EARLY AND LATE INCORPORATION OF TRITIATED THYMIDINE INTO SKIN CELLS AND THE PRESENCE OF A LONG-LIVED G_0-SPECIFIC PRECURSOR POOL

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ABSTRACT

40 min after a single injection of 50 μCi of tritiated thymidine a 3 mm punch of DBA-1 mouse skin contains about 1000 dpm. This value remains constant for at least 48 hr after injection. 50 hair follicles contain about 40 dpm, and from these values the activity calculated to reside in the basal layer of a 3 mm punch of skin is 760 dpm. These values also remain constant with time after injection. Fresh punches of skin contain much more activity. The fixative-soluble fraction (the difference between fresh and fixed values) decays slowly with time. The values for DBA-2 mice are similar. Plucking the hair from the follicles appears immediately to increase the size of the fixative-soluble fraction and decrease the fixed tissue values to about 500 dpm per punch for whole skin and about 1 dpm per 50 follicles for DBA-1. Thus almost all the activity is restricted to the epidermis. The fixative-soluble fraction returns approximately to the unplucked value between 24 and 48 hr after plucking. However, during this period the fixed tissue values are rising rapidly as stimulated cells enter S. It appears that in both strains labeled material remains available for incorporation into stimulated cells for at least 48 hr after a single injection. The amount persisting appears to decrease with time. The whole-fixed skin, the hair follicles, and the epidermis all contain cells that are capable of becoming labeled after stimulation 8–48 hr after an injection. The label in question does not become incorporated into normal cycling skin or hair follicle cells. It is concluded that the DNA precursor pool is possibly connected with G_0 cells and that both the hair follicle and the basal layer of the epidermis contain these resting cells.

INTRODUCTION

The considerable changes seen in the incorporation levels of tritiated thymidine (TdR-^{3}H) into both basal layer and hair follicle cells in mouse skin during the hair growth cycle have recently been described. The results were obtained using liquid scintillation procedures (8, 9). The changes in the levels of radioactivity in fixed tissue are the same as those seen when radioautographic techniques are used (9). A large fixative-soluble pool of tritium activity was observed at all stages of the hair

growth cycle. [1,2] The size of this pool varied slightly through the hair cycle, being particularly large immediately after stimulating the cells of both basal layer and follicle into activity by plucking the hair from the follicle.[1,3] It appears that some of this tritium pool is in a form that can be incorporated into fixed tissue and is thought to be either thymidine or its phosphorylated derivatives.[2]

Moffat and Pelc (6) described the presence of a similar pool in the hair follicle that persisted from 2 to 19 hr after injection and was capable of labeling hair follicle germ cells up to 32 hr after stimulation into activity and the initial injection of TdR-^{3}H. This thymidine pool gave diffuse labeling of frozen sections and was clearly removed by routine histological procedures. It is possible that even the frozen section techniques used may have reduced the amount of activity somewhat from the in vivo situation, since we have observed that even contact with instruments and glassware can reduce the activity detectable in fresh tissue.[2]

Late labeling has been reported to occur in many tissues (3, 4, 12, 14). These experiments have been interpreted on the basis of salvage pathways and the reutilization of thymidine from dying labeled cells. In some cases reutilization appears the only possible explanation for the observed facts (2); however some of the late labeling results could be explained on the basis of a long-lived intracellular DNA precursor pool.

The present experiments demonstrate late labeling at times when reutilization should not occur, i.e., labeling from a pool present 40 min–48 hr after an injection of TdR-^{3}H.

MATERIALS AND METHODS

Male DBA-1 mice (7–8 wk old) (Jackson Laboratories, Bar Harbor, Maine) were used. Mice not in the resting phase of the hair growth cycle characteristic of this age were discarded. The basal layer of the epidermis and the germinal region of the hair follicles were stimulated by plucking (7) an area of about 6 cm^2. The plucking procedure extracts the resting hair and its club but removes relatively few cells. Tritiated (methyl-^{3}H) thymidine at a dose of 50 μCi per mouse intraperitoneally (approximately 2.5 μCi per g) was used as a specific label for DNA synthesis (specific activity 6.0 Ci per mmole, Schwarz Bio Research Inc., Orangeburg, N.Y.).

In order to minimize any possible diurnal influences the animals were killed and skin samples were taken between 09.30 and 11.30 hr. Numerous 3 mm diameter circular punches of fresh skin were taken immediately after killing the animals.

Three fresh punches and three fixed punches (20 min in cold Carnoy's fixative followed by at least 3 hr in 70% ethanol) from each animal were placed in scintillation vials and dissolved in 1 ml of Soluene (Packard Instrument Co., Inc., Downers Grove, Ill.) at room temperature for approximately 48 hr. 10 ml of scintillation mixture were added to each vial (5.0 g 2,5-diphenyloxazole and 0.2 g 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene in 1000 ml of toluene). The vials were cooled and counted at 5°C for 20 min or to a total of 10,000 counts in a Packard Liquid Scintillation Spectrometer (model 3380). The background (23.3 cpm) was subtracted and the counts per minute (cpm) were corrected to disintegrations per minute (dpm) using an Absolute Activity Analyzer (model 544).

Other punches of fixed skin were hydrolyzed (8 min at 60°C in 1 N HCl) and Feulgen stained before 50 follicles were dissected and placed together in other scintillation vials with 0.5 ml of Soluene overnight. These vials were counted in the manner just described.

Thus from each mouse three values for dpm per 3 mm punch of fresh skin, three values for dpm per fixed skin, and one value for dpm per 50 hair follicles were obtained. Since it has been determined that a 3 mm punch of skin contains 292 $\pm$10 follicles the dpm per follicular component can be estimated and thus an estimate of the dpm per epidermal component can be calculated by subtraction (9).

The animals were divided into various groups, each group being treated according to a different experimental protocol. A minimum of four animals was used per point. In the first experiment the animals were given a dose of 50 μCi of TdR-^{3}H and were killed 40 min, 8, 24, or 48 hr thereafter. The animals had the hair clipped after death but were not plucked. In the second experiment the animals were plucked and one group was injected with 50 μCi immediately and killed 40 min later. Other animals were injected with 50 μCi 8, 24, or 48 hr after plucking and were killed 40 min after injection. In the third experiment unplucked animals were injected with TdR-^{3}H and then plucked 8, 24, or 48 hr later to stimulate dormant (resting or G_0) cells into activity. The mice

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[2] Potten, C. S., R. F. Hagemann, and J. C. Schaer. 1971. Presence of non-DNA radioactivity several days after pulse labeling with tritiated thymidine. Manuscript in preparation.

[3] Potten, C. S. 1971. Some observations on the post-plucking depression in tritiated thymidine utilisation and some tentative cell kinetic determinations. Submitted *J. Invest. Dermatol.*

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were killed 24 or 48 hr after plucking, i.e., after a time sufficient to permit the stimulated cells to enter S.

A second series of experiments was conducted using slightly different materials and methods. They differed in the following ways: (*a*) DBA-2 mice were used; (*b*) a different liquid spectrometer was used (Packard model 3375); (*c*) the vials were counted at ambient temperatures; (*d*) to quench the chemiluminescence observed at ambient temperatures three drops of concentrated HCl were added to each vial before the scintillation mixture; (*e*) absolute activity was determined using the external standard ratio method. Under these conditions the backgrounds and counting efficiencies for the two procedures were similar; about 24 cpm and 25–30%, respectively.

These DBA-2 experiments involved plucking the animals and injecting the TdR-^{3}H immediately after removing the hair. The mice were killed and samples were taken 40 min, 2, 8, 24, 48, or 72 hr after the injection.

RESULTS

The results of the DBA-1 experiments are shown in Table I. Table I is divided into three sections depending on the protocol. In the top section of Table I, results obtained when TdR-^{3}H was injected into unplucked mice and samples of skin were taken 40 min, 8, 24, and 48 hr later are shown. The fresh tissue dpm per 3 mm punch of skin and the fixative-soluble fraction (the difference) clearly decay with time. 40 min after the injection, the fresh punch is 6213 dpm higher than the fixed tissue. The fixed tissue dpm per punch, the dpm per 50 follicles, and the calculated dpm per 3 mm epidermis remain constant over the period studied. Thus the fixative-soluble activity is not apparently utilized by the cells in the follicle or basal layer that are progressing through the cell division cycle, i.e., those actively cycling. It has been found that these results can readily be reproduced. For example, if the over-all mean of numerous control samples (40 min postinjection) is taken, the values differ little from the mean values shown in Table I: fixed skin, 1028 ± 55 (44 animals); 50 follicles, 46 ± 4 (17 animals); and epidermal component, 759.

The middle section of Table I shows the results obtained when the mice were plucked, then in-

TABLE I

Early and Late Incorporation of Label into Skin Cells Stimulated into DNA Synthesis 8–48 hr after TdR-^{3}H Injection (4–6 animals per point, DBA-1 males)

Protocol			Data				
TdR-^{3}H injection	Plucked	Samples taken	dpm per punch of skin ± SE: Fresh tissue	Fixed tissue	Difference	dpm per 50 follicles	dpm per epidermal component
0 hr	—	40 min	7198 ± 590	985 ± 67	6213	39 ± 4	757
0 hr	—	8 hr p.i.*	5811 ± 317	953 ± 127	4858	33 ± 4	760
0 hr	—	24 hr p.i.	4448 ± 294	813 ± 71	3635	34 ± 4	614
0 hr	—	48 hr p.i.	3850 ± 190	900 ± 117	2950	33 ± 7	707
Immediately	0 hr	40 min p.i.	12,904 ± 1091	504 ± 43	12,400	1.2 ± 0.7	497
8 hr p.p.‡	0 hr	8 hr p.p. (40 min p.i.)	9463 ± 842	589 ± 98	8874	5 ± 3	560
24 hr p.p.	0 hr	24 hr p.p. (40 min p.i.)	11,873 ± 630	4657 ± 374	7216	258 ± 62	3150
48 hr p.p.	0 hr	48 hr p.p. (40 min p.i.)	11,776 ± 1048	6169 ± 925	5607	335 ± 78	4213
0 hr	8 hr p.i.	24 hr p.p.	7907 ± 1069	1901 ± 198	6006	45 ± 10	1638
0 hr	8 hr p.i.	48 hr p.p.	4979 ± 245	2099 ± 138	2880	73 ± 5	1673
0 hr	24 hr p.i.	24 hr p.p.	5401 ± 328	1408 ± 125	3993	27 ± 9	1250
0 hr	48 hr p.i.	24 hr p.p.	4961 ± 243	1231 ± 213	3730	36 ± 2	1021

* p.i., postinjection.

‡ p.p., postplucked.

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jected with TdR-^{3}H immediately, or after 8, 24, or 48 hr. The samples were taken 40 min after the injection of TdR-^{3}H. The fresh tissue values show that, for the first 48 hr after stimulation by plucking, the amount of fixative-soluble radioactivity per punch of skin is increased (12,400 dpm per punch compared to 6213 for unplucked skin). The dpm per punch of fresh tissue remain fairly constant over the first 48 hr after plucking, although the fixed tissue values rise rapidly after an initial lag of 10 hr. The fixed tissue levels are initially lower, remain lower for 10 hr, and then rise rapidly as the stimulated cells enter the S phase of the cell cycle. The dpm per 50 follicle values and the calculated epidermal values show the same changes with time after plucking as the whole fixed punches. These first two sections of Table I demonstrate the early labeling of skin cells before and after stimulation by plucking.

The third section of Table I shows results obtained when the mice were injected with TdR-^{3}H and then plucked 8, 24, or 48 hr later. Samples were taken 24 or 48 hr later when the stimulated cells had entered the S phase (see the middle section of Table I). The fresh tissue and the fixative-soluble fraction values are rather variable when compared with the 24 and 48 hr postinjection results in section one of the table.

When one considers that these samples were taken 32–72 hr after the TdR-^{3}H injection, there is a suggestion that the fixative-soluble pool may actually be expanded (e.g., the first sample was taken 32 hr after injection and has a difference value of 6006 dpm per punch compared to 3635 for 24 hr postinjection and 2950 dpm per punch for 48 hr postinjection—section 1, Table I). The fixed tissue values, the 50 follicle values, and the calculated epidermal values are all elevated when compared with the relevant values in the first two sections of Table I. The levels are higher when 8 hr elapse between injections and stimulation than when 24 or 48 hr elapse, suggesting that more precursor is available at the earlier times. At the time of the injection the fixed tissue values are between 800 and 900 dpm per punch.

The results of the DBA-2 experiment are shown in Table II. Plucking greatly reduces the dpm per fixed punch of skin and reduces the dpm per 50 follicles to zero. The 50 follicle values remain low for at least 8 hr after plucking and injecting TdR-^{3}H. There is an increase in the dpm per fixed punch of skin, the dpm per 50 follicles, and the dpm per epidermal component when 24 hr or more elapse between plucking and injection and the taking of the samples. The dpm per fresh punch of skin values decreases over this period. These results clearly demonstrate that material is present 24 hr or more after an injection of TdR-^{3}H that can be incorporated into cells stimulated into S by plucking.

TABLE II

Early and Late Labeling in DBA-2 Male Mice 40 min to 72 hr after Plucking and TdR-^{3}H Injection (5 animals per point)

Protocol			Data				
			dpm per punch ±SE				
TdR-^{3}H injection	Plucked	Samples taken	Fresh tissue	Fixed tissue	Difference	dpm per 50 follicles	dpm per epidermal component
Immediately p.p.*	0 hr	40 min p.i.‡	8032 ± 391	282 ± 40	7750	0	282
Immediately p.p.	0 hr	2 hr p.i.	9463 ± 179	469 ± 38	8994	0	469
Immediately p.p.	0 hr	8 hr p.i.	9272 ± 624	683 ± 68	8589	0	683
Immediately p.p.	0 hr	24 hr p.i.	5988 ± 357	865 ± 107	5123	17 ± 5	766
Immediately p.p.	0 hr	48 hr p.i.	5821 ± 304	1568 ± 249	4253	21 ± 10	1445
Immediately p.p.	0 hr	72 hr p.i.	4610 ± 235	1259 ± 77	3351	24 ± 7	1119

* p.p., postplucked.
‡ p.i., postinjection.

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DISCUSSION

Moffat and Pelc (6) observed that, when TdR-^{3}H was given shortly after plucking, it was taken up by the skin and, in the case of the hair follicles, was not incorporated into the DNA until the cells entered S some 16–32 hr later. This meant that it was held for this length of time in an intracellular pool. They also found that this pool could be removed by routine histological procedures but that it could be detected in radioautographs of frozen sections up to 19 hr after an injection. The data in Tables I and II show that tritium material is present in the basal layer and in the follicles up to 48 hr after an injection and that it will be incorporated into fixed tissue when the skin is stimulated by plucking. The incorporation is not seen until the stimulated cells enter S, which they do between 10 and 24 hr after plucking (8, 9). This clearly shows that incorporation of thymidine into DNA or its catabolization (1, 11, 13) does not remove all the injected thymidine. Some of it persists in an intracellular pool for up to 2 or 3 days. It is apparently removed by fixation and routine histological procedures. Catabolism of thymidine clearly occurs to a considerable extent since the major portion of the fixative-soluble fraction of the total fresh tissue activity can be demonstrated to be tritiated water[2] (13). Chromatographic analysis of the cold TCA-soluble fraction, the fixative-soluble fraction, the saline supernate after homogenization, and plasma appears to show the presence of small amounts of labeled material that shows similar mobility to thymine and thymidine and some phosphorylated derivatives.

Other DBA-2 experiments have been conducted which were complicated by the discovery that the hair follicles in this series of animals either had spontaneously entered the hair growth cycle or were late in terminating the previous cycle. Since in either case the basal layer of the epidermis would not have been stimulated, it still contained a significant resting cell population which provided information while the follicles did not. These experiments are being repeated and will be reported elsewhere but a few points can be mentioned from the unpublished data: (*a*) plucking animals after a 40 min exposure to TdR-^{3}H does not reduce the fixed dpm per punch.[3] Thus the reduction seen in the middle section of Table I is not due to the removal of potential TdR-^{3}H–incorporating cells. Thus also, only the 8 hr post-injection to 48 hr postplucked 50 follicle sample in the bottom section of Table I shows a significant difference from the values in section 1. However, the fixed whole punches of skin and some of the dpm per epidermal component values show significant differences. (*b*) The dpm per fixed punch and the dpm per epidermal component both show significant increases above the controls when the skin is stimulated by plucking 40–120 min after the TdR-^{3}H injection and the samples are taken 24 hr after plucking. The presence of the long-lived precursor pool can thus be demonstrated to exist from 40 min to 48 hr after injection.

Late labeling has been observed fairly frequently (3, 4, 12, 14). Reutilization of labeled DNA fragments or nucleosides from dying or dead labeled cells has been evoked to explain these results. It seems probable that salvage pathways and reutilization exist (2) but possibly do not provide the total explanation for late labeling. The existence of a long-lived intracellular pool may be more widespread than was thought, and this pool may in part account for some of the late labeling observed in the past. It seems unlikely that the results of the present experiments can be due to reutilization, since Moffat and Pelc (6) detected the pool as early as 2 hr after an injection, i.e., at a time when few labeled cells will have died and liberated their thymidine. The late labeling seen in the present experiments could be detected at times between 40 min and 48 hr after an injection (Tables I and II and unpublished data). The amount of incorporated late label appeared to decrease with time after the injection rather than increase as more cells died and liberated their label. If thymidine is a consistent fraction of fixative-soluble material, then it would decrease in quantity in proportion to the exponential decay in the size of the fixative-soluble material. This decays with a half-life of between 2 and 3 days from about 1 hr after the injection of TdR-^{3}H.[2] Late labeling due to reutilization would only be expected to contribute after a time equivalent to the half-life or transit time of a heavily labeled, rapidly proliferating tissue, such as the intestinal crypts and the bone marrow. These tissues would not release much label at times less than 40 hr.

That the fixative-soluble pool appears to expand in the skin after plucking may be due to some vascular changes induced by the plucking procedure. More of the plasma-soluble tritium activity may be carried to the skin. This plasma-soluble

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activity appears to contain DNA precursors (10). Thus, the long-lived pool appears to be distributed throughout the body and is not specific to a given tissue.

The intracellular pool of thymidine does not appear to be available to all cells of the basal layer or the follicle. The fixed skin values, the 50 follicle values, and the epidermal component values do not rise with time after the injection (Table I). These values remain constant from 40 min to times in excess of 7 days after the injection.[2] Other tissues such as the jejunal crypts, which are rapidly proliferating, show no signs of incorporating material beyond 40–60 min[2] (10). Cells are continuously entering S and, after a time equivalent to one cell cycle, initially labeled cells should reenter S; both factors should lead to a very rapid removal of the pool. Since this does not occur, the pool must somehow be restricted to certain cells only. Because the cells that respond to stimulation in the follicle and the basal layer are in a resting nonproliferating phase (G_0) and since these cells become labeled from the pool, it would appear that the pool is somehow specific to resting cells. The possibility that the fixative-soluble material which appears to contain the precursor pool may provide a measure of the relative sizes of the resting cell populations in various tissues is under investigation. The fact that the plasma contains the precursor material and that other tissues, such as the crypt cells of the intestine, cannot deplete the pool suggests that its specificity to G_0 cells may somehow be related to differences in membrane permeability of cycling or noncycling cells.

Recent experiments have shown that the major component in the fixed tissue radioactivity is labeled DNA.[2] The acid-soluble fraction in fixed skin is 26% of the total while the DNA component is 70% of the total. The acid-soluble fraction in fresh tissue is 91% of the total while the DNA component is 8% of the total. This demonstrates the importance of thorough removal of the non-DNA component from the tissue before analysis of either radioautographs or liquid scintillation data. The tissues are treated identically in the early preparative stages for radioautographs and for the liquid scintillation analysis.

The significance of these data to radioautographic cell kinetic interpretation depends to some extent on the comparability of liquid scintillation and routine radioautographic techniques. It has recently been shown that liquid scintillation results show the same relative changes with time as the fraction of labeled nuclei determined radioautographically (5, 9). Furthermore, the plasma from TdR-^{3}H–injected animals will radioautographically label cells in culture (10). Cells in culture, though not in G_0, may show a G_0-like permeability possibly because they are completely surrounded by the pool material.

The presence of a significant G_0 population in a tissue might be expected to influence cell kinetic parameters determined from a labeled mitosis curve. If G_0 cells are triggered into cycle at a steady rate, then a gradual build-up with time of labeled cells would be expected. This may be appreciable by the time a labeled mitosis curve begins to descend, i.e., as the G_1 cells at the time of the initial pulse of TdR-^{3}H reach mitosis. This may account for the fact that in vivo labeled mitosis curves rarely drop to 0% at this time. It is possible that the actual percentage of labeled mitoses at the time the G_1 cells should have reached M may be related to the proportion of G_0 cells in that population.

The present data do not allow any analysis of the distribution of label amongst the cells in the tissue in question. Although the fixed tissue values remain constant with time after an injection (Table I, section 1, and footnote 2) the label may change its distribution during this period. This does not, however, alter the fact that if cells are stimulated into cycle some time after the initial pulse, then the over-all quantity of tritium activity in the fixed tissue is increased.

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