DOSE-DETERMINED EFFECTS OF HT_3DR AS DNA LABEL UPON THE LIVER CELL REPLICATION TIME AND PATTERN IN THE GROWING RAT†

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INTRODUCTION

The use of isotopically labelled thymidine in the study of biological systems has greatly expanded the range of investigation. However, soon after its introduction it was reported that thymidine-2-^{14}C labelling of desoxyribonucleic acid (DNA) was accompanied by chromosomal alterations which could be related to the amount of radioactive label employed[1]. Since then the problem of the effects of the label upon the biological system under study, has concerned many workers[2–11]. The acute toxic effects investigated have included morphological changes in mitotic nuclei, decreased cell growth and cell death. In general the changes observed have been similar to those ascribed to gamma and X-radiation[12, 13]. The prolonged exposure to the label has been reported to induce tumours[14] and to cause foetal malformation and death[15]. During studies of the liver cell replication in this laboratory, it was found that the administration of 2 μc/g of tritiated-thymidine (H^3TDR), specific activity 0·36 c/mmole, as a DNA label, changed the nuclear ploidy pattern of liver cells of 3-week-old rats to that of 2-year-old animals within 2 weeks[16]. The present studies were undertaken to learn more about these ploidy changes.

In these experiments the replication times and pattern of liver cells, in the growing rat, have been used as the measurement systems for studying the effects of different labelling doses of H^3TDR. The results show that the incorporation of the radioactive label into DNA may disrupt the rhythm of cell replication, and delay the progress of cells in their course through DNA synthesis and into mitosis. In addition it may reduce mitoses. These effects are dose-dependent.

METHODS

Three-week-old male rats of the Wistar strain were fed Purina checkers and water *ad lib*. They were injected intraperitoneally with H^3TDR, specific activity 0·36 c/mmole, at dosage levels of: Group A—1 μc, Group B—2 μc, and Group C—10 μc/gram body weight. At stated intervals of

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0·25 to 96 hours later, groups of 2 to 6 animals were killed. Their livers were fixed in Carnoy's fluid and 5 μ sections were stained by the Feulgen technique. Strips of Kodak AR 10 film were applied in the dark and after 30 days in the cold, the slides were developed.

Under oil immersion, interphase and mitotic nuclear labelling were determined. Thirty to 100 mitoses were scored per animal. Interphase and mitotic labelling and percentage of mitoses were estimated from the random scanning of over 3000 cells per animal. The details of the methods employed for the estimation of the replication times and patterns are based upon the work of Quastler and Sherman[17] and have been presented elsewhere[18]. They are summarized as follows:

The generation time and its component parts have been estimated from the waxing and waning of mitotic labelling with time, following administration of H^3TDR. Each labelled mitosis is derived from a labelled interphase nucleus. The replication time may be found from the interval between two comparably placed points on each of two similar curves of mitotic labelling. In these studies, the points chosen have been those at the beginning of ascent of each curve. The validity of these estimates requires that the cells of each cycle be derived from those of the previous cycle and that the labelled cells should continue in phase in each division cycle. Where feasible, cell continuity has been demonstrated by recording the decreasing grain counts in successive cycles. This has been done[18] in the group A. In the other groups, where labelling is too heavy to permit accurate grain counts, estimates have been made of the relative percentages of heavily and lightly labelled interphase nuclei. The decrease in heavily labelled nuclei and the increase in lightly labelled nuclei over stated time intervals indicate that cell division has occurred. This is a cruder estimation than the method of grain counts.

The labelling of prophase, metaphase, anaphase and telophase nuclei has been scored separately. The replication time has been determined as noted above, from the prophase labelling curve. This stage was chosen because of its relative ease of recognition and because the numbers of grains over these nuclei were usually higher than those over other mitotic nuclei.

The several parts of the replication cycle were timed as follows: The time for post-DNA synthesis gap (G_2) and mitosis was determined from the time when 50 per cent of all of the mitotic nuclei were labelled. Inasmuch as the orderly time lag of labelling of each succeeding mitotic class was not sharply defined in Groups B and C, estimates of the respective times for each stage of mitosis were not possible. The time for DNA synthesis was estimated from the interval between the 50 per cent labelling of the ascending and descending limbs of the first prophase curve. Finally the post-mitotic gap (G_1) was obtained by subtracting the mean of the times for G_2 + mitosis and DNA synthesis from the total replication time.

RESULTS

Group A: One μc/g

The labelling of prophase nuclei follows an evenly spaced polycyclical pattern (*Figure 1*). The generation time is 21·5 hours in the first cycle

and 20 hours in the second. The time for G_2 and mitosis is 3·5 hours (*Table 1*). DNA synthesis occupies 9 hours. The post-mitotic gap (G_1) is 9 hours. About 4 per cent of the interphase nuclei are labelled during the first cycle and 1 per cent of the cells are in mitosis, 2 hours after H^3TDR. At

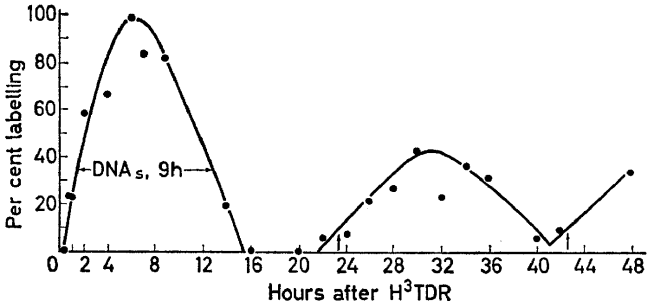

Figure 1. Prophase labelling after 1 μc/g, specific activity 0·36 c/mmole; the vertically directed arrows indicate beginning of curve ascent

Table 1. Replication times of liver cells after several dosage levels of H^3TDR (in hours)

Dose/g (μc)	*Replication time* (h)	*Cycles*	*DNA synthesis*	G_2 + *mitosis*	G_1	*Interphase labelling* (%)	*Mitosis* (2 h) (%)
1	21·5	4	9	3·5	9·0	4·2	1·1
2	30·5	2	16	4·5	10·0	5·6	0·2
10	43·0	2	34	4·0	5·0	5·7	0·2

Table 2. Decreasing percentages of "heavily" labelled interphase nuclei at several doses of H^3TDR

Dose (μc/g)	*Hours after* H^3TDR	*No. rats*	*Cells with > 41 grains* (%)	*Mitoses* (%)
1	2	4	38·0	1·1
	24	4	12·5	2·2
	48	6	2·6	0·4
	72	2	0·3	0·4
			Percentage of "heavily" labelled	
2	2	3	61·3	0·2
	24	3	45·8	0·1
	48	3	35·7	0·2
	72	3	23·8	0·5
	96	4	21·7	0·9
	2 weeks	3	18·6	—
10	2	3	58·2	0·2
	24	3	42·4	0·3
	48	3	21·8	0·5
	72	3	8·8	0·4
	96	3	8·7	0·5
	2 weeks	3	5·3	—

24 hours there are 2·2 per cent mitoses and at 48 and 72 hours the figure is 0·4 per cent.

All labelled cells are replicating in phase, the curves intercepting the abscissa in each cycle. The continuity of the cell line in this group has been demonstrated by a significantly decreasing grain count from cycle to cycle[18]. These data are represented as the decreasing per cent of interphase nuclei with 41 grains or more (*Table 2*). Three cycles of cell division are shown (*Figure 1*) and a fourth may be inferred by a significant decrease in the grain count at 72 hours.

Group B: Two μc/g

The curve of prophase nuclear labelling rises sharply but the descending limb falls only to 50 per cent at 28 hours. It rises at 32 hours to begin a second cycle (*Figure 2*). The generation time is estimated at 30·5 hours. The interval for G_2 + mitosis is 4·4 hours and for G_1 is 10·1 hours.

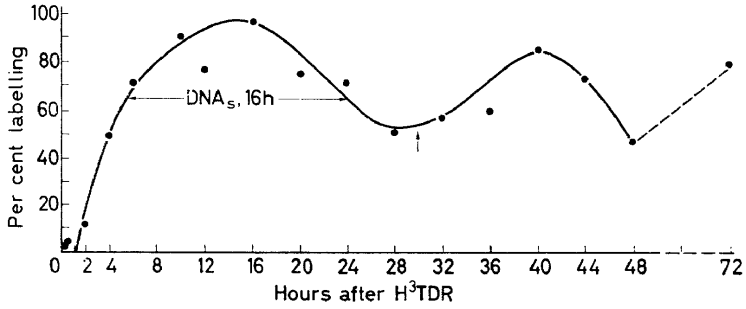

Figure 2. Prophase labelling after 2 μc/g, specific activity 0·36 c/mmole; the arrows are used as in *Figure 1*

About 5·6 per cent of the interphase nuclei are labelled and 0·2 per cent of the cells are in mitosis 2 hours after H^{3}TDR. At 24, 48, 72 and 96 hours, the percentages of mitosis are 0·1, 0·2, 0·5 and 0·9 respectively. From the prophase labelling curve, two cycles are recorded (*Figure 2*), but from the declining percentages of heavily labelled cells it is evident that cell division is continuing (*Table 2*).

Group C: Ten μc/g

The prophase nuclear labelling curve is markedly distorted (*Figure 3*). It rises to a high level within 6 hours and then remains at a plateau until 40 hours, when it falls to 64 per cent. At 44 hours it rises again and remains above 70 per cent at 72 and 96 hours.

The estimates of the replication time intervals from this curve are of questionable significance. The generation time is calculated at 43 hours and the DNA synthesis time as 34 hours. The time for G_2 and mitosis is about 4·0 hours and that for G_1 is 5 hours (*Table 1*).

The interphase labelling per cent and mitotic per cent are similar to

those at the 2 μc/g dose, 2 hours after H^3TDR. At 24, 48, 72 and 96 hours after H^3TDR the percentages of mitoses are 0·3, 0·5, 0·4 and 0·5, respectively. Cell continuity is suggested by the exponentially decreasing occurrence of heavily labelled interphase nuclei (*Table 2*). No necrotic cells are seen.

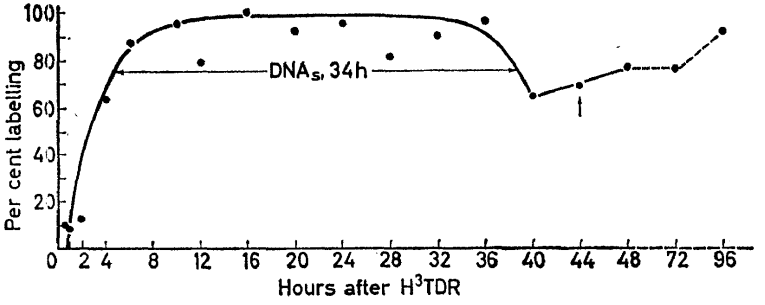

Figure 3. Prophase labelling after 10 μc/g, specific activity 0·36 c/mmole; the arrows are used as in *Figure 1*

DISCUSSION

The data in Group A (1 μc/g) show that the liver cells in the growing rat follow a distinct pattern of replication. A particular group of labelled cells passes through the replication cycle "in phase". The same cohort of cells goes from one cycle to the next in an orderly fashion. Four such cycles have been observed. Beyond that number the label becomes too dilute to be detectable. The first cycle requires 21·5 hours and the second 20 hours. Theoretically the first and second cycle curves should be identical. However, many cells are probably "lost" in the autoradiograph during the second cycle, because of dilution of the label with "cold" DNA. This would account for the lower peak of the second curve. The regular pattern of replication in this group suggests that the radioactive label has not altered this phase of the biological system under study. The decline in mitoses at 48 and 72 hours may be due to radiation.

With increasing amounts of radioactive label, the number of cells delayed in DNA synthesis and failing to enter mitosis during the first cycle increases markedly. Whereas in Group A 100 per cent of the labelled cells enter mitosis, 50 per cent of Group B and only 36 per cent of Group C make this transition. The finding that the times for G_2 + mitosis are not markedly prolonged in these groups, indicates that much of the widening in the curves of these groups may be due to prolonged DNA synthesis time. In these groups too, the rhythm of the replication cycle is so markedly altered that it is difficult to assess the reliability of the estimates of their generation times and component intervals. Although these are crude data, it is clear from the curves of the disappearance of heavily labelled nuclei that cell division is occurring. However, the markedly broadened curves in Groups B and C suggest that there is much variation in the respective time intervals among the cell population. This is in sharp contrast to the relatively uniform time intervals in Group A. At the higher dose levels (Groups B and C) the

numbers of mitoses are reduced markedly at 2 hours after H^3TDR and remain low. The levels of interphase labelling are essentially unchanged from Group A. The previously published[16] low levels of interphase nuclear labelling at 2 μc/g H^3TDR were in error. The arrest of many cells in DNA synthesis, coupled with the reduction in mitosis, would explain in large part the development of polyploidy, previously reported[16] after 2 μc/g. Unpublished studies in this laboratory have confirmed these earlier observations and have shown similar ploidy effects with 10 μc/g. It is of interest to note the many heavily labelled interphase nuclei which persist 2 weeks after labelling, in Groups B and C (*Table 2*).

The effects of H^3TDR upon ploidy, DNA synthesis, mitosis and cell replication are similar to those reported for gamma and X-radiation[12, 13, 21–24]. The present data support earlier studies which focused attention upon the DNA as the site of radiation injury[12]. It is likely that many biochemical systems are altered[20], and that these changes are manifested in the behaviour of cells as found in this and other reports. One likely effect is to increase the variation of the several time intervals of replication within the cell population. This is characteristic of the replicating hepatoma cell population[19]. Indeed, the prolonged interval in DNA synthesis in Groups B and C might facilitate the development of mutations which eventually might produce tumours[14]. The long term sequelae to these different dosage levels have not been studied, nor have the effects in organ systems, other than the liver, been investigated.

It is noteworthy that the present studies were performed in a relatively radiation-resistant cell line, in which no evidence of cell death was observed. It is suggested that in such a biological system, the effects, at different dosage levels, may be separated out more readily than in an experimental design where greater radiation-sensitivity is operative.

The data add to the already abundant evidence that the label dose of H^3TDR may alter the biological system under investigation. They indicate a limitation in the use of this valuable tool. Finally it is suggested that the measurement of the replication time is a useful technique in studying the biological effects of radiation.

The possible effect of an increase in the thymidine pool has not been excluded in the Groups B and C. Based upon calculations derived from Lajtha's estimates in the mouse[25], the body thymidine pool is expanded by about 2·5 per cent at the 1 μc/g dose and by about 25 per cent at the 10 μc/g dose. Experiments are in progress with a high specific activity H^3TDR to rule out pool dilution as a factor responsible for the effects observed.

SUMMARY

Studies have been made of the effects of different dosage levels of H^3TDR, as a DNA label, upon the replication pattern and time of liver cells in the growing rat. The dose-dependent effects are (*i*) disruption of the regular, evenly-spaced replication cycles, (*ii*) lengthening of the generation time and of the DNA synthesis time, (*iii*) increased variation of the respective time intervals of the replication cycle of the cells of the population, (*iv*) marked delay in the transition of many cells through DNA synthesis and

(*v*) reduction in the number of cells in mitosis. The last two effects contribute to the previously reported radiation-induced polyploidy. All of these effects are similar to those previously reported to follow gamma and X-radiation. No cell necrosis has been observed. The measurement of the replication time offers a useful technique in the study of the biological effects of radiation.

Note added in proof

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