DNA SYNTHESIS AND CHROMOSOMAL ASYNCHRONY

Induced Parasynchronous DNA Synthesis in Human Leucocyte Cultures and Chromosomal Asynchrony in the Early S Phase

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A number of studies investigating the chronology of DNA synthesis in human chromosomes have indicated a degree of asynchrony among the members of the complement. The most striking asynchrony of DNA synthesis demonstrated within pairs of human chromosomes is the late replication of one of the X chromosomes shown by pulse labeling with tritiated thymidine in cultures of leucocytes (3, 4, 7, 8). Morishima *et al.* (8), have presented evidence that this late replicating X chromosome is the same chromosome which produces the sex chromatin body in interphase somatic nuclei of females. Their study covered the late S phase and did not determine whether the asynchrony occurred also at the beginning of replication of these chromosomes in the early S phase.

In order to observe chromosomal synchrony or asynchrony in early S phase, it was useful to have a greater degree of synchrony of cell division than ordinarily is found in cultures of human leucocytes, and to know when a large number of cells in a culture could be labeled for autoradiography of the very beginning of DNA synthesis.

It is known that aminopterin, a folic acid analog, acts as an antagonist of tetrahydrofolic acid in the biosynthesis of thymidylic acid, and thus inhibits DNA synthesis and subsequent mitosis (5). In the present study, using leucocytes from normal human females, the degree of parasynchrony of DNA synthesis in the culture was enhanced by a period of aminopterin inhibition followed by simultaneous release from inhibition and pulse labeling of DNA with tritiated thymidine in what is presumed to be, for most of the cells, the early S phase. A period of incubation allowed cells thus labeled to complete the S phase and G_2 phase and proceed into mitosis. A high proportion of metaphases of such cells was found to have all chromosomes labeled except one, which by size and morphology was found to be in the $6-12+X$ group (2), and is presumed to be the heteropycnotic X chromosome.

MATERIALS AND METHODS

Human leucocytes from peripheral blood of normal females were cultured *in vitro* by minor modifications of the technique of Moorhead *et al.* (6). In a series of experiments several culture schedules were used in which, after 33 to 44 hours of incubation at 37 C, the cultures were centrifuged and the cells resuspended in one-third of the medium; the supernatant two-thirds of the medium was refrigerated until later use. The cells were treated with aminopterin in a final concentration of 5×10^{-6} M for periods of 10 to 18 hours. During the final 15 minutes of this period (including centrifuge and pipetting time) the cells were provided with thymidine-H^3 in the amount of 1 μc.ml of culture medium. The cells were removed from the culture medium containing aminopterin and thymidine-H^3 and resuspended in the remaining two-thirds of the original culture medium to which had now been added unlabeled thymidine in a concentration 100 times the molar concentration of the thymidine-H^3. The cells were incubated another 11 to 13 hours with colchicine added for the final 3 hours, then treated with hypotonic solution, fixed, and quickly dried on slides.

The slides were stained by the Feulgen technique and Kodak stripping film AR-10 was applied. After

3 weeks' exposure, the autoradiograms were developed and the chromosomes were stained through the film with buffered thionine pH 6 (Morishima, personal communication).

RESULTS

1. *Parasynchrony*

The increase in synchrony induced by aminopterin inhibition followed by labelling with tritiated thymidine is shown in Table I. The proportion of cells in the S phase at the time of labeling following aminopterin treatment is twice that of cells in the S phase from the control cultures. The number of labeled division figures increased from 2.1 per cent to 5.4 per cent of the cells.

2. *Chromosomal Asynchrony*

Metaphases labeled at the beginning of the S phase (*i.e.* following a long period of aminopterin inhibition) were found, in the females studied, to have a distinctive labeling pattern of one unlabeled chromosome in the entire complement (Fig. 1). In every case the unlabeled chromosome has the size and morphology of the $6-12+X$ group and is presumed to be the heteropycnotic X chromosome found to continue DNA replication after all other chromosomes have completed replication.

In many cells the chromosomes were all moderately to heavily labeled (at least 10 grains per chromosome) except for the one X chromo-

TABLE I

Parasynchrony Induced by Aminopterin

Percentages shown are for 1000 cells each from aminopterin-treated and control cultures. These results are from a single experiment using peripheral leucocytes from one normal female. After 44 hours of incubation at 37 C, aminopterin was added for 10 hours, then thymidine-H^3 for 15 minutes, followed by culturing in cold thymidine for 11 hours including a terminal colchicine treatment of 3 hours.

	Labeled		Unlabeled	
	Inter-phase	Meta-phase	Inter-phase	Meta-phase
	per cent			
Aminopterin-treated	26.0	5.4	68.1	0.5
Control (no aminopterin)	13.4	2.1	82.6	1.9

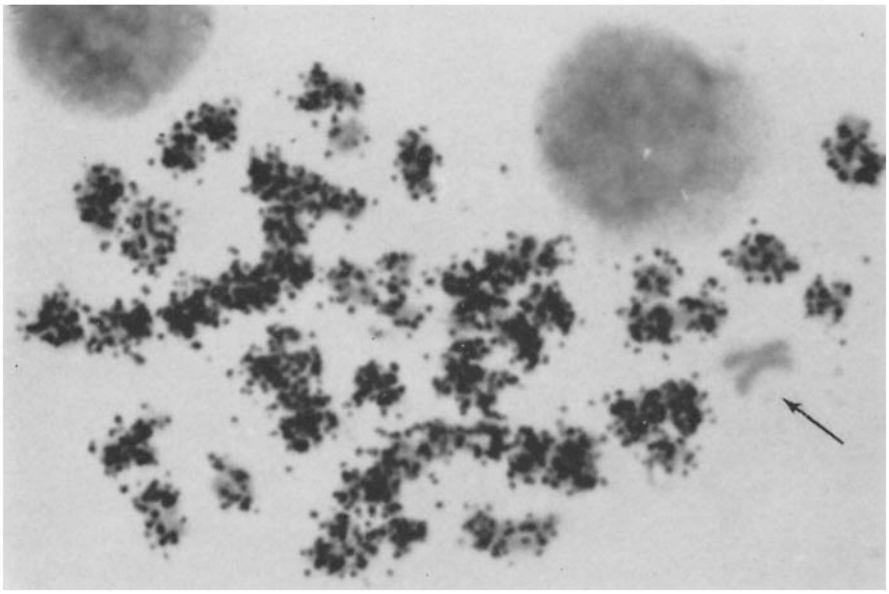

FIGURE 1 Autoradiogram of a cell from a human female leucocyte culture labeled with thymidine-H^3 for 15 minutes after 10 hours of aminopterin treatment, and fixed 11 hours later. Chromosomes stained with thionine. The unlabeled chromosome (arrow) is presumed to be the X chromosome.

some which carried 1 to 3 grains. In at least some of these, the grain(s) on the single lightly labeled chromosome could be attributable to background.

Table II summarizes the condition of labeling observed in 185 metaphases from these experiments. Only those figures were counted that showed moderate to heavy label and sufficient spreading to show chromosomes as more or less separated units. Of the 185 cells, 162 showed the distinctive labeling pattern described above. Five (of 185) cells had, in addition to the X chromosome, one or two unlabeled autosomes. These were from the 13–15 group (4 cells), the 4–5 group (1 cell), and the 16–18 group (1 cell).

TABLE II

Asynchrony of DNA Replication in Early S Phase
Cells having a moderate to heavy label in metaphase following pulse labeling with thymidine-H^3: few if any overlaps were classified and counted in four categories.

Pattern of labeling	Number of cells	Per cent
1. Fully labeled complements	18	9.7
2. All chromosomes labeled except one X chromosome	74	40.0
3. Complement moderately to heavily labeled, but one X chromosome with only 1 to 3 grains	88	47.6
4. Only one X and a few autosomes unlabeled	5	2.7
Total	185	100.0

Less than 10 per cent of the cells had all 46 chromosomes labeled comparably in the first 15 minutes after the release from aminopterin inhibition. It is possible that some or all of these had started replication before the cells were exposed to aminopterin, so that the label would represent a period in early or middle, but probably not at the very beginning, of the S phase. Complete labeling is the usual pattern found after the culture period used here, but without inhibiting substances such as aminopterin (1).

DISCUSSION

In Taylor's study (9) of chromosome duplication in the Chinese hamster the finding of asynchrony led to the hypothesis of genetic control and functional significance of timing of DNA synthesis. This has been further substantiated by the evidence of Morishima *et al.* (8) suggesting that the X chromosome continuing replication to the late S phase is the same chromosome which produces the sex-chromatin body in interphase somatic nuclei of females. The evidence presented here of one chromosome of the 6−12+X group starting replication after all other chromosomes suggests that it is the same X chromosome which produces the sex-chromatin body, starts DNA replication late, and continues DNA replication to the end of the S phase.

While differential counts of the leucocyte cell types were not made in the present study, it is believed that most of the unlabeled interphase cells are granulocytes which are not induced by phytohemagglutinin to enter mitosis. The large number of cells seen as thymidine-H^3–labeled interphases indicates a considerable variance in time from early S phase to metaphase. This is in agreement with the observations of Bender and Prescott (1); according to their data, the greatest variability is probably in the length of the S phase.

Continuing experiments, in which adjustment is made of time intervals in the culturing schedule before, during, and following aminopterin treatment, are being done and it is expected that a greater degree of mitotic synchrony can be achieved than is found here. Any increase in synchrony that can be attained without induction of chromosomal aberrations is of value in providing more metaphases for observations of either chromosome morphology or timing of DNA synthesis.

SUMMARY

Peripheral leucocytes of normal females were cultured *in vitro* and treated for a period of time with aminopterin causing a blocking of DNA synthesis. At the end of this period, thymidine-H^3 was used in a 15-minute pulse both to bypass the blockage of DNA synthesis and to label the newly synthesized DNA.

Counts of metaphases showed that aminopterin treatment had induced a degree of parasynchrony of DNA replication within cultures, doubling the number of metaphases in treated cultures as compared with untreated control cultures. Autoradiography of DNA synthesized at the beginning of the S phase revealed a characteristic pattern of early replication, with labeled DNA in every chromosome except one known to be in the 6−12+X group. This late-starting chromosome

is presumed to be the same X chromosome reported by earlier workers to be replicating late in the S phase.

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