Stem-Cell Survival and Tumor Control in the Lewis Lung Carcinoma

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SUMMARY

The stem-cell response of the Lewis lung carcinoma to single doses of cyclophosphamide has been studied by three assay techniques: in vitro colony formation, lung colony formation, and the end-point dilution assay. These three techniques have given comparable results, and the end-point dilution results showed that 50% takes could be achieved with as few as 1 to 3 cells. Studies have been made of the growth of small i.m. implants and of the time following implantation at which they could be eradicated by cyclophosphamide. The results were compared with the curability that would be expected on the basis of cell survival studies. It was found that older (and therefore larger) implants were cured than might have been expected. It seems unlikely that this discrepancy was due to additional cell kill caused by an immune response. An alternative explanation, that the surviving fraction following a dose of cyclophosphamide was lower in small implants than in larger i.m. tumors, was supported by studies of cell survival in dissectable lung colonies.

INTRODUCTION

The response of experimental tumors to irradiation or to chemotherapeutic agents may be assessed by a variety of end points, which can be divided into the main categories of tumor inhibition and regrowth studies, tumor cure rate studies, and studies of cell survival. While regrowth and tumor control studies have the advantage of directness of application, the cell survival studies offer the hope of identifying some of the cellular mechanisms involved in tumor response. In particular they can sometimes show that successful treatment is frustrated by a minority of resistant tumor stem cells (21). The major drawback to cell survival studies is, however, their artificiality. The assays involve subjecting tumors to the additional insult of trypsinization or mechanical homogenization, thereby removing each cell from its in vivo relationship with other neoplastic or connective tissue cells. It is important, therefore, to examine directly the relationship between the results of cell survival studies and tumor regrowth and control (12, 13), partly in order to substantiate the cell survival data but also to investigate the mechanisms involved in the regrowth of tumors from small numbers of surviving cells. The project described in this report was designed to investigate the relationship between cell survival and tumor control in the Lewis lung tumor. The results also have a bearing on the importance of tumor size in determining therapeutic response.

MATERIALS AND METHODS

Lewis Lung Carcinoma. A specimen of Lewis lung carcinoma was obtained from Dr. K. Hellman of the Imperial Cancer Research Fund, London, England, in December 1971. It has since been maintained within C57BL mice of the Institute of Cancer Research colony. Its histological and macroscopic characteristics are consistent with those reported by Sugiura and Stock (20). The transplantation procedure has been to chop the tumor tissue finely with crossed scalpels, make an homogenate by forcing it through a syringe with 10 volumes of balanced salt solution, and then inject 0.02 ml bilaterally into the gastrocnemius muscles of recipient mice. No such implants have failed to take.

Tumor Size Measurement. Measurement of the size of i.m. tumors was made by a calibration curve technique (19). Mice bearing single i.m. tumors that had been produced by the implantation of between $10^3$ and $10^4$ cells were killed over a range of tumor sizes. Each tumor-bearing leg was shaved and its external diameter at the tumor site was measured with calipers. Both hind legs were then disjointed at the knee, the skin was removed down to the ankle, and the legs were weighed. The difference in weight between the tumor-bearing and control legs was taken as an estimate of tumor weight. The assumption in this was that edema due to the presence of the tumor did not seriously affect the result. Chart 1 shows the calibration curve relating tumor weight to leg diameter. The scatter in the points is reasonably small although, in view of the assumption that has just been mentioned, it is possible that the data in this chart may be subject to systematic error.

Cell Suspension Technique. Mice with i.m. tumors measuring 10 to 15 mm in diameter were killed by cervical dislocation. The tumors were dissected out using sterile precautions, freed from the muscle, and weighed. They were then finely chopped with crossed scalpels in a small amount of a mixture of phosphate-buffer saline, NaCl, 8.0 g; KCl, 0.2 g; Na$_2$HPO$_4$, 1.15 g; KH$_2$PO$_4$, 0.2 g; phenol red, 0.02 g; and distilled water, 1.0 liter. A cell suspension was
prepared by trypsinization in phosphate-buffered saline containing 2.5% by volume of Bacto-Trypsin, reconstituted as recommended by Difco Laboratories, Inc., Detroit, Mich. Brief shaking of the tumor fragments in warm trypsin-free medium at the end of trypsinization greatly increased the yield of viable tumor cells. After the cells were washed in Eagle’s basal medium (Biocult Laboratories, Paisley, Scotland) they were filtered through a 400 mesh stainless steel gauze under gravity. The cell viability as judged by phase-contrast microscopy usually exceeded 90%.

High transplantation efficiency could be obtained only by adding HR to the inocula. These were usually prepared in a separate but simultaneous digestion. They were brought to a final total cell concentration of $2 \times 10^7$/ml and were given a dose in excess of 10,000 rads of $^{60}$Co $\gamma$-rays at a dose rate of about 3,000 rads/min. Every experiment included controls given $10^6$ HR alone, and growth from such inocula was observed only in 1 experiment, which was abandoned.

For i.m. implantation, the viable cell concentration was adjusted to give the required number of cells in 0.05 ml. Equal volumes of viable suspension and HR suspension were mixed and from this mixture volumes of 0.1 ml were implanted, thus containing the viable cell dose plus $10^6$ HR.

**End-Point Dilution Assay.** Graded inocula of viable cells were implanted within a range that was designed to include the TD$_{50}$. The cell concentrations usually differed by a factor of 3.16 (i.e., $\sqrt{10}$) so that 2 dilutions just covered a 10-fold range. Recipient mice were observed for 50 days after implantation and new tumors rarely appeared after 30 days, irrespective of the treatment that the cells had received. The results were analyzed by a computer program that applied the method of Porter and Berry (11). The fraction of tumor cells surviving treatment was calculated as the ratio of the TD$_{50}$ estimate for untreated tumors to the TD$_{50}$ estimate for treated tumors. Since it was possible to implant a total cell number of over $10^8$ into each implantation site, this type of assay could be used to measure survivals down to approximately $10^{-4}$.

**Lung Colony Assay.** The method was as described by Hill and Bush (6). Viable tumor cells were mixed with $10^6$ HR and $10^6$ plastic 15-μm microspheres, and they were injected i.v. in a volume of 0.3 ml. Lungs were removed and fixed in Bouin’s fixative at 21 days after injection, and the colonies were counted under a dissecting microscope. In these experiments the yield of colonies was in the range of 1 colony/$10^5$ to $10^6$ untreated viable cells. The fraction of surviving tumor cells was calculated as the ratio of the cloning efficiency (colonies per $10^6$ viable cells) of cells from treated tumors to the cloning efficiency of cells from untreated tumors. With the present line of Lewis lung tumor, this method could be used to measure survivals down to $10^{-2}$ or $10^{-3}$.

**In Vitro Colony Assay.** Colony formation in vitro was studied by the method developed for this tumor by Courtenay (manuscript in preparation, but referred to in Ref. 16). The method was a double-layer soft agar technique using a gas phase containing 5% oxygen. For the Lewis lung tumor it was possible to measure survivals down to about $5 \times 10^{-4}$ with this technique.

**Antigenicity of the Lewis Lung Tumor.** The immunological status of the tumor growing in our colony of mice was assessed by measuring the TD$_{50}$ for i.m. implantation after attempted immunization. Three main types of immunization were used. In a series of experiments performed over a period of 18 months, HR were injected either i.m. into the left hind leg or at 5 sites close to lymph nodes, (2 axillary, 2 inguinal, plus i.p.). The i.m. TD$_{50}$ in untreated mice remained below 3 cells, while in the immunized groups successive estimates of the TD$_{50}$ were 26, 92 (hind leg immunization), 99, 94, and 54 (para-lymph node immunization). The 3rd type of immunization was to allow i.m. implants in the left hind leg to grow for 7 to 10 days after which the local tumor was eradicated by irradiation with 3000 to 4500 rads of $^{60}$Co $\gamma$-irradiation. A single dose of cyclophosphamide, 300 mg/kg, was also given in order to control the metastases existing at this time and to produce a situation that was comparable with that in mice in which the curability of artificial lung metastases was to be studied. At both 7 and 83 days after this combined treatment the i.m. TD$_{50}$ (in untreated legs) was indistinguishable from controls.

**RESULTS**

**Transplantability.** For s.c. implantation the TD$_{50}$ was over $10^5$, and the addition of up to $10^7$ HR only reduced this to $5 \times 10^4$. However, i.m., the TD$_{50}$ with viable cells alone was 600 cells and the addition of $10^6$ HR reduced this to less than 3 cells. Chart 2 indicates the results of 1 experiment using cells from untreated tumors. The full line is a cumulative Poisson distribution. Its fit to the data implies uniformity of the implantation sites and that even single Lewis lung tumor cells could establish positive takes under these conditions.

**Growth Curve for i.m. Lewis Lung Tumors.** The growth of the tumors during the “silent interval” before detection was examined by a technique that has been used on experimental
A single-cell suspension was prepared and implants, ranging in decade steps from 1 to 10⁶ cells, were made into both hind legs of groups of 10 mice. HR, 10⁸, were added to each implant in all except the 10⁶ viable cell group. The times at which the leg diameters reached 10 mm were recorded and the median times are plotted in Chart 3. The results of 2 separate experiments are shown. Positive takes were observed in all implant sites except those made with a mean of 1 cell/implant, where there were 7 of 10 takes. Although there is some interexperiment variation in the TD₅₀, and therefore in the proportion of viable cells that actually grow, this result suggests that in this experiment the TD₅₀ was close to its ultimate value of 0.7 and that almost every viable cell contributed to growth. With the assumption that this holds for all sizes of inoculum, the straight line that fits these data may be taken to represent the growth curve from small implant sizes. It has a doubling time of 1.02 days and there is no evidence for growth retardation up to a size of 10⁶ cells.

Survival Curve for Single Doses of Cyclophosphamide. Mice bearing i.m. Lewis lung tumors in both hind legs were selected for treatment on Day 13 after the implantation of 0.02 ml of a tumor homogenate (1:10 tumor in Eagle's basal medium). At this time the legs were swollen from their normal width of about 5 mm to about 10 mm, and from each leg it was possible to dissect out approximately 0.5 g of tumor. Cyclophosphamide was injected i.p., usually into groups of 3 to 4 mice, and the animals were killed by cervical dislocation usually 3 hr afterwards. Cell suspensions were prepared and assayed by the 3 methods described above.

The results are shown in Chart 4. Measurements by the end-point dilution assay were made down to a survival of 10⁻⁶, and over this range the survival curve is probably indistinguishable from an exponential. There is evidence of an initial shoulder equivalent to a cyclophosphamide dose of about 20 mg/kg. Agreement between the 3 assays is good although the 2 points obtained by the lung colony assay are insufficient to provide a precise comparison. The shape of the survival curve above a dose of 300 mg/kg has not been explored because of host toxicity.

Regrowth after Single Doses of Cyclophosphamide. Tumors growing i.m. were treated when they reached a leg diameter of 8 mm (a size of about 0.2 g as judged by Chart 1); the regrowth data are shown in Chart 5. In this chart the estimated tumor sizes are relative to the size of the tumor at the time of treatment. Also indicated (on the ordinate) are the levels of cell survival associated with each cyclophosphamide dose, derived from the data of Chart 4. It is apparent that these levels of cell kill are much lower than would have been anticipated from the regrowth data alone. For instance, a backward extrapolation of the tumor weight data for the dose of 300 mg/kg might indicate a survival of about 10⁻², compared with the measured value of 10⁻⁶. The situation is reminiscent of the work of Hermens and Barendsen (5), who found that the volume response of an experimental rhabdomyosarcoma to 2000 rads of X-irradiation was much less than was indicated by in vitro estimates of the survival of clonogenic cells. We have not attempted to measure the regrowth of clonogenic cells in the Lewis lung tumors, but the broken lines in Chart 5 indicate the approximate rate at which regrowth must have taken place. These lines have been drawn at their upper ends to the point
Cyclophosphamide, Chart 4 gives the relative cell survival. $(\ln 2)/2 = 34.6$ cells initially take. For any chosen dose of diameter, and the proportion of controlled tumors was calculated. The results are shown in Chart 6 for cyclophosphamide doses of 100, 200, or 300 mg/kg. The tumor ages at which 50% control was achieved are 4, 11.5, and 16 days, respectively.

For these studies implants of 100 viable cells plus $10^8$ HR were made into the left hind legs of groups of 10 mice. Each group was treated with a single i.p. injection of cyclophosphamide at up to 24 days later. Mice were killed at 60 days after implantation or when the tumors exceeded 10-mm diameter, and the proportion of controlled tumors was calculated. The results are shown in Chart 6 for cyclophosphamide doses of 100, 200, or 300 mg/kg. The tumor ages at which 50% control was achieved are 4, 11.5, and 16 days, respectively.

These results may be compared with the expected age at which the regrowing tumors reached a leg diameter of 12 mm (a tumor weight that is 4 times the treatment size). Measurements of TD$_{50}$ in such tumors gave values that were indistinguishable from the value in untreated tumors. The slopes of the broken lines in Chart 5 can only give approximate estimates of the doubling times of the clonogenic cell population during regrowth, but the estimates (in days) are as follows: 1.2 (50), 0.8 (100), 0.75 (150), 0.8 (250), and 0.95 (300), the cyclophosphamide dose (in mg/kg) being given in parentheses. These estimates of doubling time are very short, but they are not surprising in view of the 1.02-day doubling time of the growth curve in Chart 3 and the work of Simpson-Herren and Lloyd (17) and Hill and Stanley (unpublished observations) which has shown that the average intermitotic time of cells in small Lewis lung tumors is below 18 hr.

Curability of Small i.m. Implants of Lewis Lung Tumor. For these studies implants of 100 viable cells plus $10^8$ HR were made into the left hind legs of groups of 10 mice. Each group was treated with a single i.p. injection of cyclophosphamide to achieve theoretical expectation (see text). Drug doses were 300 mg/kg (○), 200 mg/kg (■), or 100 mg/kg (□). Broken lines, theoretical expectation (see text).
survival data were obtained on i.m. tumors weighing about 0.5 g that already contained areas of necrosis; it is conceivable that cyclophosphamide was less available to some cells within such tumors than within i.m. implants of up to only $10^3$ cells. An attempt to test this hypothesis has been made by performing cell survival studies on small lung tumors. These were produced by the simultaneous i.v. injection of $10^4$ viable tumor cells plus $10^6$ HR plus $10^6$ plastic microspheres. On Day 16 after injection, the mice were divided into groups and either left untreated or given an i.p. injection of cyclophosphamide. Three or 16 hr later the mice were killed, the lungs were removed, and lung colonies of about 1- to 2-mm diameter were removed and processed for a single-cell suspension as indicated above. Each group of 4 mice yielded about 30 colonies. The estimates of cell survival obtained in this way are shown in Chart 7. The data predominantly fall below the line that was determined for the larger i.m. tumors (Chart 4). Also shown in Chart 7 are the cell survival values that may be calculated from the tumor cure data. From the data in Chart 6 one can interpolate the tumor age at which 50% tumor control was achieved. Chart 3 gives the size of the tumors at this age and the estimate of cell survival comes from the ratio of this size to the number of cells that may be assumed to give 50% regrowth probability. For instance, the dose of cyclophosphamide, 200 mg/kg, cured 50% of tumors at 11.5 days after the 100 cell implants. Taking a TD$_{50}$ of 2.0 we assume that 34.6 cells actually grew, and from Chart 3 it can be seen that by 11.5 days they would have produced $8 \times 10^4$ cells. The further assumption that the TD$_{50}$ for regrowth lies between 0.7 (the ultimate TD$_{50}$ for the regrowth of all surviving cells) and 2.0 gives a calculated range of survivals from $8.8 \times 10^{-6}$ to $2.5 \times 10^{-4}$.

These calculations show that the tumor cure data are consistent with a steeper survival curve for cells within small lung colonies than has been determined on the larger dissectable i.m. tumors. We may also conclude that, within the rather wide limits of precision of these calculations, a cell survival curve determined shortly after drug administration gives a realistic indication of the number of cells that will retain the capacity to contribute to tumor regrowth. There is no evidence that subsequent cell kill due to immunological or other mechanisms was important.

### DISCUSSION

The numerous studies on the Lewis lung tumor during the past few years have largely concentrated upon the response of its metastases. Humphreys and Karrer (7) and Karrer et al. (9) described the efficacy of chemotherapy in eliminating metastatic disease after the surgical removal of an i.m. implant; Johnson (8) performed similar experiments using irradiation treatment of the primary implant. The chemotherapeutic response of s.c. implants was studied by Mayo et al. (10), and the inhibition of metastases by ICRF 159, Triton, and cyclophosphamide has been described by Hellman and Burrage (4), Salsbury et al. (14), and Franchi and Garratini (3). The 1st reports of cell survival studies with the Lewis lung tumor were by DeWys (1, 2) who used a quantitative transplantation assay. His results indicated that the extent of cell kill decreased with increasing age (or size) of tumors treated with cyclophosphamide.

The initial objective of this work has been to develop reliable methods for measuring cell survival following *in vivo* treatment of Lewis lung tumors. The use of the end-point dilution assay has yielded the important conclusion that TD$_{50}$ values as low as 1 to 3 cells can be achieved with this tumor. Bearing in mind that the cells that are assayed have been subjected to the trauma of trypsinization and that it is difficult to reduce the standard error of TD$_{50}$ values below about 20% of the mean, these results are consistent with a majority of the extracted cells with the capacity to initiate tumor growth in the i.m. site. In concluding this, it must be recalled that the cell separation procedure has a yield of only about 10%. The cells that are brought into suspension may or may not be representative of the total cell population of the tumor. The great sensitivity of the end-point dilution assay has allowed us to measure surviving fractions after cyclophosphamide treatment down to the limit that is set by host toxicity (a survival of about $10^{-4}$). At this level there was little evidence of a resistant component in the survival curve of the type observed by Valeriote et al. (21). The 3 assays that we have used gave cyclophosphamide survival data that were indistinguishable, although the lung colony assay was not adequately tested. We have not pursued this comparison in view of the excellent agreement between *in vitro* and lung colony assay estimates of irradiation survival in the Lewis lung tumor that has been observed in this laboratory by Shipley et al. (16). In the B16 melanoma we have also observed good agreement between all 3 assays in the study of survival following cyclophosphamide (G. G. Steel and K. Adams, manuscript in preparation).

Our investigations of regrowth after the administration of

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Chart 7. Cell survival in 1- to 2-mm diameter lung colonies following single doses of cyclophosphamide. $\Theta, A$, results of 2 separate groups of investigators using the *in vitro* colony assay, 3 experiments in all. Solid line is taken from Chart 4. Bars, cell survival that would be expected on the basis of the results shown in Chart 6.
cyclophosphamide have led to the conclusion that repopulation by clonogenic cells must be very rapid. Although we have not actually measured the recovery of the clonogenic fraction in this tumor, it would seem that the posttreatment doubling time cannot be much greater than 1 day.

One of the primary objectives of this project was to examine the relationship between measurements of clonogenic cell survival and the size of microscopic tumors that can be cured. In Charts 6 and 7 are shown the theoretical estimates of the age of implants that should be cured by effect, it is largely explained by the fact that the cyclophosphamide survival curve is steeper in small tumor foci than in the larger i.m. implants. Evidence on the antigenicity of the cells within lung nodules of Lewis lung tumor is also considerably higher than in larger tumors. For nodules up to 2 mm in diameter the hypoxic fraction was below 10% and in concurrent experiments in this laboratory Shipley et al. (16) have observed that the sensitivity to \( \gamma \)-irradiation of the cells within lung nodules of Lewis lung tumor is also considerably higher than in larger tumors. For nodules up to 2 mm in diameter the hypoxic fraction was below \( 10^{-8} \) and cells removed and irradiated under well oxygenated conditions \( \text{in vitro} \) showed a lower \( D_0 \) than for cells taken from larger s.c. tumors.

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**REFERENCES**


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