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Tritiated Thymidine Labelling *in Vitro* of Human Cancer of the Breast: Counting Error and Sampling Error

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Abstract—*Tritiated thymidine labelling indices (TLIs) were determined on a number of primary cancers of the human breast. Twenty-two slides were chosen which demonstrated a wide range of TLIs and each was counted twice to assess 'counting error'. TLIs derived from successive counts of the same slide showed a coefficient of variance greater than 25% in half of the slides, but a significant difference between the two ($P < 0.05$) in only 2 of 22 pairs. When TLIs derived from paired specimens taken from different sites in each of 22 tumours were compared, there was found to be a significant difference ($P < 0.05$) in 17 of 22 pairs. This is 'sampling error' and is clearly a major source of inaccuracy when TLIs are derived from single small samples of heterogenous tumours such as cancers of the human breast.*

INTRODUCTION

TRITIATED thymidine labelling indices (TLIs) determined *in vitro* on specimens of cancer of the human breast have been found to correlate with tumour size [1, 2], histological type and grade [3], oestrogen receptor status [4, 5], receptor and menopausal status combined [6], age [7], disease-free interval [2], survival [8], and response to therapy [9, 10] with varying degrees of significance. Many authors have studied the relationship between TLI and several of the clinical and prognostic features listed above and while they have discovered one or two significant relationships in each case, there is a lack of unanimity concerning the relationship between TLI and any one of the above features. The literature is confusing and often conflicting.

Some of this confusion may result from insensitivity or inaccuracy in determining TLI. 'Counting' error and 'sampling' error have been defined and evaluated by Smallwood *et al.* (1983) and shown to be of greater magnitude than previously demonstrated [11].

The 'counting error' and 'sampling error' of TLI are evaluated in this study of cancers of the human breast.

MATERIALS AND METHODS

Specimens of tumour were obtained at the time of either frozen section or mastectomy and processed within 3 hr after storage in Eagles medium at 4°C. Specimens were taken from both the centre and the periphery of large tumours.

Technique of tritiated thymidine labelling

The hyperberic method of Meyer and Bauer was employed [12]; 0.5–1.0 mm slices were cut with a scalpel blade and incubated with 20 μg/ml tritiated thymidine (21 Ci/mmol Radiochemicals, Amersham, U.K.) in modified Eagle's medium under 3 atmospheres pressure of oxygen, for 1 hr at 37°C. Unbound thymidine was removed with 5% Trichloracetic acid and the specimens fixed in formol-saline.

After de-waxing, AR10 stripping film (Kodak Ltd.) was applied and exposure carried out in the dark at 4°C for 10 days. The film was developed in D19 (Kodak Ltd.) and fixed with 1 to 5 Hypam (Ilford Ltd.). Staining was carried out with haematoxylin and eosin.

Sections were scanned under low-power field and the region or regions where labelled cells were most frequent were counted first. An eye-piece graticule displaying a 10 × 10 grid was inserted and the area selected was studied under high-power (× 400). The total number of tumour cells in the 10 × 10 grid was counted and the number which had been labelled was noted. Further regions of the section were counted in this way until the total cell count was around 2000. The number of labelled cells was expressed as proportion of the total cell count for each section and this was recorded as the Labelling Index (TLI).

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Evaluation of 'counting error'

Twenty-two slides were chosen with a wide range of LIs and each was counted twice at random, with the identification masked. Statistical comparison of the two TLIs determined from each slide was made by the Chi-square test (with Yates' correction).

Evaluation of 'sampling error'

Comparison was made between TLIs determined from paired specimens taken from different sites in each of 22 primary cancers of the breast, using the same methods as those for 'counting error'.

RESULTS

Raw data are presented in Tables 1 and 2. In both tables the higher of each pair of results is presented in the left-hand column.

'Counting error'

There was significant difference ($P < 0.05$) between the counts in 2 of 22 pairs (Table 1). The coefficient of variance within each pair was greater than 25% in 11 of 22 and was inversely proportional to the mean LI of each pair (Fig. 1).

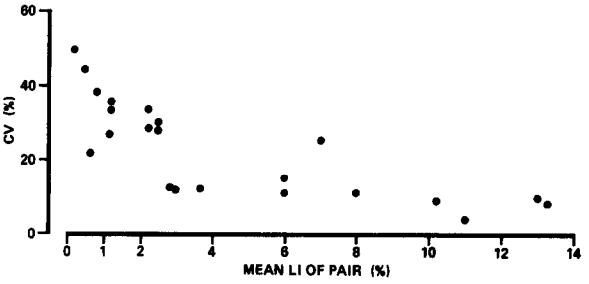

Fig. 1. Relationship of coefficient of variance (CV) and mean TLI of pair.

'Sampling error'

There was significant variation in LI ($P < 0.05$) between specimens taken from different parts of each tumour in 17 of 22 primary cancers of the breast. In six cases the differences were very significant ($P < 0.0001$, Table 2).

Effect of tumour size

There was no relationship between the degree of intratumour variation in TLI (measured by the CV within each pair of specimens) and the size of the primary tumour (Table 3).

Table 1. 'Counting error'. Comparison of labelling indices determined by repeated counts upon the same microscope slide

	Highest count			Lowest count			Significance of difference		
Tumour no.	LI%	Labelled cells	Total no. of cells counted	LI%	Labelled cells	Total no. of cells counted	Coefficient of variance	χ^2	P
1	0.27	7	2501	0.14	4	2914	48%	0.738	> 0.05
2	0.7	12	1649	0.4	7	1828	44%	1.315	"
3	0.9	19	2053	0.6	14	2043	21%	0.469	"
4	1.1	21	1986	0.7	12	1826	38%	1.340	"
5	1.5	20	1312	0.9	16	1751	35%	1.922	"
6	1.5	31	2074	1.0	20	1963	26%	1.469	"
7	1.5	31	2084	0.9	24	2667	34%	3.035	"
8	2.6	58	2234	1.7	42	2409	28%	3.605	"
9	2.7	32	1206	1.6	23	1409	33%	2.758	"
10	2.7	54	2021	2.0	49	2347	30%	1.365	"
11	2.9	70	2395	2.5	51	2066	12%	0.705	"
12	3.0	62	2017	2.0	33	1652	30%	3.765	0.05
13	3.1	59	1892	2.6	53	1994	11%	0.547	> 0.05
14	4.0	77	1928	3.4	76	2243	12%	0.113	"
15	6.5	146	2244	5.5	122	2319	15%	2.978	"
16	6.5	142	2190	5.6	143	2571	11%	1.626	"
17	8.0	86	1069	5.6	68	1217	25%	5.085	0.023
18	8.6	184	2078	7.6	219	2868	11%	2.231	> 0.05
19	11.2	338	3015	9.5	187	1785	9%	0.547	"
20	11.6	210	2669	11.0	278	2521	4%	0.368	"
21	14.2	246	1737	12.4	195	1571	10%	2.037	"
22	14.7	315	2144	12.7	314	2475	9%	3.758	"

Table 2. 'Sampling error'. Comparison of labelling indices determined from different specimens of the same tumour (primary tumours)

Tumour no.	Specimen 1 LI%	Specimen 1 Labelled cells	Specimen 1 Total no. of cells counted	Specimen 2 LI%	Specimen 2 Labelled cells	Specimen 2 Total no. of cells counted	Coefficient of variance	χ^2	P
a	0.7	12	1649	0.4	10	2413	39%	1.25	> 0.05
b	1.0	24	2326	0.7	13	1826	30%	0.85	"
c	1.5	31	2074	0.9	25	2667	32%	2.645	"
d	1.9	33	1736	1.2	16	1373	33%	2.222	"
e	2.5	51	2015	1.6	39	2392	30%	3.995	0.043
f	3.8	82	2152	2.7	41	1504	24%	2.876	> 0.05
g	4.1	110	2667	2.7	53	1917	22%	6.470	0.010
h	4.6	96	2091	3.3	65	1951	22%	3.863	0.046
i	4.7	103	2197	3.2	83	2566	26%	6.282	0.012
j	5.6	112	2002	3.3	64	1921	36%	10.96	0.0013
k	5.8	125	2142	2.7	66	2470	53%	24.73	< 0.0001
l	7.7	203	2777	3.8	86	2297	46%	31.51	< 0.0001
m	7.7	206	2663	3.0	74	2427	61%	52.75	< 0.0001
n	9.0	194	2143	4.3	24	560	50%	12.97	0.0007
o	9.1	232	2539	5.4	103	1986	39%	24.8	< 0.0001
p	9.2	216	2386	7.1	189	2665	17%	6.299	0.012
q	9.2	241	2611	7.0	172	2449	19%	7.919	0.005
r	10.9	263	2413	7.4	114	1539	27%	12.87	0.0008
s	11.3	284	2521	3.2	71	2166	78%	105.3	< 0.0001
t	11.5	209	1822	8.8	189	2150	20%	6.854	0.008
u	15.1	298	1979	12.6	262	2088	13%	4.909	0.025
v	15.3	371	2425	12.7	286	2253	14%	6.350	0.012

Table 3. Relationships between 'sampling error' and size of tumour

Tumour size		Number of tumours	Mean CV between LIs of specimens from same tumour
T_1	< 2 cm	3	20%
T_2	2–5 cm	13	34%
T_3	> 5 cm	6	38%
T_4			

There is no significant difference between the three groups ($P = 0.4$).

DISCUSSION

It is not the purpose of this paper to evaluate all the assumptions, inaccuracies and errors inherent in the technique of tritiated thymidine labelling. These have been discussed in detail elsewhere [13, 14, 15, 16]. This study shows clearly that there is both 'counting' and 'sampling' error in the use of tritiated thymidine labelling in cancer of the human breast, the latter being considerably greater. Quantification of these errors depends upon the analysis to which they are subjected; coefficient of variance, difference between 95% confidence limits and the mean and mean difference between paired samples have all been used [1, 11, 12].

Comparisons of pairs of results may be made appropriately by use of the Chi-square distribution because TLIs are fractions (expressed at %) and are parametric, i.e. a cell is either labelled or not. Coefficient of variance should not, strictly, be used to analyse pairs of results but it has been included to enable comparison to be made with other studies. It is less than ideal for analysis of the errors of TLI since it can be seen to vary inversely with TLI itself (Fig. 1). Thus a difference between paired results of nearly two-fold giving a C.V. of 48% (Table 1, tumour 1) is insignificant when compared by Chi-square whereas a difference of one sixth between paired results (CV only 24%) proves to be significant when the Chi-square is applied (Table 2, tumour v, $\chi^2 + 6.35$, $P = 0.012$.

'Sampling' error is clearly much greater than 'counting' error over all ranges of TLI studied. This is not surprising: cancer of the human breast is notoriously heterogenous. Intratumour variations in histological type and grade [3, 17] and oestrogen receptor content [18] are known to exist. Furthermore, TLI is based upon a cell count of approximately 2000; 19 of the tumours whose TLIs are given in Table 2 were 2 cm or more in diameter and therefore contained 10^9–10^{10} cells so each TLI

thus derived represents no more than one hundred-thousandth part of the whole tumour and may not logically be held to represent the tumour as a whole.

In one study of cancers of the human breast, Wolberg and Brown stated that 'there was no significant difference between samples taken from different parts of the same tumour' [19]. They do not state the cell count from which the TLIs were determined, but a later paper from the same group indicated that only 350 cells were counted, giving TLIs of very low sensitivity [20].

Meyer's group compared the TLIs of pairs of specimens, one taken by the surgeon at biopsy and one taken by the pathologist from the residual tumour in the subsequent mastectomy specimen. The differences between the pairs tended to be proportional to their mean LI, and in the seven cases in which the mean LI exceeded 2.1% 'the quotient of the difference divided by the mean of the pair did not exceed 0.57'. This statistically inevaluable statement belittles the fact that the largest absolute difference between pairs was 12.1 : 20.2, and the largest relative difference was 0.40 : 2.29. This latter pair shows a greater than 5-fold difference, or if taken as a variation from the mean, a variation of nearly three-fold either way [3, 12].

Smallwood *et al.* (1983) evaluated sampling error using two different methods. By one method a cross-section was taken from the middle of each tumour and this was cut into many 1 mm cubes, from which 'pool' two samples each of five 1 mm cubes were labelled and the TLIs compared. The difference between the two TLIs for each tumour showed a median value of 0.38%, and in no case was the difference greater than 0.5% [11].

By a second method five separate samples were taken from each tumour and labelled, the five TLIs showed a coefficient of variance which ranged from 4.36 to 14.8% [11].

'Counting error', defined in this paper as the variation in TLI produced when one observer counts the same 5 μ tissue section on more than one occasion, has two components. The first is actual observer error: inaccurate counting of the total number of cells in each high-power field (even with a grid incorporated in the eye-piece counting can be difficult), inconsistency in deciding which cells are to be considered labelled [21] and inconsistency in deciding whether certain cells are tumour or stroma.

The second component is, even within a single tissue section, one of sampling. A typical thin section measuring a few millimetres square often consists of considerably more than 2000 cells. Repeated counts upon the same section, though each may total 2000 cells, are not necessarily the same 2000 cells on each occasion and since most sections of breast cancer demonstrate obvious variations in labelling index from one high-power field to the next, this is a manifestation of sampling error. The only way to reduce this component would be to count every single cell in each tissue section which, in the absence of a method of marking each cell that it has been counted, would be practically extremely difficult and very time-consuming.

Counting error produced a coefficient of variance of only 4% in one high-labelling tumour [12]. Smallwood *et al.* could be 95% confident of counting to only within 0.5% of the true value and this error appeared to increase with high values of TLI [11].

CONCLUSIONS

This study confirms the work of others, especially Smallwood *et al.* (1983), in demonstrating that TLIs derived from all single samples of cancers of the human breast are subject to both 'counting' and 'sampling' error [11]. 'Sampling' error is substantial and is a reflection of the intratumour variation in TLI in cancer of the human breast. A possible solution to this problem would be to count samples from several different parts of each tumour; further studies would be required to determine whether the mean TLI or the highest TLI thus derived were best related to the observed growth rate or clinical behaviour of the tumour. This analysis of multiple samples would make labelling studies very time-consuming. TLIs derived from single small samples of human cancers of the breast are clearly only a very crude index of proliferative activity, but are often presented in the literature without any comment upon how inaccurate or unrepresentative they may be. Counting error and sampling error are both well-recognised by those working in the field of thymidine labelling but have rarely been drawn to the attention of the more general reader in the subject of breast cancer.

If TLIs are quoted, particularly those derived from heterogenous tissues such as breast cancer, their inaccuracy should be stressed and analysis of both counting error and sampling error should accompany their publication.

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