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Distribution of Cardiac Output during Development of Two Metastasizing Murine Tumors*†

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Abstract—*The study of cardiac output (CO) distribution to tumors and metastases is of interest for a better understanding of tumor biology and for pharmacological approaches. A radioactive microsphere method was developed to assess CO distribution in C57BL/6J mice bearing syngeneic 3LL or BALB/c mice with JWS. At the initial stages of cancer growth, the CO relative fractions per g of tissue (%CO/g) to 3LL and JWS were similar to those in surrounding tissues. In both tumors a positive, significant correlation was found between tumor weight and tumor CO fraction, but %CO/g was lower in 3LL 2 and 3 weeks after transplantation, whereas it did not change in JWS. Indeed, much larger necrotic areas developed in 3LL than in JWS. The %CO/g to the lungs increased in both models when metastases were not yet visible; subsequently, the appearance of lung nodules was accompanied by a decrease of %CO/g in JWS and a further increase in 3LL. This corresponded to the much higher ratio of metastatic to intact lung tissue in JWS than in 3LL. In fact, isolated lung metastases had a significantly lower blood supply than the surrounding tissue. This might be due to a different vascularization pattern and/or smaller amounts of vasodilator substances being produced by metastatic nodules; the latter is suggested by lower generation of prostacyclin activity in isolated lung metastases than in intact pulmonary tissue.*

INTRODUCTION

INFORMATION about blood supply to experimental tumors and their metastases would be very useful to increase knowledge of the biology of cancer cell growth and dissemination, and their possible pharmacological modulation.

Although several studies have been conducted on blood flow in experimental tumors [1–6], no data are apparently available about blood supply to metastatic nodules. Moreover, rat and rabbit tumors have been investigated much more than mouse tumors, presumably due to difficulties in setting up the method for blood supply measurement in very small animals.

Most metastasis models and pharmacokinetic data on antitumoral drugs are available for the mouse; since it has so far been technically impossible to evaluate directly blood supply to organs and tissues in mice, we have set up a method to measure the cardiac output distribution in tumor-bearing mice [7].

In this paper we report data on cardiac output distribution to primary tumor, metastases and other tissues at various stages during development of two different murine metastasizing tumors.

MATERIALS AND METHODS

Animals

Male C57BL/6J mice, obtained from Charles River Breeding Laboratory (Calco, Italy) and

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weighing 19–23 g at the start of the experiment, were used. The animals were kept in plastic cages in an air-conditioned room with a constant day-light rhythm and free access to food pellets and water.

Tumors

The Lewis lung carcinoma (3LL) was maintained by intramuscular passages in C57BL/6J mice every 3 weeks. Cell suspensions were prepared by mechanical homogenization in phosphate-buffered saline (Dulbecco) in a Virtis homogenizer, washed and resuspended in saline [8]. Animals were transplanted i.m. with 2×10^5 cells into the left hind limb. Characteristics of this spontaneously metastasizing lung tumor were previously described [8].

The JW sarcoma (JWS) arose in 1974 as a spontaneous lung tumor in a BALB/c mouse [9] and was maintained in ascitic form by weekly passages of cells collected from the peritoneal cavity. When tumoral cells were implanted s.c. a solid tumor was obtained which metastasized to the lungs. The characteristics of this tumor were described elsewhere [10]. In our study BALB/c mice were injected subcutaneously into the upper part of the back with 5×10^4 cells suspended in saline.

Method for assessment of cardiac output (CO) distribution

The determination of CO distribution has been made by a radioactive microsphere technique [11].

This is based on the principle of measuring the distribution of an indicator during the first transit [12]. When the microspheres are injected in the circulation (usually in the left ventricle or the left atrium) their distribution to any organ can be measured by counting the radioactivity present in that organ, since they are too large to pass through the capillary beds. When the following assumptions are fulfilled the distribution of microspheres to an organ reflects the distribution of cardiac output to that organ: (1) the microspheres are mixed uniformly with the blood so that their distribution is proportional to flow; (2) the microspheres are sufficiently large so that they are removed by the capillaries on the first transit; (3) the number of microspheres present in any sample must be sufficient to minimize random variability [13]. In previous experiments [7] the conditions were sought to fulfil these requirements when the microsphere technique was applied to the mouse. When microspheres were injected into the mouse left ventricle (previously cannulated) 100% trapping by capillary beds in the first passage was obtained. Then the ratio of activity in any organ to the total radioactivity injected represented the fraction of CO to that organ:

$$\%CO = \frac{\text{radioactivity in the organ}}{\text{total body radioactivity}} \times 100.$$

In the experiments reported here the mixing of microspheres with the blood was controlled in each animal by measuring the radioactivity present in two symmetrical organs, the kidneys. As shown in Fig. 1, the correlation between %CO to the right and left kidneys was 0.97 both in C57BL/6J mice bearing the 3LL and in BALB/c bearing the JWS. Possible recirculation of microspheres in tumor-bearing mice and shunting of the beads through tumor vasculature were controlled in a group of 6 mice bearing 3LL tumors of 6.3–8.5 g: the radioactivity detected in atrial blood during 2 min following microsphere injection ranged between 0.03 and 0.16% of the total radioactivity injected, thus representing a negligible fraction which cannot significantly interfere with %CO measurements in the lungs. Moreover, radioactivity present in blood collected from the inferior abdominal vena cava, which draws blood from the primary 3LL tumor, was found to be 0.01–0.02% of the total radioactivity injected and 0.28–0.70% of the radioactivity measured in the corresponding tumor tissue.

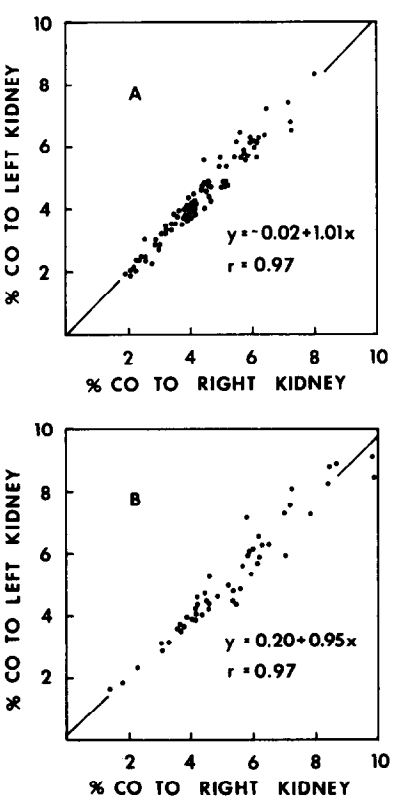

Fig. 1. Regression analysis of CO fractions to the right and left kidneys. (A) C57BL/6J mice bearing 3LL; (B) BALB/c mice bearing JWS.

The number of microspheres present in all samples of tissues, organs or tumor always largely exceeded, even in the smallest samples, the minimal number required to avoid random variability [13].

Experimental procedure

The animals were anesthetized by intraperitoneal injection of urethane (1.25 g/kg body wt). The left ventricle was cannulated via the right carotid artery with a Portex PP 10 catheter, the end of which had been sharpened to less than 0.4 mm. That the right carotid catheter was actually placed in the left ventricle was confirmed by blood pressure records using a Grass polygraph. About 150,000 ^{57}Co-labeled microspheres (15 μm diameter) suspended in 50 μl of rat serum were injected into the left ventricle during 20–30 sec; the catheter was flushed with an additional 50 μl of saline. Four minutes later the animals were killed by an overdose of urethane and their organs dissected, weighed and placed in counting vials for radioactivity measurement in a Packard well-type scintillation counter. The following organs and tissues were taken: heart, lungs, kidneys, spleen, small intestine, brain, right hind limb muscle, skin surrounding the tumor and tumor. The residual bodies were also placed into counting vials, to calculate the total radioactivity administered. In addition, metastases were isolated from the surrounding pulmonary tissue of 17 3LL-bearing animals 3 weeks after tumor implantation and the radioactivity was measured separately. The infiltrative growth of lung metastases in JWS made it very difficult to separate them from the surrounding tissue; therefore the CO fraction was determined only in total lungs of JWS-bearing mice. Radioactivity was also measured separately in necrotic and living tumoral tissue of 7 mice with 3LL 3 weeks after implantation. Careful dissection of necrotic and non-necrotic portions of the tumor was performed under microscopic guidance. In contrast, JWS had very small necrotic foci (6 weeks after implantation) which could not be separated from living tumoral tissue.

Because environmental temperature changes may induce redistribution of CO [13], all the experiments were performed at a constant temperature of 22–23°C.

Experimental groups

Cardiac output distribution has been determined in the following groups of mice (labeled as in the Figs): C57BL/6J: 0, control animals ($n = 21$); 1, 1 week after 3LL implantation ($n = 19$); 2, 2 weeks after 3LL implantation ($n = 17$); 3, 3 weeks after 3LL implantation ($n = 19$). The CO distribution was also studied in a further group of 10 mice, 11 days after 3LL implantation; BALB/c: 0, control animals ($n = 16$); 2, 2 weeks after JWS implantation ($n = 14$); 4, 4 weeks after JWS implantation ($n = 14$); 6, 6 weeks after JWS implantation ($n = 6$).

Microsphere and drugs

Tracer Sephadex beads (15 ± 1.5 μm in diameter) labeled with ^{57}Co and mixed in Ficoll 10% solution in 0.9% saline (Pharmacia Fine Chemicals, Uppsala, Sweden) were used. The bead suspension was centrifuged and the supernatant discarded and replaced by rat serum. In this way the administration of microspheres did not disturb significantly the animals' conditions, as controled in both normal [7] and tumor-bearing mice [14].

Heparin (Liquemine, Roche) was administered, before the left ventricle cannulation, into the right carotid artery of mice at a dose of 1000 U/kg body wt for prevention of blood clotting.

Statistical analysis

Differences in cardiac output fractions among the experimental groups were analyzed using the Duncan new multiple range test. Other comparisons between means were evaluated using the umpaired or paired Student's t test. All these statistical analyses were done after logarithmical transformation of the data. Various types of correlations were analyzed by the minimum squares method using a Hewlett Packard calculator.

RESULTS

Cardiac output distribution in 3LL-bearing and control mice

Table 1 shows the evolution of the weight in tumors, lungs (with metastases), liver and spleen of mice during development of 3LL.

Figure 2 reports the values of CO distribution in control C57BL/6J mice and in mice studied 1, 2 and 3 weeks after 3LL implantation. The CO fractions to muscle, skin, tumor and to organs whose weight changed during tumor growth have also been calculated per g of tissue (%CO/g) in Fig. 3.

After the first week no significant differences were observed between control and tumor-bearing mice in the distribution of CO to the various organs; the value of %CO/g to the tumor (2.37 ± 0.23) was close to the value measured in the hind limb muscle (2.62 ± 0.35). In the following weeks the %CO to primary tumor progressively increased and a positive, significant correlation

Table 1. Evolution of the weight (g) of the primary tumor and of some of the host's organs during 3LL growth (means ± S.E.)

	Control ($n=21$)	Time after transplantation 1 week ($n=19$)	2 weeks ($n=17$)	3 weeks ($n=19$)
Lungs	0.154 ± 0.005	0.156 ± 0.007	0.163 ± 0.004	0.199 ± 0.010*†‡
Liver	1.255 ± 0.040	1.223 ± 0.050	1.381 ± 0.066	1.422 ± 0.070*†
Spleen	0.083 ± 0.003	0.099 ± 0.005	0.230 ± 0.013*†	0.302 ± 0.014*†‡
Tumor		0.483 ± 0.037	5.092 ± 0.367†	8.538 ± 0.427†‡

*$P < 0.05$ vs control group.
†$P < 0.05$ vs the 1-week group.
‡$P < 0.05$ vs the 2-week group.

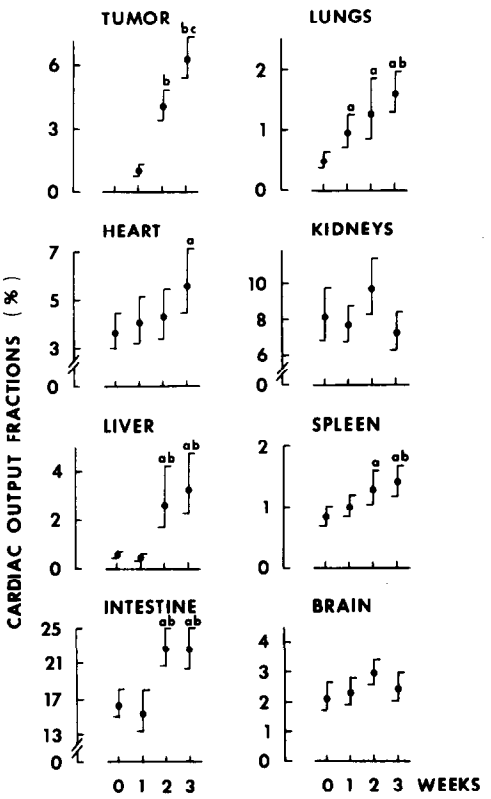

Fig. 2. Cardiac output fractions (%CO) to 3LL primary tumor and to the main host organs. 0, C57BL/6J tumor-free mice; 1, 2 and 3, weeks after i.m. 3LL implantation; a, b and c indicate P < 0.05 vs 0, 1 and 2 respectively, according to the Duncan new multiple range test applied to the logarithmically transformed data. Vertical bars represent the 95% confidence limits.

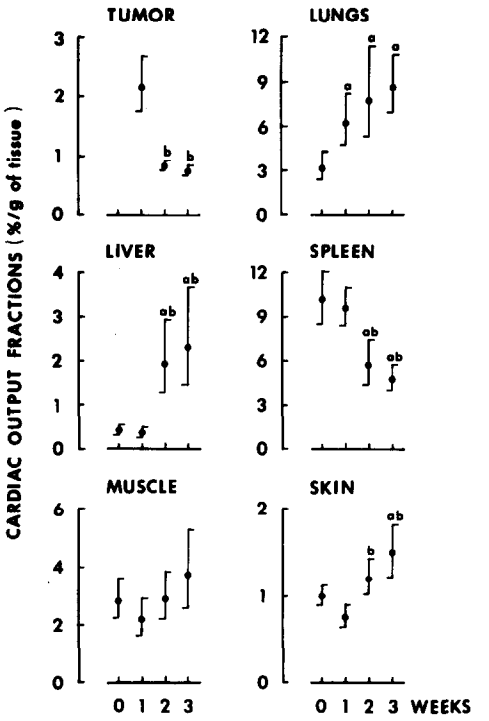

Fig. 3. Cardiac output relative fraction (%CO/g of tissue) to 3LL primary tumor and some host tissues. 0, C57BL/6J tumor-free mice; 1, 2 and 3, weeks after i.m. 3LL implantation; a, b and c indicate P < 0.05 vs 0, 1 and 2 respectively, according to the Duncan new multiple range test. Vertical bars represent the 95% confidence limits.

was found between %CO to the tumor and tumor weight ($y = 0.688 \pm 0.68x$, $r = 0.90$, $P < 0.01$).

However, the %CO/g at 2 and 3 weeks of tumor growth was smaller than that measured 1 week after implantation (Fig. 3). In an additional group of 10 mice studied 11 days after implantation the %CO/g was already found to be significantly lower than that measured after 1 week ($P < 0.05$). The decrease in relative CO distribution to tumor tissue was also evident when the %CO/g was plotted against the corresponding tumor weight; indeed, the blood supply per g of tissue appeared drastically reduced to tumors weighing more than 1 g (Fig. 4).

After 3 weeks %CO/g to the living 3LL tissue was about 10-fold higher than that to the necrotic areas (Table 2). It was, however, lower ($P < 0.05$) than %CO to the 1-week tumor. Both %CO and blood flow to the lung increased during tumor growth. %CO/g to the metastases, isolated from the lung after 3 weeks, was significantly lower ($P < 0.001$) than that to the surrounding pulmonary tissue (Table 3). However, it was higher than that to the living primary tumor tissue both at initial (1 week) and later stages of growth.

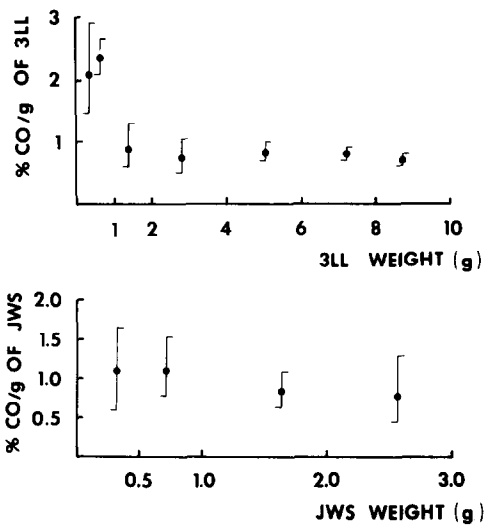

Fig. 4. Fraction of cardiac output per g of tumor weight (%CO/g) to 3LL and JWS, plotted against the corresponding tumor weight. Each point represents the mean fractional supply to 3–6 tumors in that weight range. Vertical bars represents the 95% confidence limits.

During tumor growth both %CO and %CO/g to the liver were significantly increased; %CO to the spleen increased too but, due to splenomegaly, %CO/g was progressively decreased ($P < 0.01$).

Radioactivity found in the small intestine 2 and 3 weeks after tumor implantation was higher than in control mice. An increase was also found after 3 weeks in the heart and skin surrounding the tumor.

CO distribution in control and JWS-bearing mice

Table 4 shows the evolution of tumor, lung (with metastases), liver and spleen weight of mice during development of JWS.

Figure 5 shows the value of CO distribution in control mice and in mice studied 2, 4 and 6 weeks after JWS implantation. The corresponding %CO/g are shown in Fig. 6.

The %CO to JWS progressively increased during tumor growth; there was a significant positive correlation between tumor weight and %CO to the tumor ($y = 0.088 + 0.934x$, $r = 0.65$, $P < 0.01$), but no correlation with CO/g. The latter was lower after 4 weeks than after 2 or 6 weeks (Fig. 4).

At all the stages of tumor growth studied no significant differences were found between %CO/g to JWS and that to the skin surrounding the tumor. The %CO to the lungs (with metastases) at 4 and 6 weeks was smaller than that after 2 weeks of tumor growth.

At 2 and 4 weeks no differences were observed between control and JWS mice in CO distribution to the other tissues.

Although there were no significant changes in

Table 2. Cardiac output distribution in 3LL primary tumor 3 weeks after implantation

	Weight ± S.E. (g)	%CO	%CO/g
Living tissue	3.44 ± 0.24	5.55 (4.66–6.62)	1.64 (1.31–2.06)
Necrotic tissue	4.37 ± 0.20	0.45 (0.13–1.54)	0.10 (0.03–0.34)

Mean values and their 95% confidence limits (in parentheses) are shown ($n = 7$).

Table 3. Cardiac output distribution in 3LL tissues

	n	Weight ± S.E. (g)	%CO	%CO/g
Primary (1 week)	12	0.39 ± 0.03	0.78 (0.5–1.13)	2.07 (1.47–2.92)
Primary (3 weeks)	19	7.82 ± 0.25	6.26 (5.35–7.32)	0.75 (0.67–0.85)
Metastatic lung	17	0.21 ± 0.01	1.55 (1.31–2.09)	8.12 (6.33–10.40)
Metastasis-free lung	17	0.15 ± 0.05	1.47 (1.17–1.85)	10.12 (7.92–12.93)
Isolated metastases	17	0.06 ± 0.01	0.17 (0.12–0.24)	3.64 (2.79–4.76)*

Mean values and their 95% confidence limits (in parentheses) are shown.

*The unpaired Student's t test using logarithmically transformed data gives statistically significant differences with respect to primary 1-week ($P < 0.05$) and primary 3-week tumors ($P < 0.001$) and metastasis-free lung ($P < 0.001$).

Table 4. Evolution of the weight (g) of primary tumor and some of the host's organs weight during JWS growth (means ± S.E.)

	Time after transplantation			
	Control ($n = 16$)	2 weeks ($n = 14$)	3 weeks ($n = 14$)	6 weeks ($n = 6$)
Lungs	0.163 ± 0.021	0.164 ± 0.009	0.213 ± 0.016	0.410 ± 0.053*†‡
Liver	1.012 ± 0.042	1.212 ± 0.064	1.264 ± 0.064	1.189 ± 0.084
Spleen	0.124 ± 0.007	0.150 ± 0.010	0.184 ± 0.009*	0.159 ± 0.013
Tumor		0.495 ± 0.058	1.843 ± 0.186	2.225 ± 0.147†‡

*$P < 0.05$ vs control group.
†$P < 0.05$ vs 2-week group.
‡$P < 0.05$ vs 4-week group.

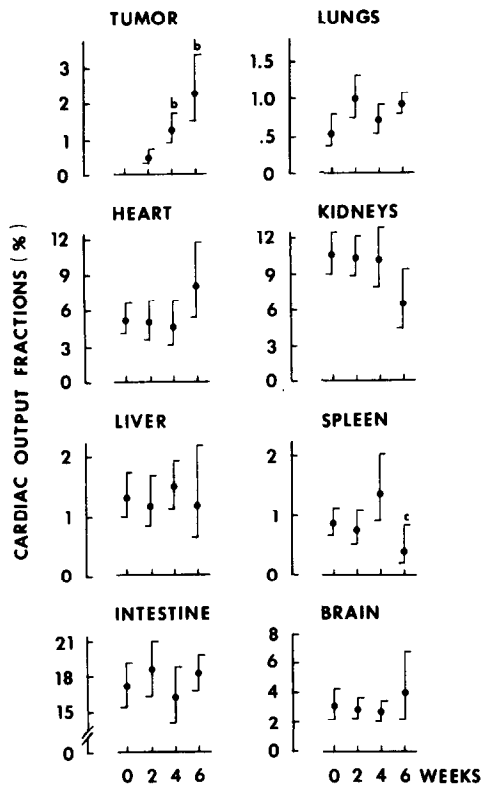

Fig. 5. Cardiac output fraction (%CO) to JWS primary tumor and to the main host organs. 0, BALB/c tumor-free mice; 2, 4 and 6, weeks after s.c. JWS implantation; a, b and c indicate P < 0.05 vs 0, 2 and 4 respectively, according to the Duncan new multiple range test. Vertical bars represent the 95% confidence limits.

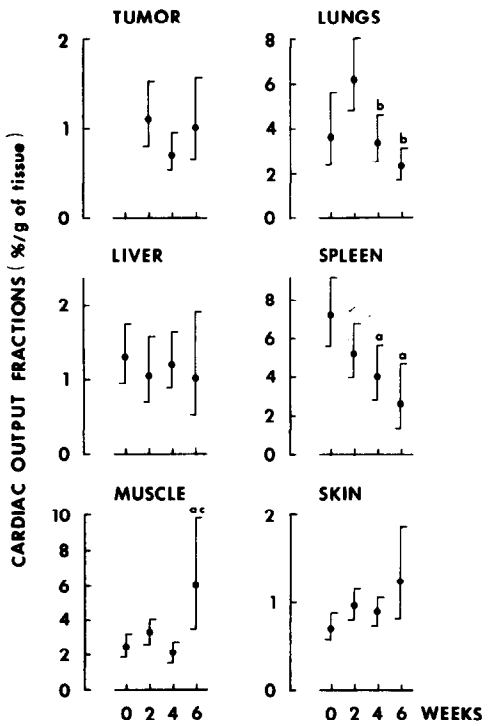

Fig. 6. CO relative fractions (%CO/g of tissue) to JWS primary tumor and some host tissues. 0, BALB/c tumor-free mice; 2, 4 and 6, weeks after s.c. JWS implantation; a, b and c indicate P < 0.05 vs 0, 2 and 4 respectively, according to the Duncan new multiple range test. Vertical bars represent the 95% confidence limits.

%CO to the spleen, its %CO/g progressively decreased due to the development of splenomegaly during tumor growth.

DISCUSSION

The microsphere technique in mice

This study has evaluated the changes in cardiac output distribution to tumoral tissues and other organs at various times during development of two murine experimental tumors metastasizing to the lungs (3LL and JWS). For this purpose a radioactive microsphere technique has been used. Such a method is widely employed to measure organ blood flow and CO distribution in some animal species and recently it has been applied to evaluation of tumoral blood flow in the rabbit [15] and the rat [6, 16]. From previous studies [7] two main limits emerged in interpretation of the results: (1) since a blood reference sample cannot be collected, only semi-quantitative data on blood flow are obtained, e.g. on the fraction of CO arriving to each organ: from CO values the organ blood flow data, in ml/min, can be easily obtained; (2) the need to work with anesthetized mice. It has been shown that anesthesia may alter

the tumor blood flow [4]; we cannot exclude such an interference in our model.

On the other hand, this method has some advantages with respect to the other methods for measurement of CO distribution. Indeed, the use of ^{86}Rb does not provide information on the CO fraction to the brain and over-estimates those to the lungs and liver [17, 18].

Quantitative tumoral blood flow data can be obtained with the clearance of ^{133}Xe, but at the same time blood flow to surrounding tissue and to the main organs cannot be determined. Despite the above-mentioned limitations in working with anesthetized mice, the latter are under a 'standardized' condition; the administration of indicators into the tail vein or intratumorally to unanesthetized mice, in contrast, stresses the animals; as a consequence, tumoral and organ blood flow can be modified in an uncontroled manner.

Moreover, with the microsphere technique it is possible to make two determinations of CO distribution in the same animal, thus allowing evaluation of the acute effects of drugs or other conditions. A more detailed description of the application of this method in mice has been reported previously [7].

As it appears from our results, standard errors were of a reasonable size, indicating that the method gives consistent results on the various tissues, including the tumors.

CO distribution to primary 3LL and JWS

At the initial stage of tumor growth the JWS had a smaller %CO/g than the 3LL. This difference could be due to the different tumor characteristics or to the different host tissue: subcutaneous for JWS and muscular for the 3LL. Gullino and Grantham [1] and Edlich and co-workers [19] have found that tumor blood supply is independent of the host tissue, while Young and co-workers [20] have recently reported different blood flow to the same tumor implanted in different tissues.

It is interesting that the relative CO to JWS and to the surrounding skin were similar; the same was true for the %CO to 3LL (at the initial stage of growth) when compared to that to the surrounding muscle. The higher relative fraction of CO to the carcinoma than to the sarcoma found in our study is in agreement with data from other authors [2, 21].

After 11 days and 2 weeks of implantation the %CO/g to 3LL significantly decreased (to less than 50% with respect to the first week). After 2 weeks necrosis could be observed microscopically (L. Morasca, personal communication). Thus it seems that the initial decrease in %CO/g to 3LL tissue was not secondary to necrosis but, rather, preceded it.

It has been recently reported [20] that in the V2 carcinoma of the rabbit necrosis is independent of the tumor blood flow; our results in a different tumor and animal species do not appear to support such a view.

Indeed, the fall in %CO/g to 3LL and its high growth rate might be important contributory factors in the appearance of tumor necrosis. Three weeks after implantation about 50% of the tumoral mass was necrotic; at this final stage the value obtained for %CO/g of total tumoral tissue could result from the flow to the macroscopically necrotic areas (virtually 0) and the flow to different areas of living tissue, some of which were already bearing microscopically visible necrotic foci.

At variance with 3LL, the relative CO fraction to JWS did not change markedly during tumor development. The growth rate of these tumor cells is much lower than that of 3LL cells and virtually no necrosis is visible during the whole growth period.

CO distribution to metastatic foci

Different changes in CO distribution to the lungs were observed during growth of 3LL and JWS; both of these tumors give spontaneous metastases to the lungs, although with a different growth pattern. Relative CO fractions to the lungs of both 3LL- and JWS-bearing mice increase at the initial stages of primary tumor growth, long before the appearance of visible lung metastases, presumably as a tissue reaction to the first tumoral cells disseminating from the primary tumor. Subsequently, the %CO to the lungs increased in 3LL and was unchanged in JWS-bearing mice; the relative CO fraction per g of tissue was also increased in 3LL lungs (whose weight only slightly increased) but was decreased in JWS lungs, whose weight was more than doubled at 6 weeks.

The possibility that the increase in CO fraction to the lungs of 3LL mice might be due to the shunting of microspheres through the vasculature of the primary tumor has been reasonably ruled out on the basis of *ad hoc* experiments (see Materials and Methods).

The increase in %CO to 3LL metastasis-bearing lungs appeared to be mainly linked to lung tissue surrounding metastases; indeed, the %CO/g to isolated metastatic nodules (which can be easily enucleated) was much lower than that to the surrounding tissue (although higher than the %CO/g to the primary tumor).

This might be due to a different vascularization pattern and/or smaller amounts of vasodilatory

substances being produced by metastatic nodules; the latter is suggested by lower generation of prostacyclin (PGI_2) activity in isolated 3LL lung metastases than in pulmonary tissue [22, 23].

On the other hand, JWS lung metastases grew in an infiltrative manner and a great mass of fibrous, poorly vascularized, reactional tissue could be microscopically observed around tumoral cells. This could explain the marked increase in lung weight and the decrease in the relative CO fraction per g of tissue observed after 4 and 6 weeks.

CO distribution to host organs and tissues

The tumor development was accompanied by modifications in the CO distribution to host organs and tissues; in this respect differences were also observed between the two tumor models.

Fractions to the heart, liver, spleen, small intestine and skin increased in 3LL-bearing mice, while only the fraction to the hind limb muscle increased in JWS-bearing mice. An increase in blood flow to most organs and tissues was reported in rats bearing Guérin carcinoma [5], and blood supply was increased to the muscle and skin (the only tissues studied) in hamsters bearing amelanotic melanoma [3] but only in the heart of rats bearing MT-W9B mammary carcinoma [6]. It is obviously rather difficult to draw conclusions on tumor-induced modifications from the comparison of different experimental models in different animal species using different techniques for blood flow measurement.

The surprising 6-fold increase in the hepatic CO fraction in mice with 2- and 3-week-old 3LL is very difficult to explain; liver blood flow regulation mechanisms are poorly understood. This increase might be due to the recirculation (shunting) of microspheres arriving at the intestinal tract. However, some of our experimental data could help to rule out this possibility. Escape of microspheres from the intestinal tract through arteriovenous shunts would lead to an underestimation of blood flow in this area. In contrast, we have found a simultaneous increase of both hepatic and intestinal %CO, the mechanism of which remains unclear.

Increase in cardiac work due to anemia in 3LL-bearing mice [8] could account for the increase in %CO to the heart observed in both models.

The increase in CO fraction to the spleen in 3LL was not sufficient to maintain the relative %CO/g due to the striking increase in spleen weight.

In conclusion, using the radioactive microsphere technique in two murine tumors, it has been possible to: (1) follow the evolution of CO relative fractions to the tumor at different stages of growth and dissemination; (2) evaluate the pattern of CO distribution to the lungs invaded by the 3LL metastasis model; and (3) study the effect of the presence of the tumor and metastases on %CO to host organs and tissues. It would be of special interest to utilize the information derived from this study in the evaluation of anticancer drug distribution and activity in tumor and host's tissues. Moreover, the possibility should be considered that the blood supply to the tumor and metastases remarkably influences, in the models we have studied, the lodgement of circulating tumor cells.

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