated mice to 18 days. The difference of 8 days in
growth time was maintained as X-rays were added in increasing
doses (Chart 1a). For example, an increase in growth delay of
8 days was obtained by a 10 to 20% increase in X-ray dose at
a reasonably high X-ray dose level, in contrast to the doubling
of the CY dose required for the same effect. This contrast is
not surprising because X-rays can act only locally whereas CY
is a systemic treatment intended to deal primarily with métas-
tases, in addition to any effect it may have on the primary
tumor. The X-rays, being used as a localized beam, were not
limited in dose as much as a systemic drug is. Although CY
alone was relatively ineffective against this solid transplanted
tumor, it certainly increased the effectiveness of the X-ray
treatments, especially of the highest X-ray dose, as shown in
Charts 1b and 2. X-ray doses above about 70 grays would
have caused unacceptable local necrosis.

Because the growth delay was increased beyond about 35
days by irradiating the transplantable tumor, some mice had to
be sacrificed for gross métastases in their axillary lymph nodes

Chart 3. Proportions of mice at risk which exhibited metastases (mets) before
120 days, versus X-ray (X) dose (a) and CY dose (b). Symbols as in previous
charts. Bars, 66% confidence ranges. Gy, grays.

Chart 4. Average time of sacrifice (sac) due to gross métastases (mets) (i.e.,
ot local recurrence) versus X-ray (X) dose versus and CY dose (b). Symbols as
before except that the lower pair of lines shows the average (Avge) sacrifice time
excluding (exc) cured mice. Incl., including; Gy, grays.

Chart 5. Regrowth delay (a) and percentage (b) of mice free of disease at
120 days versus interval in days between giving the X-ray and the CY doses.
△, ▽, ×, as in Chart 1b, with CY (188 mg/kg). △, ▽, × with CY (94 mg/kg).
Instead of for local growth of the tumors. All the metastases became significant between 17 and 92 days, most of them between 35 and 65 days. Growth delays were longer when higher doses were given, but the longest growth delay was observed at 94 days. There was therefore no distinction in time scale between sacrifices due to local growth (i.e., recurrence after treatment) or to metastasis, although each sacrifice was due to one or the other and was thus recorded. As the average growth delay was increased beyond about 65 days, a proportion of the mice had their tumors locally controlled, i.e., they did not recur up to the end of the experiment at 120 days. In these mice, no metastases were found, even at the PM dissection after 120 days. Since this corresponds to a disease-free interval of about 30 days beyond the latest appearance of either metastases or local recurrence, the mice could be regarded as cured. A small number of cured mice kept to 150 days without local recurrence or metastases confirmed this description.

Chart 2 illustrates the steeper increase in the proportion of mice cured with dose of X-rays than of CY. The only high proportion of mice cured, i.e., over 50%, was when the highest dose of X-rays was given simultaneously with either of the 2 dose levels of CY. Although X-rays had the larger effect on the local tumor, a high proportion of cures was not obtained without the use of CY as well.

Chart 3 illustrates the detail of this result. The proportion of mice with metastases decreased more steeply with X-ray dose than with CY dose. No mice were made free of metastases unless a dose of at least 50 grays had been given locally to the transplanted tumor. If the largest dose of X-rays was used with no CY, 27% of these mice did not show metastases and indeed were completely cured. Adding CY (94 or 188 mg/kg) simultaneously with the 70-gray dose of X-rays increased the clearance rate of metastases to 70 or 78%, respectively, and the cure rates to 63% (10 of 16) or 78% (14 of 18). The CY undoubtedly helped significantly, but the dependence on dose of CY was less steep than that on dose of X-rays.

When a gap was introduced between the CY and the X-rays, in either sequence, worse results were obtained, although when the gap was as short as 4 days with the highest dose of X-rays, the results were not significantly worse than when both agents were given simultaneously (Charts 5 and 6). Where there were differences between giving X-rays before or after CY, the effectiveness of X-rays after CY was less than when given before, e.g., with CY (94 mg/kg) in Charts 5 and 6.

The X-ray doses delivered directly to the axillary regions, by scatter from the irradiated tumor and thoracic wall, were small: 0.35, 0.5, and 0.7 grays for the tumor doses of 35, 50, and 70 grays, respectively. Even the highest of these axillary doses would not sterilize as many as 50% of any mammalian cells, except lymphocytes or marrow cells which are known to be more radiosensitive than most other mammalian cells. The present tumor cells cannot be unduly radiosensitive because 50% local control required an X-ray dose as large as about 60 grays. Therefore, the decrease in metastatic incidence with X-ray dose cannot have been due to direct radiation killing of the axillary deposit of malignant cells.

Tables 1 and 2 show that giving CY 4 days before X-rays almost always gave better local control but worse (higher) incidence of metastases than simultaneous administration gave. This is consistent with the importance of the X-ray dose in the prevention of seeding of metastases; waiting 4 days after the size of 7.5 mm was reached before giving X-rays was a significant disadvantage in terms of eventual incidence of metastases. It is important that the results for the 4-day interval were not significantly worse than for simultaneous administration, especially when X-rays were given first. Four days is, however, a sufficiently long interval to avoid the well-known enhancement by CY of radiation injury in some normal tissues (16). This point is worthy of further study.

It cannot, of course, be expected that the importance of local X-rays in preventing metastases will be as great in all types of tumor, but the conditions for which this may be so can be discussed. In the present tumor system, a full range of results from local recurrence, through delay in growth of the primary but death due to metastases, through elimination of both the primary tumor and of metastases to full cure was obtained by appropriately high doses of X-rays and CY. If metastases were seeding out of the tumor after treatment had started, then the thoroughness of the local treatment would indeed be expected to affect the incidence of distant metastases.

We are led to the conclusion that a thoroughly good curative treatment of the solid transplanted tumor, not a "spontaneous primary" in the present experiments but intended to model a primary tumor in human cancer, does increase the metastasis-free rate. This is not so surprising if one considers the opposite. A failure to control the primary tumor would allow it to seed out through delay in growth of the primary tumor and of metastases to full cure was obtained by appropriately high doses of X-rays and CY. If metastases were seeding out of the tumor after treatment had started, then the thoroughness of the local treatment would indeed be expected to affect the incidence of distant metastases.

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References


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