The Radiosensitivity of Lymphosarcoma Cells as Determined by the Liver Colony Method

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Abstract. The liver colony method is based on the observation that intravenous injection of an appropriate number of LS/BL cells into isologous non-irradiated hosts leads to the formation of colonies of proliferating cells in the livers of these animals. The relationship between the number of cells injected and the number of colonies appearing in the livers was determined. This technique was used to measure the radiation sensitivity of LS/BL cells and it yielded a D_0 of 1.05 Gy. The results show that LS/BL cells have a similar radiation sensitivity as other mammalian cells. The liver colony assay used to determine the radiation sensitivity of the lymphosarcoma LS/BL cells can also be used whenever the number of viable tumour cells in a suspension is to be estimated.

Key words: Liver-colony assay — Radiosensitivity — Lymphosarcoma cells

Introduction

Radiation damage to cells can be quantitatively measured by their colony-forming ability. The principle of evaluation is based on the all-or-nothing effect, which means that an irradiated cell is either alive or dead (Tremp 1981). Since the introduction of colony forming ability determination of haematopoietic cells as a quantitative assay of irradiation damage (McCulloch and Till 1960; Till and McCulloch 1961), this model has been extended for investigating tumour cell populations (Bruce and Van der Gaag 1963; Bush and Bruce 1964; Pluznik and Sachs 1964; Wodinsky et al. 1967; Metcalf et al. 1969; Tanaka et al. 1970; Boranić et al. 1973).

Our previous investigation (Jurášková and Drášil 1978) of the radiation sensitivity of lymphosarcoma LS/BL cells have been based on the cell-dose-host survival-time method originally introduced by Maruyama and Brown (1964). In this method, different number of tumour cells are injected intravenously into recipient animals, the host survival time is scored and used for the estimation of

lethal number of tumour cells. If irradiated tumour cells are used the host survival time increases, and by determining the values at different doses, the radiosensitivity of the cell can be established. Our results obtained by this method provided evidence that during the years of transplantation and progressive adaptation to ascites growth, the values of D_0 increased slightly from 0.8—1.0 Gy (passages No. 50—200) to 1.4—1.6 Gy (passages No. 400—600).

In further experiments we tried to estimate the radiosensitivity of LS/BL cells using the spleen-colony assay. The present paper reports on the development of the liver-colony assay as compared to the spleen-colony method. This idea assay has been prompted by our previous findings (Drášil and Jurášková 1964) that numerous metastatic nodules in the liver of host mice were observed within two weeks after an intravenous injection of tumour (EAT) cells.

Material and Methods

LS/BL cells originated from a radiation-induced lymphosarcoma in a C57BL female mouse in 1963 (Jurášková and Drášil 1978) and were carried as an ascites tumour by weekly intraperitoneal inoculations (10^6 cells per mouse). The present experiments were performed using transfer generations 850 to 900. Tumour transfers and further experiments were carried out on 12- to 14-week-old female C57BL mice weighing approximately 25 g.

Calcium- and magnesium-free Hanks's balanced salt solution was used for washing and dilution of the cells.

Cells for the experiments were obtained on the 6th day of their intraperitoneal growth. Washings from several donors were harvested, diluted to the desired cell concentration and 0.5 ml were injected intravenously into the tail vein.

Cell suspensions (4.8×10^5 cells/ml) were irradiated at a temperature close to the freezing point, using a TuR X-ray unit operated at 180 kV with added filtration to yield a beam with an HVL of 1.02 mm Cu. The dose rate was approximately 0.46 Gy/min (48 R/min) as measured with a Victoreen 550 dosimeter. The interval between the irradiation and the intravenous administration of cells was 20 to 40 minutes.

The method used to measure the colony-forming activity of cells in vivo is based on the observation that intravenous injection of an appropriate number of competent cells into animals results in the formation of macroscopic colonies in the spleens of these animals. These colonies are easily recognized after a short fixation in Bouin's solution. In our experiments the colony-forming activity of LS/BL cells in both the spleen and the liver of recipient animals was tested. For the spleen colony assay, LS/BL cells were harvested from the peritoneal cavity of 6-day tumour-bearing animals and diluted to the proper cell concentration. Groups of 15 recipient mice were injected intravenously the cell suspensions. Spleen-colonies were evaluated on the parietal surface of the spleen on the 9th day after the transplantation of LS/BL cells. For the liver-colony assay the recipient animals were killed on the 12th day. After a short fixation in Boiun's solution the macroscopically visible colonies on the surface of the liver left anterior lobe were counted.

For histological analysis the spleens and livers were fixed with the Helly solution and sections were stained with haematoxylin-eosin.

Survival curve parameters and their standard deviations were estimated by regression analysis using a PDS/3 Nuclear Chicago computer.

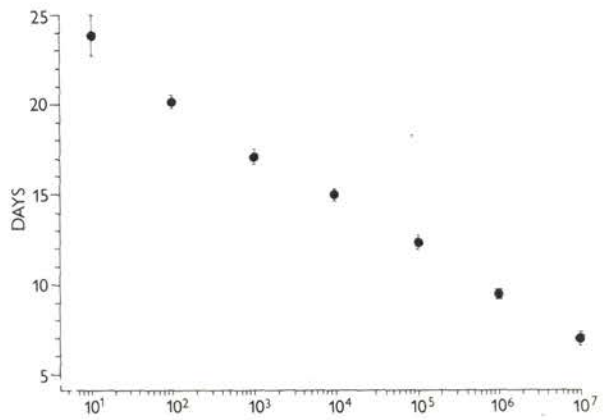

Fig. 1. Mean survival time (ordinate) of hosts given LS/BL cells intravenously (abscissa).

Results

In order to find the optimal size of the lymphosarcoma cell inoculum for the spleen and liver colony assay, mice were inoculated intravenously with $10^1 - 10^7$ LS/BL cells and kept for survival. In Fig. 1 the mean survival time is plotted against the cell numbers in the inoculum. All inoculated mice died except the group 10^1, where 2 of 10 inoculated mice survived. At the time of this experiment the tumour was in about its 850th passage.

The survival curve showed the usual linear dose-response relationship as it is observed with exponentially growing tumour cells. According to these results inocula of 10^4 cells or less, were used in our further experiments so that a sufficiently long survival time was obtained to enable spleen and liver colony assays.

For the spleen colony assay, LS/BL cells were injected intravenously to groups of 15 irradiated (6 Gy) mice. Nine days later 12 of the surviving animals were killed and the mean number of colonies was estimated. The type of the colonies was determined in serial histological sections (Jurášková et al. 1964). After the inoculation of 5×10^3; 1×10^4; and 2×10^4 of LS/BL cells per mouse, the mean numbers of spleen colonies were 2.86 ± 0.47; 1.31 ± 0.50 and 1.62 ± 0.42 respectively. The number of endogenous colonies in the group of control (only irradiated) mice was 2.67 ± 0.71. This experiment demonstrated that there was no relationship between the injected LS/BL cell and the mean number of spleen colonies.

Histological examination of spleens showed an extensive leukemic infiltration and an almost complete replacement of normal elements by leukemic cells. The colonies appeared as scarcely distinguishable pale nodules on the surface of the spleen (Fig. 2 *A*). Discrete easily recognizable colonies (Fig. 2 *B*) were rarely

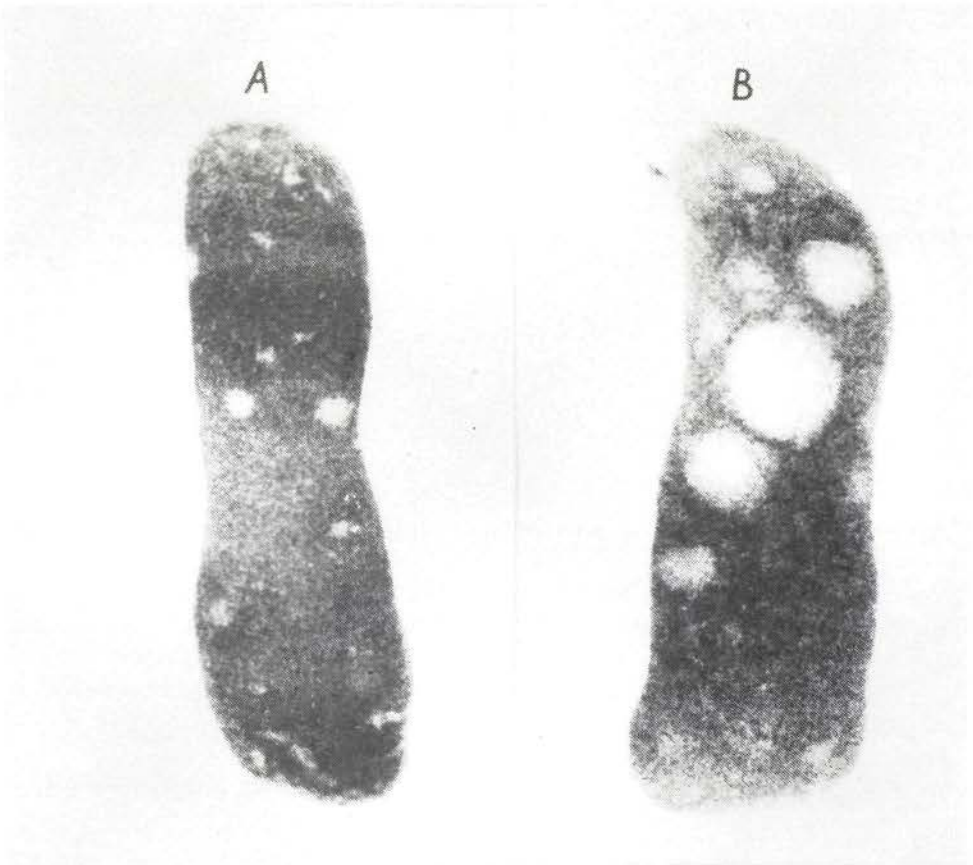

Fig. 2. Spleen colonies in C57BL mice nine days after inoculation with 2×10^4 LS/BL cells per mouse.

observed and they were in most cases of the haematopoietic origin. As shown in Table 1 haematopoietic colonies in the spleens of LS/BL cells inoculated mice predominated over the leukemic ones. To prevent the admixture of haematopoietic colonies it was necessary to enhance the preconditioning exposure of recipient animals: however doses higher than 6 Gy followed by the injection of tumour cells resulted in the death of animals within the first week after the treatment. When using non-irradiated recipients the colonies on the surface of greatly enlarged and hyperaemic spleens were scarcely recognizable.

Since the livers of the recipient mice are invaded by LS/BL cells to a lesser extent as compared to the spleen we tried to find out whether there is any possibility of testing the colony-forming ability in. Our preliminary experiments had shown that in the livers of mice injected with a small number of LS/BL cells

Table 1. Colony numbers and types in histological preparations of the spleen of irradiated mice inoculated with 5×10^3 LS/BL cells

Type of colony	*Spleen No.*												Number of colonies
	1	2	3	4	5	6	7	8	9	10	11	12	
Myeloid	1	–	–	1	–	–	–	1	1	1	1	–	6
Erythroid	–	–	2	1	1	1	–	1	3	–	3	–	12
Megakaryocytic	–	–	2	2	–	3	–	–	4	1	–	1	13
Leukemic	–	1	–	1	–	–	2	2	1	–	1	–	8

easily recognizable pale nodules appeared (Fig. 3). Histologic examination of these nodules revealed that they consisted of leukemic cells only. No admixture of heamatopoietic cells could ever be observed.

Cell suspensions were prepared from the discrete colonies removed from unfixed livers and they were injected intraperitoneally into non-irradiated mice. The animals developed progressive disease until death occurred between the 7th

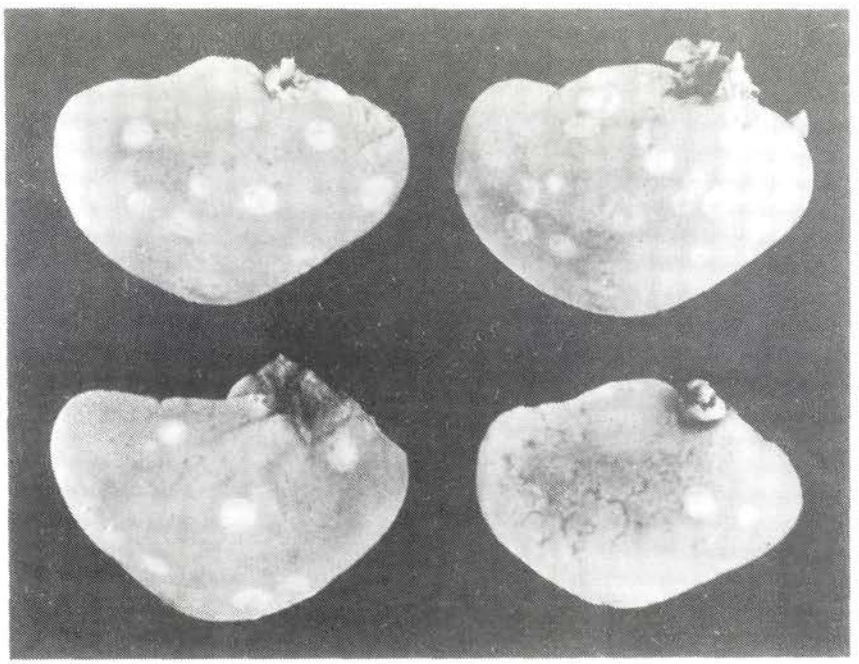

Fig. 3. Liver colonies twelve days after inoculation with LS/BL cells.

Table 2. Relationship between the numbers of injected LS/BL cells and the number of colonies formed in the left anterior liver lobe of non-irradiated (*A*) and irradiated (*B*) recipients

Number of injected cells	Liver colony counts	Mean ± S. D.
A		
5×10^2	2, 1, 2, 1, 6, 3, 2, 2	2.4 ± 0.56
10^3	6, 8, 4, 7, 2, 10, 9, 2	6.0 ± 1.25
5×10^3	7, 25, 27, 20, 28, 18, 21, 16	20.2 ± 2.42
B		
2.5×10^3	14, 2, 7, 7, 18, 6, 12	9.4 ± 2.07
5×10^3	10, 12, 17, 7, 16, 12, 10, 10	11.8 ± 1.18
10^4	50, 36, 25, 30, 26	33.4 ± 4.58

and 12th posttransplantation day. Cells harvested from the peritoneal cavity of the recipients on the 6th day were proved to be LS/BL cells. There is no doubt that the nodules observed in the livers were colonies of cells resulting from the proliferation of viable LS/BL cells.

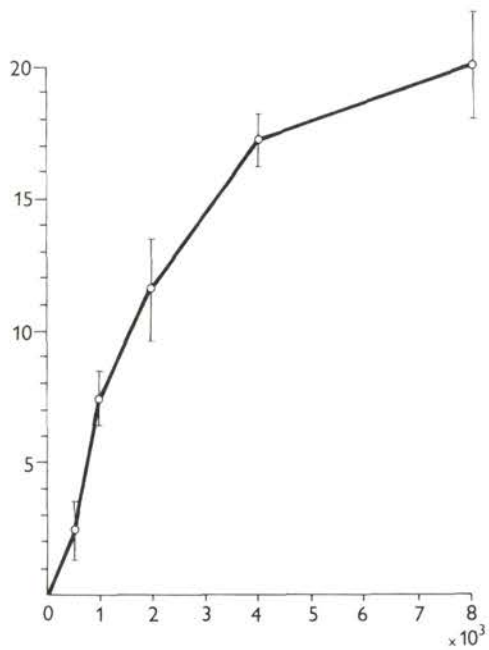

Fig. 4. The relatioship between the number of injected LS/BL cells and the number of colonies formed in the liver.

In further experiments we tried to determine if there was any association between the number of liver colonies and that of inoculated cells. Table 2 summarizes the results of colony counts from the livers of non-irradiated (*A*) and irradiated (*B*; 5 Gy) animals injected with different numbers of LS/BL cells. The results provided evidence for linear relationship between the number of liver colonies and that of inoculated cells (up to 4×10^3 cells per mouse). They also indicated that irradiation of recipient animals was not necessary as the number of colonies forming per 5×10^3 injected cells was 1.7 times higher in the livers of non-irradiated recipients than in the irradiated ones.

In another experiment non-irradiated animals were inoculated with increasing numbers of LS/BL cells (from 5×10^2 to 8×10^3). After injection of doses exceeding 10^4 LS/BL cells the liver colonies became confluent and not distinguishable. The results of this experiment shown in Fig. 4 proved again the linear relationship between the number of liver colonies and that of injected cells (up to 4×10^3 cells per mouse).

To test the possible influence of heavily irradiated cells, used as a feeder-layer in experiments performed in vitro, the suspensions of LS/BL cells were exposed to doses of 50 Gy in order to inactivate them. This suspension was then used as a diluent in preparing LS/BL dilutions; each aliquot contained 10^6 heavily irradiated cells in addition to the known number of viable cells. The results showed that under these experimental conditions there was little if any contribution of the cells killed to the take of the viable cells in the liver.

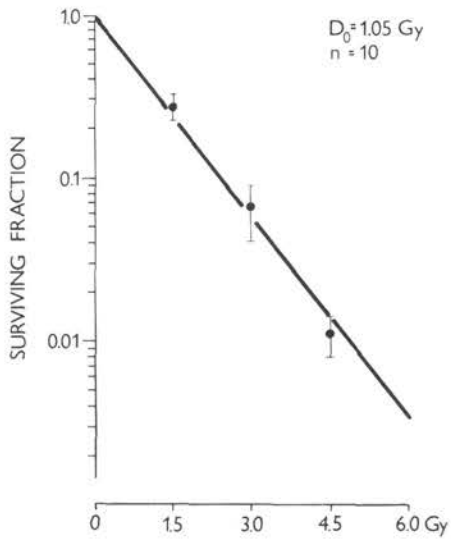

Fig. 5. X-ray survival curve for LS/BL cells.

To construct an X-ray survival curve for LS/BL cells the relationship between the number of injected cells and that of liver colonies was used. In vitro exposure of aliquots of LS/BL cell suspensions to X-rays was found to decrease the number of viable cells with increasing radiation dose. The resulting survival curve is characterized by a D_0 of 1.05 Gy and an extrapolation number of 1.0 (Fig. 5).

Discussion

The relationship between the intravenous inoculation with murine leukemic cells and the production of macroscopic colonies in the spleens of recipient animals has been reported by a considerable number of authors (Axelrad 1963; Bruce and Van der Gaag 1963; Bush and Bruce 1964; Wodinsky et al. 1967; Thomson and Axelrad 1968; Tanaka et al. 1970; Boranić et al. 1973). In applying this technique to lymphosarcoma LS/BL cells little if any relation between the injected cells and the number of macroscopicall visible spleen colonies could be observed in our experiments. The colonies of LS/BL cells mostly appeared on the surface of enlarged and hyperaemic spleens as scarcely distinguishable pale nodules. The histological examination of the spleens showed an extensive infiltration with leukemic cells. Irradiation of recipient animals enhanced the visibility of spleen colonies; under such conditions however the admixture of endogenous haematopoietic colonies had to be evaluated on the histological specimens.

As we failed in utilizing the spleen colony assay for measuring the growth of transplanted LS/BL cells we tried to find out whether the liver would be a more suitable environment for the production of leukemic colonies. The present experiments indicate that the liver colony assay may be used as a method for determining the proportion of viable lymphosarcoma LS/BL cells in inoculated suspensions. In the livers of recipient animals discrete colonies are formed and they may be easily counted after a short-term fixation. Histological examination of liver colonies in both irradiated and non-irradiated hosts revealed that they consisted entirely of lymphosarcoma cells. After removing and transplanting these colonies to host animals the clones of LS/BL cells could be established.

Heavily irradiated cells mixed with tumour cell suspensions were found to increase markedly the number of lung-colonies (Hill and Bush 1969). In our experiments the admixture of heavily irradiated cells to the injected suspension did not produce any effect on the number of liver-colonies. Nor did the use of whole-body irradiated recipients increase the colony numbers (Table 2 *B*). Finally, the assay has the advantage of being less time-consuming: it takes less than 2 weeks to complete it.

When evaluating the colony-forming efficiency the invasive character of LS/BL cells should be taken into account as shortly after transplantation these cells spread to virtually all the organs of the host. Only a proportion of viable LS/BL

cells arriving at the liver give rise to the colonies that may be counted. These considerations make it likely that each colony derives from a very small number of cells, possibly form one cell. After injection of 5×10^3 LS/BL cells 20 colonies were counted on the surface of one side of the left liver lobe. However, when counted in the entire liver the number of colonies will be much higher.

Since the tumor growth in vivo and the radiosensitivity are related due to the increase in D_0 during the logarithmic growth phase reaching a plateau after the 4th day after transplantation (Belli and Andrews 1963), we always harvested cells from the peritoneal cavity on the 6th day of their growth. The experimental results showed that the radiation sensitivity of LS/BL cells irradiated in vitro and proliferating in vivo, expressed as the dose required to reduce the number of viable cells to 37 % (D_0) is 1.05 Gy. These results are in a good agreement with others reported for murine leukemias (ranging from 1.3 to 1.6 Gy) (Hewitt and Wilson 1959; Bush and Bruce 1965; Silini and Maruyama 1965; Belli et al. 1967). Results of our earlier radiation experiments using the cell-dose — host survival time method yielded D_0 values for LS/BL cells ranging from 1.1 to 1.6 Gy. The difference could be ascribed to the lesser precision of the earlier method, where survival of host animals was used as the end point. Summarizing our results, we can say that the D_0 value for LS/BL cells is similar to the values reported for murine leukemic cells and to that for normal and malignant human cells in tissue culture (between 1.0 and 1.5 Gy; Puck et al. 1957). Thus our results support the hypothesis concerning the similarity between the radiation sensitivities of human cells grown in tissue celture and mouse lymphoma cells transplanted in vivo (McCulloch and Till 1960). The liver colony assay that has been used to determine the radiation sensitivity of the lymphosarcoma LS/BL cells can also be used whenever the number of viable tumour cells in a suspension is to be estimated.

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