PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 508-512 (1977)

Ribonucleoside and Nucleotide Components of Normal Human Blood Lymphocytes and Platelets (39705)

S. H. PROSS,[1] T. W. KLEIN AND C. W. FISHEL

Department of Medical Microbiology, University of South Florida Medical School, Tampa, Florida 33620

Introduction. The analysis of intracellular nucleotide levels has contributed to the understanding of several physiologic and biologic regulatory processes in various cells and tissues (1-3). Major attention has been directed to studies of variation of the cyclic nucleotides, cAMP and cGMP (4, 5), with relatively little information available concerning the levels of the ribonucleoside or the 5′-mono-, 5′-di-, and 5′-triphosphates. Perhaps one reason for the "neglect" is the rather complicated procedures necessary for the detection and quantitation of the noncyclic nucleotides. Advances in the techniques of high pressure liquid chromatography have tended to minimize these complications.

The present studies were designed to identify and quantitate the nucleosides and noncyclic nucleotides present in human blood platelets and lymphocytes. Since the lymphocytes can be divided into two basic populations (T and B cells) that differ according to many criteria including ontogeny, surface receptors, immunologic function (6), and adherence to nylon wool columns (7), chromatographic profiles of the two populations were determined.

Materials and methods. Platelet isolation. Blood (50 ml) from healthy adult volunteers was collected into syringes containing 4.5 ml of heparin (1000 USP units/ml). The blood was centrifuged at 300*g*, the platelet-rich plasma (PRP) was carefully removed, and the number of platelets was determined by a thrombocounter (Coulter Electronics, Inc., Hialeah, Fla.). The platelets were sedimented by centrifugation at 1800*g*, frozen immediately in an alcohol-dry ice bath, and stored at −80°.

Lymphocyte isolation. The platelet-poor plasma (PPP) was added back to the sedimented blood. Tubes consisting of 10 ml of blood and 10 ml of 0.15 *M* NaCl underlayered with 15 ml of Lymphoprep (Gallerd-Schlesinger Chem, Carle Place, N.Y.) were centrifuged at 400*g* at the interface, 40 min, 22° (7). The layer containing lymphocytes was removed and centrifuged, and the sedimented cells were washed 2× with Hank's balanced salt solution (HBSS, Flow Laboratories, Rockville, Md.) and resuspended in 2-ml volumes of BME-FCS consisting of Basal Medium Eagle (BME, Flow Laboratory, Rockville, Md.) plus 5% heat-inactivated fetal calf serum (Grand Island Biological Company, Grand Island, N. Y.). Viabilities were determined by exclusion of 0.4% trypan blue (Grand Island Biological Company, Grand Island, N.Y.).

Lymphocyte subpopulations. Nylon wool columns were prepared as described in Trizio and Cudkowicz (8) with all operations performed at 37°. Two milliliters of lymphocyte preparation were added to the column and incubated for 45 min. "Effluent" cells were obtained by passage of 25 ml of BME-FCS through the column. The column was then washed with 100 ml of media and "eluted" cells were obtained by pressing the wool with forceps.

Erythrocyte rosettes. Nonimmune (E) and complement-binding (EAC) rosettes were prepared as previously described (9). Sheep red blood cells (SRBC) were obtained each week from Lee Laboratories, Grayson, Ga. For E rosettes, SRBC were washed, and a 0.5% suspension was made in one part HBSS and one part heat-inactivated, SRBC-absorbed, fetal calf serum. Lymphocytes (1×10^6/0.25 ml) and SRBC (0.25 ml) were incubated for 5 min at 37°, centrifuged at 200*g* for 5 min at 22°, and incubated overnight at refrigerator temperatures. For enumeration of rosettes, the cells

[1] Correspondence should be sent to S. H. Pross, Ph.D., Medical Center, College of Medicine, Department of Medical Microbiology, Box 10, 12901 North 30th Street, Tampa, Florida 33612.

ISSN 0037-9727

were gently suspended by rotating the tube, and 100 μl of cells were mixed with 50 μl of 10% methylene blue. For EAC rosettes, heat-inactivated hemolysin (Difco, Detroit, Mich.; 0.02 ml) at one-half the minimum agglutinating dose was added to 10 ml of a 2.5% suspension of SRBC, and incubated at 37° for 30 min. Mouse serum as complement source (0.2 ml) was added to 1.8 ml of the EA suspension, incubated for 30 min at 37°, and washed 3× in HBSS. The EAC suspension indicator cells (0.25 ml) were incubated with lymphocytes (1×10^6/0.25 ml) for 15 min at 37° and centrifuged at 200*g*, 37°, for 5 min. The cells were suspended on a vortex, and 200 μl of cells were mixed with 50 μl of 10% methylene blue and counted in a hemocytometer. A minimum of 300 cells were counted by hemocytometer with those containing three or more attached SRBC being scored as rosettes.

Nucleoside/nucleotide extraction. Nucleotides were extracted by adding 1 ml of cold 12% TCA/5 × 10^9 platelets or 1 ml 12% TCA/1 × 10^8 lymphocytes as described by Scholar *et al.* (1). Proteins were pelleted by centrifugation at 1800*g* for 5 min at 4° and the supernatant was removed. The pH was brought up to approximately 6 by four to five extractions with cold-water-saturated diethyl ether. The ether was bubbled off and the samples were lyophilized and stored at −80°. Before the run, samples were reconstituted in 0.15 ml of H_2O (lymphocytes) or 0.3 ml of H_2O (platelets). Volumes of 50 μl (lymphocytes) or 25 μl (platelets) were added to the column. A column extract resulted from a minimum of approximately 1 × 10^7 lymphocytes of 6 × 10^8 platelets.

Liquid chromatography. The general procedures of separation and analysis have been described by Brown (10). We used a Perkin-Elmer (Norwalk, Conn.) LCS 1220 with the LCS 55 detector operated at 260 nm. The column was partisil sax 10/25. The mobile phase consisted of linear gradient of 0.01 *M* $NH_4H_2PO_4$, pH 2.8, to 0.5 *M* $NH_4H_2PO_4$, pH 4.8; 5%/min. The flow rate was 2 ml/min at 40°. The Perkin-Elmer 56 recorder was utilized and the quantitations determined by the PEP-1 data system which compared the response factors (RF) of unknown sample peaks with standard peaks of known concentrations.

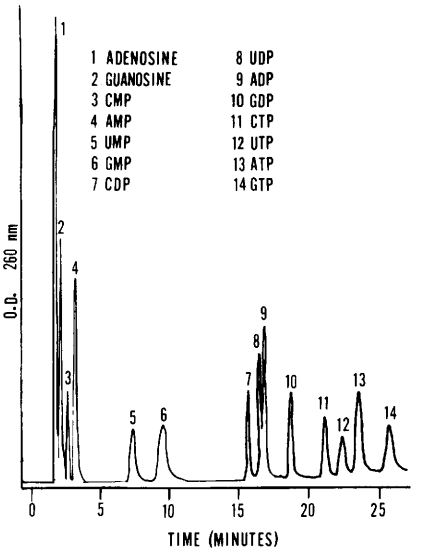

FIG. 1. Standard mixture of ribonucleosides and nucleotides (each 1–2 n*M* in a 10-μl volume loaded onto the column). Retention times in minutes are as follows: (1) adenosine, 1.52; (2) guanosine, 1.86; (3) CMP, 2.88; (4) AMP, 2.93; (5) UMP, 7.20; (6) GMP, 9.42; (7) CDP, 15.62; (8) UDP, 16.56; (9) ADP, 16.84; (10) GDP, 18.78; (11) CTP, 21.22; (12) UTP, 22.48; (13) ATP, 23.69; (14) GTP, 25.89.

Results. Figure 1 shows the retention times of a standard mixture of ribonucleosides and 5′-mono-, 5′-di-, and 5′-triphosphates under normal operating conditions. Standards were run before each experiment and the times proved to be very reproducible. Figure 2 shows a sample chromatogram of (A) platelets and (B) nylon wool effluent cells. Table I shows the concentrations (nmole/10^9 platelets) of human platelet nucleosides and nucleotides. The ratio of ATP/ADP was 1.6:1, in agreement with others (1, 11). The GTP:GDP ratio was 1:1, also in agreement with others (1, 11). It should be noted, however, that it was possible to identify components not previously reported. Thus, AMP, GMP, CTP, guanosine, and adenosine can be easily quantitated in addition to the previously studied 5′-di- and 5′-triphosphates.

Peripheral blood lymphocytes of normal adults were separated into two populations according to differential adherence to nylon wool columns. The efficiency of the nylon wool column was determined and presented

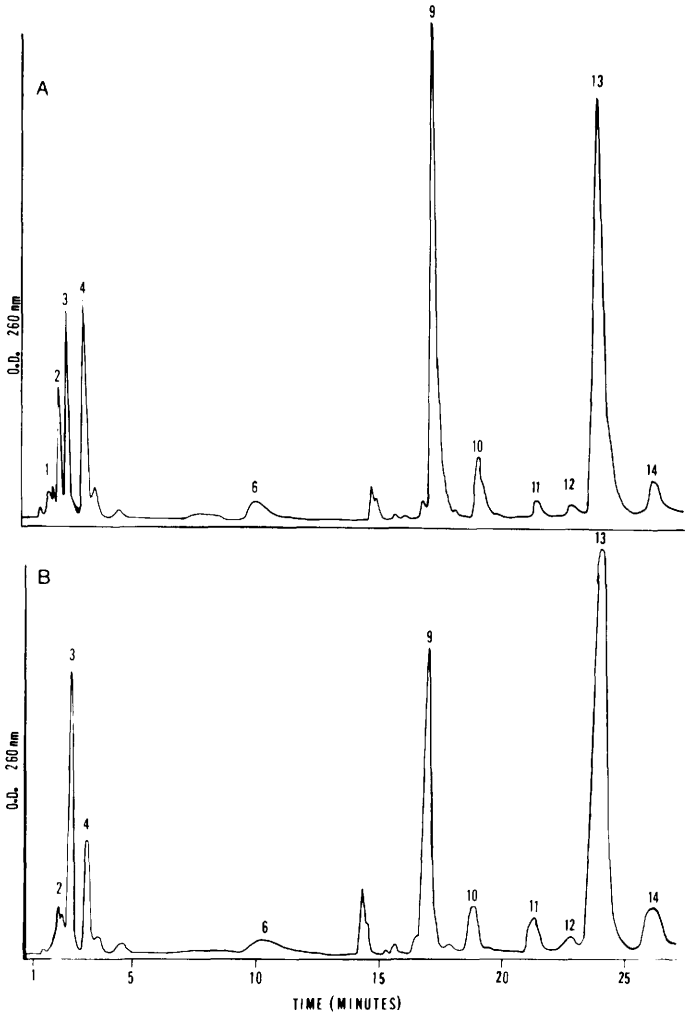

FIG. 2. A. Platelet sample. Following lyophilization, residue was redissolved in 0.3 ml of cold deionized water and 25 μl was loaded on column. B. Effluent cell sample. Residue was redissolved in 0.15 ml of cold deionized water and 50 μl was loaded on column. The peaks can be identified by referring to the legend for Fig. 1.

in Table II. E rosettes were used as a measure of T lymphocytes; EAC rosettes were used as a measure of B lymphocytes (9). This procedure removed about half of the EAC-rosette-forming cells from the effluent fraction relative to whole blood (18.9 vs 9.7%) and reduced the number of E-forming cells in eluted fraction by about 2/3 (50.7 vs 15.2%). The T cells were always 95% viable. The viability of the B cells averaged 85%.

Table III shows the nucleotide concentrations in the effluent and eluted cells. The low numbers of B cells in peripheral blood forced a pooling of the samples. The ATP/ADP ratio in the effluent cells was 3.3 whereas in the eluted cells it was 1.5. The GTP/GDP ratio in the effluent cells was 1.8, while that of the eluted cells was 0.5. In addition to the adenosine and guanosine nucleotides, it was possible to quantitatively estimate the concentration of CMP, CTP, and UTP in these cells.

Discussion. High-pressure liquid chromatography employed in the present investigation has provided a method to identify and quantitate nucleotide components previously undetected even by similar techniques. Scholar *et al.* (1) have shown the presence of adenosine and guanosine 5′-di-

and 5′-triphosphates in platelets; however, AMP, GMP, CMP, GTP, UTP, guanosine, and adenosine were not reported. Goetz *et al.* (11) have quantitated AMP and GMP in platelets, but were unable to separate GDP from UTP.

Nylon wool columns provide a quick way to partially purify E- and EAC-forming cells. The data suggest a different nucleotide profile in effluent and eluted cells from nylon wool with a higher concentration of the triphosphates present in the effluent components. It should be remembered, however, that the effluent cell data represent the average of nine individual experiments, whereas the eluted cell data represent two pools of cells. Also, the viability of the effluent cells, as determined by trypan blue exclusion, was excellent (>95%); the viability of the eluted cells averaged 85% (ranged from 82–90%).

The large majority of peripheral blood lymphocytes are T cells. Although the concentrations of ATP, CTP, and UTP noted in the effluent cells compared favorably to those reported by Scholar *et al.*, (1), we found higher concentrations of mono- and diphosphates. The reason for this variation is not clear, although it most probably is due to column differences. It should be noted that varying concentrations of the adenosine and guanosine diphosphate have been reported by several laboratories (1, 11, 12).

Experimental evidence has shown that the normal nucleotide levels of certain types can be altered through biochemical or pharmacological mechanisms (12). The role of the adenylates as regulatory effectors has been well-documented (14). Nucleotides (particularly cyclic nucleotides) have been implicated in T cell–B cell interactions, E rosetting, and mitogen stimulation (15, 16). Correlations between disease states and subtle changes in nucleotide composition have been proposed (1, 2). It may be that the establishment of standard values of nucleotide concentrations in normal blood elements will aid in recognizing variations in these pools resulting from pathological states.

It is obvious that one cannot utilize the procedures described in this report for an analysis of cyclic nucleotides in concentrations less than approximately 200 pmole. If the samples are concentrated to a point that

TABLE I. RIBONUCLEOSIDE AND NUCLEOTIDE CONCENTRATIONS IN HUMAN PLATELETS (NMOLE/10^9 PLATELETS).

Component	$\bar{X}$[a]	SE
AMP	4.904	0.431
ADP	19.281	1.341
ATP	31.887	2.272
GMP	2.677	0.518
GDP	4.565	0.373
GTP	4.892	0.474
CMP	15.311	0.233
CTP	1.410	0.285
UTP	2.040	0.256
Guanosine	1.305	0.884
Adenosine	0.165	0.019

[a] Mean ± standard error of the mean of nine samples.

TABLE III. NUCLEOTIDE CONCENTRATIONS (NMOLE/10^7 CELLS) OF LYMPHOCYTES FROM NYLON WOOL.

	Effluent cells[a]		Eluted cells[b]	
Component	$\bar{X}$	SE	$\bar{X}$	SE
AMP	0.915	0.223	3.689	0.911
ADP	2.998	0.503	2.368	1.111
ATP	9.837	1.201	3.677	1.411
GMP	0.670	0.201	0.362	0.050
GDP	0.977	0.138	1.221	0.362
GTP	1.767	0.304	0.589	0.446
CMP	1.200	0.116	5.889	3.775
CTP	1.200	0.116	0.485	0.063
UTP	0.817	0.182	0.178	0.178
Guanosine	0.236	0.050		

[a] Average based on nine samples individually run.
[b] Average based on two pools of samples (pool 1 had six samples; pool 2 had three samples).

TABLE II. SEPARATION OF LYMPHOCYTE SUBPOPULATIONS BY NYLON WOOL.

	Recovery from column[a] (%)	E (%)	EAC (%)	E/EAC	EAC/E
Before nylon wool	–	50.7 ± 5.2[b]	18.9 ± 2.9	2.7	0.4
Effluent cells	56.3	56.9 ± 6.9	9.7 ± 2.6	5.7	0.2
Eluted cells	10.5	15.2 ± 6.4	30.5 ± 7.7	0.50	2.0

[a] Percentage of total cells placed on wool.
[b] Mean ± standard deviation.

cAMP or cGMP would be detectable, the other nucleotides would overlap. Attempts are underway to develop a method to convert nucleotides (other than the cyclic) to nucleosides to offset the above problem and this will be reported at a later date.

Summary. Ribonucleoside and nucleotide profiles of human peripheral blood platelets, nylon wool effluent, and nylon wool eluted cells were obtained by high pressure liquid chromatography. AMP, ADP, ATP, GMP, GDP, GTP, CMP, CTP, and UTP components were present in all cell types. Guanosine was found in platelets and effluent cells. Adenosine was detected in platelets only. The use of high pressure liquid chromatography provides a rapid, reproducible means for analyses of nucleotides in blood elements.

We would like to acknowledge the expert technical assistance of Mrs. Betty Nager.

1. Scholar, E. M., Brown, P. R., Parks, R. E., Jr., and Calabresi, P., Blood **41,** 927 (1973).
2. Nakai, G. S., and Craddock, C. G., Cancer Res. **25,** 575 (1965).
3. Holmsen, H., and Weiss, H. J., Blood **39,** 197 (1972).
4. Atkinson, J. P., Wedner, H. J., and Parker, C. W., J. Immunol. **115,** 1023 (1975).
5. Smith, R. S., Sherman, N. A., and Coffey, R. G., Int. Arch. Allergy. **47,** 586 (1974).
6. Miller, R. G., Gorczynski, R. M., Laflew, L., MacDonald, H. R., and Phillips, B. A., Transplant. Rev. **25,** 59 (1975).
7. Boyum, A., Scan. J. Lab. Clin. Invest. **21** (Suppl. 97), 77 (1968).
8. Trizio, D., and Cudkowicz, G., J. Immunol. **113,** 1093 (1974).
9. WHO/IARC, Scand. J. Immunol. **3,** 521 (1974).
10. Brown, P. R., J. Chromatog. **52,** 257 (1970).
11. Goetz, U., DaPrade, M., and Platschet, A., J. Pharm. Exp. Therap. **178,** 210 (1971).
12. Bishop, C., Rankine, D. M., and Talbott, J. H., J. Biol. Chem. **234,** 1233 (1959).
13. Robison, G. A., Butcher, R. W., and Sutherland, E. W., *in* "Cyclic AMP," Academic Press, New York (1971).
14. Atkinson, D. E., Science **150,** 851 (1965).
15. Bach, M. A., and Bach, J. F., Eur. J. Immunol. **3,** 778 (1973).
16. Watson, J. J., Exp. Med. **141,** 97 (1975).

Received June 28, 1976. P.S.E.B.M. 1977, Vol. 154.