Effect of Phytohemagglutinin on Enzymes of Thymidine Salvage Pathway of Cultured Chronic Lymphatic Leukemic Lymphocytes

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RESPONSE OF N-L when cultured with PHA to form large blastlike, dividing cells is accompanied by a variety of metabolic changes. During the first hours there are increases in glycolysis, RNA and protein synthesis. Increase in DNA synthesis is not prominent until the end of the first day of culture, while mitosis becomes active in the second to third day. A significant increase in total LDH activity with increase in the proportion of M-LDH occurs during the first day of culture. DNA polymerase activity, on the other hand, although showing an increase during the first day, does not become prominent until the second to third days of culture, paralleling the timing of increased DNA synthesis. This led to the suggestion that induction of DNA polymerase is a factor in the control of DNA synthesis. In a study of enzymes of the TdR salvage pathway it was shown that increased TdR kinase activity in cultures of N-L with PHA also paralleled the timing of DNA synthesis, with maximal increases in enzyme activity occurring with the peak of DNA synthesis. Increased TdR kinase activity had also been found to be associated with the onset of DNA synthesis in synchronized cell cultures. TdR kinase and DNA polymerase may be important factors in the control of DNA synthesis.

The response of CLL-L to PHA in cultures varies. Blast-cell development is usually greatest in cultures of lymphocytes from cases with the lowest peripheral WBC counts and least in cultures from those with the highest WBC counts. Perhaps the number of blast-cells formed depends on the number of relatively normal lymphocytes present. In previous studies it was shown that the degree of blast-cell response of CLL-L to PHA was related to the levels attained of increased glycolysis, of increased LDH activity and proportion of M-LDH, and of increased DNA polymerase activity. In the present study activities of enzymes of the TdR salvage pathway were investigated in cultures...
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of CLL-L to determine if their activities bear a relationship to responsiveness of the cells to PHA.

MATERIALS AND METHODS

Leukemic blood was drawn from patients at the Veterans Administration Hospital, Hines, Ill., through the courtesy of Dr. William Donnelly, Hematology Section Chief, and his staff.

The procedures used in this study were previously described in detail, including: cell collection and separation of lymphocytes on glass-bead columns, cell cultures, cell storage at low temperature, preparation of enzymes, incubation, separation of products with paper chromatography, and counting of radioactivity.

Separated lymphocytes (98-100%) were cultured in 16 x 125 mm. screw-cap tubes in a volume of 7 ml at a concentration of 1 x 10^6 N-L per ml or 2 x 10^6 CLL-L per ml. The medium consisted of 20 per cent autologous plasma, 30 per cent Hanks BSS and 50 per cent Medium 199. PHA-P (Difco) was added at a concentration of 7 µl/ml of medium. N-L cultures were maintained for three days and CLL-L for five days. The cells after culture were washed twice with Hanks BSS and the pellet quick-frozen, and stored at -98°C. For assay the cells were sonicated for five minutes in 0.05 M Tris buffer, pH 7.5. Supernatants, after centrifugation at 10,000 g, for 20 minutes at 4°C, were used as the enzyme preparations. The enzyme reactions were done in total volumes of 30 µl which contained the supernatant from 7-10 x 10^6 cells and in final concentration: 0.1 M Tris buffer, pH 7.5, MgCl₂, 2.7 mM, K₂HPO₄, 1.33 mM, phosphoenolpyruvate (Calbiochem) 2.33 mM, pyruvate kinase (Calbiochem) 1.5 µg. (c-4.5 EU), ATP 2.7 mM (Sigma), mercaptoethanol 0.0015 µl, and either 0.083 mmoles of TdR-H³ (Schwarz, Sp. Act. 6 c./mmole), or 2.66 mmoles of TMP-C¹⁴ (Schwarz, Sp. Act. 7.5 mc./mmole). The concentrations of ATP and Mg (equimoler) used were shown to be optimal for phosphorylation of TdR or TMP by extracts of lymphocytes. Tubes were incubated for one hour at 37.5°C.

The solvents used for chromatography were: (a) isobutyric acid, 1 N NH₄OH and 0.1 M EDTA (50:30:1), and (b) ethyl acetate, H₂O, and formic acid (60:35:5). Ascending chromatography for 16 hours with solvent (a) separated TTP, TDP, DHT, and dU, but gave a single spot for TdR and T. Ascending chromatography for six hours with solvent (b), on the other hand, separated TdR, T, dU and DHT while the other compounds migrated together. All parts of the chromatogram not included in the identifiable spots were counted so that no major unidentified area of radioactivity could be overlooked. Of approximately 200,000 CPM of TdR-H³, or 24,000 CPM of TMP-C¹⁴ added to each reaction tube, almost 100 per cent were recovered.

RESULTS

Illustrated in Figs. 1-3 are typical examples of results of incubations of supernatant extracts of a minimum of three complete runs each, of cultures of N-L, high and low-count CLL-L. N-L (Fig. 1) showed the characteristic strong blast-cell response to the addition of PHA to cultures, low-count CLL-L (Fig. 2) gave a good response, while high-count CLL-L (Fig. 3) failed to respond.

When results using TdR-H³ as the radioactive substrate (Figs. 1A, 2A and 3A) are compared with those found starting with TMP-C¹⁴ (Figs. 1B, 2B and 3B), considerable information is obtained about the relative activities of the various enzymes present. High T production from TdR with extracts of both N-L and CLL-L indicated they had considerable TdR phosphorylase activity. The much lower T production from TMP was evidence of relatively low 5'-phosphatase activity. With TdR-H³ as the initial radioactivity, the sum of the TMP, TDP and TTP produced was a measure of TdR kinase activity, while TDP and TTP production resulted from the activities of TMP and TDP.
Fig. 1.—Results of incubations of reaction mixtures (see text) for one hour at 37°C with supernatant extracts of 7.5 × 10⁶ cells per tube from run of cultured N-L which gave strong blast-cell response to PHA. Radioactivity in reaction mixture (A) TdR-H³ and (B) TMP-C¹⁴.

Fig. 2—As Fig. 1, but cells CLL-L from case with low peripheral WBC (26,000 per mm.³) with good blast-cell response to PHA in culture.

kinases. Both TMP and TDP kinases were present in both N-L and CLL-L in amounts adequate to phosphorylate most of the TMP available.

N-L or CLL-L cultured without PHA showed little or no apparent change in enzyme activity (Figs. 1–3). In the case of cells, on the other hand, which showed morphological development to blast-cells in cultures with PHA, there
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was a distinct alteration in enzyme pattern and products formed. This appeared to be mainly attributable to increased TdR kinase activity. In most runs there was little change evident in the first day of culture, but during the second and third days production of TMP + TDP + TTP from TdR increased substantially with concomitant fall in T production. The degree of response appeared to parallel the extent of blast-cell development. In some runs small but insignificant increases in TdR kinase activity were found during the first day of culture with PHA of N-L, but not with CLL-L. CLL-L from cases with high peripheral WBC counts which did not respond to PHA with blast-cell formation also failed to give evidence of increased TdR kinase activity (Fig. 3A). In the case of N-L (Fig. 1B) or CLL-L (Fig. 2B) which responded to PHA, some increase in phosphatase activity is perhaps indicated by slight decreases in TDP + TTP production from TMP with corresponding increases in T. With CLL-L (Fig. 3B) which failed to respond to PHA, the apparent increase in 5'-phosphatase activity in PHA-cultures was more substantial; the portion of the total counts in TMP + TDP + TTP was 17 per cent less than controls while the T fraction doubled.

DISCUSSION

The end products obtained after incubation of TdR-H\(^3\) or TMP-C\(^{14}\) with cell extracts represent the net balance of the activities of the various enzymes present. In these complex cellular mixed enzyme systems, changes in concentrations of certain small molecules such as ATP\(^{10}\) can affect the enzyme balance and alter the end products formed, perhaps by affecting individual
enzymes in different ways. Similarly, the results obtained here might have been due to the action of products of the complicated sequence of metabolic events called into play by PHA-induced activation. Changes in pool size of various members or precursors of the TdR salvage pathway as a result of PHA activation might easily have eventuated in alteration in the activities of individual enzymes. In any case, starting with TdR-H, a net increase in phosphorylated TdR nucleotides resulted in N-L or CLL-L which responded to PHA in culture. This might result from increased TdR kinase activity or reduced phosphatase activity. Since some increase rather than decrease in dephosphorylation of TMP-C was found in cells cultured with PHA, it appeared that the findings were the result of increased TdR kinase activity.

Changes in TdR kinase activity related to the timing of DNA synthesis have been shown in synchronized HeLa cell cultures. Enzyme activity increased with DNA synthesis and fell with cell division. These findings were interpreted as representing actual increases and decreases in amount of enzyme. An alternative explanation, analogous to one offered by Mazia for DNA polymerase in sea urchin embryos, was that the enzyme was fixed in an inactive complex with a nuclear particulate fraction, becoming active only after release to the supernatant with DNA synthesis. Conflicting evidence was found by Littlefield in studies of cultured mouse fibroblasts, which led to the conclusion that TdR kinase was synthesized continuously in proportion to total protein synthesis, while enzyme preservation depended on stabilization by TdR.

Increased DNA polymerase and TdR kinase activities were shown by Lieberman et al. to occur in primary cultures of rabbit kidney cortex cells about the time of DNA formation. This apparently also holds true for lymphocytes responding to PHA. Cells from cases of high-count CLL which failed to show morphological response to PHA in culture did not show increased activities of TdR kinase or DNA polymerase. The higher levels of 5'-phosphatase activity found in these cells in PHA cultures may have contributed to these results. Increased TdR kinase and DNA polymerase would appear to be essential for increased DNA synthesis in response to PHA rather than a mere accompaniment of increased overall protein synthesis in blast-cell development.

Extracts of both N-L and CLL-L under the experimental conditions converted a large proportion of TdR to T. Gallo et al. showed that with similar enzyme preparations production of TdR from T could be obtained when deoxyribose-1-phosphate was added to the reactions. With response to PHA a fall in T production did occur. This apparently resulted from the removal of TdR by phosphorylation combined with relatively low phosphatase activity.

PHA perhaps acts at allosteric sites on repressors or inducers of operator genes involved in enzyme synthesis. The TdR salvage pathway is affected by a variety of factors which may exert regulatory effects. Of special importance are those which influence the activities of TdR kinase since it appears to be a key enzyme in the salvage pathway. TdR kinase is inhibited by the end products of the pathway TTP and is influenced by the concentrations of ATP and
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Mg\(^{++}\) and of TdR.\(^{30,31}\) PHA may act to alter a balance between these and other factors to produce its effects on cultured lymphocytes.

**Summary**

N-L and CLL-L prior to cell culture gave evidence of high activities of TdR phosphorylase, TMP and TDP kinases, while TdR kinase and phosphatase activities were relatively low. Cells cultured without PHA showed no appreciable alteration in enzyme activities during three to five days of culture. Response to PHA with blast-cell formation in cultures of N-L or CLL-L (low-count cases) was associated with increased TdR kinase activity which became prominent at the time of increased DNA polymerase activity and DNA synthesis during the second to third day of culture. Cells from cases of CLL-L with high WBC counts which failed to respond to PHA showed no increase in TdR kinase activity but did show increased phosphatase activity. Increased TdR activity and increased DNA polymerase activity may both be needed for increased synthesis of DNA to occur in lymphocytes responding to PHA.

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**Abbreviations**

- N-L: normal lymphocytes
- CLL-L: chronic lymphatic leukemic lymphocytes
- PHA: phytohemagglutinin
- LDH: lactic dehydrogenase
- M-LDH: muscle type LDH
- TdR: thymidine
- T: thymine
- TMP, TDP, TTP: thymidine 5'-mono-, di- and tri-phosphate
- dUMP: deoxyuridine 5'-monophosphate
- dU: deoxuridine
- DHT: dihydrothymine

**References**

8. Rabinowitz, Y., McCluskey, I. S., Wong, P., and Willhite, B. A.: DNA polymerase activity of cultured normal and leu-
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