Pyrimidine Metabolism in Man. II. Studies of Leukemic Cells

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The central role of ribonucleic acids and deoxyribonucleic acids in protein synthesis and genetic function, respectively, has led to a continued interest in their metabolism in neoplasia. A number of antineoplastic agents have been developed as structural analogs of purine or pyrimidine precursors of the nucleic acids. These agents have shown their greatest clinical effectiveness in the treatment of leukemia. Only a few studies have been reported on the quantitative aspects of nucleic acid metabolism in leukemic cells. Using suspensions of whole cells Winzler and his colleagues have studied the incorporation of a number of isotopic precursors of purines and pyrimidines into the nucleotide or acid-precipitable fractions of the cells. Observations have been made on the effect of various antineoplastic agents on the uptake and utilization of these precursors by the intact leukemic cell. Recently methods have been developed which allow measurements of certain individual enzymes involved in pyrimidine synthesis to be made on leukocyte sonicates and erythrocyte hemolysates. This report describes the pattern of these enzymatic activities observed in leukemic cells.

Experimental Procedure

The methods developed for the measurement of aspartate carbamyltransferase, dihydroorotase and dihydro-orotic dehydrogenase (fig. 1) have been described in detail. In summary, leukocytes and erythrocytes were separated by dextran sedimentation and washed repeatedly. Aliquots of cell suspensions were counted. Leukocytes were destroyed by sonication in a Raytheon 10 kc. Oscillator at maximal intensity for 2.5 minutes and erythrocytes by freeze-thaw hemolysis. Each measurement of an enzyme activity involved the incorporation of a C14-precursor of high specific activity into the product, which was then isolated and analyzed by the carrier technic. Enzyme assays were developed after determining optimal conditions of time, pH, and substrate concentration (fig. 1).

Aspartate carbamyltransferase.—C14-l-aspartate .015 M; carbamylphosphate .010 M; 30 min. incubation; Tris buffer pH 9.0, 0.20 M.
Dihydro-orotase and dihydro-orotic dehydrogenase.—C14-dihydro-orotate 10–3 M, 120 min. incubation; Tris buffer pH 8.2, 0.25 M.
5-Carboxymethylhydantoinase.—C14-5-carboxymethylhydantoin 10–3 M, 120 min. incubation; Tris buffer pH 8.2, 0.25 M.

Following the addition of nonisotopic carrier and protein precipitation the product
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(carbamyl aspartic acid or orotic acid) was isolated by chromatographic procedures, the specific activity determined and the total yield calculated by the standard carrier formula:

$$\mu M \text{ product formed} = \frac{\mu M \text{ carrier} \times \text{specific activity of the isolate}}{\text{specific activity of the precursor}}$$

For comparative purposes all results are expressed as millimicromoles (m\(\mu\)M) synthesized per 10\(^8\) leukocytes or per 10\(^9\) erythrocytes.

Patients studied.—Normal controls were obtained from laboratory personnel. A brief clinical description of the patients studied is outlined below.

MYELOCYTIC LEUKEMIA. Patient 1. A 42 year old woman with painful splenomegaly was diagnosed as having chronic myelocytic leukemia. Hemoglobin 10.0 Gm. per cent; leukocytes 276,000 per cu.mm. with polymorphonuclears 26 per cent, band forms 46 per cent, metamyelocytes 7 per cent, myelocytes 17 per cent, nucleated RBC 1 per cent, lymphocytes 1 per cent, basophils 2 per cent. There was a good symptomatic and hematologic response to x-ray therapy.

Patient 2. A 69 year old man with chronic myelocytic leukemia of 3 years duration had been treated twice with splenic irradiation and had received 2 courses of Myleran. His last therapy with Myleran had been completed 3 weeks prior to this study: Hemoglobin 8.0 Gm. per cent, leukocytes 13,400 per cu.mm. with polymorphonuclears 53 per cent, band forms 16 per cent, metamyelocytes 3 per cent, promyelocytes 1 per cent, myeloblasts 1 per cent, lymphocytes 17 per cent, basophils 5 per cent, eosinophils 3 per cent, monocytes 1 per cent. He was considered to have chronic myelocytic leukemia in partial remission.

Patient 3. A 69 year old man with known chronic myelocytic leukemia of 2 years' duration had been treated intermittently with Myleran. Within the month prior to entry his leukocyte count had risen rapidly to 361,000 per cu.mm, with polymorphonuclears 24 per cent, myelocytes 39 per cent, promyelocytes 9 per cent, myeloblasts 15 per cent, nucleated RBC's 7 per cent, basophils 3 per cent, lymphocytes 3 per cent. Hemoglobin, 9.4 Gm. per cent. He was considered to have chronic myelocytic leukemia in relapse. Response to splenic irradiation was slow and incomplete.

Patient 4. A 79 year old woman entered the hospital with weakness, fever, mild congestive failure and phlebitis: Hemoglobin, 11.4 Gm. per cent; leukocytes 260,000 per cu.mm with myeloblasts 86 per cent, promyelocytes 5 per cent, myelocytes 4 per cent, polymorphonuclears 3 per cent and lymphocytes 2 per cent. The diagnosis of acute myelocytic leukemia was made. The patient died from a pulmonary embolus within 48 hours.

Patient 5. A 43 year old woman entered the hospital because of weakness and easy bruising of 3 months duration: Hemoglobin, 7.2 Gm. per cent; leukocytes, 43,000 per cu.mm with myeloblasts 20 per cent, promyelocytes 60 per cent, myelocytes 12 per cent, polymorphonuclears 6 per cent, and lymphocytes 2 per cent. The patient was considered to have acute or subacute myelocytic leukemia. There was temporary response to 6-mercaptopurine and later to prednisone, but the patient died 6 months later with acute leukemia and pneumonia.

Di Guglielmo Syndrome. A 12 year old boy presented with weakness, weight loss, ecchymoses and moderate hepatosplenomegaly. Hemoglobin was 7.8 Gm. per cent and nucleated cells, 8700 per cu.mm with 50 to 60 per cent being nucleated erythrocytes. Reticulocyte count was 8.8 per cent. Bone marrow aspiration revealed erythroid hyperplasia with moderate megaloblastosis. Over the next 8 months prior to his death he was treated with vitamin B\(_12\), 6-mercaptopurine, prednisolone and transfusions, with only temporary response. At the time of study he was not receiving treatment: Hemoglobin, 3.0 Gm. per cent, nucleated cells 9800 per cu.mm with nucleated erythrocytes 55 per cent, polymorphonuclears 16 per cent, metamyelocytes 3 per cent, lymphocytes 21 per cent and unidentified blast forms 5 per cent. At autopsy there was extensive infiltration of the spleen, liver, kidneys, lymph nodes and marrow by immature cells of the erythrocytic series. It was felt by all observers that his clinical course, cytologic picture and autopsy findings were consistent with the Di Guglielmo syndrome.

MYELOPROLIFERATIVE DISORDERS. Patient 1. A 68 year old woman was followed in the
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clinic because of polycythemia vera, marked splenomegaly and changes suggestive of early myeloid metaplasia. Leukocyte alkaline phosphatase was increased approximately fivefold. At the time of study: Hemoglobin, 21.4 Gm. per cent, leukocytes, 21,300 per cu.mm with polymorphonuclears 78 per cent, band forms 13 per cent, lymphocytes 5 per cent, monocytes 2 per cent, eosinophils 1 per cent.

Patient 2. A 64 year old man with pulmonary fibrosis, emphysema and hepatosplenomegaly presented with the following hematologic findings: Hematocrit 32 per cent; leukocytes, 140,000 per cu.mm with polymorphonuclears 75 per cent, band forms 12 per cent, monocytes 4 per cent, lymphocytes 4 per cent, monocytes 5 per cent, and nucleated RBC's 5 per cent. Leukocyte alkaline phosphatase was slightly elevated. He was considered to have myeloid metaplasia.

LYMPHOCYTIC LEUKEMIA. Patient 1. A 16 year old girl entered the hospital because of enlargement of lymph nodes and pruritis of several months' duration: Hemoglobin, 11.8 Gm. per cent; leukocytes, 9000 per cu.mm with lymphocytes 80 per cent, lymphoblasts 10 per cent, polymorphonuclears 7 per cent, eosinophils 2 per cent, monocytes 1 per cent. Biopsy of a lymph node revealed malignant lymphoma, lymphoblastic type.

Patient 2. A 70 year old man with psoriasis and rheumatoid spondylitis was known to have lymphocytic leukemia with lymphadenopathy and splenomegaly for 18 months: Hemoglobin, 11.1 Gm. per cent, leukocytes, 141,000 per cu.mm. with small lymphocytes 97 per cent and polymorphonuclears 3 per cent.

INFECTION. Five patients with pneumonia and leukocytosis from 15,000 to 25,000 cu.mm. were studied. All had a “shift to the left” in the polymorphonuclear series.

RESULTS

5-Carboxymethylhydantoinase (fig. 1).—The enzyme 5-carboxymethylhydantoinase, which catalyzes the reversible ring closure of carbamylaspartate to its corresponding hydantoin, has been described only in Zymobacterium oroticum. It was previously found to be absent in normal leukocytes, erythrocytes and rat liver. An attempt was made to determine whether this enzyme were present as a qualitative defect in neoplasia. In repeated studies, no enzymatic activity was obtained in leukemic cells.

Myelocytic leukemia.—Enzymatic studies were carried out on sonicates of the isolated leukocytes of five patients with myelocytic leukemia, whose case summaries have been presented above. The results which were obtained are presented in the bar graphs of figure 2, in comparison with normal values for these enzymatic activities in leukocytes. The patients were arbitrarily placed in the sequence of progressive increase of activity of aspartate carbamyltransferase. This same sequence is maintained in the graphs showing the activities of dihydro-orotase and dihydro-orotic dehydrogenase. In general, activities of the three enzymes varied in parallel. From the case summaries it is apparent that cytologic evidence of immaturity of leukemic cells tends to parallel the increase of enzymatic activity per cell.

Di Guglielmo syndrome.—Studies were carried out on the erythrocytes of a single patient with the Di Guglielmo syndrome. The enzymatic activities found are presented in figure 3. Of particular note is the finding of dihydro-orotic dehydrogenase in the patient’s erythrocytes, an enzyme which is normally absent from mature erythrocytes. Aspartate carbamyltransferase and dihydro-orotase were elevated approximately threefold in comparison to normal erythrocytes.
Fig. 1.—Outline of the enzymatic reactions studied in normal and leukemic cells. CAP, carbamylphosphate; 1-ASP, 1-aspartic acid; CAA, carbamylaspartic acid; 5-CMH, 5-carboxymethylhydantoin; DHO, dihydro-orotic acid; OA, orotic acid.

Myeloproliferative disorders.—A striking and consistent difference has been found in the cellular enzymatic activities of alkaline phosphatase in leukemic cells as opposed to the leukocytes of the myeloproliferative disorders.5 In two patients who were considered to have polycythemia vera and myeloid metaplasia, respectively, on clinical grounds and by leukocyte alkaline phosphatase determinations, studies were carried out in an attempt to find whether other enzymatic differences could be demonstrated between the leukocytes of the myeloproliferative disorders and leukemia. The results are summarized in figure 4. It is apparent that the enzymatic activities per cell are somewhat increased in these disorders, as in leukemia, the difference being one of degree. Further studies were carried out on leukocytes from patients with the leukocytosis of infection to determine whether immaturity of cells per se, "a shift to the left," is associated with an increase of enzymatic activity. The results of five such studies are also presented in figure 4. Here the only significant abnormality was an elevation of dihydro-orotic dehydrogenase to about twice the normal control value.

Lymphocytic leukemia.—The method utilized for isolation of leukocytes tends to concentrate polymorphonuclear leukocytes at the expense of lymphocytes. The normal control values for enzyme activities in leukocytes are therefore valid only for myelocytic leukemia. For comparative purposes, however, observations were made on lymphocytes from two patients with lymphocytic leukemia. The findings are summarized in figure 5. It is apparent that in comparison with normal polymorphonuclear leukocytes the lymphocytic leukemic cell has similar activities of aspartate carbamyltransferase and dihydro-orotase, but marked increase of dihydro-orotic dehydrogenase (fourfold to sevenfold).
Fig. 2.—Comparison of the enzymatic activities of aspartate carbamyltransferase, dihydro-orotase and dihydro-orotic dehydrogenase in the leukocytes of five patients with myelocytic leukemia with levels of activity found in normal polymorphonuclear leukocytes (mean ± S.D. of 18 determinations).

Discussion

The leukocyte offers many advantages in the study of metabolic and neoplastic diseases. It is the only nucleated cell which can readily be obtained in man. Easily isolated as a viable, free floating cell, the leukocyte can be studied intact in vitro without the damage accompanying cell preparations.
from other tissues. Following destruction enzymatic activities can be determined and conveniently expressed per unit number of cells. Finally, normal leukocytes may be metabolically compared with the abnormal cells of a number of disease states. There are accompanying limitations in the small amount of "tissue" obtained and in the heterogeneity of the cell population studied—heterogeneity of cell type and of cell age. The study reported here is largely concerned with the myelocyte. The influence of immaturity of blood cells on certain enzymatic activities was the major variable investigated. Such studies on the biochemistry of the leukemic cell were begun almost fifty years ago.

The enzyme 5-carboxymethylhydantoinase was found to be absent from leukemic cells. This enzyme, which cyclizes 1-carbamylaspartic acid to its corresponding hydantoin, has been demonstrated only in *Zymobacterium oroticum*, an organism isolated by enrichment culture with orotic acid. It is absent from many other microorganisms, yeast and the ascites cell tumor. Its function in pyrimidine synthesis in *Zymobacterium oroticum* has remained obscure.

In the myelocytic leukemic cell the activities of aspartate carbamyltransferase, dihydro-orotase and dihydro-orotic dehydrogenase were increased several fold above the normal values for the mature polymorphonuclear leukocyte. The increased enzymatic activity of dihydro-orotic dehydrogenase was particularly marked, being as much as 14 times elevated in acute leukemia. In general, increased enzymatic activity tended to parallel cytotologic evidence of immaturity of cells, as might have been anticipated. It is to be noted that a general nonspecific increase of enzymatic activities is not found in neoplastic cells. Leukemic cells have reduced activities of a number of enzymes in the glycolytic pathway. Reduction of alkaline phosphatase content of the leu-
Fig. 4.—Enzymatic activities in the leukocytes of two patients with myeloproliferative disorders compared with five patients with the leukocytosis of infection (pneumonia) and 18 normal controls. Abbreviations are the same as in figure 3.

kemic cell is of sufficient consistency to be of diagnostic importance. The significance of the striking increase of these enzymes involved in pyrimidine synthesis is not apparent from these studies. Using suspensions of whole cells, Winzler has demonstrated that the leukemic cell incorporates a number of isotopic precursors of nucleic acids into the acid-precipitable fraction at rates far in excess of those found with normal leukocytes. The current studies demonstrate more specifically changes in some of the individual enzymes leading to this over-all increase of nucleic acid synthesis.

The mechanism which leads to increase of some enzymatic levels in the neoplastic cell is, of course, unknown. This is part of the larger problem of appropriate control of reaction rates within the cell. One such mechanism for control of enzyme activity is that of the negative feedback reaction, in which the product of a series of reactions inhibits the synthesis of the proximal enzymes involved in its own formation. This has been clearly demonstrated for pyrimidine biosynthesis in the classic studies of Yates and Pardee on E. coli mutants. This has added significance for the current studies in that it has been postulated that some types of neoplasia, including leukemia, represent the metabolic results of acquired enzyme defects. This is carcinogenesis by enzyme deletion. Dameshek has discussed this theory in relationship to the Di Guglielmo syndrome. If such a hypothetical block occurred somewhere in the pyrimidine sequence, it would result in "pyrimidine starvation," with a compensatory increase in formation of the proximal enzymes. In the leukemic cell the greatest increase of enzymatic activity was usually found in dihydroorotic dehydrogenase. In E. coli with "pyrimidine starvation," aspartate carbamyltransferase demonstrated the greatest rise of activity. Re-
Fig. 5.—Enzymatic activities in the lymphocytes of two patients with lymphocytic leukemia. Although of different cell type, these values are compared with the average of five studies on myelocytic leukemia and 18 normal controls.

cient studies in pernicious anemia, in which there is probably a block in the later stages of pyrimidine synthesis secondary to $B_{12}$ deficiency, have demonstrated a pattern which is consistent with that found in $E. coli$ with genetic blocks in pyrimidine synthesis.\(^15\) This evidence from the pattern of increased enzymatic activities found, indirect and incomplete as it is, would suggest that release from negative feedback control is not the \textit{modus operandi} of enzymatic changes in the leukemic cell. The results are far from conclusive in this regard.

In the mature erythrocyte there is a block in pyrimidine synthesis in the absence of dihydro-orotic dehydrogenase. The mature erythrocyte is unique in that it does not contain ribonucleic acid and therefore is unable to carry out protein synthesis. Dihydro-orotic dehydrogenase was previously found to be present in the nuclei of leukocytes and nucleated avian erythrocytes.\(^8\) It is therefore of interest that in the Di Guglielmo syndrome with the appearance of nucleated erythrocytes in the peripheral blood this enzyme is found as a qualitative difference between the normal and abnormal erythrocyte. Presumably this enzyme is lost with the nucleus in the maturation sequence unique in the erythrocyte.

A number of antineoplastic agents have been developed as structural analogs of nucleic acid precursors. Winzler has demonstrated the feasibility of studying the action of some of these agents on the uptake of nucleic acid precursors by leukemic cells in vitro. Assay methods such as those described here allow direct testing of such agents on individual enzyme systems in leukemic cells.

The studies reported here measure the enzyme activities (presumably a direct function of enzyme content) obtained from fragmented cells. It is
understood that these technics of necessity negate many factors which control rates of reaction in the uninjured cell. Such a study should be supplemented by data concerning the steady state concentration of individual pyrimidine precursors within the intact cell.

Summary

1. The activities of three enzymes involved in pyrimidine synthesis— aspartate carbamyltransferase, dihydro-orotase and dihydro-orotic dehydrogenase—were studied in sonicates of circulating leukocytes from 5 patients with myelocytic leukemia, 2 with lymphocytic leukemia, 2 with myeloproliferative disorders and 5 with infection. The erythrocytes from one patient with the Di Guglielmo syndrome were studied.

2. Neoplastic cells showed increased activities of all three enzymes tending to parallel the cytologic evidence of immaturity. The increase of dihydro-orotic dehydrogenase was the most striking abnormality. Leukocytes from patients with infection or with myeloproliferative disorders showed similar but much less marked alterations in the enzyme pattern.

3. Dihydro-orotic dehydrogenase, absent from mature erythrocytes, was present in the nucleated erythrocytes in the Di Guglielmo syndrome.

4. The enzyme 5-carboxymethylhydantoinase, previously found in some bacteria, was absent from normal and abnormal hemic cells.

Summario in Interlingua

1. Le activitate de tres enzymas interessate in le synthese de pyrimidina—carbamyltransferase de aspartato, dihydro-orotase, e dishydrogenase dihydro-orotic—esseva studiate in sonicatos de circulante leucocytos ab 5 patientes con leucemia myelocytic, 2 con leucemia lymphocytic, 2 con disordines myeloproliferative, e 5 con infection. Le erythrocytos ab un patiente con le syndrome de Di Guglielmo esseva studiate.

2. Cellulas neoplastic exhibiva augmentos del activitate de omne le tres enzymas, con un tendentia de parallelismo con le evidentia cytologic de immaturitate. Le augmento de dishydrogenase dihydro-orotic esseva le plus frappante anormalitate. Leucocytos ab patientes con infection o disordine myeloproliferative exhibiva simile sed minus marcate alterationes in le patrono enzymatic.

3. Dishydrogenase dihydro-orotic, que es absente in erythrocytos matur, esseva presente in le erythrocytos nucleate del syndrome de Di Guglielmo.

4. Le enzyme 5-carboxymethylhydantoinase, previemente incontrate in certe bacterios, esseva absente ab cellulas hemic normal e anormal.

REFERENCES


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