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Letter to the Editor

Estimation of Malignant Tissue Blood Flow With Radioactively Labelled Microspheres

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IN THE paper entitled "Blood flow to Lymphatic Metastases in Concious Rats" [1], the relative blood flow of the SMT-2A mammary adenocarcinoma, when transplanted into either the inguinal or axillary mammary gland, was compared to that of the spontaneous lymph node metastases. The SMT-2A tumor was chosen for this investigation because after transplantation into mammary tissue it spontaneously metastasizes via the lymphatics, a route very common with human mammary tumors. To determine the normal and malignant tissue blood flows in conscious unrestrained rats, the radioactively labelled microsphere technique was employed. Drs. Endrich, Schosser and Messmer [2] have raised three questions concerning the microsphere technique as utilized in this study.

They first state "With a venous reference sample, one could distinguish between nutritional and non nutritional flow in a malignant tumor. This differentiation has not been performed in the above study...". The distinction between nutritional and non nutritional blood flow is not as clearly defined in a tumor as in normal tissue. All vessels in a tumor provide some degree of nutrition, although some are more efficient than others. Endrich *et al.* suggest that the proportion of trapped and still circulating microspheres should have been assessed by obtaining a venous reference sample. Such a reference sample will not provide meaningful results unless a single vein collects the total venous outflow from the tumor and there is no mixing with venous blood from the surrounding normal tissue. Unless these criteria are met, the reference blood sample will contain microspheres which have passed through a–v shunts in the normal tissue in addition to those which were not trapped in the tumor. Since neither the transplanted nor the metastatic tumors used in this study have such a vascular arrangement, a venous reference sample will only provide ambiguous results.

They secondly state "Since reliable estimates of cardiac output and regional blood flow can be made only with at least 400 microspheres in both the arterial reference sample and the tissue specimen, the above calculations strongly suggest that the microsphere technique was not adequately used in the study of Jirtle." The average number of microspheres injected in these experiments was $74{,}645 \pm 4173$ and the cardiac output was 96.0 ml/min (95% confidence interval: 86.2–107.2 ml/min). The average number of spheres in the withdrawn blood sample was 396, not 357, as calculated by Endrich *et al.* In order for the 95% confidence limits to be within 10% of the mean, 384 spheres are required. Thus, in this study the theoretical precision is slightly greater than that suggested by Buckberg *et al.* [3]. Even if 357 rather than 396 microspheres were present in the reference blood sample, the 95% confidence limits would be within 10.4% rather than 10% of the mean. Though 357 is about 10% lower than the 400 spheres Buckberg *et al.* suggested, the difference in the precision is a mere 0.4%.

The average number of spheres contained in the outer regions of the tumor was 260 ± 20. With this number of spheres in the tissue sample, the 95% confidence limits would be within 12% of the mean for repeated measurements on the same animal. Thus, a 35% reduction in the number of spheres in a sample results only in 2% less precision. This demonstrates a very important point. That is,

the theoretical precision changes very slowly as a function of sphere number until one has considerably less than 400 spheres in the sample. This is clearly shown in Fig. 1.

The number of microspheres in the inner region of the tumor was 84 ± 9. To increase the average number to 400, as suggested by Endrich *et al.*, the animals would have to be injected with 350,000 × 25 μm spheres. We have purposely limited the number of spheres we inject to approximately 70,000, because a greater number of spheres often causes disorientation in the animals. Additionally, Tsuchiya *et al.* [4] have shown that cumulative injections of more than 100,000 microspheres produced significant systemic hemodynamic alterations. The alternative suggestion by Endrich *et al.*, to remove a larger sample, is also not possible when one is interested in determining blood flow as a function of tumor size. The best method of dealing with this problem is to utilize more experimental animals. The rationale for this approach is provided below.

The measure of precision, as calculated by Buckberg *et al.* [3], assumes that the number of microspheres observed follows a Poisson distribution and the only source of variation in the measurement is the random variability associated with this distribution; the variance is equal to the mean. This assumption is only appropriate for describing the precision of repeated measurements from one animal, as in the paper by Buckberg *et al.* [3]. However, when tissue blood flows are estimated averaging the observations obtained from several animals, the animal to animal variation overwhelms the Poisson variation. As a result, the sample standard deviation should be used to assess the actual experimental error. This conclusion applies regardless of the number of microspheres observed in a tissue sample.

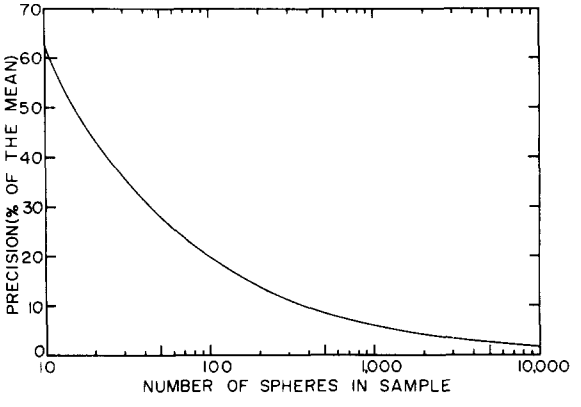

Fig. 1. Theoretical precision of the 95% confidence interval for the mean number of spheres versus the number of spheres in the sample. The results are based on the Poisson distribution [3].

Although the experimental error may increase with fewer spheres, one can, however, compensate for this by increasing the sample size. Buckberg *et al.* [3] also clearly stated this when they wrote "Use of small numbers of microspheres will be associated with large errors and low precision of estimate, but should not lead to a systematic over or underestimate of mean flow to any organ. With small numbers and large errors, however, more experiments will be needed to demonstrate the significance of any given difference." Thus, even though the number of spheres in a sample is low, a significant difference in blood flow estimates can be demonstrated if a sufficient number of animals are observed.

For the experiments described by Jirtle [1], the blood flow measurements were first normalized by a log transformation [5], then confidence interval estimation and statistical tests were performed. Table 1 can be used to determine the sample size required to detect a specified difference between two hypothesized blood flows, depending on their associated standard deviation. The reference values given in Table 1 for differences and standard deviations refer to the log-transformed ($\log_e$) data. The column R supplies the corresponding ratio of the actual blood flow values which one could detect. For example, if one wished to detect a two-fold difference in blood flow (R = 2.0, $\Delta = 0.7$), and estimated that the variation which would be observed after log transformation was 0.3, then the required number of animals in each group would be 6. With this

Table 1. Number of observations required to detect a specified difference between two mean blood flow measurements after log transformation *

R†	Δ‡	Standard deviation§					
		0.1	0.2	0.3	0.4	0.5	0.6
1.1	0.1	23‖	86	> 100	> 100	> 100	> 100
1.2	0.2	7	23	49	86	> 100	> 100
1.3	0.3	4	11	23	39	60	86
1.6	0.5	3	5	10	16	23	33
2.0	0.7		4	6	9	12	17
3.0	1.1		3	4	5	6	8

*Based on a two-tailed t-test with significance level (α) 0.05, power ($1-\beta$) 0.9.

†$R = e^{\Delta}$ = ratio of mean blood flows.

‡$\Delta = \log_e R$ = difference between log-transformed mean blood flows.

§Standard deviation of log transformed data; assumed the same in the two groups.

‖Number of observations in *each* group.

number of observations one would have a 90% chance of detecting a two-fold difference at a significance level of 0.05. These calculations are based on the use of a two-tailed *t*-test.

By observing sufficient numbers of animals we were able to demonstrate that the blood flow to the inner region of the tumor was significantly lower than that to the outer region, even though the average number of spheres in the necrotic tissue was 89. Additionally, we were able to show that the relative blood flow to the outer viable region of the tumor was significantly greater than that to muscle, skin and mammary gland.

Endrich *et al.* finally state "The size of microspheres was randomly assessed by referring to experiments published earlier". Our goal was to develop an animal tumor-host model which closely mimics the vascular relationship that exists between human primary and metastatic mammary tumors and their surrounding normal tissues. Because of this constraint, tissue blood flow must be measured by an indirect method. We have chosen 25 μm diameter radioactively labelled microspheres to measure tumor blood flow for reasons previously described [5]. If 15 μm spheres are used they underestimate tumor blood flow for all but the smallest tumors. If 50 μm spheres are utilized tumor blood flow is also underestimated because of their preferential axial-streaming. Thus, the use of 25 μm diameter microspheres is a compromise between these two extreme situations. If a significant number of the 25 μm microspheres would have preferrentially shunted through the vascular bed of large SMT-2A tumors, the estimated relative blood flow for both the outer and inner regions would have decreased with increasing tumor weight. In fact, the estimated relative blood flows for both regions are uncorrelated with weight, indicating that over the weight range investigated, the probability of 25 μm microspheres shunting through the tumor is not dependent upon tumor size.

In conclusion, when utilizing microspheres to estimate tissue blood flows, desired statistical precision must be balanced with the maximum number of microspheres which can be injected without causing physiological alterations. Additionally, a microsphere diameter must be chosen which is effectively trapped in the tissue of interest and also distributes itself in the vessels in a manner similar to that of erythrocytes. To estimate the blood flow of the SMT-2A mammary adenocarcinoma we chose to inject approximately 70,000 microspheres 25 μm in diameter. In view of the opposing constraints outlined above and the fact that a desired statistical precision can be attained by adequate sample sizes, we feel that the microsphere technique was appropriately utilized in this study [1].

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