J. Cell Sci. 5, 575–582 (1969)
Printed in Great Britain

COMPARISON OF MULTIPLE INJECTIONS WITH CONTINUOUS INFUSION OF TRITIATED THYMIDINE, AND ESTIMATION OF THE CELL CYCLE TIME

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SUMMARY

Labelled nuclei were counted in stratified squamous epithelium in mice killed after 24 h intraperitoneal administration of tritiated thymidine to label cells synthesizing deoxyribonucleic acid. Multiple injections produced the same result as an infusion of tritiated thymidine given after 24 h infusion of saline, but infusion of tritiated thymidine alone produced a different result. Thus, cell proliferation was depressed during the first 24 h of continuous infusion but was normal during the second 24 h.

Comparison of proliferation of the oesophageal epithelium at the level of the thyroid and at the level of the diaphragm showed no difference between the two.

Comparison of male with female mice given 72-h infusions of tritiated thymidine showed that cell proliferation occurred at the same rate in both.

The cell cycle time was estimated in the epithelium of the oesophagus and tongue by comparison of mice given a single injection with mice given multiple injections of tritiated thymidine.

INTRODUCTION

Evidence has been presented that continuous infusion for a few days does not subject rats to significant stress (Blenkinsopp & Blenkinsopp, 1967). However, unpublished results of continuous infusion of tritiated thymidine in rats and mice suggested to the present author that animals were being subjected to significant stress during the first 24 h of infusion, and the present work was planned to investigate this. The method chosen was to compare the results of nuclear labelling in the stratified squamous epithelium of the oesophagus and tongue of mice after 24 h exposure to tritiated thymidine by multiple injections, continuous infusion alone, or continuous infusion preceded by 24 h infusion of saline.

In addition, the opportunity was taken to compare cell proliferation in the oesophagus at two levels (at the level of the thyroid and at the level of the diaphragm), to compare cell proliferation in male and female mice (using 72-h infusions of tritiated thymidine), and to estimate the cell cycle time by comparing mice given a single injection with mice given multiple injections of tritiated thymidine.

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MATERIAL AND METHODS

Thirty-two inbred *C3H/He* mice (age 8 weeks) were used; 28 were male (weight 24 g) and 4 were female (weight 20 g). The mice were divided into 8 groups of 4 mice (groups A–H), and were given tritiated thymidine (specific activity 5·0 Ci mM^{-1}) intraperitoneally. Each mouse received a single injection of 1·5 μCi g^{-1} body weight at 09.00 G.M.T., or multiple injections for 24 h (0·5 μCi g^{-1} body weight per injection, injections being given at 09.00, 16.00, 23.00, 06.00 and 08.50 G.M.T.), or a continuous infusion for 24, 48 or 72 h (begun with an initial dose of 0·5 μCi g^{-1} body weight and continued with 1·5 μCi g^{-1} body weight/24 h.) The infusion technique used was that of Mendelsohn (1962). Details of administration of tritiated thymidine are given in Table 1.

Table 1. *Plan of experiments: month, time, and type of administration of tritiated thymidine*

Group	Month	Time of first exposure (G.M.T.)	Type of administration of tritiated thymidine	Sex
A	March	09.00	Multiple injections (24 h)	Male
B	March	09.00	Infusion (24 h)	Male
C	March	09.00	Infusion (24 h) preceded by saline infusion	Male
D	November	10.00	Infusion (24 h)	Male
E	November	10.00	Infusion (48 h)	Male
F	November	10.00	Infusion (72 h)	Male
G	November	10.00	Infusion (72 h)	Female
H	April	09.00	Single injection	Male

The mice (group H) given a single injection of tritiated thymidine received at the same time a single injection of colchicine (2 mg kg^{-1} body weight), and were killed 4·8 h later; evidence has been presented elsewhere that this procedure gives the same number of labelled nuclei as killing the animals within 1 h of the injection of tritiated thymidine (Blenkinsopp, 1968*a*). The mice given multiple injections (group A) were killed 10 min after their last injection. The infused mice were killed at the end of their tritiated thymidine infusion.

The oesophagus and the anterior third of the tongue were removed from each mouse at sacrifice and fixed in Bouin's solution. Autoradiographs were prepared from transverse paraffin sections (5 μm) with Ilford K 5 nuclear emulsion, and these were exposed for 4 weeks at 0–4 °C, developed, and stained with haematoxylin. More than 800 epithelial nuclei were counted in each oesophagus and on the ventral surface of each tongue, and these were subdivided into basal and superficial, labelled and unlabelled. Nuclei were considered labelled if they were overlaid by 4 or more grains.

RESULTS

The results of the counts are given in Table 2, and the appropriate comparisons are shown in Table 3 (using the Student *t* test and the data for total labelled nuclei). Comparisons made using the Student *t* test and the data for labelled basal nuclei gave the same answers. There was no difference in number of labelled nuclei (and thus no difference in rate of proliferation) between the two levels of oesophagus studied, and there was no difference between male and female mice.

The proportion of nuclei labelled in the oesophagus and tongue was lower in the mice given tritiated thymidine infusion only than in mice given multiple injections or mice given tritiated thymidine infusion after saline infusion (the ratios being 1 : 1·64 and 1 : 1·60 respectively). This suggests a reduction equivalent to about 9 h non-proliferation. There was no significant difference between the results of the last two procedures. Thus the setting-up of a continuous infusion caused sufficient stress to reduce cell proliferation, whereas the continuance of the infusion did not reduce proliferation.

Table 2. *Mean number* (± *standard deviation*) *of labelled basal, labelled superficial, total labelled, and total superficial nuclei, per* 100 *total basal nuclei*

Epithelium	Group of mice	Labelled basal nuclei	Labelled superficial nuclei	Total labelled nuclei	Total superficial nuclei
Oesophagus	A	72·2 ± 6·5	5·4 ± 1·9	77·6 ± 7·8	77 ± 13
	A*	67·9 ± 10·6	3·6 ± 1·4	71·5 ± 11·9	73 ± 6
	B	40·4 ± 3·1	0·9 ± 0·6	41·3 ± 3·7	78 ± 3
	C	63·1 ± 11·6	2·9 ± 0·8	66·0 ± 12·4	74 ± 7
	D	28·5 ± 5·0	1·7 ± 0·6	30·2 ± 4·7	67 ± 4
	E	84·3 ± 5·5	18·0 ± 1·3	102·3 ± 4·9	72 ± 10
	F	88·6 ± 2·9	25·1 ± 5·0	113·7 ± 6·4	61 ± 6
	G	85·7 ± 9·6	23·0 ± 5·5	108·7 ± 13·7	62 ± 8
	H	21·5 ± 4·2	0	21·5 ± 4·2	64 ± 15
Tongue: ventral surface	A	49·5 ± 10·1	1·2 ± 0·7	50·7 ± 10·7	62 ± 5
	B	35·0 ± 4·5	0·9 ± 0·2	35·9 ± 4·6	69 ± 6
	C	56·4 ± 10·2	1·4 ± 0·8	57·8 ± 10·9	72 ± 5
	D	17·1 ± 2·6	0·5 ± 0·3	17·6 ± 2·4	61 ± 7
	E	55·2 ± 3·2	2·5 ± 1·6	57·7 ± 4·3	72 ± 11
	F	69·7 ± 10·3	8·1 ± 6·4	77·8 ± 16·6	74 ± 13
	G	63·5 ± 11·5	6·7 ± 3·5	70·2 ± 10·2	70 ± 5
	H	11·9 ± 1·4	0	11·9 ± 1·4	56 ± 12

* Sections taken just above the diaphragm; all other sections of oesophagus were taken at the level of the thyroid.

The difference between 24 h infusion of tritiated thymidine in March and the same in November (Table 2, groups B and D) is probably at least in part a reflexion of the different times at which the infusions were begun (Table 1), as each 24-h infusion is an index of cells labelled in 24 h plus cells in the phase of deoxyribonucleic acid synthesis (*S*) at the beginning of the infusion, and the number of cells in *S* shows wide diurnal variation. Thus no conclusion can be drawn as to seasonal variation in cell proliferation.

The infusions for 24, 48 and 72 h were planned to permit estimation of the cell cycle time, but owing to the depression of cell proliferation at the beginning of infusion this cannot be done. Even if this depression had not occurred, the calculation would have had to take into account loss of labelled nuclei and re-utilization of label, and it would not have been reliable.

The cell cycle time (Tc) has been estimated from the values for total labelled cells in the mice given a single injection (group H) and those given multiple injections of tritiated thymidine for 24 h (group A). The method used for this calculation is given in the appendix. It requires estimates for the duration of the post-synthetic gap (T_{G2}) and of mitosis (T_M). In the oesophagus of these mice T_{G2} was found to be 2·8 h (Blenkinsopp, 1969), and from Cameron & Greulich (1963, fig. 2) $T_{G2}+\frac{1}{2}T_M$ appears to be 2·5 h in mouse oesophagus. Cameron's (1966) figure 12 suggests that $T_{G2}+\frac{1}{2}T_M$ is 3 h in mouse tongue. In the hamster cheek pouch, $T_{G2}+\frac{1}{2}T_M$ was 2·4 h (Brown &

Table 3. *Comparison between groups, using the Student t test and the data for total labelled nuclei*

		Oesophagus		Tongue: ventral surface	
Group	Type of administration of tritiated thymidine	Total labelled nuclei (mean)	P	Total labelled nuclei (mean)	P
A	Multiple injections (24 h)	77·6	0·50 > P > 0·40	—	—
A*	Multiple injections (24 h)	71·5			
A	Multiple injections (24 h)	77·6	P < 0·001	50·7	0·05 > P > 0·025
B	Infusion (24 h)	41·3		35·9	
B	Infusion (24 h)	41·3	0·01 > P > 0·005	35·9	0·01 > P > 0·005
C	Infusion (24 h) preceded by saline infusion	66·0		57·8	
C	Infusion (24 h) preceded by saline infusion	66·0	0·20 > P > 0·10	57·8	0·40 > P > 0·30
A	Multiple injections (24 h)	77·6		50·7	
D	Infusion (24 h), November	30·2	0·01 > P > 0·005	17·6	P < 0·001
B	Infusion (24 h), March	41·3		35·9	
F	Infusion (72 h), male	113·7	0·60 > P > 0·50	77·8	0·50 > P > 0·40
G	Infusion (72 h), female	108·7		70·2	

* Sections taken just above the diaphragm; all other sections of oesophagus were taken at the level of the thyroid.

Oliver, 1968), and in the mouse forestomach T_{G2} was 1–2 h (Wolfsberg, 1964) or 3·5 h (Frankfurt, 1967). T_M appears to be about 0·5 h in most tissues (Knowlton & Widner, 1950; Widner, Storer & Lushbaugh, 1951). Error in the estimation of T_{G2} and T_M will make only small differences to the value obtained for Tc, so the present author has assumed that T_{G2} is the same in the tongue as in the oesophagus (that is 2·8 h) and that T_M is 0·5 h. The mice given a single injection of tritiated thymidine were not killed immediately after the injection, the availability time of tritiated thymidine is 0·5 h, and T_S is about 8·5 h (Blenkinsopp, 1969): the number of labelled nuclei in these mice was therefore corrected for the calculation by using the factor 8·5/9·0. Tc was 61 h in the oesophageal epithelium and 84 h in the epithelium of the ventral surface of the tongue.

The proportion of labelled nuclei found outside the basal layer after 24-h labelling

can be calculated by the method given in the Appendix: oesophagus 12%, tongue 9%. The observed values (from Table 2) are lower: oesophagus 7%, tongue 2·4%; this is presumably the result of difficulty in distinguishing cells which have just left the basal layer.

DISCUSSION

Variation in rate of cell turnover at different sites of the same epithelium is a possible explanation for the discrepancies between different estimations of the rate. Such variation is known to occur in the skin (Blenkinsopp, 1968*b*) and in the tongue (Cameron, 1966). Dormer & Moller (1968) found that various parameters of cell kinetics in mouse forestomach differed according to the site examined; however, the over-all labelling index for each of the 6 forestomachs covered an acceptably small range, as did the labelling indices from several slides prepared from the same forestomach. Bertalanffy (1960) found that in rats cell turnover appeared to be more rapid in oesophageal epithelium at the level of the thyroid than at the level of the cardia (respectively 11:9 mitoses per 100 nuclei per 24 h, after colchicine injection). In the present work the rate of cell turnover in oesophageal epithelium did not vary with the site examined.

There is considerable difficulty in using single injection techniques for the investigation of cell proliferation in female animals, because of the marked variation which occurs during the oestrous cycle (Bullough, 1946). Techniques using 'continuously' available tritiated thymidine seem well suited to this type of study, and the present work has shown that over a period of 3 days proliferation occurred at the same rate in mature female as in male mice of the same age.

The finding that setting up a continuous infusion reduced cell proliferation is not entirely surprising; the continuance of such an infusion might also be expected to have some effect, but the evidence given here shows that it has no more effect than multiple injections, and if the persistence of normal levels of blood eosinophils is a reliable criterion of the absence of stress then neither a continued infusion nor multiple injections have any effect at all (Blenkinsopp & Blenkinsopp, 1967). However, the available evidence is limited, and a more direct study of this is now in progress.

The estimation of *Tc* by the technique used here has the major disadvantages of assuming homogeneity of an identifiable proliferating population. On the other hand, it may provide a useful check on other methods and it may be of value in the study of cells which multiply so slowly that the labelled mitoses technique cannot be used (Brown & Oliver, 1968). The values for *Tc* obtained in this work (oesophagus 61 h, tongue 84 h) are longer than those previously found by a different technique in the same inbred strain of mice (oesophagus 58 h and tongue 53 h, Blenkinsopp, 1968*b*; oesophagus 41 h, Blenkinsopp, 1969) but are still within the range indicated by a comparison of other workers' results (table 5, Blenkinsopp, 1968*b*). There is as yet insufficient evidence to decide whether the differences are due to seasonal variation, the technique used, or a significant lack of homogeneity in the proliferating population.

I am grateful to Dr C. W. Gilbert for the Appendix, to Miss Susan Pearce for technical assistance, and to the British Empire Cancer Campaign for financial assistance.

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(*Received* 3 *March* 1969)

APPENDIX

NOTATION

x	proportion of basal nuclei labelled in a flash label.
y	ratio of total number of nuclei (inside and outside basal layer) labelled in 24 h to number of nuclei in the basal layer.
z	proportion of total labelled nuclei in the basal layer after 24 h labelling.
T_1	time of beginning of S, from the last cell division.
T_2	time of end of S.
Tc	cell cycle time.
T_{24}	time of continuous labelling (24 h).
$\phi(\tau).\mathrm{d}\tau$	the phase distribution, the proportion of basal nuclei with ages between τ and $\tau+\mathrm{d}\tau$.
w	$2\exp(-0{\cdot}693T_2/Tc)$.
v	$\phi(Tc)\,[Tc-T_2] = 0{\cdot}693(Tc-T_2)/Tc$.

THE MODEL

Cells in the basal layer are assumed to be in continuous cell cycle with a constant cell cycle time Tc. Cells migrate from the basal layer randomly during the cell cycle at a rate which just counters cell multiplication so that the basal population remains constant. The phase distribution of cells in the basal layer is that appropriate to an exponentially growing population with random loss and is (Lajtha & Gilbert, 1967):

$$\phi(\tau) = 1{\cdot}386 \exp(-0{\cdot}693\tau/Tc)/Tc. \quad (1)$$

The cells which have migrated from the basal layer do not undergo further cell division, nor do they pick up label, but they retain any label they had before leaving the basal layer. The period of continuous labelling is assumed to be less than Tc so that all cells entering S are unlabelled.

Flash label

The proportion of nuclei labelled in a flash label is the proportion of nuclei in the S-phase and is:

$$x = \int_{T_1}^{T_2} \phi(\tau).\,d\tau$$
$$= 2\,[\exp(-0{\cdot}693T_1/Tc) - \exp(-0{\cdot}693T_2/Tc)],$$

and so $$x+w = 2\exp(-0{\cdot}693T_1/Tc) \quad (2)$$

hence $$(Tc-T_1)/Tc = \ln(x+w)/\ln(2). \quad (3)$$

Continuous label

During the continuous labelling, labelled nuclei are being produced at constant rates by two mechanisms. Those nuclei entering the S phase become labelled at a rate $\phi(T_1)$ and labelled nuclei entering mitosis produce an extra labelled nucleus on division: this rate is $\phi(Tc)$, but labelled nuclei do not reach division before a time $Tc-T_2$. Though some of the labelled nuclei migrate from the basal layer they still remain in the adjacent layers and are included in the score for y. Hence the net increase of labelled nuclei during the 24 h of continuous label is $y-x$ and so

$$y-x = [\phi(T_1)+\phi(Tc)]\,T_{24} - \phi(Tc)\,[Tc-T_2].$$

$$\phi(Tc) = 0{\cdot}693/Tc$$

from (1).

$$\phi(T_1) = 0{\cdot}693\,(x+w)/Tc$$

from (1) and (2),

so $$y-x = 0{\cdot}693\,[1+x+w]\,T_{24}/Tc - v,$$

and hence $$Tc = \frac{0{\cdot}693\,(1+x+w)}{(y-x+v)}T_{24}. \quad (4)$$

Proportion of labelled cells in the basal layer

Some labelled nuclei migrate out of the basal layer, and they do so in a random manner with a half time of Tc. Labelled nuclei are being produced at a steady rate and so the proportion of them that still remain in the basal layer at the end of the continuous labelling period is

$$z = [1 - \exp(-A)]/A,$$

where $A = 0{\cdot}693\ T_{24}/Tc$.

CALCULATION

The value of Tc can be calculated from the experimental values of x and y by successive approximations. First $Tc - T_2$ is assumed zero, so that $v = 0$ and $w = 1$, and then Tc is calculated from equation (4). Using this value of Tc and the known value of $Tc - T_2$ ($T_{G2} + T_M$), v and w can be estimated and so the next approximation for Tc can be derived from equation (4).

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