RNA Synthesis in Cultures of Normal Human Peripheral Blood

By Lois B. Epstein and Frederick Stohlman, Jr.

There has been increasing acceptance of the concept that small lymphocytes can transform both in vitro and in vivo into larger cells capable of division.\(^1\)\(^9\) Repeated observations have shown that an increase in basophilic cytoplasm is an essential aspect of this transformation. Furthermore, electron microscopic studies of small lymphocytes growing in tissue culture have shown increase in ribosomal content in the transformed small lymphocytes.\(^6\) These observations suggested the present study of RNA synthesis during the transformation of small lymphocytes in tissue culture.

Methods

Blood was obtained from nine normal adult males. Up to 110 ml. normal venous blood was withdrawn into a previously heparinized syringe (0.2 ml. of 0.5 Gm. per cent phenol-free heparin in sterile isotonic saline for each 10 ml. blood). The syringe was rotated gently and the blood was transferred to a collection flask containing 0.02 ml. phytohemagglutinin \(P^*\) and 0.2 ml. phytohemagglutinin \(M^*\) for each 10 ml. blood and left in an ice bath undisturbed for 45-60 minutes. It was then centrifuged at 0-5 C. in an International Centrifuge at 400 rpm for 4 minutes. An additional 2 minutes each was allowed for acceleration and deceleration. The supernatant leukocyte-rich plasma was withdrawn and 4 ml. aliquots were distributed into 2 oz. St. Louis culture bottles. Sixteen ml. of tissue culture medium 199, with penicillin and streptomycin, were added to each culture bottle. After gentle rotation the cultures were incubated at 37 C. in room air for 0 to 96 hours.

When larger amounts of blood than 110 ml. were desired, a Fenwal pack containing appropriate amounts of phenol-free heparin solution and phytohemagglutinin \(M^*\) and \(P^*\) was used. The pack was rotated several times, placed in an ice bath for 45 minutes, and then centrifuged as described above. The supernatant leukocyte-rich plasma was withdrawn and 4 ml. aliquots were distributed into 2 oz. St. Louis culture bottles. After gentle rotation the cultures were incubated at 37 C. in room air for 0 to 96 hours.

When cultures were prepared without phytohemagglutinin, the heparinized blood was allowed to remain in sterile centrifuge tubes at a 45° angle in an ice bath for 50 minutes. The tubes were then centrifuged at 1000 rpm for 2 minutes, allowing ½ minute each for acceleration and deceleration. The leukocyte-rich plasma was withdrawn and 4 ml. aliquots distributed into the culture bottles. Phytohemagglutinin was then added to control bottles prepared from the same leukocyte-rich plasma. Irrespective of the method of preparation of the leukocyte-rich plasma, the final concentration of leukocytes in the culture bottles varied from 250,000/ml. to 1,500,000/ml.

The time at which cultures were placed in the incubator was designated as zero. To study RNA synthesis, 2 \(\mu\)C. of \(H^3\)-cytidine\(^\dagger\) were added for each ml. of culture at times varying from 0 to 96 hours. After gentle rotation, the cultures were incubated with \(H^3\)-cytidine for 2 hours. Duplicate or triplicate cultures were set up for each time interval.

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\(\dagger\)Specific activity 1.50 c./nM, Schwarz BioResearch, Inc.
The cultures were harvested by centrifugation at room temperature at 800 rpm for 8 minutes, and the supernatant was withdrawn. Smears were prepared from the cell buttons and fixed in methyl alcohol.

Parallel control cultures were prepared in which DNA synthesis was evaluated by the incorporation of tritiated thymidine. Two μc. of H3 thymidine were added for each ml. culture and the cultures were treated as previously described for the H3-cytidine experiments.

The slides for autoradiography were fixed in absolute methyl alcohol for 10 minutes and then dipped for 3 seconds at 43°C in NTB-2 Kodak Nuclear Track Emulsion to which 2 drops of Tween20 had been added. After drying in room air, they were placed in black slide boxes with a desiccant and stored in a freezer for 1 week. The slides were then developed by passage through D-19 Kodak Developer, water, and Kodak fixer for 3 minutes each, and were then washed in running water for 20 minutes. The autoradiographs were passed through successive solutions of (1) 50 per cent methyl alcohol and 50 per cent citrate phosphate buffer, pH 5.8 (85 ml. 0.1 M citric acid plus 115 ml. 0.2M Na2HPO4 diluted to 1 liter), (2) the pH 5.8 citrate phosphate buffer, and (3) absolute methyl alcohol, for 3 minutes each, and then stained with an alcoholic, pH 5.8 Giemsa solution for 8 minutes. After a final rinse in the pH 5.8 buffer, the slides were air-dried. In the final preparations, macromolecular polynucleotides are the only tritiated compounds present intracellularly.20 During the initial 2 hours, cytidine is incorporated primarily into RNA and to a substantially lesser extent into DNA. Under the conditions of culture used in the present study, there was no DNA synthesis during the first 24 hours. It can be inferred, therefore, that all the labeling observed after 2-hour incubation with cytidine reflected RNA synthesis.

Five hundred mononuclear cells on each slide were counted, and the per cent of the total cells with 0, 1 or more, 6 or more, and 11 or more grains was determined. Evaluation of background indicated that cells with 6 or more grains had significant label.

RESULTS

Initially, the curves for DNA synthesis were repeated to establish that the growth patterns were similar to those previously reported. The agreement was good(fig. 1A). DNA synthesis occurred in ~0.2 per cent of the mononuclear cells during the first 20 hours of culture. The per cent of mononuclear cells synthesizing DNA increased rapidly after 24 hours so that by 48 hours 13 per cent of the mononuclear cells were in DNA synthesis. After 72 hours, 45-50 per cent, and after 96 hours ~55 per cent of the cells were synthesizing DNA. The incorporation of H3-thymidine occurred predominantly in large "blast-like" mononuclear cells and only rarely in medium-sized mononuclear cells.

Figure 1B illustrates the results of the experiments in which incorporation of H3 cytidine was used as a measure of RNA synthesis. From these results (fig. 1A, B) it is evident that RNA synthesis preceded DNA synthesis by 24 hours. There was a rapid rise in the per cent of mononuclear cells in RNA synthesis in the initial 24 hours, which by 48 hours began to level off. Approximately 2 per cent of the mononuclear cells were in RNA synthesis at time zero, ~55 per cent at 24 hours, ~70 per cent at 48 hours, and ~80 per cent at 72 hours. The predominant cells in RNA synthesis, like those in DNA synthesis, were the "blast-like" mononuclear cells. However,

*Specific activity 1.09 c./mM, Schwarz BioResearch, Inc.
Fig. 1A.—The percentage of mononuclear cells in DNA synthesis in culture as measured by tritiated thymidine uptake. The dashed line represents the results of similar experiments performed by MacKinney et al.1

Fig. 1B.—The percentage of mononuclear cells in RNA synthesis in culture as measured by tritiated cytidine uptake.
RNA synthesis also occurred in some small lymphocytes, whereas DNA synthesis did not. The large "blast-like" cells appeared to incorporate far more $H^3$-cytidine than did the small lymphocytes.

Figure 2 illustrates the results of experiments in which RNA synthesis was compared in cultures with and without phytohemagglutinin. At 10 hours the phytohemagglutinin-containing cultures from subjects R. G. and A. S. had 16 per cent and 19 per cent of mononuclear cells labeled with 11 or more grains; cultures prepared without phytohemagglutinin had less than 1 per cent. At 24 hours the figures were 29 per cent and 47 per cent with phytohemagglutinin but only 1 to 2 per cent without. At 48 hours the figures were 69 per cent and 66 per cent with and <1 per cent without. A similar pattern suggesting a negligible amount of RNA synthesis in cultures prepared without phytohemagglutinin is seen on examination of the bar graphs for 6 or more grains.

Repeated observations of cultures prepared with phytohemagglutinin showed that the lymphocytes grew in large clumps of cells. No such clump-
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The above data suggest that phytohemagglutinin induces RNA synthesis which results in a transformation of small lymphocytes into larger basophilic cells capable of DNA synthesis and division. In the absence of phytohemagglutinin, there was virtually no RNA synthesis nor evidence of transformation of small lymphocytes. The initiation of RNA synthesis ~24 hours prior to DNA synthesis and the absence of DNA synthesis in cultures without phytohemagglutinin point to the fact that RNA synthesis and transformation of small lymphocytes are necessary steps in the initiation of DNA synthesis.

The mechanism by which phytohemagglutinin induces RNA synthesis is unknown. Several studies point to its action as an antigenic stimulus. It has been shown, for example, that tuberculin will induce lymphocyte transformation in tuberculin-sensitive individuals. Further, mixtures of homogeneous leukocytes will initiate lymphocytic transformation and proliferation; isogenic leukocytes, however, are ineffective. These studies, together with Nowell’s observation that prednisolone will block the transformation of lymphocytes by phytohemagglutinin, point to an antigenic mechanism for the initiation of RNA synthesis and eventual DNA synthesis and growth in peripheral leukocyte cultures. However, cells from newborns which presumably are immunologically incompetent will grow in culture. This casts some doubt on the thesis that phytohemagglutinin acts solely as an antigen in the conventional sense. Further, in cultures containing phytohemagglutinin, the growth starts earlier; the mitotic index is higher; and gamma globulin production occurs in a greater number of cells than in those cultures with conventional antigens such as tuberculin. These observations suggest that factors other than antigenic induction of RNA synthesis are affecting cell growth in cultures with phytohemagglutinin. It is possible that a surface effect on the leukocyte with the subsequent clumping which is seen in phytohemagglutinin cultures provides a more favorable medium for growth after the initial induction of RNA synthesis.

SUMMARY

RNA and DNA synthesis were measured in cultures of normal human peripheral blood using tritiated cytidine and thymidine and autoradiographic technics. RNA synthesis preceded DNA synthesis by 24 hours. RNA synthesis occurred predominantly in the large and medium-sized “blast-like” cells, but did occur, to a lesser extent, in the small lymphocytes. RNA synthesis did not occur in the absence of phytohemagglutinin, nor did DNA synthesis. Mecha-
nisms of action of phytohemagglutinin are discussed with particular reference to its possible antigeneric nature.

**Summario in Interlingua**

Le synthese de acido ribonucleic (ARN) e de acido disoxyribonucleic (ADN) esseva mesurate in culturas de normal sanguine peripheric human con le uso de tritiate cytidina e thymidina in technicas autoradiographic. Le synthese de ARN precedeva le synthese de ADN per 24 horas. Le synthese de ARN occurreva predominantemente in le cellulas “blastoide” de magnitude major e intermedie, sed illo non occurreva (o occurreva solmente in basse grados) in micre lymphocytos. Le synthese de ARN non occurreva in le absentia de phytohemagglutinin, e le mesmo valeva pro le synthese de ADN. Mechanismos del action de phytohemagglutinin es discutite con referentias particular a su natura possibilemente antigeneric.

**REFERENCES**

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