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Quantitative Studies on Cancer Dissemination[1]

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SUMMARY

This paper describes an approach to estimating in a quantitative way the cancer cells present in blood and tissues of tumor-bearing animals. By using two transplantable tumors of mice, i.e., Ehrlich carcinoma and Sarcoma 180, in the ascites form, a relationship compatible with linearity was observed between the logarithm of cancer cells and either the weight of the tumor or the probit of the percent of tumor take. The number of cells necessary to obtain 50% of takes was considerably different when the cancer cells were included in fragments of different tissues or in the blood clot.

The number of cancer cells present at 7 and 12 days after transplantation in the blood and lung of mice bearing an intracerebral tumor was calculated by using the proposed method.

INTRODUCTION

An important aspect of cancer dissemination is represented by the appearance of cancer cells in blood and tissues before the formation of visible metastases. This problem has been studied with different approaches, but the lack of proper quantitative methodology has somewhat hampered the value and the significance of the results obtained. Cytologic methods to detect cancer cells have been favored in the last few years because they allow direct counting of cancer cells after suitable concentration and staining. Other methods (sedimentation, flotation, and filtration), which depend on the separation of cancer cells from normal cells in tissues, had only limited success because of the lack of reproducibility and low sensitivity (6).

The technic of separating cancer cells from normal cells by electrophoresis is still under investigation, but it requires a rather high number of circulating cancer cells because of the low sensitivity of the method (5). Other authors have injected radioisotope-labeled cancer cells intravenously and have successfully followed their fate and localization (2–4, 11, 12, 15). Finally a bioassay method has recently gained favor (1, 7–10). This method consists of transplanting into normal animals blood, tissue fragments, or tissue homogenates obtained from tumor-bearing animals. The growth of a tumor within a given period of time is taken as proof that at least one cancer cell was present in the transplanted tissue under study. Thus the dissemination of cancer cells could be followed in the body with the added advantage of allowing differentiation of living from dead cells. Previous work by Goldie (7), Greene (8, 9), and Karrer (10) has given evidence that cancer cells are present particularly in blood and lung of animals bearing various types of tumors. However, no systematic efforts have been made to establish the sensitivity of the bioassay method in a quantitative way. The purpose of this paper is to study such an aspect in connection with previous findings concerning the dissemination of tumors implanted intracerebrally (13, 14).

MATERIALS AND METHODS

Female Swiss mice of the average weight of 20 gm were kept in Makrolon cages (size, 20 x 26 x 13 cm) at room temperature of 22°C and relative humidity of 60%, with free access to water and food during the experiments. Ehrlich carcinoma and Sarcoma 180 in the ascites form, maintained in the same strain of animals for more than 250 serial passages, were diluted with phosphate buffer solution and transplanted into the brain of mice according to a technic previously described (13). The cancer cells were always counted under a microscope. The number of leukocytes present in the ascites was not included among the number of cells given in the following text or in the tables.

As previously reported (13), the presence of cancer cells in blood and tissues was detected after subcutaneous transplantation of known amounts of blood clot or tissues into normal mice. The evaluation of the presence of cancer cells in blood or tissues was made by establishing the percentage of tumor takes and the average of tumor weights. The nature of this technic required the use of a large number of mice for each determination (about 50 or more mice per point). From each donor mouse two blood fragments and two lung fragments were obtained, and each fragment of each tissue was transplanted into a recipient mouse.

Vital staining performed with neutral red indicated that both Ehrlich carcinoma and Sarcoma 180 cells were viable at room temperature for the time required to perform the transplantation (less than 2 hours).

RESULTS

Take of Cancer Cells Implanted Subcutaneously. The influence of blood or tissues on the take of a given number of cancer cells implanted subcutaneously was evaluated. For this purpose 40 ± 10 mg of blood clot, lung, liver, and brain, taken from normal mice, were injected with a volume of 0.01 ml

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containing 10, 100, 1,000, 10,000 or more cancer cells; the tissue was then transplanted subcutaneously into normal recipient mice. A group injected subcutaneously with the same number of cancer cells suspended in phosphate buffer in the same volume without any tissue substrate served as control. Charts 1 and 2 report the percent of takes present 30 days after the subcutaneous transplantation. A linear relationship between the percent of takes, expressed in probits, and the logarithms of numbers of implanted cancer cells is evident. These data are summarized in Table 1, where the equations of the regression lines for the various tissues inoculated with either tumor are reported. Table 1 shows also the number of cells necessary to obtain the take in 50% of the implanted mice (ED_{50}) for the various tissues. This concentration of cells, together with 95% fiducial limits, indicates that the virulence of cancer cells is clearly modified depending on the tissue in which they have been incorporated.

It should be noticed that leukocytes were present in addition to cancer cells in the ascites, which amounted to 6.3% of the total cells for Sarcoma 180 and 9.5% for Ehrlich carcinoma. It is presently unknown if the number of leukocytes influence the ED_{50}.

Although there is no parallelism among the various regression lines, the ratio between the ED_{50} obtained with cancer cells suspended in buffer solution and the ED_{50} calculated for the cells injected in the different tissues represents an estimate of this change in virulence. For instance, the ED_{50} of Ehrlich cells suspended in buffer or injected in the brain is 5, 10, and 15 times higher than the ED_{50} obtained injecting the cells in lung, liver, or blood respectively.

The difference is even higher for Sarcoma 180. In this case the ED_{50} of cancer cells injected in blood or lung is respectively 74 and 13 times lower compared with the cells suspended in buffer. It may also be added that in all the experimental conditions the ED_{50} of Sarcoma 180 cells was always higher than the ED_{50} of Ehrlich carcinoma cells.

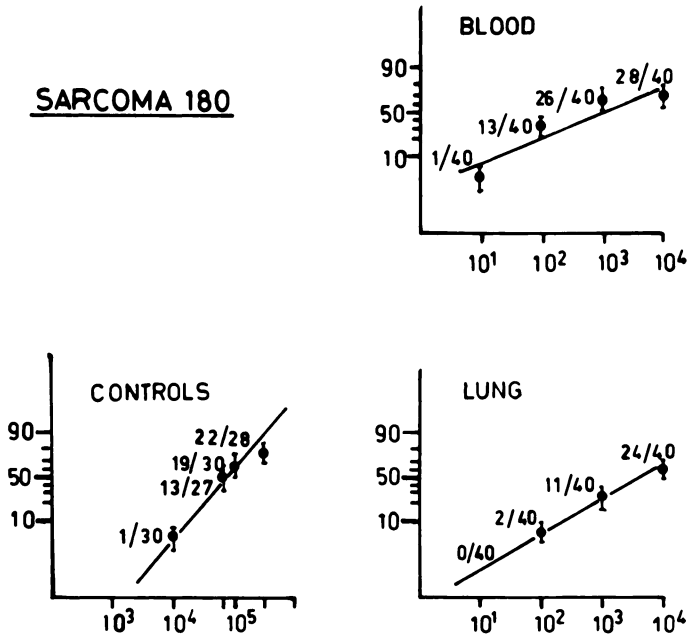

Chart 1. Takes of Sarcoma 180 cells diluted in buffer phosphate (controls) or included in various tissues and transplanted subcutaneously into normal mice. On the ordinates (probit scale), the percent of takes; on the abscissae, number of cancer cells (log scale). The numbers represent ratios between the number of mice showing tumors and the number of animals injected with cancer cells. The vertical bars represent the standard errors calculated on the percent of takes obtained in groups of 8–10 mice each. Data obtained 30 days after transplantation.

Tumor Weight after Subcutaneous Implantation of Cancer Cells

Using the experimental conditions above described, the weight of the tumor was determined 30 days after subcutaneous transplantation of cancer cells in each tissue. Table 2 shows that, for Sarcoma 180 and Ehrlich carcinoma injected in blood or lung, there is a significant correlation between the number of cells and the resulting weight of the tumors. For cancer cells transplanted in buffer solution, such correlation was not present, perhaps because the number of animals used was insufficient.

Attempts to Estimate the Number of Cancer Cells in Blood and Lung of Mice Bearing an Intracerebral Tumor

In two different experiments 100,000 cells of Sarcoma 180 or Ehrlich carcinoma were injected intracerebrally. Seven and 12 days after tumor transplantation, fragments of about 40 mg of blood clot and lung obtained from both series of animals were transplanted subcutaneously in normal mice.

Table 3 reports the takes and the tumor weights evaluated 30 days after this transplantation and the estimated numbers of cancer cells present in the blood or the lung of intracerebrally tumor-bearing animals. These values have been obtained by using the regression lines reported in Table 1 for the percent of takes and in Table 2 for the weight of the tumors. The numbers of cancer cells calculated according to both methods are in satisfactory agreement, although there is, as expected, a large variability. It is evident that at any time the number of cancer cells present in lung is higher than that present in blood. Furthermore, after intracerebral transplantation the number of cancer cells tends to increase for both tumors and for both tissues.

DISCUSSION

Known concentrations of Sarcoma 180 or Ehrlich carcinoma cells were transplanted either suspended in buffer or inoculated in tissue fragments subcutaneously into normal animals, and the number of takes and tumor weight were determined. The ED_{50} was about 11 times higher for Sarcoma 180 than for Ehrlich carcinoma when cells were suspended in buffer.

Transplantation of tissue fragments (blood, lung, liver, brain) containing cancer cells resulted in a considerable decrease of the ED_{50} except for brain. The lung decreased the ED_{50} by a factor of about 13 and 5 respectively for Sarcoma 180 and Ehrlich carcinoma. The liver was also effective because it reduced the ED_{50} about 10 times for Ehrlich carcinoma. The

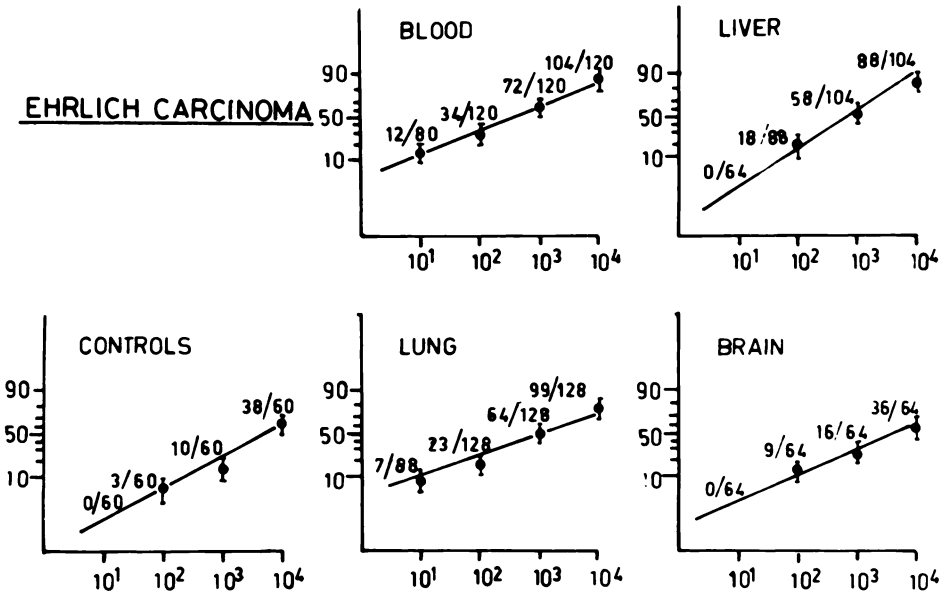

Chart 2. In these experiments Ehrlich carcinoma cells were used according to the conditions reported under Chart 1.

inclusion of cancer cells in blood clots resulted in a striking decrease of the ED_{50} for both tumors, i.e., about 15 times for Ehrlich carcinoma and about 74 times for Sarcoma 180.

A factor which could not be controlled in these experiments was the number of cells actually present in the tissue fragments or blood clots at the moment of the transplantation. Actually the effect of blood clot could be due to the fibrin network, which retained the cancer cells better than the other tissues. Therefore the figures given as ED_{50} may, in fact, be only approximated because of the differential escape of cells from the tissues or the blood clot during the necessary manipulations. In any case, it is evident from the results obtained that the virulence of the tumor cells seems much increased when they are incorporated with transplanted tissues. The reasons for such an effect are presently unknown, although several possible explanations could be suggested. It may be due to a weakening effect of the transplanted tissue on the immunologic defenses of the host or to the fact that the cells are less readily cleared out from the subcutaneous site or simply because of the nutritional medium provided to the cells by the tissues.

The relationship between the probit of percent of takes versus the logarithm of number of cells was compatible with linearity in a range from 10 to 200,000 cancer cells injected in a fragment of 40 ± 10 mg of tissue or blood clot. The high sensitivity of this bioassay permits the detection of Ehrlich carcinoma cells in blood in concentrations as low as 10 cells per 0.04 ml of blood. This may represent an advantage over cytologic methods, considering that in this volume of blood about 2.4×10^8 normal cells are present. Although the bioassay procedure is quite sensitive, the degree of precision is relatively low because a 10-fold increase in the inoculum of cancer cells only results in a moderate increase of the percent of tumor takes. Satisfactory results were obtained also when the parameter of the weight of the tumor rather than the percent of takes was used. Both parameters were then used to evaluate the number of cancer cells disseminated from a tumor implanted intracerebrally in mice.

Good agreement was found by using the two methods, and therefore only one set of data will be used for the following considerations. According to the parameter of tumor weight, about 9 and 62 cancer cells/cu mm are present in the blood of

Table 1

Tissue used to include cancer cells	Total no. of mice	Regression line (in probit)	Coefficient of regression (r)	ED_{50} (and 95% confidence limits)	$\frac{ED_{50}\text{ buffer}}{ED_{50}\text{ tissue}}$
Ehrlich					
Buffer phosphate	240	$y = 1.62 + 0.89x$	0.99	6,270 (1,850–21,230)	
Blood	440	$y = 3.14 + 0.71x$	0.99	416 (260–650)	15
Lung	472	$y = 2.99 + 0.65x$	0.98	1,200 (510–2,980)	5
Liver	360	$y = 1.68 + 1.19x$	0.96	620 (420–900)	10
Brain	256	$y = 2.26 + 0.72x$	0.98	6,400 (1,300–31,000)	
Sarcoma 180					
Buffer phosphate	111	$y = -5.49 + 2.16x$	0.98	71,800 (47,800–109,000)	
Blood	160	$y = 2.73 + 0.76x$	0.96	970 (410–2,280)	74
Lung	160	$y = 1.49 + 0.94x$	0.99	5,400 (1,600–18,400)	13

Relationship between number of cancer cells included in normal tissues and tumor take obtained 30 days after subcutaneous transplantation.

Table 2

	Weight of tumors in gm ± S.E. after						Total no. of tumors	Regression line ($y = a + bx$)	Test for independence[a]			
	10^1	10^2	10^3	10^4	5×10^4	10^5			r	P	t	P
Sarcoma 180												
Blood	0.18	0.47 ± 0.08	0.54 ± 0.14	1.19 ± 0.22	1.77 ± 0.29	2.43 ± 0.31	119	$y = -1.28 + 0.68x$	0.42	<0.01	4.9	<0.01
Lung		0.17 ± 0.03	0.30 ± 0.05	0.60 ± 0.09	1.11 ± 0.21	1.40 ± 0.36	85	$y = -1.30 + 0.52x$	0.49	<0.01	4.6	<0.01
Ehrlich carcinoma												
Blood	0.44 ± 0.06	0.46 ± 0.06	0.93 ± 0.09	2.26 ± 0.15			245	$y = -0.76 + 0.69x$	0.45	<0.01	8.7	<0.01
Lung	0.36 ± 0.06	0.39 ± 0.06	0.50 ± 0.05	1.10 ± 0.06			186	$y = -0.37 + 0.34x$	0.37	<0.01	7.1	<0.01

Relationship between the logarithm of cancer cells included in normal tissues and weight of the tumor (same experimental conditions reported in Charts 1 and 2).
[a]The relationship between the log of cancer cells and the weight of the tumor was tested with two methods: the correlation coefficient (r) and the "t" test calculated on the "b" of the regression line.

Table 3

Tumor implanted intracerebrally	Day after intracerebral transplantation	Tissue transplanted for bioassay	Results of bioassay			Estimated number of cancer cells (per cu mm or mg) according to	
			No. of takes/ No. of transplantations	% takes	Tumor weight (gm ± S.E.)	% takes	Tumor weight
Sarcoma 180	7	Blood	28/59	47	0.63 ± 0.16	20	16
	12	Blood	32/45	71	1.20 ± 0.14	125	105
	7	Lung	28/60	46	0.61 ± 0.16	105	125
	12	Lung	47/57	82	1.05 ± 0.12	>250	700
Ehrlich carcinoma	7	Blood	60/95	63	1.00 ± 0.11	32	9
	12	Blood	69/89	77	1.59 ± 0.15	112	62
	7	Lung	59/94	62	0.96 ± 0.12	100	220
	12	Lung	73/96	76	1.44 ± 0.14	450	>500[a]

Estimated number of cancer cells present in blood and lung of mice bearing Sarcoma 180 or Ehrlich carcinoma transplanted intracerebrally.
[a]The precise number has not been calculated because the result of the bioassay was higher than the experimental data of the reference line.

mice bearing intracerebral Ehrlich carcinoma at the 7th and 12th day after tumor transplantation. At these times, in animals bearing an intracerebral Sarcoma 180, about 16 and 105 cancer cells per cu mm were found. It must be emphasized that these figures represent only a crude estimate because of the large variability observed. Considering that the blood volume of a mouse is about 2 ml, it follows that the total number of cancer cells present in blood rises in 5 days (7th to 12th day) from about 18,000 to 144,000 cells (Ehrlich carcinoma) and from 32,000 to 210,000 (Sarcoma 180), which corresponds to an average increase of about 25,000 and 35,000 cancer cells respectively per day. It is likely that this increase is due to a continuous flow of cancer cells from the tumor growing in the brain. However, it cannot be denied that some cancer cells present in blood may actually come from proliferating cells deposited in lung.

Previous studies have indicated that other tissues such as liver, kidney, and lymph nodes of intracerebral tumor-bearing animals contain only a relatively low number of cancer cells (13). The number of cancer cells present in the lung of mice implanted intracerebrally with Sarcoma 180 was about 125 and 700/mg of tissue at 7 and 12 days after tumor transplantation. Considering an average weight for lung of about 150 mg and assuming homogeneous distribution, it follows that a total number of about 18,750 and 105,000 cancer cells were present in lung tissue at the considered times. If these data are compared with the total number of cancer cells present in the blood (32,000 and 210,000) at the same times, it is evident that the cells estimated to be in the lung cannot be due only to the cells present in the blood of the pulmonary tissue.

Similar considerations can be made for Ehrlich carcinoma. The results obtained in this investigation indicate the possible value of this bioassay technic for a quantitative estimate of the number of cancer cells involved in the various phases of cancer dissemination.

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