Acid-soluble Nucleotides in Leukemic Cells[1]

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SUMMARY

Acid-soluble nucleotides were separated and characterized from various leukocyte populations: normal leukocytes; acute myeloblastic and chronic myelocytic leukemia; acute lymphoblastic and chronic lymphocytic leukemia, and polycythemia rubra vera. The following nucleotides were identified: DPN, TPN, CMP, CDP, CTP, UMP, UDP, UTP, GMP, GDP, GTP, AMP, ADP, ATP.[3] Increased amounts of UDPG and UDPAG were found in acute myeloblastic and chronic myelocytic leukemic cells.

Acid-soluble nucleotides are present in human normal and leukemic leukocytes and erythrocytes. Bishop et al. (3), Willoughby and Waisman (21) and Ondarza (13) analyzed normal and leukemic whole blood. The following compounds were found: DPN, uric acid, AMP, TPN, ADP, ATP, UDPAG, UDPG, GTP, and UTP. Karon et al. (6) analyzed acid-soluble components in ribonucleic acid (RNA) from human leukemic cells and found no unusual components. Human erythrocytes were analyzed by Bartlett and Shafer (2, 17), Corsini et al. (4), Morell et al. (11), and Öckerman (12). The following compounds were found: DPN, uric acid, AMP, ADP, ATP, TPN, IMP, UTP, GTP, CMP, glycogen, glucose, hexose monophosphates and diphosphates, inorganic phosphate, 2,3-diphosphoglycerate, and unidentified nucleotides. Mizuno et al. (10) found the following substances in bovine thrombocytes: cytidine diphosphoethanolamine, CMP, DPN, AMP, ADP, GDP, ATP, GTP and UTP.

Analysis of acid-soluble nucleotides in a relatively pure population of normal or leukemic human leukocytes has not been made. Therefore, it was the purpose of this investigation to identify and quantitate the major acid-soluble nucleotides in normal and leukemic human leukocytes.

[3] The abbreviations used are as follows: DPN and TPN, di- and tri-phosphopyridine nucleotide; AMP, ADP and ATP, adenosine-5'-mono-, di-, and tri-phosphate; UDPG, uridine diphosphoglucose; UDPAG, uridine diphosphoacetylglucosamine; UMP, UDP, and UTP, uridine-5', mono-, di-, and tri-phosphate; CMP, CDP, and CTP, cytidine-5'-mono-, di-, and tri-phosphate; GMP, GDP, and GTP, guanosine-5'-mono-, di-, and tri-phosphate; IMP, inosine-5'-monophosphate; TEAB, triethylammonium bicarbonate; CML, chronic myelocytic leukemia; CLL, chronic lymphocytic leukemia; AML acute myeloblastic leukemia; ALL, acute lymphoblastic leukemia; DEAE-cellulose, diethylaminoethyl-cellulose.

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MATERIALS AND METHODS

Fractionation of whole blood and preparation of acid-soluble extract.—Approximately 30 ml of venous blood from leukemic patients were drawn into a chilled 50 ml centrifuge tube (containing crystalline sodium heparin as the anti-coagulant and sodium fluoride to a final concentration of 0.01 M to inhibit phosphatase), which was kept submerged in an ice-slush. All subsequent procedures were carried out at 4°C. The blood was layered onto 15 ml of serum bovine albumin of exacting specifications (specific gravity 1.079, pH 7.4, 280–285 mOs., adjusted to 4°C) in a 50 ml conical centrifuge tube. Advantage was taken of the specific gravity difference between leukocytes and erythrocytes; albumin density centrifugation for 30 min at 2900 rpm in a PR-1 International refrigerated centrifuge separated the leukocytes into a thin layer between the plasma and albumin layers (1). Small lymphocytes tended to settle on top of the erythrocyte layer. In this situation the erythrocytes were lysed as described below. The leukocyte layer was removed with a small curved tip pipet and washed several times in normal saline, by centrifugation at 1000 × g for 5 min, to remove platelets.

Large quantities of blood were available from patients undergoing phlebotomy for treatment of hemochromatosis, or, in one case, erythemia secondary to Eisenmenger's syndrome; these leukocytes will be referred to as normal. The leukocytes were separated by the method of erythrocyte sedimentation utilizing a polyvinylpyrrolidone (PVP) solution.[4] The erythrocyte contamination was lysed without apparent damage to leukocytes by hypotonic lysis; this consisted of suspending in a 0.3% NaCl solution, buffered at pH 5.0 with citrate, for 10 min and adjusting the tonicity and pH to normal prior to centrifuging (James G. Hirsch, Rockefeller Institute, personal communication).

Cold perchloric acid (6%) was added, volume for volume, to the leukocytes, and the mixture was thoroughly homogenized and centrifuged. The supernatant was

[4] PVP, 25 gm, and Na citrate (5½ H₂O), 15.1 gm, in 500 ml of distilled water. One part PVP to 4 parts blood.

decanted and the residue re-extracted twice with 3% cold perchloric acid, 0.5 volume to 1 volume of residue. The combined supernatant solution was neutralized with concentrated KOH to approximately pH 7.0. The resulting potassium perchlorate was centrifuged; the supernatant was removed and lyophilized to a small volume and stored at $-20°C$ until used.

Preparation of columns.—Columns of Dowex 1-X8, 200–400 mesh (chloride form) were prepared as described by Hulbert *et al.* (5). Columns measuring 3 mm i.d. and 60 cm in length (Microchemical Specialties Company, Berkeley, Calif.) were packed under nitrogen pressure (10–15 psi) to about 40 cm above the fritted disc and continuously washed with distilled water until ready for use.

Elution and analysis.—Two parallel-sided cylinders measuring 4.6 cm and 8.0 cm in diameter were used to obtain a concave gradient (14). Later experiments were performed with a 9-chambered Varigrad (Buchler Instruments, Fort Lee, N. J.), which allowed for better elution control. Seven chambers were utilized containing 50 ml each from chamber 1 through 7: (*a*) water; (*b*) water; (*c*) 1.0 M TEAB; (*d*) water; (*e*), (*f*), and (*g*) 2.0 M TEAB (15). Well washed Tygon tubing was used (i.d., $\frac{1}{32}$ inch; o.d., $\frac{5}{32}$ inch; wall, $\frac{1}{16}$ inch). Flow rate was carefully controlled at 4.0 ml/hr with a Harvard model 600–1200 peristaltic pump (Harvard Apparatus Company, Inc., Dover, Mass.). The concave gradient gradually increased to 1.0 M, after which the column was washed with an approximately 2.0 M solution of TEAB to elute completely ATP and GTP. The Varigrad, when utilized, gradually increased to approximately 2.0 M TEAB. Approximately 150 to 160 3.0-ml fractions were collected in a Gilson model D 15² fraction collector with an N.I.L. drop counter (Gilson Medical Electronics, Middleton, Wis.). The Varigrad required approximately 100 3.0-ml fractions. A single run required approximately 120 hr. The entire system was refrigerated at 4°C. The absorption at 260 mμ and 280 mμ of each fraction was determined in a Beckman DU spectrophotometer using a 1.0-cm light path quartz cell. The 280/260 ratio tentatively identified the compound. The material from each peak was lyophilized and dissolved in small amounts of pH 7.0 distilled water; aliquots were taken for analyses. Each peak was chromatographed on Whatman No. 1 paper for ascending chromatography in the following solvents: (*a*) isobutyric acid/concentrated ammonium hydroxide/Versene; (*b*) ethanol/ammonium acetate; (*c*) ammonium sulphate/sodium acetate/isopropanol (18). UDPG and UDPAG were identified in the following solvents: (*a*) 7.5 volumes of 95% ethanol plus 3 volumes of 1 M ammonium acetate (pH 7.5); (*b*) same as (*a*) but with 1 M ammonium acetate buffer, pH 3.8. Later chromatographic analyses were performed on DEAE-cellulose thin-layer chromatography (16). Another aliquot was dissolved in 0.1 N HCl and 0.1 N NaOH. These aliquots were analyzed in a Beckman DK-2A recording spectrophotometer or a Beckman DB recording spectrophotometer for their characteristic spectra and for quantitation purposes. In those peaks which contained more than one compound, each was eluted after paper chromatography or thin-layer chromatography and characterized, when sufficient material was available.

ATP and GTP were quantitated by the method of Loring (7). Reference compounds were obtained from Sigma Chemical Company, St. Louis, Mo., and California Corporation for Biochemical Research, Los Angeles, Calif.

RESULTS

The following nucleotides were present in normal and leukemic human leukocytes: DPN, TPN, CMP, CDP, CTP, UMP, UDP, UTP, GMP, GDP, GTP, AMP, ADP, ATP, UDPAG, and UDPG. Typical chromatographic profiles from the various types of leukocytes are presented in Chart 1. The initial breakthrough is not included. Quantities shown on the ordinate represent only one typical sample of a particular leukocyte population; this varied with the total quantity of cells analyzed.

The criteria for identification of the nucleotides are shown in Table 1. Table 2 summarizes the quantitative data. UDPG and UDPAG were detected only in acute and chronic leukemic leukocytes. The initial breakthrough probably contained purine and pyrimidine bases and nucleosides, but adequate identification could not be made on any of the compounds.

DISCUSSION

There appear to be at least 16 nucleotides in human leukocytes, with UDPG and UDPAG being present in somewhat greater quantities in leukocytes from patients with AML and CML. There appears to be no good correlation between UDPG and UDPAG content with cellular maturity. The minor nucleotides, such as CDP, CTP,
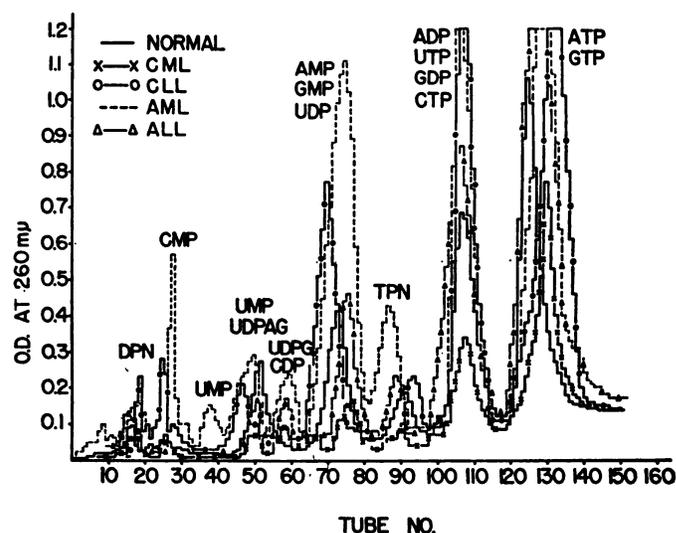

Chart 1.—Typical chromatographic profiles of nucleotides from various leukocyte populations. Abbreviations used are: DPN and TPN, di- and triphosphopyridine; CMP, CDP, CTP, cytidine-5'-mono-, di-, and triphosphate; UMP, UDP, UTP, uridine-5', mono-, di-, and triphosphate; UDPG, uridine diphosphoglucose; UDPAG, uridine diphosphoacetylglucosamine; AMP, ADP, ATP, adenosine-5'-mono-, di-, and triphosphate; GMP, GDP, GTP, guanosine-5'-mono-, di-, and triphosphate; CML, chronic myelocytic leukemia; CLL, chronic lymphocytic leukemia; AML, acute myeloblastic leukemia; ALL, acute lymphoblastic leukemia.

TABLE 1
NUCLEOTIDES IN LEUKOCYTES

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
R_F of nucleotide[a]	53 (55)[b]	52 (51)	33 (33)	36 (39)	60 (60)	35 (35)	21 (20)	22 (18)	26 (25)	27 (30)	30 (29)	29 (28)	48 (47)	23 (20)	41 (41)	20 (22)
R_F of nucleotide[c]	15 (15)	15 (15)	17 (19)	7 (7)	15 (15)	12 (11)	46 (34)	43 (28)	10 (10)	10 (11)	10 (4)	— (52)	10 (10)	10 (9)	8 (5)	7 (6)
R_F of nucleotide[d]	38 (38)	82 (86)	78 (77)	75 (73)	33 (34)	77 (72)	76 (71)	77 (78)	80 (79)	57 (53)	80 (78)	53 (53)	42 (38)	83 (82)	46 (44)	67 (65)
R_F of nucleotide[e]		74 (76)					70 (69)	— (75)	83 (82)			(67)	50 (53)	89 (89)		68 (68)
R_F of nucleotide[f]							45 (41)	32 (33)								
R_F of nucleotide[g]							46 (42)	35 (35)								
E_{max} pH 2,mμ	330[h]	279	260	—	256	—	263	260	260	—	—	320[i]	255	257	255	255
E_{min} pH 2, mμ	290[h]	245	237	—	239	—	243	240	248	—	—	300[i]	244	232	229	229
E_{max} pH 11, mμ	—	—	263	—	258	—	262	260	262	—	—	—	257	257	259	259
E_{min} pH 11, mμ	—	—	244	—	238	—	247	243	247	—	—	—	239	240	232	235
Probable identity	DPN[j]	CMP	UMP	CDP	AMP	GMP	UDPAG	UDPG	UDP	GDP	CTP	TPN	ADP	UTP	ATP	GTP

[a] Isobutyric acid/water/concentrated ammonium hydroxide/Versene (100:55.8:4.2:1.6).
[b] Normal control values in parenthesis.
[c] Ethanol/ammonium acetate (140:60)
[d] Ammonium sulphate/sodium acetate/isopropanol (160:36:4).
[e] Diethylaminoethyl thin layer: 1 M sodium acetate/saturated ammonium sulphate/isopropanol.
[f] 95% Ethanol/1 M ammonium acetate, pH 7.5 (7.5:3).
[g] Same as a but with 1 M ammonium acetate buffer, pH 3.8.
[h] DPN oxidized form, cyanide complex, using 1.0 M KCN.
[i] TPN oxidized form, cyanide complex, using 1.0 M KCN.
[j] DPN and TPN, di- and tri-phosphopyridine; CMP, CDP, CTP, cytidine-5'-mono-, di-, and tri-phosphate; UMP, UDP, UTP, uridine-5'-, mono-, di-, and tri-phosphate; AMP, ADP, ATP, adenosine-5'-mono-, di-, and tri-phosphate; GMP, GDP, GTP, guanosine-5'-mono-, di-, and tri-phosphate; UDPAG, uridine diphosphoacetyl glucosamine; UDPG, uridine diphosphoglucose.

TABLE 2

Diagnosis	No. of sub-jects	Break-through	DPN[a] TPN	Cytidine nucleotides (CMP, CDP, CTP)	Uridine nucleo-tides (UMP, UDP, UTP)	UDPG	UDPAG	Adenosine nucleo-tides (AMP, ADP, ATP)	Guanosine nucleo-tides (GMP, GDP, GTP)
Normal	9	1.8 ± 0.8[b]	0.15 ± 0.16[c]	0.02 ± 0.03[c]	0.30 ± 0.31[c]	—	—	0.95 ± 0.63[c]	0.50 ± 0.41[c]
CML[d]	6	2.3 ± 2.2	0.13 ± 0.14	0.04 ± 0.04	0.09 ± 0.04	0.08 ± 0.07[c]	0.17 ± 0.22[c]	0.68 ± 0.38	0.22 ± 0.16
CLL	6	0.7 ± 0.4	0.07 ± 0.11	0.07 ± 0.00	0.85 ± 1.45	—	—	1.14 ± 0.99	0.15 ± 0.09
AML	5	2.2 ± 0.6	0.27 ± 0.95	0.04 ± 0.02	0.53 ± 0.51	0.12 ± 0.10	0.23 ± 0.12	1.24 ± 1.30	0.32 ± 0.03
ALL	3	1.7 ± 0.9	0.19 ± 0.06	0.01 ± 0.02	0.23 ± 0.02	—	—	1.50 ± 0.16	0.32 ± 0.03
PRV	6	1.8 ± 0.6	0.13 ± 0.06	0.06 ± 0.09	0.84 ± 0.37	—	—	2.38 ± 1.06	0.27 ± 0.11

[a] DPN and TPN, di- and tri-phosphopyridine; CMP, CDP, CTP, cytidine-5'-mono-, di-, and tri-phosphate; UMP, UDP, UTP, uridine-5', mono-, di-, and tri-phosphate; UDPG, uridine diphosphoglucose; UDPAG, uridine diphosphoacetylglucosamine; AMP, ADP, ATP, adenosine-5'-mono-, di-, and tri-phosphate; GMP, GDP, GTP, guanosine-5'-mono-, di-, and tri-phosphate.
[b] ×10⁻³ O.D. units 10⁶ cells (O. D. units = O. D. × volume).

$\times 10^{-3}$ O.D. units 10^6 cells (O. D. units = O. D. $\times$ volume).
[c] ×10⁻³ μmoles 10⁶ cells (± S.D.)
[d] CML, chronic myelocytic leukemia; CLL, chronic lymphocytic leukemia; AML, acute myeloblastic leukemia; ALL, acute lymphoblastic leukemia; PRV, polycythemia rubra vera.

UMP, GMP, and GDP were identified only by their characteristic R_F values in 3 or more solvent systems because of the paucity of material. No deoxyribonucleotides were identified.

As seen from Table 2, there was great variability of individual nucleotide content. This may have been due to the therapy which some patients received, although no adequate correlation could be made, or to the variable amounts of minor nucleotides which were unavoidably measured along with the major nucleotides. There appears to be no correlation of nucleotide patterns with different methods of leukocyte separation. Most of the samples were handled alike except for the hypotonic lysis utilized in normal leukocytes and leukocytes from CLL. The lysis did not consistently affect nucleotide content in any manner.

The initial peak (not too evident in Chart 1), which elutes just prior to DPN in the majority of leukocytes, contains cytosine as evidenced by the characteristic 280/ 260 ratio and the ultraviolet spectra. This may be in the form of cytidine diphosphoethanolamine (10) because of its position of elution; it could not be characterized further because of the paucity of material.

Studies on glycogen levels and glycogen synthetase activity in the various types of human leukemic leukocytes are presently in progress, in order to determine any possible correlation with the increased amounts of UDPG and UDPAG found in acute and chronic leukemic leukocytes. Various aspects of glycogen metabolism in leukemic cells have been investigated by other workers. For instance, Valentine et al. (19) found a lower mean glycogen level in CML cells than normal. Miller and VanderWende (9) found normal levels of glycogen synthetase (necessary for glycogen storage) in 2 patients with CML. Recently, Yunis and Arimura (22) analyzed glycogen metabolism in normal and chronic myelocytic and lymphocytic human leukocytes and found that glycogen phosphorylase activity in these leukemic cells did not differ significantly from

normal. VanderWende, Miller, and Glass (20) measured glycogen degradation and synthesis *in vitro* in normal and chronic myelocytic leukemic human cells. Glycogen degradation was practically equal in both groups. Luganova and Seyts (8) found that the rate of glycogen renewal as measured by uniformly labeled ^{14}C glucose is lowest in normal leukocytes, $1\frac{1}{2}$ times greater in polycythemia rubra vera, and 15 times greater than normal in chronic myelocytic leukemic cells. Therefore, there appears to be no good correlation between glycogen synthetase, increased UDPG content, and glycogen content in chronic myelocytic leukemic cells. UDPG was increased in acute myeloblastic leukemic cells, and studies are now in progress to assess the glycogen synthetase activity because these cells are very low in glycogen content.

ACKNOWLEDGMENTS

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