DNA Replication Patterns of Normal Human Leukocyte Cultures

Time Sequence of DNA Synthesis in Relation to the H3-Thymidine Incorporation Over the Nucleolus

By Manuel Ribas-Mundo

In recent years many papers dealing with DNA synthesis in short-term cultures of human peripheral blood1-6 have appeared. There has been increasing evidence that the small lymphocytes under stimulation of phytohemagglutinin1,5-9 and other substances10-12 are able to proliferate and produce mitosis, but very few researchers have tried to investigate the time sequence of the DNA synthesis in the interphase nuclei of these proliferating lymphocytes. Prescott and Bender3 have described two patterns of H3-thymidine incorporation in the interphase nuclei of the leukocyte cultures — i.e., a diffuse and a localized pattern of labeling. Further, Lima-de-Faria and Reitao4 have demonstrated the incorporation of H3-thymidine on the heterochromatin situated around the nucleolus of the leukocytes cultivated in vitro.

Stimulated by these studies and by our previous observation13 that the labeling of the interphase nuclei which synthesize DNA is not done at random and that in some cells H3-thymidine is incorporated in the nucleolus, we have undertaken an autoradiographic study of normal human peripheral blood cultures to determine (1) which patterns of H3-thymidine incorporation can be demonstrated in the nuclei of PHA-stimulated lymphocytes, and (2) the time sequence of the observed patterns of labeling.

Materials and Methods

For both short-term cultures of peripheral leukocytes and autoradiographic studies we have followed the method described by W. Schmid.14 We will only summarize the most important facts.

Culture of Peripheral Blood Leukocytes

Ten ml. of peripheral blood were sedimented in 15 ml. centrifuge tubes containing 0.1 ml. of commercial heparin (1,000 units/ml.) at 37 C. without phytohemagglutinin. One and one-half ml. of the supernatant plasma rich in leukocytes was introduced into a prescription bottle which contained 7 ml. Eagle’s Basal Medium. 700 units of penicillin, 0.7 ml. streptomycin and 0.2 ml. of phytohemagglutinin P (Difco). The rest of the donor’s blood was centrifuged 10 min. at 3,000 rpm, and 1.5 ml. of plasma was added to the culture. The

From the Department of Hematology (Dir.: Prof. P. Farreras) of the Clinic for Internal Medicine (Dir.: Prof. A. Pedro-Pons), Faculty of Medicine, University of Barcelona, Barcelona, Spain.

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M. Ribas-Mundo, M.D.: Department of Hematology of the Clinic for Internal Medicine, Faculty of Medicine, University of Barcelona, Barcelona, Spain.

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cultures were incubated at 37 C. in a 5 per cent CO₂ 95 per cent air atmosphere in prescription bottles.

**Labeling**

Tritiated thymidine (Schwarz Bioresearch, SpA 1.9 c./mM) was added to the cultures 6 hours prior to fixation at a final concentration of 1 μc./ml. The excess of isotope not incorporated was removed at harvest with the culture medium and the cells were washed with a hypotonic solution.

**Fixation and Spreading of the Cells**

Two hours before termination of the culture, Colcemid (Ciba) was added to a final concentration of 0.03 μg./ml. This was done to study the labeling in the metaphases, and does not interfere with the DNA synthesis. At harvest the cells were treated with a hypotonic solution (1 per cent sodium citrate) for 10 minutes and fixed with 50 per cent acetic acid. After at least 20 minutes of fixation the preparations were made by squashing.

**Autoradiography and Staining**

Kodak stripping-film AR-10 was used. The preparations were exposed for 8 to 14 days. They were then developed in Kodak developer D-19b at 20 C. for 2 minutes, rinsed in water, fixed for 2 minutes in acid fixer, and washed in running water for 10 minutes.

The cells were stained through the film for 4 minutes with a diluted and buffered solution of Giemsa blood stain (distilled water 100 ml., M/10 citric acid 3 ml., M/5 Na₂HPO₄ 3 ml., methyl alcohol 3 ml., and stock solution of Giemsa's blood stain 5 ml.). Thereafter the preparations were rinsed, dried, and mounted with Euparal. Some preparations were stained with the methyl-green-pyronin stain.¹⁵

To determine the patterns of H³-thymidine incorporation, peripheral blood was obtained from 7 normal adult persons (4 females and 3 males). The cultures were incubated 72 hours and labeled with H³-thymidine 6 hours prior to fixation. The patterns of 500 labeled interphase nuclei of each case were recorded, and some cells were photographed. Subsequently the silver grains were removed with potassium ferricyanide (7.5 per cent) 3 to 4 minutes and sodium thiosulphate (20 per cent) 5 minutes; then the slides were restained with Giemsa or methyl green-pyronine. The same cells were localized and photographed again. Some slides from three cases were hydrolyzed with HCl for 8 minutes at 60 C., stained by Feulgen reaction, stripped again with Kodak stripping-film AR-10, exposed for 8 to 14 days, and developed as previously described. The labeling patterns of the interphase nuclei were again controlled.

To study the time sequence of the patterns of H³-thymidine incorporation, 18 leukocyte cultures from 3 normal subjects (2 females, 1 male) were prepared and fixed after 24, 42, 50, 56, 72, and 96 hours of culture. Each culture was labeled with H³-thymidine 6 hours prior to fixation, and the method described above was used.

**Results**

**DNA Replication Patterns**

The study of the labeled interphase nuclei from peripheral blood lymphocytes stimulated with PHA after 72 hours “in vitro” culture and 6 hours H³-thymidine incubation, revealed three different patterns of labeling (Fig. 1): (1) 17.8 per cent (11–21 per cent) of the labeled interphase nuclei showed a light, homogeneous labeling over the whole nucleus and no labeling over the nucleoli; (2) 57.7 per cent (50–65 per cent) of the labeled interphase nuclei shared a heavy labeling equally distributed over the whole nucleus and nucleoli; (3) 24.5 per cent (22–33 per cent) of the labeled interphase nuclei showed a heavy labeling over the nucleoli and a moderate labeling on the nucleus diffusely or “spottily” distributed with preponderance at the periphery.
Fig. 1.—Three white blood cells after 72 hours of culture, labeled with H³-thymidine 6 hours prior to fixation. Autoradiographic Stripping film Kodak AR-10. Giemsa stain. I left: First pattern of labeling. Light, homogenous labeling on the nucleus and no labeling over the nucleolus (nucleolus-negative phase). I right: The same cell without silver grains. II left: Second pattern of labeling. Heavy labeling on nucleus and nucleoli (intermediate phase). II right: The same cell without silver grains. III left: Third pattern of labeling. Heavy labeling over the nucleolus and moderate labeling at the periphery of the nucleus (nucleolus-positive phase). III right: The same cell without silver grains.
Some interphase nuclei presented a pattern of labeling intermediate between two of the three patterns described. When the density of silver grains was clearly lower over the nucleoli than over the nucleus the cell was classified in the first group. When the density of silver grains was much higher over the nucleoli than over the rest of the nucleus, the cell was classified in the third group. The cells with similar density of silver grains over both nucleus and nucleoli were classified in the second group.

In the third pattern of labeling, three further subgroups (Fig. 2) were distinguished: (1) the label accumulated over the nucleoli and diffusely around the periphery of the nucleus; (2) the label accumulated over the nucleoli and in some limited areas at the periphery of the nucleus; (3) the label accumulated over only one nucleolus or a pole of a nucleolus, and the nucleus had a heavily labeled spot, mostly at the periphery.

In the female blood leukocyte cultures, this last pattern was found in 4 per cent of the labeled cells. This heavily labeled spot was seen sometimes near to one nucleolus. It was considered as a manifestation in the interphase nuclei of the late replicating X chromosome of the females, and it was very evident in a previously described case of Iso-X-chromosome. This observation led us to suppose that the incorporation of H\textsuperscript{3}-thymidine over the nucleolus corresponded to a late stage of the DNA replication.

To prove that the label was accumulated over the nucleoli the silver grains were removed and the slides restained with Giemsa or methyl green-pyronine stain. The heavily labeled regions of the previously photographed cells corresponded exactly with the nucleoli.

The same patterns of labeling were also seen when the slides were treated with HCl hydrolysis, stained by the Feulgen reaction and stripped again. Thus, we suppose that the labeling accumulated over the nucleoli was not due to the incorporation of H\textsuperscript{3}-thymidine in the RNA or to the accumulation of an acid-soluble thymidine metabolite in the nucleoli, but to the incorporation of H\textsuperscript{3}-thymidine in the DNA.

**Time Sequence of the DNA Replication Patterns**

Figure 3 illustrates the results of this experiment. After 24 hours of culture only 0.3 per cent of the cells were in DNA synthesis. Due to the low number of cells replicating DNA we did not take the DNA replication patterns into account. The percentage of cells in DNA synthesis increased rapidly from the 42nd hour of culture, 6 per cent; to the 50th hour, 27 per cent; and the 56th hour of culture, 40 per cent. After 72 and 96 hours of culture, 51 and 48 per cent of the cells, respectively, were synthesizing DNA. These data confirm the results from similar experiments previously reported.

The distribution of the three patterns of labeling revealed that after 42 hours of culture, 57 per cent of the labeled cells showed the first pattern of labeling; the second pattern was observed in 39 per cent of the labeled cells and the third pattern appeared in only 4 per cent of the labeled cells.

After 50 hours of culture the first pattern was observed in 20 per cent of the labeled cells, the second pattern had risen to 72 per cent of the labeled cells,
Fig. 2.—Three white blood cells after 72 hours of culture, labeled with H\textsuperscript{3}-thymidine 6 hours prior to fixation. Autoradiographic Stripping film Kodak AR-10. Giemsa stain. A left: Third pattern of labeling, subgroup A. Heavy labeling over the nucleoli and at the periphery of the nucleus. A right: The same cell without silver grains. B left: Third pattern of labeling, subgroup B. Heavy labeling over the nucleolus and some scarce labeling on the nucleus. B right: The same cell without silver grains. C left: Third pattern of labeling, subgroup C. Heavy labeling over one nucleolus and scarce labeling over the other. (arrow): Heavy labeled spot at the periphery of the nucleus (female blood leukocyte culture). C right: The same cell without silver grains.
Fig. 3.—Percentage of cells in DNA synthesis as measured by \( \text{H}^3\)-thymidine uptake during culture, and percentage of labeled cells showing one of the three patterns of labeling. Mean values of three cases.

and the third pattern appeared only in 8 per cent of the labeled cells. After 56 hours of culture, there was a slight decrease of the first and second pattern to 15 per cent and 63 per cent respectively and a rapid rise in the per cent of labeled cells showing the third pattern, 22 per cent. This distribution of patterns persisted approximately constant at 72 hours—17 per cent, 55 per cent, 22 per cent—and 96 hours—11 per cent, 69 per cent, 20 per cent—of culture.

From these results it is evident that the first pattern of labeling corresponds to the beginning of the DNA synthesis. The great majority of cells synthesizing DNA move from the first to the second pattern between the 42nd and the 50th hour of culture. Only after 56 hours of culture could we see an important percentage of labeled cells moving into the third pattern. Simultaneously, the first mitoses appeared. These results suggest that the three patterns of labeling were consecutive and depended on the asynchronous replication of the DNA. Thus, the incorporation of \( \text{H}^3\)-thymidine over the nucleolus corresponded to a late stage of the DNA replication.

The percentage of labeled cells increased rapidly between the 42nd
hour culture (6 per cent) and the 56th hour culture (40 per cent). We believe that during these 14 hours the lymphocyte culture, stimulated with PHA, replicates DNA as a semisynchronized culture. But after 56 hours of culture the percentage of labeled cells and of patterns of labeling remained fairly constant, suggesting that the culture becomes asynchronous. Thus, we can calculate approximately the duration of the three patterns of labeling considering an S period of at least 12 hours and a G2 period of 4 hours. These calculations yield a duration of 2 to 2.7 hours for the first pattern, 6.5 to 8.8 hours for the second pattern, and 0.5 to 3.5 hours for the third pattern of labeling.

In previous experiments we found that with a pulse labeling of 10 minutes and 30 minutes prior to fixation it was difficult to differentiate clearly the three patterns of labeling. An incubation of at least 2 hours was necessary with H3-thymidine to have evidence of one of the three patterns in every labeled cell.

**Discussion**

Prescott and Bender described two labeling patterns of the interphase nuclei of leukocytes in short-term culture—a diffuse pattern and a “spotty” pattern—and suggested that the “spotty” labeling could be “characteristic of a particular interval of the total DNA synthesis period (perhaps the beginning and the end) in every cell.” Lima-de-Faria and Reitalu, working also with short-term leukocyte cultures, described the large heterochromatic regions of the nucleus as containing a much higher number of silver grains per unit area than did the euchromatic regions of the nucleus and proved that these autosomal heterochromatic regions were associated with the nucleolus. The same authors pointed out that DNA synthesis of the euchromatic regions was not synchronous with the DNA synthesis of the heterochromatic regions: “...there is a period in DNA synthesis when the euchromatin alone replicates.” From our previously described investigations it is evident that DNA replication of the chromatin in the short-term leukocyte cultures is asynchronous.

The first pattern of labeling shows the diffuse distribution of the replicating euchromatin when the DNA synthesis begins. The density of silver grains is low, and the grains are homogeneously distributed throughout the nucleus whereas the nucleoli remain unlabeled (nucleolus-negative phase). The second pattern of labeling shows a further stage of the euchromatin replication when the whole nucleus and nucleoli are covered by silver grains (intermediate phase). The third pattern of labeling represents the DNA replication of the “nucleolus associated chromatin” and some other late replicating regions of the periphery of the nucleus (nucleolus-positive phase). This is the late replication pattern which appears in an important percentage of labeled cells only after 56 hours of culture. Thereafter, 4 per cent of the labeled cells in the female leukocyte cultures at 72 hours show a later replicating region frequently on the periphery of the nucleus, whereas the “nucleolus associated chromatin” has already partially completed its DNA replication.

The three main patterns of labeling described here were previously de-
scribed by Harris\textsuperscript{17} in rat fibroblast cultures (although we would classify his example [Fig. 1C] as third phase subgroup b of labeled nucleus and nucleolus). This indicates that the three patterns of labeling are not restricted to leukocyte cultures, and we have seen them also in the human fibroblast cultures labeled with H\textsuperscript{3}-thymidine.\textsuperscript{16} Harris suggested that the pattern of nucleolar labeling alone was the first to be detected in a synchronized culture, and therefore the synthesis of DNA would probably initiate at the nucleolus. Other authors\textsuperscript{19-21} have described the nucleolar labeling with H\textsuperscript{3}-thymidine; they were either in agreement with Harris or did not indicate the time of appearance of the nucleolar pattern. The present studies therefore indicate that DNA synthesis begins diffusely throughout the nucleus and ends at the nucleolus-associated chromatin and some localized regions of late replicating chromatin.

**Nucleolar Labeling with H\textsuperscript{3}-thymidine**

Firtet and Verly\textsuperscript{22} were the first authors to describe the incorporation of H\textsuperscript{3}-thymidine around the nucleolus: “... the periphery of nucleoli often contains more grains than other areas of similar size in the nuclei.” Harris\textsuperscript{17} observed the incorporation of H\textsuperscript{3}-thymidine in the nucleoli of fibroblasts cultures; Bogoroch and Siegel\textsuperscript{19} proved that DNAase removed the label from this site, suggesting that the label was truly DNA, but they were not sure if this DNA was in the nucleolus or around it. Lima-de-Faria and Reitalu\textsuperscript{4} demonstrated the labeling of heterochromatic regions around the nucleolus but concluded that they were not part of the nucleolus which is Feulgen negative and does not incorporate H\textsuperscript{3}-thymidine. On the contrary, Altmann et al.\textsuperscript{29} confirmed the nucleolar DNA synthesis and stated that it was not synchronous with the DNA synthesis of the nucleus. Our observations allow us to assert that the silver grains appear over the nucleolar regions, but our methods do not provide a solution if the H\textsuperscript{3}-thymidine uptake occurs only around or also in the nucleolus.

The problem has been probably solved by Bernhard and Gramboulan,\textsuperscript{23} who demonstrated, by the use of histochemical technics and electron microscope, the presence of chromatin in the nucleoli. Sections through nuclei which had incorporated H\textsuperscript{3}-thymidine revealed labeling also in some of the nucleoli: “... an unexpected finding was the localization of thymidine in nucleoli.” It was also observed a selective further incorporation of thymidine along the nuclear membrane in some cells with thymidine incorporation in the nucleoli. After treatment with RNAase the label remained unchanged. Gramboulan and Gramboulan\textsuperscript{21} confirmed these results and concluded that the nucleolar-associated chromatin, which is around the nucleolus, infiltrates the inside of the nucleolus, forming a network of chromatin. Thus, the nucleolus-associated chromatin and the intranucleolar chromatin form a functional and morphologic unit.

Based on these investigations we think that the nucleolar labeling seen in the third pattern corresponds to the DNA synthesis of the intranucleolar and nucleolus associated chromatin. Bernhard and Gramboulan\textsuperscript{22} indicate that H\textsuperscript{3}-
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Thymidine incorporation in the nucleoli was observed more often in the KB-tumor cells and in cells infected with a DNA-virus (SV40); these DNA-virus-infected cells presented also a considerable increase of intranucleolar DNA. Further studies of Caspersson et al. suggest that "the increased cytochemical variability of populations of neoplastic cells may well be related to disturbances in the nucleolus-associated chromatin or in the euchromatic genes regulating its function." Thus, some disturbances of the nucleolar and nucleolus-associated chromatin could be possibly found by studying the DNA replication patterns of neoplastic and leukemic cells.

SUMMARY

Three patterns of DNA replication in the interphase nuclei of short-term leukocyte cultures are described: (1) light, homogenous labeling on the whole nucleus and no labeling over the nucleolus (nucleolus-negative phase), (2) heavy labeling equally distributed over the whole nucleus and nucleoli (intermediate phase), (3) heavy labeling over the nucleoli and moderate labeling at the periphery of the nucleus (nucleolus-positive phase).

These three patterns of labeling appear consecutively during the process of DNA synthesis. Thus, the nuclear and nucleolar associated chromatin replicates during the late stage of DNA synthesis. The existence of nucleolar chromatin is discussed. Study of H3-thymidine incorporation in the nucleoli of leukemic and neoplastic cells may develop data regarding the possible metabolic disturbances of these cells.

SUMARIO IN INTERLINGUA

Es describite tres configurationes del replication de acido deoxyribonucleic in le nucleo interphasal de cultures a breve tempore de leucocytos human: (1) un leve homogenee marcase al superficie del nucleo total e nulle marcase supra le nucleolo (phase nucleolo-negative); (2) forte marcase de distribution uniforme supra le nucleo total e le nucleolos (phase intermedia); e (3) forte marcase supra le nucleolos e moderate marcase al peripheria del nucleo (phase nucleolo-positive).

Iste tres configurationes del marcase appare consecutivemente durante le processo del synthese de acido deoxyribonucleic. Assi le chromatina associate con le nucleo e le nucleolos se replica durante le stato tardive del synthese de acido deoxyribonucleic. Le existentia de chromatina nucleolar es commentate. Le studio del incorporation de thymidina a tritium ad in le nucleolos de cellulas leucemic e neoplastic va forsan producer datos relative al possibile disturrbationes metabolic in tal cellulas.

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MANUEL RIBAS-MUNDÓ