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AN IN VIVO METHOD OF STUDYING THE KINETICS OF CELL PROLIFERATION IN NORMAL HUMAN EPIDERMIS

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Abstract. A method is described for the evaluation of DNA synthesis time and cell cycle duration for the basal cell of normal human epidermis. The method is based on in vivo double injection of tritiated thymidine, intradermally. Since it requires only two biopsies and the injection of small quantities of tritiated thymidine, the method would seem preferable to others. The DNA synthesis time found was 7 hours 35 min; the entire germinative cell cycle duration was 206 hours. The advantages deriving from the described procedure are extensively discussed.

An analysis of the papers published between 1960 and 1971, concerned with the kinetics of cell proliferation in the skin, reveals that most of the work has been done on laboratory animals. This is probably due to the fact that the techniques for studying the cell cycle are hardly feasible in man. However, the need to obtain more data on some parameters of the kinetics of cell proliferation in normal human epidermis, in vivo, appeared to warrant further investigation. As Table I shows, the results of measuring the duration of DNA synthesis and of the entire germinative cell cycle in the epidermis of different mammals, including man, did not agree.

MATERIALS AND METHODS

Six male patients between 62 and 85 years of age were informed of the nature of the experiment and volunteered to participate. All had been hospitalized for long-term care and none was suffering from any severe degenerative disease. They were tested by being injected intradermally with 0.1 ml of tritiated thymidine (0.003 mCi = 3 μCi, N.E.N., spec. act. 22.3 Ci/mM), in two areas of abdominal skin (*A* and *B*), not less than 15 cm apart. Area *A* was injected at 4.00 p.m. and again at 6.00 p.m.; area *B* was injected at 6.00 p.m. only. Both areas were biopsied at 7.00 p.m. The needles used for intradermal injection were the smallest available; the second injection was made at the same point as the first injection. Those subjects in whom a slight hemorrhagic effusion occurred after the first injection were excluded, since we used only those subjects showing ischemic, regular wheals. To verify that the DNA synthesis and/or the entire cell cycle duration could be altered by the trauma of the first injection, we injected physiological saline and, 2 hours later, tritiated thymidine in a control subject, 63 years old. The biopsies were fixed in Bouin fluid and embedded in paraffin. Serial sections, 4–5 μm thick, were coated with Kodak AR 10 stripping film and developed after 25 days of exposure at 4°C. The counting of the labelled basal cells was made on a Reichert light microscope equipped with an 8× eye-piece and an immersion objective of 100/1.25. A correction factor of 1.4 was introduced in all percentages (9). We chose such factor in view of the fact that the greater thickness of our histological sections (5 μm) was correspondent to the lower thickness of the sections used by Iversen & Evensen (9). We preferred to maintain the above-mentioned correction factor which had been proved to be correct in previous experiments. The duration of the S phase[1] was obtained as follows. According to Pilgrim & Maurer (11) and to Baserga & Lisco (1), the ratio between the difference of percentage of labelled cells in the two areas (A–B) and the percentage of labelled cells occurring in the area labelled once (B) is equal to the ratio of the interval of time (Δ_t)/total length of the S phase (T_s). Therefore, from the relationship:

$$\frac{(A-B)}{B} = \frac{\Delta t}{T_s};\ T_s = \frac{B\Delta t}{(A-B)}$$

Assuming that the growth fraction of the basal cell population is between 0.95 and the unity (8), since $N_s : T_s = N_{g.c.} : T_{g.c.}$ the entire germinative cell cycle time was given by the formula:

$$T_{g.c.} = \frac{T_s N_{g.c.}}{N_s}$$

[1]

T_s = S phase (DNA synthesis) time
N_s = number of labelled basal cells
$N_{g.c.}$ = total number of basal cells
$T_{g.c.}$ = duration of the entire germinative (basal) cell cycle
Δt = interval of time (2 hours)

Table I. *Duration of DNA synthesis time and of the entire germinative cell cycle in the epidermis of mammals*

Author	Animal	Remarks	"S" Phase (hrs)	Cell cycle duration (hrs)
(13, 1960)	Mouse, rat	in vivo	4–5	24–120
(14, 1961)	Mouse	in vivo	30	586
(11, 1962)	Mouse	in vivo	7.1	—
(9, 1962)	Hairless mouse	in vivo	5.3	110
(4, 1964)	Mouse	in vivo	8	150
(3, 1967)	Man	in vitro	5.9	142–166
(16, 1969)	Man	in vivo	16	308
(7, 1971)	Man	in vitro	10	213

RESULTS

The mean percentage of labelled cells was 3.79% $\pm$0.75 in area B, which had been injected only once. This percentage rose to 4.80% $\pm$0.99 in area A, which had been injected a second time, 2 hours after the second injection. Thus, the number of labelled cells increased by 0.50% per hour. The "*t*" test for paired comparison resulted in a highly significant difference between *A* and *B* ($t = 9.5514$; $P < 0.01$). The DNA synthesis time for the normal basal cell of human epidermis, in vivo, was 7 h, 35 min $\pm$1.13 hours. The time for the entire germinative cell cycle duration was 206.67 $\pm$ 50.92 hours. The percentage of labelled cells in the control subject was 3.13% in the area injected with tritiated thymidine and 2.94% in the area previously injected with physiological saline. To obtain these data, it was necessary to count more than 10 000 cells per biopsy, due to the well known irregular, wave-like arrangement of the basal layer of human epidermis. When we processed our data, we obtained figures in terms of hours, minutes and seconds; however, we mentioned these crude data only in the above results and not in Table II.

DISCUSSION

Weinstein & Frost (16) demonstrated that the duration of the S phase in the normal human basal cell, in vivo, is 16 hours and that the entire germinative cell cycle duration is 308 hours. We believe that the technique of labelled mitoses used by these authors is substantially one of the most correct. However, it requires the injection of large amounts of tritiated thymidine and, moreover, a certain number of very co-operative patients. Their findings were, in fact, obtained from more than 10 biopsies performed in the same subject within 27 hours. On the other hand, the in vitro double-labelling methods used on human skin, yielded contradictory data: a DNA synthesis time of 5.9 hours was reported by Christophers & Schaumlöffel (3), while Heenen & Galand (7) reported 10 hours; the cell cycle duration was 142–166 hours and 213 hours respectively (see Table I: 3 and 7).

We believe that in vivo methods are preferable to in vitro techniques, for several reasons. First, no matter how perfect the laboratory conditions may be, it is impossible to duplicate the in vivo conditions. Second, the multiple trauma involved in cutting the specimens into small pieces for tissue culture purpose, probably alters the mitotic rhythm of the tissue. Moreover, to be valid, in vitro methods require that all conditions of the experiment be controlled. For example, it has been demonstrated that labelled cells cannot be found more than 150 μm from the surface of the tissue culture specimen; to increase the depth

Table II. *DNA synthesis time and duration of the entire germinative cell cycle in epidermis of man*

T_s = Duration of "S" phase; $T_{g.c.}$ = Duration of entire germinative cell cycle; Δ_t = interval of time (2 hours)

No.	Name	Age	*A*%	*B*%	$\frac{A-B}{\Delta_t}$	$T_s = \frac{B \cdot \Delta_t}{A-B}$		*T*
1	G. G.	62	4.15	3.45	0.34	9.99	9.54'	288
2	B. U.	70	4.78	3.75	0.51	7.26	7.12'	193
3	L. E.	78	4.09	3.20	0.44	7.17	7.06'	223
4	G. A.	81	6.76	5.29	0.73	7.16	7.06'	135
5	C. G.	63	4.61	3.51	0.54	6.40	6.24'	182
6	P. E.	85	4.46	3.55	0.45	7.81	7.48'	219
			4.80$\pm$0.99	3.79$\pm$0.75	0.50$\pm$0.13		7.35' $\pm$ 1.13'	206$\pm$50

of the labelled zone, a high grade of partial oxygen pressure must be used (15). If these conditions are lacking, some of the nuclei in DNA synthesis do not remain labelled; therefore the reliability of the in vitro method for quantitative estimation is doubtful. To be sure that all the DNA synthesizing cells could incorporate the tritiated thymidine that we injected intradermally, the biopsy was taken "inside" the wheal caused by the injected labelled compound.

In a recent report, Heenen & Galand proposed an in vitro double-labelling method that needs two different doses of tritiated thymidine to identify heavily and weakly labelled cells in the same specimen. Granted that the labels "heavy" and "weak" represent a subjective opinion, we believe that the number of grains visible on the histological sections of the nuclei will depend on whether the section has been made through the centre of the nucleus. In other words, the same cell may appear heavily labelled in one section and weakly labelled in the next, because the more tangential the section, the less will be the number of grains.

One of the purposes of the present study was to keep the experimental conditions as safe as possible, even though we are aware that the injection of tritiated thymidine is not completely free of side effects (5). Therefore, we used the smallest possible quantity of the labelled compound, injecting single doses of 3 μCi. The use of small doses was justified by other considerations. It has been shown that ^{3}H-Thymidine induces mitotic depression in the labelled cell population and supranormal mitotic activity in the unlabelled part of the cell population. Cold thymidine, on the contrary, does not affect these parameters (6). The use of small doses of the labelled compound should therefore overcome or, at least, reduce, the above-mentioned side effects within manageable limits. Moreover, performing the same procedure in both areas of skin helps to restrict the error to reasonably constant limits.

Following Baserga & Wiebel (2) we chose the interval of 2 hours between the first and second injection because it was shorter than the presumable duration of the "S" phase. Studies on the metabolism and fate of tritiated thymidine in man have established that the period when the labelled compound is available for incorporation into nuclear DNA is extremely short and that, afterwards, it is no longer available (12). The 2-hour interval we chose, therefore, seems to be long enough to ensure that all the tritiated thymidine had disappeared from the tissue by the time of the second injection.

To standardize our technique and to minimize the variations that arise from sex, age and activity, we chose male, elderly, bed-resting patients. Moreover, we performed our experiments between 4.00 p.m. and 7.00 p.m., although we are aware that the influences of the circadian rhythm are negligible in man (10). The control performed in a subject 63 years old, demonstrated that the effects of the double injection are negligible as far as the cell cycle is concerned.

The method described here does not make it possible to verify the variations of the labelling index that naturally occur in the same individual, nor the so-called "growth fraction". Such verifications could be obtained only by performing more biopsies in the same subject, but this would militate against the safety criteria expressed above. However, we did obtain a low standard deviation and a statistically low average difference between A and B (0.022 and 0.787 respectively); moreover, the variability coefficient was 2.83%. These data demonstrated that the individual variability in the two areas investigated remains within a narrow range and that no wide variations are apparent in the same subject. It has been shown that the growth fraction of the basal cells of mouse skin is between 0.95 and unity, since the labelling index is 95% of the interfollicular basal cell population after repeated labelling (8). It seems reasonable to believe that the growth fraction of human skin may be contained within the above limits and therefore to assume that the growth fraction cannot be less than 0.95, which is not statistically different from unity.

CONCLUSIONS

The different phases of the cell cycle (mitotic duration, G1, S, G2 phase) can be measured only by counting the percentage of labelled mitoses throughout the entire period of the cell cycle. However, such a procedure is not easily accomplished in man, in vivo, because it requires a large number of biopsies and the injection of large amounts of tritiated thymidine. Although

derived from other similar double-labelling techniques (1, 11), the method presented here seems to fulfil the need for development of techniques for cell kinetic studies on human epidermis, in vivo. We suggest that it can be used for in vivo investigations in man, although it is limited to the S phase and to the entire cell cycle duration. However, it requires only two biopsies and only small quantities of the radioactive compound to be injected. Presumably, many pathological conditions could be studied by means of the above method; the pharmacological effects of cytostatic drugs acting on the DNA synthesis could also be profitably studied in man. In our opinion, no method that allows us to study some of the parameters of the cell cycle should be neglected.

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