

IN VITRO AUTORADIOGRAPHIC STUDIES OF CELL PROLIFERATION**IN THE GASTROINTESTINAL TRACT OF MAN**

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Up to the present time the radiobiological effects of labeled thymidine have restricted its use in man to patients with limited life expectancy. This fact explains the relative paucity of data concerning autoradiographic estimates of the generative cycle duration in human tissues.

But as Kissel *et al* (1) have pointed out, the autoradiographic technique is the only way of studying the rhythm of cellular division. Therefore methods have been proposed to avoid the radiobiological hazard linked to using tritiated thymidine in clinical studies of human cancer growth. For example, *in vivo* studies have been performed by perfusing the tissues during surgical interventions (1). In addition, *in vitro* studies have been used without any injection of labeled precursors to the patients (2,3). However, these studies let one observe the labeling pattern and labeling index of the biopsies without true measurement of the generation time or S phase (length of phase of desoxyribonucleic acid synthesis).

We undertook the present work to set up a procedure for measuring mitotic parameters which could be applied to several human tissues without any risk to the patient. The method we use takes advantage of the very elegant double-labeling technique proposed by Wimber and Quastler (4) and by Maurer *et al* (5) who applied it to animal tissues (6,7). We hoped the technique, which requires a single tissue sample and a relatively short treatment duration, would give valuable information when applied *in vitro* to biopsies maintained under simple survival conditions. This paper reports the preliminary observations made in the case of the human rectum. The rewarding results seem sufficiently encouraging to justify this communication.

METHODS

Rectal biopsies were collected from patients with no intestinal disease and were placed immediately in the incubation medium in an ice bath. After rinsing,

each biopsy was divided into 2–4 fragments about 1-mm thick. The samples were incubated in 25-ml flasks containing 2 ml of the medium. The flasks were stoppered with rubber stoppers and placed in a shaking water bath at 37°C.

The incubation medium was Eagle Medium (Powder Medium 3ME; Diploid, purchased from GBI, Chagrin Falls, Ohio) supplemented with 10% calf serum. The pH was adjusted to 7.2–7.4 by adding drops of a 5% sodium bicarbonate solution. We prepared three different media: one containing 1 $\mu\text{C}/\text{ml}$ ^3H thymidine (Amersham, 5.0 curies/mM), a second containing 10 $\mu\text{C}/\text{ml}$ of ^3H thymidine and a third without labeling.

The specimens were successively incubated for 15 min in 1 $\mu\text{C}/\text{ml}$ ^3H thymidine, then 60 min in “cold” medium (without labeled precursors) and finally 15 min in 10 $\mu\text{C}/\text{ml}$ ^3H thymidine. Whenever the flasks were opened, the medium was bubbled with carbon dioxide. This experimental procedure was taken as approximating a treatment in which two pulse-labelings are realized at a 1-hr interval (see below). After the end of incubation, tissue specimens were washed in “cold” medium, rinsed in 0.9% NaCl and fixed in Bouin’s fixative.

Autoradiography. Specimens embedded in paraffin were cut to a thickness of 3–5 microns. Sections were covered with nuclear emulsion (Ilford K₅ in gel form) by dipping the slides in the melted emulsion. The autoradiographs were exposed 3–6 weeks, developed in Dektol (2 min) at room temperature and fixed in Kodak Rapid Fixer. After rinsing in tap water for 1 hr, the slides were kept overnight in alcohol 70. The tissues were then stained with hematoxylin-eosin, dehydrated, cleared in xylol and mounted in DPX mounting medium (Michrome).

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TABLE 1. MITOTIC PARAMETERS OF HUMAN RECTAL BIOPSIES : IN VITRO MEASUREMENTS

Patient	Biopsy	S phase duration (hr)	Labeling index (%)	Generation time (hr)
A 17	1	8.8	8.2	108.5
	2	9.0	6.9	131.0
		mean:8.9		mean:120
A 18	1	8.2	8.8	93.0
	2	7.3	8.3	90.4
		mean:7.85		mean:91.7
A 19	1	7.0	8.7	80.5
	2	7.4	10.0	74.0
		mean:7.2		mean:77.2
A 20	1	8.5	8.0	118.0
	2	7.7	8.0	96.2
	3	8.0	8.0	100.0
	4	7.3	8.0	91.5
		mean:8.05		mean:101.5
A 21	1	7.3	5.3	136.5
	2	8.0	7.3	109.8
	3	7.3	5.3	137.9
	4	6.3	4.7	135.5
		mean:7.2		mean:129.9

Measurement of S phase duration and generation time. As already mentioned, we used the double-labeling technique (4,5) which consists of two successive, brief exposures to labeled thymidine—the first one different from the second so that their effects in labeling the cell nuclei can be recognized. The labeling is accomplished either by using ^3H and ^{14}C thymidine or by using two very different doses of ^3H thymidine, i.e., a weak one and a high one. Knowing the time interval between the two pulse-labelings (1 hr in our case) and the ratio of the number of cells labeled by the second dose to the number of cells reached by the first (and thus appearing as weakly labeled cells), one can estimate the S phase duration using the formula (6):

$$N_h/N_w = S/t$$

in which N_h is the number of heavily labeled cells, N_w is the number of weakly labeled cells, S is the S phase duration and t is the time interval between the two ^3H thymidine exposures.

The generation time can then be estimated on the basis of the labeling index using the formula:

$$\text{TG} = (S/\text{LI}) 100$$

in which TG is generation time, S is S phase duration and LI is labeling index (number of labeled cells per 100 cells). The validity of this formula has been discussed by several authors (8,9). It has been shown for several tissues that the double-labeling technique gives results that are in fair agreement with those obtained with the more classical method of "wave of labeled mitoses" of Quastler and Sherman

(9) as well for S phase duration (5) as for generation time (10).

RESULTS AND DISCUSSION

The results obtained for biopsies from five patients are summarized in Table 1; Fig. 1 shows the type of labeling observed. The labeled precursors seem to diffuse very slowly into the specimen so that only the crypts adjacent to the cut surface of the biopsies appear labeled in the autoradiographs. We therefore restricted our counts to these labeled regions, and the results obtained seem very reproducible.

The values of the S phase duration and generation time established by this method have been compared with data in the literature for *in vivo* determinations of mitotic parameters of the human rectum. The values of 7–9 hr obtained for the S phase duration in our tests differ somewhat from the reported values of 10–11 hr from *in vivo* determinations (11) on the same tissue. However, these larger values are based on the wave of labeled mitoses after ^3H thymidine injection (9). They were

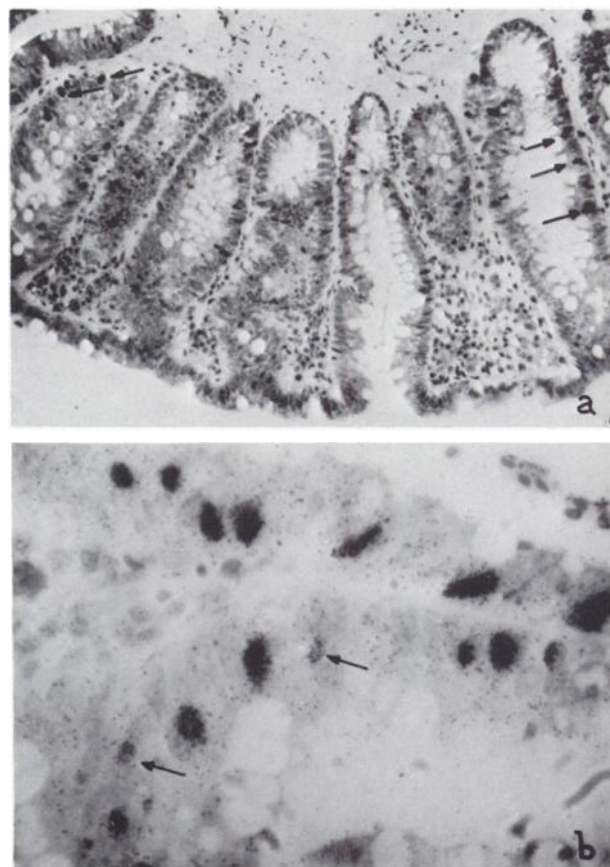


FIG. 1. Autoradiographs show distribution of labeling in crypts of human rectal biopsy after *in vitro* incubation with ^3H thymidine. In (a) distribution of labeled nuclei is shown (arrows); in (b) higher magnification shows two poorly labeled cells (arrows) among unlabeled and heavily labeled nuclei. (Ilford L4—3-week exposure).

estimated from the duration between the 37% points on the ascending and descending limbs of the curve of labeled mitoses and have therefore probably been overestimated. In fact a recent mathematical study (13) of this method showed that the 50% points constitute a better reference for this estimation of S phase. The estimated durations of generation cycle we obtained (77–130 hr) are compatible with the proliferation rate of 1–2 cells/100 cells/hr measured *in vivo* by Lipkin (11) and with the turnover rates of 6–8 days ascribed to the human rectal mucosal epithelium on the basis of *in vivo* observations following ³H thymidine injection (12).

CONCLUSIONS

In this preliminary report we have measured the S phase (phase of desoxyribonucleic acid synthesis) and cell cycle duration of the human rectal mucosal epithelium incubated *in vitro*. The results obtained are quite reproducible and seem compatible with the available data in the literature concerning estimates made after *in vivo* labeling of this tissue. Therefore the procedure seems to constitute a way of avoiding the biological hazards of using ³H thymidine when studying the proliferation of human tissues.

Other studies have used *in vitro* incubation of biopsies to avoid injections of labeled thymidine in human beings (2,3). However, the present work is the first to our knowledge that brings forward kinetic data about human biopsies incubated *in vitro*. This has been possible thanks to the double-labeling technique (4,5). Our study suggests that the procedure warrants further investigation as a way of studying the proliferative parameters of all the human tissues that can be biopsied. Further work is now in progress in this field.

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