Experimental Cell Research **27**, *221–229 (1962)*

DNA SYNTHESIS AND MITOSIS IN CULTURES OF HUMAN PERIPHERAL LEUKOCYTES

M. A BENDER and D. M. PRESCOTT

Biology Division, Oak Ridge National Laboratory,[1] *Oak Ridge, Tenn., U.S.A.*

Received September 4, 1961

ADVANCES in human cytogenetics [13] have created a demand for a rapid and simple technique for obtaining diploid mitotic figures. A very appealing and ready source of cells is peripheral blood. However, although tissue cultures of human peripheral leukocytes have been reported several times [7, 10], none of these reports resulted in acceptance of the method for chromosome analysis. The recent discovery that the red cell agglutinin, Phytohemagglutinin (Difco), tremendously increased the number of mitoses in three-day leukocyte cultures, together with the development of an excellent method for fixing and mounting the cells [9], has given the leukocyte culture method wide acceptance as a source of material for both cytogenetic investigations and clinical cytodiagnosis.

Experimental studies on human leukocyte chromosomes require information concerning the timing of DNA synthesis, time of the onset of mitosis, duration of the mitotic cycle, and the number of mitoses undergone by individual leukocytes. In addition, and perhaps even more importantly, this information has a direct bearing on the problem of the mitotic potential of circulating leukocytes. This potential is undoubtedly responsible for the capacity of transplants of peripheral leukocytes to induce recovery in lethally irradiated mammals [3, 5]. It is also probable that a mitotic potential is necessary for the immunological competence of such cells [4].

For these reasons, we have studied DNA synthesis and mitosis in human peripheral blood leukocyte cultures, using tritiated thymidine.

MATERIALS AND METHODS

Culture of peripheral blood leukocytes.—In the method of Hungerford *et al.* [7], the separation of the leukocytes from whole blood depends upon the agglutination of the red cells with Phytohemagglutinin. This procedure is unreliable because of the variability of the Difco Phytohemagglutinin, and variations among individual

[1] Operated by Union Carbide Corporation for the U.S. Atomic Energy Commission.

donors. We have, therefore, adopted the simple method of "buffy coat" separation. Sterile blood (5 ml culture) is drawn by venipuncture and placed in sterile, screw cap, 15-ml centrifuge tubes containing 0.1 ml of commercial heparin (1000 units ml). The tubes are spun at maximum speed in an International clinical centrifuge for 10 min at room temperature. The leukocytes form a dense layer (buffy coat) lying on top of the packed erythrocytes. The serum, buffy coat, and the top 1 mm of erythrocytes are withdrawn with a sterile Pasteur pipette and added to a milk dilution bottle containing the culture medium. The cell clumps are then broken up by vigorous pipetting.

The culture medium is composed of 10 ml of mixture 199, 4 ml of freshly inactivated type AB-positive human serum, 700 units of penicillin G, 0.7 mg of streptomycin, and 0.1 ml of Phytohemagglutinin per culture.

Our procedure is less complicated than that recommended by Moorhead *et al.* [9], in that we do not count the cells, adjust the medium volume to give uniform cell density, or allow for the volume of autologous serum added to the culture along with the leukocytes.

The unsealed cultures are incubated at 37°C in a 5 per cent CO_2-95 per cent air atmosphere.

Labeling.—Tritiated thymidine (Schwarz, 3.0 C/mM) was added to the cultures at various times to a final concentration of 1 μc/ml. The tritiated cultures were incubated for 25 min, decanted into sterile centrifuge tubes, and spun down for 5 min. The radioactive medium was discarded, and the cells were resuspended in fresh medium and returned to the incubator. This operation resulted in at least a thousand-fold dilution of the tritiated thymidine.

Fixation and mounting of the cells.—The cytological methods used are adapted from the procedures outlined by Moorhead *et al.* [9]. Metaphases were collected by colchicine treatment. Five hours before the culture was to be fixed, colchicine was added to a final concentration of 10^{-7} *M*. Just prior to fixation the cells were centrifuged out of the medium and resuspended in balanced salt solution (BSS). After an additional centrifugation all but 1 ml of the BSS was removed. The cells were resuspended, and 4 ml of warm distilled water was added slowly with constant agitation. The cells were left in this hypotonic environment for 10 min and then centrifuged for 5 min. The hypotonic solution was discarded, and approximately 5 ml of freshly made 3:1 (v/v) methyl alcohol:glacial acetic acid fixative was carefully added without disturbing the cells. When this is done, fixation proceeds slowly from the top to the bottom of the cell pellet. When an appreciable volume of red cells was present, it was found advisable to agitate the fixative (without disturbing the pellet of unfixed cells). After ½ hr the cells were suspended in the fixative with a Pasteur pipette and any lumps broken up by vigorous pipetting. The cells were washed by centrifugation in three changes of fresh fixative and finally resuspended in 0.5 to 1.0 ml. When the concentration of cells was unusually high, even further dilution was sometimes necessary.

The fixed cells were mounted by placing single drops on the *clean* wet surface of a slide. After the excess water was wiped off, the slides were thoroughly air-dried. This schedule yields large numbers of well-spread metaphase figures.

Autoradiography and staining.—The slides were dipped in NTB2 liquid emulsion (Kodak) and exposed for 1 to 6 weeks. They were developed in D11 for 2 min at 20

to 22°C, rinsed in water, fixed for 5 min, and washed in running water for 20 min. The cells were stained through the emulsion with Giemsa stain [6] at about pH 7.0 for 30 min, rinsed, dried, and mounted.

RESULTS

Level of DNA synthesis in whole, fresh blood.—Bond *et al.* [2] reported that a very small fraction of peripheral leukocytes of freshly drawn human blood are engaged in DNA synthesis. We have confirmed this observation. Tritiated thymidine was added to a 5 ml sample of whole, heparinized blood and left for ½ hr. The blood was centrifuged, and the buffy coat and serum removed. The leukocytes were washed twice in fresh medium by centrifugation. They were then added to the standard culture medium together with an additional 2 ml of inactivated type AB-positive human serum. The cultures were treated with colchicine and the cells fixed 72 hr after culture initiation. Autoradiographs showed that very few nuclei had incorporated tritiated thymidine and further, that the few cells that had were very lightly labeled, indicating a very low rate of DNA synthesis *in vivo*. All of the labeled cells were either of a large or a smaller mononuclear type (Fig. 1). Bond *et al.*[2] have identified these cells as monocytes and lymphocytes. Mitotic figures were numerous in our material but none contained label. Therefore, cells in DNA synthesis (in the S period) in the whole blood at the time of venipuncture contribute few if any cells to the population that is in mitosis from 66 to 72 hr later.

Time of appearance of first mitosis in cultures.—In order to determine the time of the onset of mitosis, two experiments were performed in which 30 ml of blood was drawn from a single donor and made into six cultures. The cultures were fixed at 12-hr intervals after 6 hr of colchicine treatment; thus, the intervals of 6 to 12 hr, 18 to 24 hr, etc., up to 66 to 72 hr were sampled for mitotic activity. No mitoses were seen in material fixed at 12, 24, and 36 hr. Numerous mitoses accumulated between 42 and 48 hr. Mitoses became even more frequent in the last two collection periods.

Culture life span.—To determine the life span of our leukocyte cultures, an experiment was performed in which a 30-ml blood sample was divided into six cultures which were subsequently treated with colchicine and fixed at alternate 3- and 4-day intervals over the next 3 weeks. At the end of 1 week a semiweekly medium replacement schedule was initiated. Dividing cells were numerous at 3, 7, and 10 days. The number of mitoses decreased slightly at 14 days, and there were no mitoses in the 17- and 21-day cultures. The general appearance of the cells was excellent during the first 14 days. By 17 days, however, the cultures appeared moribund, with fewer cells and

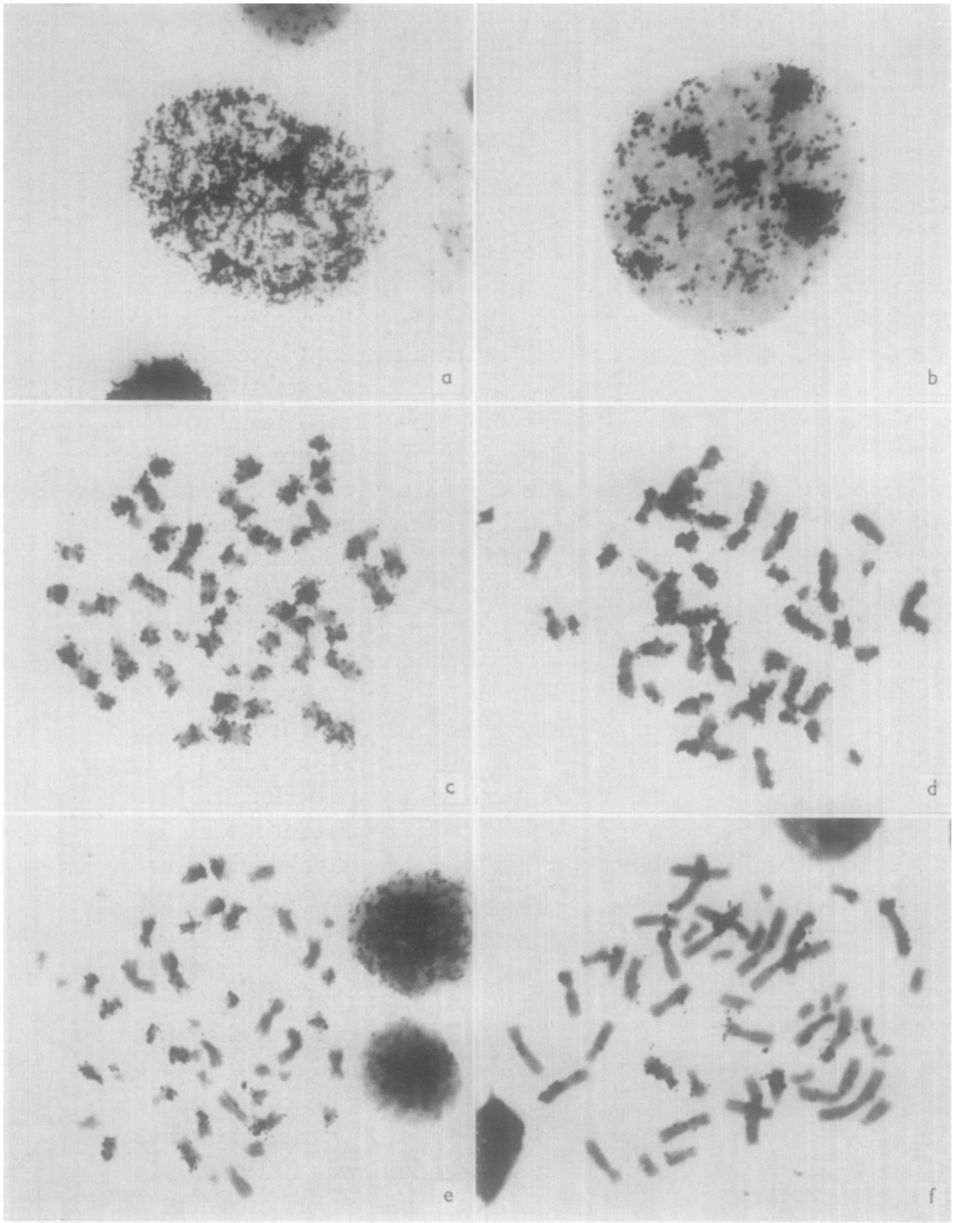

Fig. 1.—Autoradiographs of tritiated thymidine-labeled human peripheral leukocytes from cultures fixed 72 to 154 hr after initiation. Magnified 1350. (*a*) Large and small monocytes showing a moderate level of labeling. (*b*) Localized labeling in a large monocyte. (*c*) Chromosomes of a first post-DNA-labeling metaphase. Both chromatids of each chromosome are labeled.

many pycnotic nuclei. At 21 days there were practically no healthy cells remaining.

Time of DNA synthesis.—When whole blood is cultured immediately after X-irradiation, only chromosome-type aberrations are seen, whereas chromatid-type aberrations are obtained when cultures are irradiated shortly before the advent of mitosis [1]. DNA synthesis must thus occur sometime between the initiation of the culture and mitosis; most, if not all, of the circulating leukocytes that divide in our *in vitro* system are in the pre-DNA-synthesis (G_1) growth phase at the time the blood is drawn. Experiments were therefore designed to determine the time of DNA synthesis.

A 35-ml sample of fresh blood was divided among seven cultures. Every 6 hr one culture was exposed to tritiated thymidine for 30 min. All cultures were treated with colchicine from the 42nd to the 48th hr after culture initiation and then fixed. Both interphases and mitoses were scored for labeling.

Only occasional, very lightly labeled interphase nuclei were observed in cultures treated at 6, 12, and 18 hr. In cultures treated at 24 hr, both the number of labeled nuclei and the intensity of labeling were greater. When cultures were treated at 30 hr, an even higher percentage of nuclei was labeled. The general intensity of labeling was high, and some nuclei were very heavily labeled. With treatment at 36 hr and thereafter, the percentage of labeled interphase nuclei remained the same, as did the intensity of labeling of individual nuclei.

In cultures labeled at 6 and 12 hr after initiation, and fixed at 48 hr, metaphases were extremely rare, suggesting a tritium-induced mitotic delay. In cultures treated at 18 hr and thereafter, the usual level of mitotic activity was observed. With treatment at 18 hr, 75 per cent of the mitotic figures were labeled. With treatment at 24 hr or later, all mitotic figures were labeled. Thus, the total time between the beginning of DNA synthesis and metaphase (S plus G_2) can at least be as short as 30 hr and possibly as short as 18. The uncertainty is, of course, due to the 6-hr experimental intervals. The figure of 30 hr is derived from the fact that 25 per cent of the cells can enter DNA synthesis after 18 hr and still reach metaphase by 48 hr. The minimum estimate is derived from the fact that some of these cells may enter DNA synthesis as late as 24 hr and reach metaphase as early as 42 hr. Since no unlabeled metaphases were seen in 100 metaphases from cultures labeled

---

(*d*) Chromosomes of a second post-DNA-labeling metaphase. Only one chromatid of each chromosome is labeled. (*e*) Chromosomes of a third post-DNA-labeling metaphase. Only about half of the chromosomes contain a labeled chromatid. (*f*) Chromosomes of a fourth post-DNA-labeling metaphase. Only about one-fourth of the chromosomes contain a labeled chromatid.

at 42 hr and fixed at 48 hr, all cells were still engaged in DNA synthesis 6 hr before metaphase. Therefore, the post-DNA-synthesis period (G_2) in these cells must be shorter than 6 hr. Subtracting this maximum estimate of G_2 (6 hr) from the minimum possible value for S plus G_2 (18 hr), we conclude that S can be no shorter than 12 hr. However, from the fact that some of the cells dividing in the 42- to 48-hr interval were engaged in DNA synthesis as early as 6 hr, we may conclude that in some cases the S period may be as long as 30 hr (42 minus 6 minus the 6-hr G_2).

The foregoing observations demonstrate that intensive DNA synthesis does not occur until 18 to 30 hr after initiation of the culture and further, that every cell entering mitosis between 42 and 48 hr, i.e., in the first wave of mitoses, has passed through a period of DNA synthesis *in vitro*. This confirms the expectation based on the X-ray induced aberration study that virtually every leukocyte capable of dividing *in vitro* was in a pre-DNA-synthesis period (G_1) at the time the blood was drawn.

No asynchrony of DNA synthesis among chromosomes within a single complement was seen in this material, although asynchrony of synthesis within individual chromosomes was evident. Localized labeling of interphase nuclei was also observed (Fig. 1). This phenomenon will be discussed more fully in a subsequent paper.

Repeated mitoses.—Since a high level of mitotic activity persists in leukocyte cultures for at least 14 days (see p. 223), we designed an experiment to determine whether an individual cell traverses more than one division cycle. A 40-ml blood sample was divided into eight cultures, all of which received 30 min exposure to tritiated thymidine at 48 hr. The first culture was treated with colchicine and then fixed at 72 hr, an additional culture was colchicine-treated and then fixed every 12 hr thereafter up to 156 hr. Two criteria were used to determine how many mitoses a cell had undergone after DNA labeling.

First divisions are readily distinguished from all subsequent divisions because in the first metaphase after labeling both chromatids are labeled, while in subsequent mitoses no more than one chromatid in a given pair is labeled [14]. Further dilution of the labeled DNA by newly synthesized, unlabeled DNA enables us to distinguish between second, third, and fourth division. In the third metaphase post-DNA-labeling, only about half the chromosomes contain labeled chromatids, while at fourth metaphase only about one-quarter of the chromosomes are labeled [12]. A concomitant decrease in the number of silver grains over interphase nuclei was seen. Fig. 1 shows examples of labeled first, second, third, and fourth divisions.

At 72 hr only one of 75 labeled metaphases did not show label in both chromatids. At 84 hr approximately two-thirds of the labeled metaphases showed label in only one chromatid. By 96 hr, and in subsequent samples, there was only an occasional metaphase labeled in both chromatids. At 108 hr, approximately one-third of the metaphases showed label in only half the chromosomes of a complement. In the 120-hr sample a few metaphases with only one-quarter of the complement labeled appeared. Subsequent samples showed a mixture of all the above labeling types.

It follows from these observations that at least four divisions of an individual leukocyte can occur.

The fact that first division still occurs as late as 156 hr in cells labeled at 48 hr shows that the S plus G_2 period can be in excess of 100 hr.

DISCUSSION

The results of Bond *et al.* [2], which are confirmed here, show that a small fraction of the leukocytes in peripheral blood synthesizes DNA *in vivo*. The leukocyte culture technique clearly demonstrates that a much larger fraction of the leukocyte population of peripheral blood has a mitotic potential. In a separate study of X-ray-induced chromosome damage [1], only chromosome-type aberrations, instead of a mixture of chromosome and chromatid types, were found when human blood was irradiated *in vitro* shortly after collection. This finding strongly suggested that the leukocytes that were capable of dividing in our *in vitro* system were in the pre-chromosome replication (and presumably pre-DNA synthesis, or G_1) phase at the time the blood was drawn.

The present experiments provide a detailed picture of the relationships between the initiation of the culture, DNA synthesis, and mitosis, and confirm that the freshly drawn leukocytes are in the G_1 phase.

We originally considered two possible explanations for the induction of DNA synthesis and mitosis in these cultures. The transfer of leukocytes to an *in vitro* situation might release them from some *in vivo* suppression or control, or some "factor" in the culture medium might stimulate mitosis. In experiments in which two cultures, one with and one without Phytohemagglutinin, were made from the same blood sample, only the culture supplemented with Phytohemagglutinin showed any mitoses. This finding both substantiates the second of the above hypotheses and shows that the "factor" is in fact the Phytohemagglutinin. In view of the hypothesis that antibody production is intimately related to mitosis [8] and the demonstration that

peripheral leukocytes are immunologically competent [4], it is attractive to speculate that the stimulating action of Phytohemagglutinin may be a consequence of its antigenicity.

The present experiments establish limits for the lengths of G_1, S, and G_2 of a minimum of 24 hr, a minimum of 12 hr, and a maximum of 6 hr, respectively, in cells dividing in the first wave of mitoses. We also know that S can be as long as 30 hr. Since at least two morphologically distinct types of cells are synthesizing DNA in culture, some of this variation in timing may be ascribed to heterogeneity of the population. Nevertheless, the observed variability in the time required to double DNA among cells of the same genetic constitution is of interest. The observed lengths of G_1, S, and G_2 are of the same order of magnitude as those observed in other mammalian cell types [14]. Also, the position of the S period in interphase is the same as in other mammalian cells; a short and relatively constant G_2 seems to be characteristic of all vertebrate cells [11]. This observation suggests that there may be a relatively direct causal relationship between the completion of DNA synthesis and the initiation of mitosis. Unfortunately, virtually nothing is known about the events occurring during G_2.

Several circumstances may contribute to the long and variable G_1 period in this system. It is possible that the leukocytes that divide *in vitro* are more or less permanently arrested in G_1 while *in vivo*; the readjustments necessary following *in vitro* release from this arrest may contribute to the observed length and variability of this period. It is also possible that the length of time spent by a cell in arrested G_1 *in vivo* might affect the time required to complete this phase *in vitro*. Our lack of knowledge about the events occupying G_1 makes further speculation about the factors affecting its length unprofitable.

In our experiments human leukocyte cultures have a limited life span (about two weeks), although we have shown that individual cells can divide at least four times. We can offer no explanation for this situation, but such limited life spans are not without precedent in the field of tissue culture.

While we have observed no clear cases of asynchrony of DNA synthesis among different chromosomes of a single cell in these experiments, asynchronous synthesis of DNA *within* individual chromosomes was quite obvious. An extended description of this phenomenon and of the chromatid segregation of labeled DNA in these and other mammalian cells will be presented elsewhere [12].

SUMMARY

DNA synthesis and mitosis in human peripheral blood leukocyte cultures have been studied using tritiated thymidine. In whole, unmanipulated blood the level of DNA synthesis was very low, and mitoses were undetectable. In culture, Phytohemagglutinin induced a very great increase in the level of DNA synthesis and mitosis. The first wave of mitosis started at about 42 hr, and every mitosis was preceded by an *in vitro* DNA duplication. In these experiments the initial G_1 phase occupied a minimum of 24 hr, the S period occupied a minimum of 12 hr, and the G_2 phase occupied a maximum of 6 hr. Although individual cells underwent at least four mitoses, the cultures had a life span limited to about two weeks.

REFERENCES

1. BENDER, M. A and GOOCH, P. C., *Proc. Natl. Acad. Sci. (Wash.)* **48**, 522 (1962).
2. BOND, V. P., CRONKITE, E. P., FLIEDNER, T. M. and SCHORK, P., *Science* **128**, 202 (1958).
3. GOODMAN, J. W., *Ann. N.Y. Acad. Sci.* **97**, 95 (1962).
4. GOODMAN, J. W. and CONGDON, C. C., *Am. Med. Ass. Arch. Pathol.* **72**, 18 (1961).
5. GOODMAN, J. W. and HODGSON, G. S., *Blood* **19**, 702 (1962).
6. GUDE, W. D., UPTON, A. C. and ODELL, T. T., JR., *Stain. Technol.* **30**, 161 (1955).
7. HUNGERFORD, D. A., DONNELLY, A. J., NOWELL, P. C. and BECK, S., *Am. J. Human Genetics* **11**, 215 (1959).
8. MAKINODAN, T., *in* Encyclopaedia of Medical Radiology, (ed. A. ZUPPINGER) Berlin: Springer Verlag, in press, 1961.
9. MOORHEAD, P. S., NOWELL, P. C., MELLMAN, W. J., BATTIPS, D. M. and HUNGERFORD, D. A., *Exptl. Cell Research* **20**, 613 (1960).
10. OSGOOD, E. E. and KRIPPAEHNE, M. L., *Exptl. Cell Research* **9**, 116 (1955).
11. PRESCOTT, D. M., *Intern. Rev. Cytol.* in press.
12. PRESCOTT, D. M. and BENDER, M. A, manuscript in preparation (1961).
13. STERN, C., Human Genetics. W. H. Freeman and Co., San Francisco, 1961.
14. TAYLOR, J. H., *in* Cell Physiology of Neoplasia. The University of Texas Press, Austin, 1960.