DNA Polymerase and Carbohydrate Metabolizing Enzyme Content of Normal and Leukemic Glass Column Separated Leukocytes

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STUDIES OF THE enzyme content of leukocytes reported in the past\textsuperscript{1-9} have been performed on mixed cell suspensions. Some attempts to estimate the enzyme values for individual cell types have been made\textsuperscript{2, 3, 8} on the basis of complex calculations after multiple assays of mixed cell suspensions with varied cell differential counts.

The technique of separation of leukocytes on glass bead columns\textsuperscript{11, 12} now permits a more direct approach to this problem. The present study reports the results of assays of DNA polymerase and of five representative carbohydrate metabolizing enzymes (glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, lactic dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase and isocitric dehydrogenase) performed on normal and leukemic leukocytes which were separated on glass columns.

MATERIALS AND METHODS

The Blood: Normal blood was obtained from medical students and laboratory technicians. Leukemic blood was drawn from patients at the Veterans Administration Hospital, Hines, Illinois, through the courtesy of Dr. William Donnelly, Hematology Section Chief, and his staff. Slides and records of each case were reviewed and only those with clear-cut diagnoses were used. Criteria for the diagnosis of lymphosarcoma cell leukemia were similar to those recently described by Schwartz et al.\textsuperscript{10}

Leukocyte Collection and Separation: The method used for separation on glass bead columns of heparinized, dextran-sedimented normal and leukemic leukocytes was previously described in detail.\textsuperscript{11, 12} Viable normal lymphocytes and granulocytes were separated almost free of other leukocytes. Monocyte collections were contaminated with granulocytes but were concentrated greatly. In nonleukemic lymphosarcoma, lymphosarcoma leukemia and chronic lymphocytic leukemia separations of lymphocytes and granulocytes, as in the normal, approached 100 per cent.\textsuperscript{12} In the case of granulocytic leukemia (8 cases), because of the more complex cell differential, separations were less perfect, but marked concentration of one cell type or another was usually obtained. Of particular value in this regard were two patients whose disease was followed through various stages ranging from remission to blast crisis. Results of the separation of the normal and leukemic cells used in the present study were detailed in the previous reports.\textsuperscript{11, 12}
DNA POLYMERASE AND CARBOHYDRATE METABOLIZING ENZYMES

Cell Storage: Cells after separation were washed twice with normal saline, quick-frozen with dry ice and alcohol, and stored at -98 C. in a Revco freezer. Cells assayed for their enzyme content at intervals up to a year after freezing showed a loss of activity of less than 10 per cent, for each of the enzymes studied. All assays were done on such frozen cells.

Enzyme Preparation: Assays of the carbohydrate metabolizing enzymes were performed with a homogenate prepared by sonication for 5 minutes in a Ratheon S102A sonicator. The cells were suspended in a volume made up to 100 µl. by the addition of 0.05 M Tris buffer (pH 7.4) in a 3 or 5 ml. cellulose nitrate tube. This was stoppered or closed with parafilm and placed into the sonicator cup which contained about 5 ml. of water.

DNA polymerase was assayed after pelleting the sonicate at 6000 g for 30 minutes in an International HRI centrifuge. The supernates contained about 90 per cent of the enzyme activity. Isocitric dehydrogenase activity of the high speed supernate was demonstrated to be similar to that of the whole homogenate. In several instances, in order to conserve material, isocitric dehydrogenase was assayed on the supernate.

DNA Polymerase Assay: DNA polymerase was assayed by a procedure modified from the Littlefield et al.13 microadaptation of the methods of Bollum and Potter14 and Lehman et al.15 Each reaction tube contained in a total volume of 25 µl.: 1.25 µmoles Tris buffer (pH 7.4); 0.05 µmoles MgCl₂; 0.05 µmoles K₂HPO₄; 0.025 µmoles ATP; 0.05 µg. pyruvate kinase; 0.01 µmoles phosphoenolpyruvate; 10 nmoles each of dCTP, dGTP and TTP; 2 nmoles ATP-H3 (Schwarz, Sp. Act. 1.25 C/mnole); and enzyme from 2 x 10⁶ cells. After incubation at 38 C. for 60 minutes the reaction was terminated by the addition of cold perchloric acid to 0.5 N; 400 µg. of cold DNA was added as carrier. The precipitate was washed twice by dissolving in 1 N NaOH and reprecipitating with perchloric acid. The final precipitate was dissolved in 1 ml. of Hydroxide of Hyamine (Packard) and 19 ml. of scintillation mixture (toluene 1000 ml., POPP 50 mg., PPO 2 g.) was added for counting in a liquid scintillation counter.

Carbohydrate Metabolizing Enzyme Assays: The five enzymes studied were assayed by the microfluorometric methods of O. H. Lowry and associates.16-18 The detailed procedures were followed with minor changes. In the isocitric dehydrogenase assays 4 x 10⁵ cells were used per reaction tube, while 1-2 x 10⁶ cells were used for the other enzymes. Incubation was at 38 C. for 5 minutes for all 5 enzymes. These conditions were in a range to give results which were demonstrated to be proportional to enzyme concentration and to time of incubation.

Red Blood Cell Contamination: Contamination of initial suspensions from bloods with normal leukocyte counts was usually 1-2 RBC/WBC, and was much lower with high-count leukemic bloods. Contamination of “lymphocyte” tubes was somewhat higher than initial suspensions, while the “poly” tubes were almost completely free of RBC. Since the enzyme content of RBC was only 1/20 or less than that of the leukocytes, their contribution to the leukocyte assays could be ignored.4 The method of Fallon et al.19 for removing RBC by hemolysis was tested but gave erratic results. In some experiments there was a moderate to great loss of enzyme activity after a 30-second hemolysis, while in others there was none. The method was therefore only used in a few instances where RBC contamination was excessive. This was apt to occur when the initial leukocyte count was very low.

Calculation of Results: Results for most of the individual cell types were obtained directly, although a few, particularly the intermediate members of the granulocytic series, involved some simple calculation. The latter consisted of the substitution of a directly assayed value for cell x into an equation where x + y had also been assayed, permitting the simple calculation of cell y.
RESULTS

Results of the enzyme assays of normal and lymphoproliferative disease leukocytes are given in Table 1, while Figures 1–6 show the results in granulocytic leukemia.

With all the enzymes studied, results for mixed cell suspensions were the sum of the enzyme activity of the component cells. This was demonstrated experimentally with assays of recombined column separated cells. The results shown in Table 1 reflect the fact, which was consistently evident throughout this work, that increased numbers of high enzyme content cells so raised the values of mixed cell suspensions that changes occurring in low enzyme cells might be obscured (cf. isocitric dehydrogenase of lymphocytes).

DNA Polymerase

DNA polymerase activity (table 1) in mature PMN leukocytes was almost absent, while in lymphocytes it was relatively high. Lymphocytes and monocytes contributed most of the enzyme activity found in initial suspensions. In the lymphoproliferative diseases DNA polymerase levels were moderately elevated above the normal average due to increased lymphocyte enzyme content. In granulocytic leukemia (fig. 1) DNA polymerase activity was very high in the immature forms, but fell very sharply with cell maturity until values approached zero.

Carbohydrate Metabolizing Enzymes

Glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, lactic dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase gave assays which paralleled each other fairly closely, although varying in absolute values. Isocitric dehydrogenase, on the other hand, showed a different enzyme pattern.

Glucose-6-Phosphate Dehydrogenase: Assays of normal PMN leukocytes (table 1) were about 4 times normal lymphocytes. The number of granulocytes in the initial suspension had a pronounced influence on the enzyme level. This was reflected in the markedly lowered values of initial suspensions seen in the lymphoproliferative diseases in association with reduced PMN leukocyte numbers. It was only when the PMN leukocytes were removed that the real differences between normal and abnormal lymphocytes were demonstrated. Isolated PMN leukocytes from the leukemic cases had about the same enzyme levels as the normal. Lymphocytes from lymphosarcoma leukemia and chronic lymphatic leukemia, on the other hand, had an enzyme content which was significantly lower than that of normal lymphocytes (p<0.01).

Figure 2 shows values for glucose-6-phosphate dehydrogenase obtained for the various cell types in granulocytic leukemia. Myeloblasts had the lowest assays, while enzyme content increased with cell maturity to reach a peak in the adult cells.

Assays of 6-phosphogluconate dehydrogenase gave results (table 1) which paralleled those with glucose-6-phosphate dehydrogenase, although absolute values were about one-third of the latter. Mature PMN leukocyte enzyme
content was about 4 times that of normal lymphocytes. Leukemic lymphocytes again had reduced enzyme content, while PMN leukocytes were normal. Assays (fig. 3) of 6-phosphogluconate dehydrogenase in granulocytic leukemia also showed a pattern similar to that of glucose-6-phosphate dehydrogenase; enzyme content increased with cell maturity.

Results with lactic dehydrogenase (table 1) also paralleled glucose-6-
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<th>Diagnosis</th>
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*Mean μmoles converted per 10^10 cells per minute ± standard error of the mean, except DNA polymerase is μmoles.
phosphate dehydrogenase. Lactic dehydrogenase gave absolute values about four-sevenths of the latter. Although myeloblast values (fig. 4) were fairly high, enzyme content, nevertheless, almost doubled with cell maturity. Lactic dehydrogenase isoenzymes were not assayed (see Discussion).

Results of glyceraldehyde-3-phosphate dehydrogenase assays (table 1) also roughly paralleled those with glucose-6-phosphate dehydrogenase, although lymphocyte values were higher relative to PMN leukocytes. As with the preceding enzymes, normal and leukemic PMN leukocytes gave similar assays, while leukemic lymphocytes showed significant reduction in enzyme content. In granulocytic leukemia (fig. 5) myeloblasts again had the lowest values, while enzyme content increased with cell maturity to reach a peak in adult PMN leukocytes.

Isocitric dehydrogenase assays did not parallel the results with the other carbohydrate metabolizing enzymes studied. Table 1 shows that absolute values were in a much lower range—one-tenth to one-twentieth of the values for glucose-6-phosphate dehydrogenase. As with the latter, PMN leukocyte values were several times those of lymphocytes. Values obtained for lymphatic leukemic lymphocytes, however, were not below the normal, but were even slightly elevated. Figure 6 shows that a different pattern of enzyme activity was obtained during granulocyte maturation than with the other enzymes (cf. figs. 2-5). Myeloblasts gave fairly high assays. Values reached a peak in myelocytes and metamyelocytes but fell sharply in adult PMN leukocytes.

**DISCUSSION**

This study has demonstrated the feasibility of assaying the enzyme content of individual white cell types from normal and leukemic blood after separation
on glass bead columns. Such cells could be stored at low temperatures (-98 C.) for long periods prior to assay. The use of microassay methods, either fluorometric or radioisotope tracer, usually permitted assay of all the enzymes on cells from the same blood sample.

Most of the results reported were assayed directly, except for intermediate
members of the granulocytic series and monocytes. In the latter, some simple calculation was required. Antonioli pointed out that application of algebraic calculation in determining values for individual cell types requires that the activity of each be constant and that the activity of the mixture be a function only of the number of cells of each type present. Results obtained experimentally in the present study indicated that this assumption was apparently valid, at least with respect to the enzymes studied. This would also tend to lend some supports to reports based primarily on such calculations. The estimates of Ghiotto et al. for glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and isocitric dehydrogenase were generally in keeping with the assays determined more directly in the present report.

Any evaluation of changes in enzyme content reported in assays of initial mixed cell suspensions must of necessity take into account two factors: (1) changes in the differential cell count and (2) changes in enzyme content of the individual cell types. Failure to consider both factors may lead to a distorted or even erroneous impression of the true changes occurring in the leukocyte enzyme content in a given blood. Assays of initial suspensions in lymphosarcome cell leukemia and chronic lymphatic leukemia gave results with glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, lactic dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase which strongly reflected the diminished numbers of high enzyme PMN leukocytes, but exaggerated the actual low values of the leukemic lymphocytes. It was only when the granulocytes were removed that the true lowering of the leukemic lymphocyte enzyme content could be detected.
In the case of isocitric dehydrogenase the fallibility of reliance on initial mixed cell suspension assays was demonstrated. In this case fall of initial suspension values in lymphatic leukemia resulted entirely from reduced numbers of relatively high enzyme PMN leukocytes and served to hide the fact that the lymphocyte values were actually somewhat higher than normal.

Mature normal and leukemic PMN leukocytes gave similar assays with each of the enzymes studied. Enzyme levels in the granulocytic series, at least, were clearly related to cell maturity. Mature PMN leukocytes contained a fairly constant complement of each enzyme, while the enzyme content of the immature cells varied. In the lymphatic leukemias altered lymphocyte enzyme content could not always be correlated with changes in cell maturity or with variation in morphologically definable cell types. Lymphocytes, of course, are not as readily divided into developmental cell types as are granulocytes.

Changes in enzyme content in mixed cell suspensions after therapy, regardless of its nature, were found chiefly to be related to altered differential cell counts. Changes in enzyme content of individual cell types were not detected. In general, assays of carbohydrate enzymes of initial suspensions tended to rise during remission because of increased numbers of mature high enzyme PMN leukocytes. In the case of DNA polymerase, on the other hand, remission resulted in lowering of initial suspension enzyme content due to rise in numbers of mature enzyme-poor cells.

Isocitric dehydrogenase content was much lower overall than that of any of the other carbohydrate enzymes studied. Low values for isocitric dehydrogenase, perhaps, bear some relationship to the high aerobic glycolysis attributed to leukocytes. Studies are in progress of the aerobic glycolysis of the myelocytes to determine if their relatively low glycolytic enzyme assays, but high isocitric dehydrogenase levels, are associated with a lower aerobic glycolysis than that found in adult PMN leukocytes.

Levels of DNA polymerase appeared to parallel the capacity of a given cell type to divide. It has been demonstrated that members of the granulocytic series as mature as metamyelocytes are capable of division. Highest DNA polymerase values were found in myeloblasts, while enzyme content fell in the intermediate cells until practically none was present in the nondividing PMN leukocytes.

The moderate elevation of DNA polymerase levels found in the lymphocytes in the lymphatic leukemias is noteworthy. Most of these cases (80-90 per cent), as has been noted by others, fail to give a positive phytohemagglutinin reaction. Normal lymphocytes, on the other hand, despite their somewhat lower DNA polymerase content, regularly gave a positive reaction. This inability to produce Nowell’s blast-like mitotic cell is thus apparently not the result of a lack of DNA polymerase. The elevated DNA polymerase levels in the leukemic lymphocyte, however, may be due to small numbers of very high enzyme-containing immature cells which are overlooked on the stained smears. Some lymphoblasts are usually found in slide chambers with inverted phase microscopy. These cells, if their enzyme content were very high, could obscure an actual lowered enzyme content in the bulk of the leukemic lymphocytes.
In the present study isoenzymes of lactic dehydrogenase\textsuperscript{27} were not assayed. The findings of Starkweather et al.\textsuperscript{28} indicated that extensive changes in content of the isoenzymes of lactic dehydrogenase occurred with alterations in leukocyte and erythrocyte maturity in leukemia. It would, therefore, be of interest to extend these findings with assays of separated cells. Total lactic dehydrogenase content may fluctuate without reflecting corresponding increases or decreases in the isoenzymes, but rather may represent a mean of alterations in individual isoenzyme content.\textsuperscript{29}

**Summary**

1. Enzymes of normal and leukemic glass column separated leukocytes were assayed with fluorometric (glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, lactic dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase and isocitric dehydrogenase) and radioisotope (DNA polymerase) micromethods.

2. Most results were obtained by direct assay of the specific cell type. Some required simple calculation. It was demonstrated that the enzyme activity of mixed cell suspensions was the sum of the enzyme content of the component cells, at least for the enzymes studied.

3. Assays of mixed cell suspensions may give an erroneous picture. Thus changes in differential cell counts, as well as alterations in enzyme content of individual cells, must be taken into account for correct interpretation.

4. Changes in enzyme content of mixed cell suspensions after therapy were due chiefly to alterations in the differential cell count rather than to changes in the enzyme content of individual cell types.

5. The patterns of assays of the carbohydrate metabolizing enzymes studied, except isocitric dehydrogenase, paralleled each other. Highest values were found in mature PMN leukocytes and lowest in blasts. With isocitric dehydrogenase absolute values were much lower, while the highest assays were found in myelocytes.

6. DNA polymerase content correlated well with the ability of a cell to divide. It was highest in blasts and lowest in mature PMN leukocytes.

**Summario in Interlingua**

1. Le contento de enzymas in leucocytos normal e leucemic separate per un methodo a columna de vitro esseva studiate con micromethodos fluorometric e radioisotopic. Fluorometria esseva usate pro dehydrogenase de glucosa-6-phosphato, dehydrogenase de 6-phosphogluconato, dehydrogenase lactic, dehydrogenase de glyceraldehyda-3-phosphato, e dehydrogenase isocitric. Polymerase de acido desoxyribonucleic esseva studiate con le uso de radioisotopos.

2. Le majoritate del resultatos esseva obtenite per un essayage directe del specific typo cellular. In certe casos, un simple calculation esseva requirite. Esseva demonstrate que le activitate enzymatic in mixte suspensiones cellular esseva le summa del activites enzymatic del cellulases componente, al minus pro le enzymas includite in le studio.

3. Le essayage de mixte suspensiones cellular pote resulitar in un impression erronee. Assi, alterationes in le numerationes differential de cellulas e etiam...
alterationes in le contento enzymatic de cellulas individual debe esser prendite in consideration pro un interpretation correcte.

4. Le alterationes notate posttherapeuticamente in le contento enzymatic de mixte suspensiones cellular esseva causate primarimente per alterationes in le numerations differential de cellulas e non per alterationes in le contento enzymatic de typos individual de cellulas.

5. Le configurationes observate in le studios del enzymas metabolisante hydrato de carbon, con le exception de dehydrogenase isocitric, esseva mutualmente in parallela. Le valores le plus alte esseva trovate in matur leucocytos polymorphonuclear; le valores le plus basse, in blastocytos. Le valores absolute pro dehydrogenase isocitric esseva multo plus basse. Le nivellos le plus alte esseva trovate in myelocytos.

6. Le contento de polymerase de acido deoxyribonucleic se monstrava ben correlationate con le capacitate del cellula de divider se. Iste contento esseva le plus alte in blastocytos e le plus basse in matur leucocytos polymorphonuclear.

REFERENCES


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