CELL KINETICS OF URETHANE-INDUCED MURINE PULMONARY ADENOMATA: II. THE GROWTH FRACTION AND CELL LOSS FACTOR

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Summary.—Continuous labelling of urethane induced pulmonary adenomata in adult male A2G mice at intervals up to 20 weeks showed that the growth fraction fell progressively from 18% at 7 weeks to 7% at 20 weeks. This fall appears to be wholly responsible for the decrease in production of adenoma cells with age.

A fraction labelled mitoses curve was constructed for pulmonary adenomata at 14 weeks post urethane. Only the first peak was apparent, giving median t_2 and t_S values of 2 and 9 respectively. The cell cycle time was calculated at 45 h and the growth fraction at 6·2%, whilst the cell loss factor was estimated at 31%. Other cell loss values were calculated from data on the rate of entry into DNA synthesis obtained previously by double labelling. These values remained constant with time at 83–95%, suggesting that cell loss is in some way linked to cell production. However, the development of polyploidy in adenoma cells could not be eliminated. No areas of necrosis were seen in the adenomata at any time although karyorrhexis occurred in isolated cells.

The labelling characteristics of alveolar wall cells in the same lung sections as the adenomata did not vary with time and the continuous labelling curve gave a growth fraction of 1·8%, a DNA synthetic time of 10 h and a cell cycle time of 30 h.

IN THE CELLS comprising urethane induced pulmonary adenomata the rates of entry into metaphase (R_M) and into DNA synthesis (R_S) decreased as survival of the mice lengthened (Dyson and Heppleston, 1975). This fall in adenoma cell production took place concurrently with a hyperbolic decrease in growth rate and a linear increase in the doubling time. On the available evidence, it was not possible to say whether cell production or cell loss was the more important factor influencing the growth of the neoplasms, or whether the fall in cell production was due primarily to a decrease in growth fraction (I_P), increase in cell cycle time (t_C) or both. The duration of DNA synthesis (t_S) increased in the adenomata as the mice aged, raising the possibility that t_C might also lengthen. In an attempt to determine the effects of t_C and I_P on cell production, a series of continuous labelling experiments was performed at intervals during the development of the adenomata.

Continuous labelling (CL) experiments *in vivo* usually entail injection of tritiated thymidine (^{3}H-TdR) at regular intervals shorter than t_S over a period greater than the combined durations of the $G2$, M and $G1$ phases, *i.e.* $> t_1 + t_2$ (where $t_1 = G1 + 0·5\ M$ and $t_2 = G2 + 0·5\ M$). All cells entering DNA synthesis during this period become labelled and under ideal conditions there is a marked flexion point in the CL curve when all cycling cells are labelled. The time taken for I_L to reach this flexion point is equal to $t_1 + t_2$; t_S and t_C may then be calculated using the simultaneous equations method (Baserga and Malamud, 1969). At the flexion point, the CL index gives an approximate estimate of I_P. If, however, significant decycling of labelled

cells occurs I_P will be over-estimated since non-proliferative labelled cells will be included; the lower I_P, the more decycling and the greater will be the overestimate. Taken in conjunction with information on the duration of cell cycle phases obtained from a fraction labelled mitoses (FLM) study, this I_P value can be used in an iterative procedure to estimate decycling probabilities and so estimate I_P more accurately (Steel, Adams and Barrett, 1966). The combining of FLM and CL data can also be used to test various theoretical models of cell loss.

The most direct and informative procedure for kinetic analysis of a cell population is by construction of a fraction labelled mitoses (FLM) curve. Ideally, this method requires a relatively high proliferative rate so that sufficient mitoses (usually about 100) can be counted at each sampling interval to give a precise curve. In the case of urethane induced adenomata the proliferative rate is high only during the first few weeks post urethane (PU), during which the neoplasms are small and difficult to find in lung sections. When the adenomata are large enough to be readily identified their proliferative rate is much reduced. As a compromise, it was considered that a FLM curve could most usefully be constructed at 14 weeks PU. The difference between the rate of adenoma cell production (K_B) and the observed growth rate (K_G) gives an estimate of the rate of cell loss (K_L) and an indication of its contribution to the diminishing growth rate.

MATERIALS AND METHODS

Specific pathogen-free (SPF) male A2G mice (Laboratory Animals Centre, Carshalton) were injected intraperitoneally (i.p.) with urethane (British Drug Houses) at a dose of 1 mg/g body weight when 3–4 months old.

The CL experiments were performed at 7, 11, 14 and 20 weeks PU, using 10–11 mice for each experiment. ^{3}H-TdR of specific activity 5 Ci/mmol (Radiochemical Centre, Amersham) was injected i.p. into each mouse (0·5 μCi/g body weight) at 10.30 hrs and one animal killed 40 min later. The remaining mice received another ^{3}H-TdR injection 5 h after the first, and again one mouse was killed 40 min later. This procedure was repeated at 5-hourly intervals until a total of 50 h of continuous labelling had been achieved in each experiment.

For the FLM experiment 42 male, SPF derived A2G mice survived 14 weeks after urethane treatment. They were then given a single i.p. injection of ^{3}H-TdR at 10.30 hrs and killed at intervals over the succeeding 60 h.

The mice were killed by cervical dislocation, the left lung fixed in Carnoy's fluid and serial paraffin sections prepared for determination of the CL indices by stripping film autoradiography as described previously. Dipping film autoradiography with Ilford K2 emulsion was employed for the FLM study and at least 100 metaphases were counted at each sampling interval, an objective which often necessitated scanning several adenomata. The pulse labelling index (I_S) was determined by counting 3000 adenoma nuclei in mice killed 1–2 h after ^{3}H-TdR injection.

RESULTS

Continuous labelling

The findings in the 7, 11, 14 and 20 week observations on *adenomata* are given in Fig. 1a, c, b and d respectively, the curves being drawn by eye. The lack of marked flexion points on the CL curves make it difficult to derive an exact $t_1 + t_2$ value, but it was judged to remain approximately constant at 35–40 h. The I_L value at the flexion points, which approximates to I_P, fell as the adenomata aged. The I_P estimates at 7, 11, 14 and 20 weeks were 18, 12, 10 and 7% respectively. These figures indicate the overall pattern of change though they probably overestimate I_P and underestimate its rate of fall in view of the fact that decycling of labelled cells should be increasing as I_P is progressively falling with time.

The CL pattern for *alveolar wall cells,* in the same lung sections as the

adenomata, was essentially similar at the various time intervals and all the results were therefore combined (Fig. 2). At $t_1 + t_2$ of 20 h and an I_P of 1·8% were taken from the point where the curve starts to flatten. Beyond this point the CL curve still has a slight positive gradient, suggesting either that some decycling of labelled cells occurred

or that labelled leucocytes were emigrating from capillaries into the alveolar wall. Assuming that the alveolar wall tissue was in steady state during the CL experiments, t_S and t_C may be calculated from 2 equations: $t_C = t_S + (t_1 + t_2)$, and the pulse labelling index $(I_S) = t_S . I_P/t_C$. Taking the 40 min I_L value of 0·6% as equivalent to I_S, t_S can be calculated
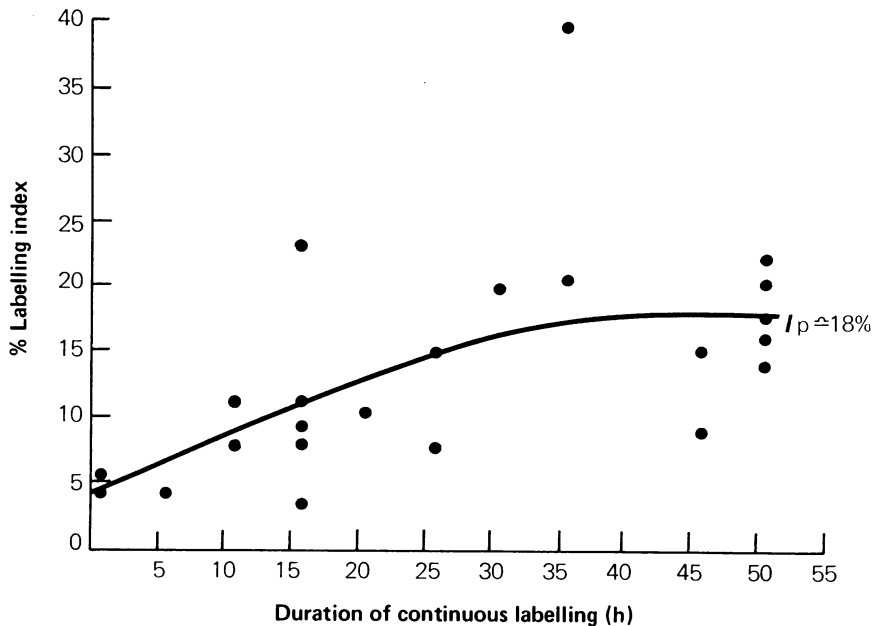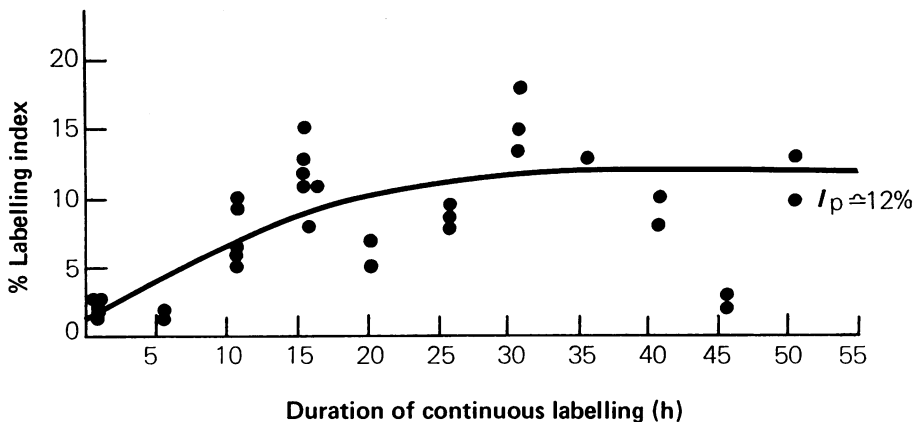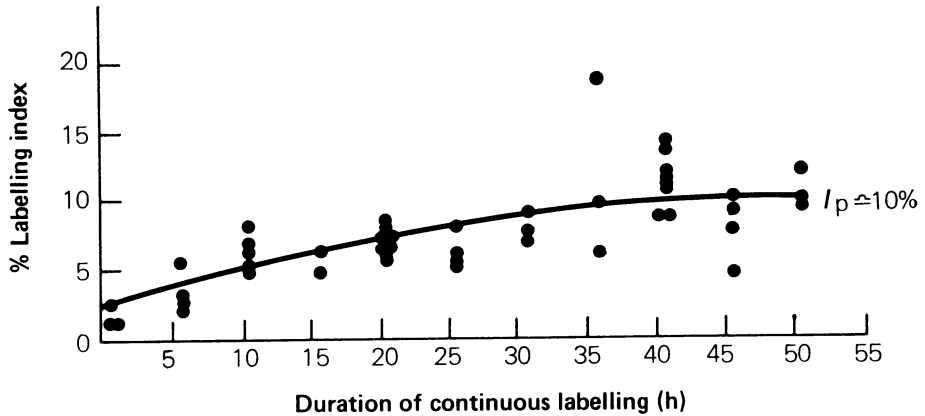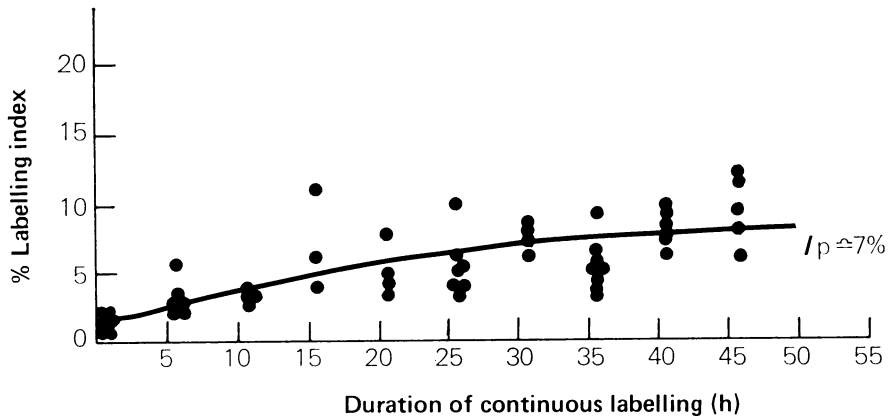

Fig 1 (a)

Fig 1 (b)

FIG 1 (c)

FIG 1 (d)

FIG. 1.—Continuous labelling curves for adenomata at various intervals after urethane treatment: (a) 7 weeks, (b) 11 weeks, (c) 14 weeks and (d) 20 weeks.

at 10 h and t_C at 30 h since $t_1 + t_2$ was estimated to be 20 h.

Fraction labelled mitoses

The *adenoma* curve (Fig. 3) was first drawn by eye but a similar curve was then obtained by means of a Gilbert (1972) computer programme. Only the first peak is apparent, the curve rapidly flattening from about 20 h, indicating that there is considerable variability in t_1 between adenoma cells, and that the initially synchronized cohort of labelled

cells has rapidly become randomly distributed throughout the component phases of the cell cycle. Compared with the variability in t_1, the precision of the peak shows that there is little variation in t_2 and t_S. The FLM curve gives median t_2 and t_S values of 2 and 9 h respectively. The plateau FLM value of 0·20 represents the ratio of t_S to t_C, *i.e.* the theoretical labelling index ($I_{S(T)}$) for cycling cells alone. t_C by calculation is therefore 45 h and t, by subtraction, 34 h. The observed pulse labelling index
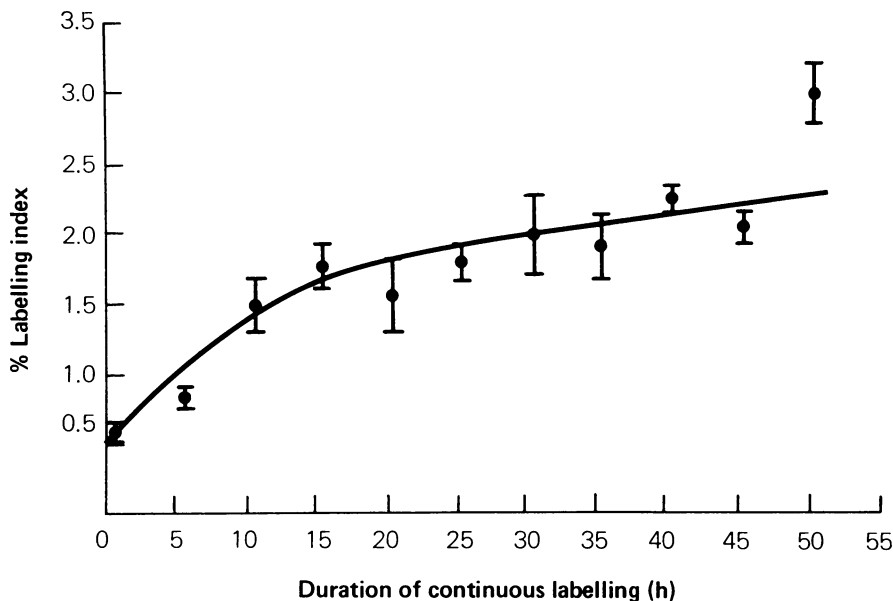
FIG. 2.—Continuous labelling curve of alveolar wall cells from the combined results at all intervals.
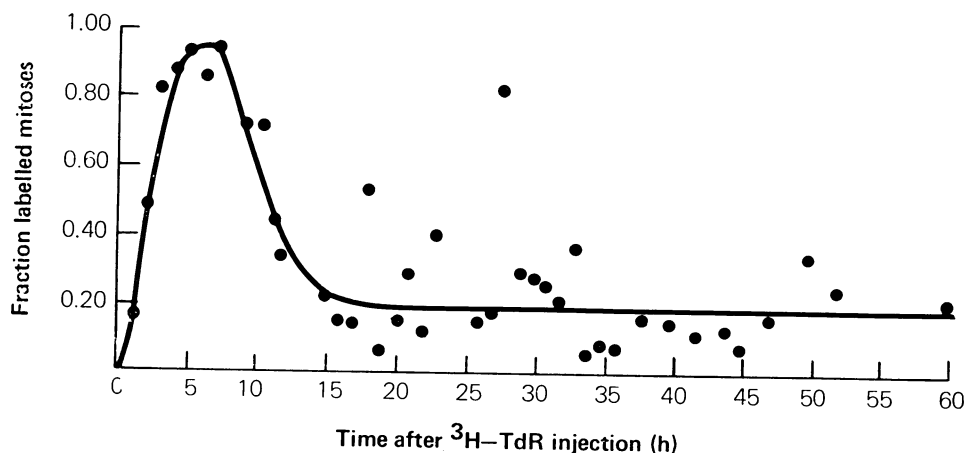

FIG. 3.—Fraction labelled mitoses curve for adenomata 14 weeks after urethane injection.

(I_S) was found to be 1·23% (s.e. ± 0·10%), and I_P was then calculated at 6·2% from the ratio of I_S to $I_{S(T)}$.

DISCUSSION

Although *alveolar walls* include more than one cell population undergoing renewal, it is believed that most of the mitotic activity (70–80%) is contributed by the non-vacuolated cells (Bertalanffy, 1964; Kauffman, 1969) which evidently correspond to alveolar macrophage precursors. Thus the t_S of 10 h and the t_C of 30 h are probably applicable to monocytes or promonocytes. Pilgrim and Maurer (1962) by double isotope labelling and Shorter, Titus and Divertie (1966) by a limited FLM study estimated t_S

to be 7·5 h and 8 h respectively in un-
treated alveolar tissue. The correspon-
dence with our result of 10 h is sufficiently
close as to suggest that the early pro-
liferative effect of urethane on alveolar
wall cells (Kauffman, 1969; Dyson and
Heppleston, 1975) had subsided by the
time the mice were killed.

The adenoma CL data show that
I_P decreases markedly with age, and
therefore suggests that decycling is occur-
ring. This means that the absolute
growth fraction values may be unreliable
since the method overestimates this para-
meter if decycling occurs. The lack of
precise flexion points on the CL curves
prevents the derivation of t_C values and
hence the assessment of the t_C contribu-
tion towards the decrease in cell pro-
duction. However, when the R_S values
obtained previously by double labelling
are plotted against the I_P estimates, a
straight line is obtained which extra-
polates back to the origin (Fig. 4). R_S
thus appears to be directly proportional
to I_P and consequently changes in R_S
may largely be accounted for by altera-
tions in I_P without invoking marked
changes in t_C. In many solid experi-
mental tumours it has been found that

t_C remains constant with age whilst I_P
falls and cell loss increases (Lala, 1971).
CL data obtained from rapidly growing
transplanted animal tumours, taken in
conjunction with accurate estimates of
cell cycle phases obtained from double
peak FLM curves, elucidated the factors
of decycling and cell loss in tumours
(Steel et al., 1966). The CL and FLM
values for urethane induced adenomata
are, however, too variable to merit the
application of computer modelling tech-
niques to assess decycling probability.
Although it is not easy to obtain cell
kinetic data on a slowly growing benign
tumour such as the urethane induced
pulmonary adenoma, such information as
can be secured, though less precise than
in the case of the more rapidly proliferat-
ing malignant neoplasms, goes some way
to explain the basis for benign behaviour
and demonstrates the applicability of
kinetic principles derived from rapidly
growing transplantable tumours to pri-
mary tumours in their natural environ-
ment.

The observed growth rate (K_G) of the
adenomata is the resultant between the
rates of cell production (K_B) and of cell
loss (K_L), i.e. $K_G = K_B - K_L$, and may
be calculated for any time period by the
formula $K_G = 2\cdot181/t$ derived previously.
A K_B value at 14 weeks PU can be
derived from the t_C and I_P estimates
obtained in the FLM study since

$$K_B = \ln (1 + I_P)/t_C$$
$$= \ln (1\cdot062)/45$$
$$= 0\cdot1338\% \text{ per h.}$$

The double labelling-derived R_S values
referred to earlier can also furnish K_B
estimates by assuming that R_S is ap-
proximately equal to R_M. Such an as-
sumption is reasonable in view of the
long t_Cs and low I_Ps during the develop-
ment of the adenomata, factors which
tend to produce rectangular steady state
age distributions. Rather than calculate
K_L, the effect of cell loss on the growth
potential of adenomata is better demon-
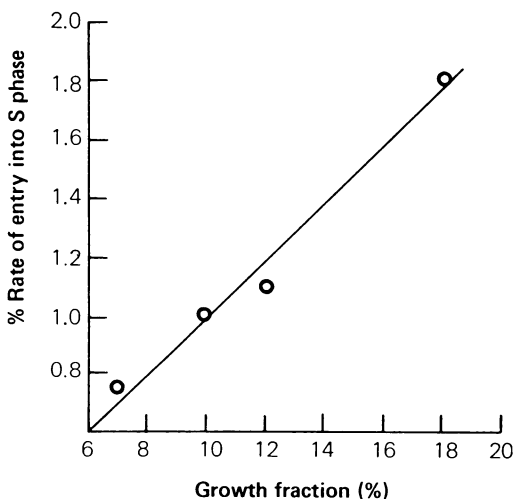strated by expressing K_L as a percentage

FIG. 4.— Comparison of the rate of entry of
adenoma cells into DNA synthesis with the
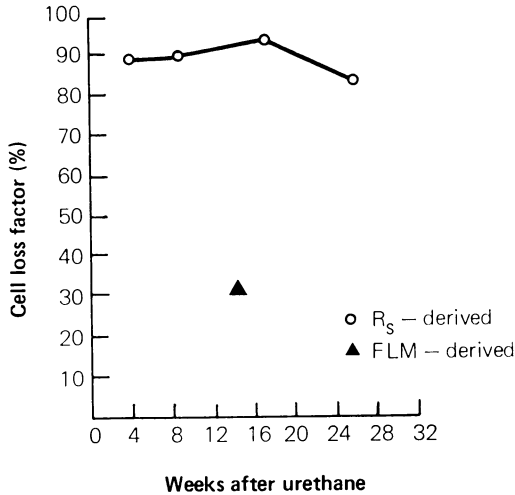growth fraction.

FIG. 5.—The cell loss factor for adenomata in relation to survival.

of K_B (Steel, 1968). Thus, the cell loss factor

$$(\phi) = K_L/K_B \, . \, 100$$
$$= (1 - K_G/K_B) \, . \, 100.$$

The variation in ϕ with time is shown in Fig. 5, from which it is evident that the ϕ values derived from R_S remain relatively constant at 83–95%. Since K_B has been shown to fall with age, it follows that K_L must also be falling *pari passu* to maintain a constant ϕ. Cell loss, as indicated by the double labelling studies, may thus be linked in some way to cell production. The possibility also arises that a proportion of cells which take up ^{3}H-TdR during DNA synthesis might fail to pass through mitosis, either from phase specific cell loss (as in the S phase) or by the formation of polyploid cells. Either of these phenomena could help to explain the discrepancy between the FLM derived ϕ of 31% and the R_S-derived ϕs of 83–95%.

Cell loss is not readily detected histologically. No areas of necrosis were seen at any stage in the development of adenomata, the tumours being well vascularized. Apart from occasional collections of lymphocytes at the periphery of some adenomata, there was no morphological evidence of an immune reaction by the host. Pulmonary adenomata are benign neoplasms and we have never observed metastasis, by which cells could be lost. Karyorrhexis was seen in isolated nuclei and could indicate that apoptosis or spontaneous cell death (Kerr, Wyllie and Currie, 1972) was occurring. It should be noted that cell loss does not need to achieve a high level to have an appreciable effect on growth potential, since the rate of cell production is also low throughout the development of adenomata.

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