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*STIMULATION OF MITOSIS IN ADULT MICE BY ADMINISTRATION OF THYMIDINE**

BY RICHARD C. GREULICH, IVAN L. CAMERON, AND JACK D. THRASHER

DEPARTMENT OF ANATOMY, SCHOOL OF MEDICINE, UNIVERSITY OF CALIFORNIA, LOS ANGELES

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Implicit in the application of tritiated thymidine to studies of cell proliferation and tissue renewal is the assumption that its administration in small doses has no pharmacodynamic effect upon subsequent cell divisions. However, a search of the literature fails to reveal any systematically derived experimental evidence in its support.

Only in isolated and somewhat specialized situations has this problem even been touched upon experimentally. For the most part, such studies have involved examination of populations either of plant cells,[1, 2] microorganisms,[3] or mammalian cell types[4–6] with the primary object of assessing tritium radiation effects rather than pharmacodynamic ones. Taken as a whole, we believe the available results have provided no conclusive evidence for or against a direct action of thymidine on dividing mammalian cells *in vivo*.

A part of the activity of this laboratory has been concerned with a comparative evaluation of mitotic indices in tissues of embryonic and adult mice. In the course of these studies, we have had occasion to derive values for the mitotic index of a given adult tissue on the basis of histological quantitation (both under normal conditions and following colchicine treatment) or by analysis of autoradiograms following administration of tritiated thymidine. Apparent discrepancies in the results of these methods have led us to postulate that tritiated thymidine might exert a stimulatory effect upon cell division when administered in doses (weights) commonly employed for *in vivo* mammalian experiments. In view of the present widespread applications of tritiated thymidine and the fundamental importance of the results obtained by its use, it appeared appropriate to inquire further into its action in the intact animal. Consequently, the experiment reported herein was undertaken, the results of which indicate that after a single injection of normal or tritiated thymidine at the 8.5–10 μg dose level, there ensues an almost immediate and prolonged stimulation of mitotic activity in the duodenum of the adult mouse.

Materials and Methods.—A total of 43 male mice of the AHE strain, 7 months old and weighing between 28 and 34 gm, were utilized in the experiment. For a month previously, the animals had been maintained in quarters controlled with respect to temperature (78–80°F) and light (13 hr darkness, 11 hr illumination per day).

Twenty-eight of the animals, constituting the experimental group, received a single dorsal subcutaneous injection of normal or tritiated thymidine. Of these, 14 received a volume of

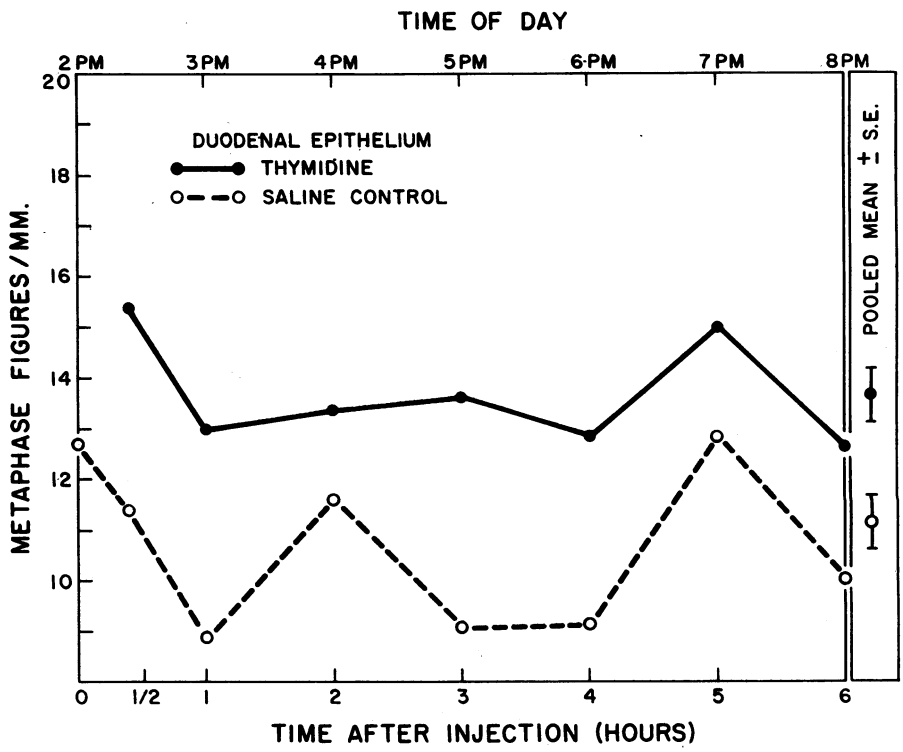

FIG. 1.—Post-injection frequency of metaphase figures plotted against time in saline-injected control animals (lower curve) and in experimental animals given a single subcutaneous injection of 8.5 μg tritiated thymidine or 10.0 μg normal thymidine (upper curve). Each experimental point (solid circle) represents the mean value of four individuals, two of which received tritiated thymidine and two of which received normal thymidine. Each control point (open circle) represents the mean value of two individuals, with the exception of the half-hour point, which is a single animal. The pooled mean values are plotted at the right with the respective standard error of their means indicated.

0.1 ml containing 10 μg normal thymidine (Schwarz BioResearch, Inc., Mount Vernon, N. Y.; Lot #TDN 6016) as a 10 mg% solution in normal saline. The remaining 14 experimental animals were given an injection of 0.05 ml saline diluted tritiated thymidine solution (Schwarz, Lot #*HTDN 1012, specific activity 0.36 curies/mM) containing approximately 8.5 μg thymidine, or approximately 12.5 μc in terms of tritium activity.

The 15 animals of the control group each received a single subcutaneous injection of 0.1 ml normal saline. The injection procedure and subsequent manipulation of the living animals were carried out in the animal quarters. Injections were given within the 15-min period from 2:00–2:15 P.M. in order to minimize diurnal differences in mitotic activity.

Two of the control animals were killed by a blow on the head immediately after injection of normal saline. At intervals thereafter of one-half, one, two, three, four, five, and six hours, animals from both control and experimental groups were killed in a similar manner. At each interval, two experimental animals which had received normal thymidine and two which had received tritiated thymidine were killed. Only one control animal was killed at the half-hour interval, whereas two control animals were killed at each subsequent interval.

Lengths of approximately 1 cm of proximal duodenum, just distal to the pyloric-duodenal junction, were removed from each animal immediately after its death. These samples were incised longitudinally, were flattened serosal side down on stiff card, and were fixed in Carnoy's fluid for 16 hr. The fixed tissues were trimmed and embedded in paraffin and oriented in the blocks so that the resultant histological sections would pass perpendicularly through the mucosa.

Four slides, each containing several 5-micron sections from different regions of the paraffin block, were prepared from each animal for histological quantitation of mitotic activity and were stained with hematoxylin-eosin. The slides were coded to minimize bias and were then examined by three observers.

The microscope of each observer was provided with an ocular reticle, which at oil immersion magnifications delimited a virtually square field of view measuring approximately 100 microns on a side. Each observer counted the number of metaphase figures visible in successive, usually adjacent, 100-micron strips of duodenal wall, including in his counts all metaphases found in the area between the base of the crypts of Lieberkühn and the villal tips. Care was exercised to count only those fields of the section which were cut perpendicular to the basal region. Portions of the sections cut in other planes, or portions in which fixation shrinkage or processing artefact was apparent, were disregarded.

Each observer counted a total of 100 metaphase figures or 100 fields (effectively 10 mm of duodenal base length) from each animal, whichever figure was last arrived at. The three sets of raw data thus obtained for each animal were then pooled and averaged and were finally expressed as the average number of metaphase figures per millimeter of duodenal base length. Ultimately, the average values for the four experimental animals at each time interval were also averaged, as were those of the pairs of control animals at each time (excepting the half-hour interval), and were plotted graphically (Fig. 1).

Additional 5-micron sections were cut from the blocks containing the tissue of the tritium thymidine–treated animals. These were prepared for autoradiography and were covered with Eastman Kodak Nuclear Track Emulsion, Type NTB2, according to the technique of Messier and Leblond.[7] Autoradiographic exposures ranged from 14 to 18 days, following which the slides were developed for two minutes in full-strength developer ("Dektol") at 18°C. After fixation and washing, the autoradiograms were strained in Harris' hematoxylin.

Results.—Analysis of variance for within-group deviation at the different time intervals (Table 1*A*) revealed no significant differences within control or experimental groups, despite the fluctuations apparent in the time plot of the averaged data (Fig. 1). Consequently, significance in the difference between the grand means of control and experimental animals was sought by the students' "*t*" test, irrespective of time of sacrifice and, in the case of the experimental animals, irrespective of whether thymidine had been administered in the normal or tritiated form.

TABLE 1

A. ANALYSIS OF DATA FROM EXPERIMENTAL AND CONTROL GROUPS FOR SIGNIFICANT VARIATION AS A FUNCTION OF TIME

Group	Variance	$\Sigma(x^2)$	*df*	Mean Square	*F*	*p*
Experimental	Between times	27.88	6	4.67	0.57	>0.05
	Within times	171.00	21	8.14		
Control	Between times	33.09	6	5.51	3.56	>0.05
	Within times	12.42	8	1.55		

B. COMPARISON OF MEANS OF POOLED EXPERIMENTAL AND CONTROL GROUPS BY THE STUDENTS' "*t*" TEST

Group	Mean	S.D.	$\Sigma(x^2)$	*df*	*t*	*p*
Pooled experimental	13.7	± 2.67	198.8	27	3.96	<0.001
Pooled control	10.6	± 1.75	46.1	14		

Reference to Table 1*B* reveals that a high degree of significance ($p < 0.001$) exists in the difference between means of the experimental and control groups. Thus, thymidine administration at the 8.5–10 μg dose level produced an increase in the size of the metaphase population in duodenal epithelium within a half-hour after injection, and in terms of mean difference, this increase averaged 29.2 per cent throughout the six-hour period of observation following administration of the drug.

Examination of autoradiograms of duodenal samples from tritium thymidine–treated animals failed to reveal any indication of abnormality in the DNA-synthetic process or in the subsequent division of labeled cells. A half-hour after injection, only interphase and some prophase cells appeared to be labeled, whereas in successive later observations, other mitotic figures were also seen to become labeled in orderly progression. After one hour, many labeled prophase and metaphase figures were observed, as well as an occasional anaphase. After two hours, labeled metaphases were very common, and although labeled anaphase and telophase figures were less frequent than metaphases, they were also very evident. By three hours, labeled cells in all stages of the mitotic cycle, as well as labeled pre- and post-mitotic cells, comprised the reactive cell population.

Discussion.—The above findings are clear-cut in their demonstration of the marked effect of a small dose of tritiated or normal thymidine upon the process of cell replication *in vivo*. A logical explanation of these findings is difficult and must in any case remain speculative pending further study. However, it seems pertinent to explore and contrast the possible toxic origin of this effect with what may be termed its physiological origin.

Possible toxic action of thymidine: It does not appear that thymidine acts as a mitotic spindle poison. If this were the case, the numbers of visible metaphase figures would tend to accumulate during the first few hours after its administration, much as in the case of colchicinized tissues.[8] Figure 1 reveals, however, that following the initial increase a half-hour after injection, the number of visible metaphases is essentially stable at the various time intervals. Our autoradiographic observations further support this conclusion in that all stages of mitosis became labeled in an orderly sequence after administration of tritiated thymidine (see below).

An alternative mechanism of toxic origin might be suggested as being responsible for increasing the number of metaphase figures. According to this, thymidine would increase the duration of the metaphase stage of mitosis or increase the duration of the entire mitotic sequence. It should be emphasized that this mechanism implies the continued availability of circulating thymidine over the six-hour period of observation. However, current evidence [9–11] concerning the rate at which exogenous thymidine is either cleared from the body or bound into newly synthesized DNA indicates that the chance of its being available in significant amounts for more than one hour after injection is very slight. Moreover, in the autoradiograms, the rapidity with which labeled interphase cells were seen to pass through prophase and metaphase to the anaphase and telophase stages of division suggests that this mechanism, if operative at all, is of minor importance.

In conclusion to this section, neither histological examination nor autoradiographic analysis has provided any indication of disturbance in structure or in the orderly sequence of events leading to cell division in the duodenal epithelium following thymidine treatment. Consequently, a basis for the increase in duodenal mitotic activity observed in the experimental animals should be sought in terms other than those of toxicity.

Possible physiological mode of action of thymidine: A wide range of dosage levels of tritiated thymidine has been utilized in prior *in vivo* mammalian experiments. In our laboratory, for example, using mice of approximately 30 gm body weight,

we have usually employed a dose of 25 μc for routine autoradiographic study of cell population kinetics. Assuming a specific activity of 0.36 curies/mM, this is equivalent to a weight of almost 17 μg of thymidine. By adapting these values to the calculations of Lajtha and Oliver,[12] it can be demonstrated that, fully utilized, this weight of thymidine would provide the entire thymidine complement of approximately 1×10^7 new cells, assuming equimolar distribution of the component nucleotides in DNA.

While it is true that thymidine utilization in DNA does not approach the 100 per cent level following injection,[11] it is equally likely that not every thymidine residue in the newly-synthesized DNA of a dividing cell would be derived from exogenous thymidine. Consequently, the actual number of cells involved may be less than, or far more than, the derived figure. In either case, any effect of exogenous thymidine would be manifested primarily in rapidly dividing populations of cells (such as the duodenal epithelium) in which DNA synthesis is frequent, and which, at any instant in time, probably represent a quite small fraction of the adult total-body cell population. As a result, the calculated number of cells potentially affected by microgram quantities of exogenous thymidine attains a considerable significance and introduces the possibility that should the effect be one of increased mitotic activity, it might be demonstrable by histological methods. This possibility appears to be borne out by the present data.

It is well established that DNA synthesis requires the simultaneous presence of its four component nucleotides.[13] Of these, presumably only thymidine nucleotide is not readily available as a free metabolite under normal circumstances, but must be formed by the methylation of deoxyuridine monophosphate in the presence of tetrahydrofolic acid.[14] Studies of dividing cell systems *in vitro* have revealed that free nucleosides added to the medium are immediately utilized for nucleic acid synthesis.[15] Folic acid antagonists have been shown to interrupt DNA synthesis in cell cultures, while the introduction of thymidine immediately brings these cells back into the DNA-synthetic phase and ultimately precipitates a marked spurt in cell division.[16]

In view of this evidence and that of the present study, we offer as a tentative hypothesis that the methylation reaction is rate-limiting in DNA synthesis *in vivo* as well as *in vitro* and can be bypassed by the addition of the finished product, i.e., thymidine. This possibility has previously been suggested and discussed by Friedkin[17] but without direct experimental support.

If such a hypothesis is correct in its major premises, it will require that figures which have been derived previously regarding the relationships of DNA-synthesis time and cell population generation time be reappraised. All of these calculations, so far as we are aware, have resulted from studies involving the use of tritiated thymidine, frequently in far larger concentration than has been employed in the present study.

Finally, our present data do not permit precise interpretation of the observed prolongation of the thymidine effect upon mitotic activity. In the light of our general hypothesis, however, it would seem likely that microgram quantities of thymidine effectively shorten DNA synthesis time *in vivo* and that the resulting increase in the number of cells entering mitosis would as a consequence be demonstrable over a period of time equal to the shortened DNA synthesis time, i.e.

approximately seven hours.[9] The detailed time relationships of this stimulation and its possible dose dependency are currently being investigated.

Summary.—Mitotic activity of the duodenal epithelium of adult male mice has been examined at intervals up to six hours after subcutaneous administration of 10 μg of normal thymidine or of 8.5 μg (12.5 μc) of tritiated thymidine. A comparison to saline-injected control animals has revealed an immediate and highly significant increase in the number of metaphase figures in the experimental animals, which was maintained throughout the six-hour period of observation and averaged 29.2 per cent during this period.

Consideration of the mechanisms which might be involved in thymidine action *in vivo* suggests that the results represent a physiological, rather than toxic, action and that the addition of free thymidine at this dose level *in vivo* shortens the average DNA synthesis time in duodenal epithelium and so permits an increase in the rate of cell entry into mitosis.

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