The Volume Distribution of Human Lymphocytes* (34043)

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(Introduced by L. M. Meyer)

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Since the development of electronic gating for the measurement of cell volume, erythrocyte (1, 2) and platelet (3) volume distribution have been defined. Leukocyte volume measurements, on the other hand, have been impaired by the heterogeneous cell populations normally found in whole blood or plasma-cell suspensions. While volume measurements of these cell suspensions may provide useful information (4, 5), they do not define volume distribution for specific cells.

Separation of leukocytes may be accomplished by several means (6–8). In this laboratory the method of Rabinowitz (7) has provided high yields of lymphocytes and reproducible results. This report describes volume distribution characteristics of lymphocytes from healthy subjects and from patients with diseases in which lymphocyte size is thought to be altered.

Materials and Methods. Blood was obtained from allegedly healthy laboratory and hospital personnel and from patients with acute lymphocytic leukemia (ALL), chronic lymphocytic leukemia (CLL), and infectious mononucleosis (IM). Leukemia patients in remission and those in relapse were included in the study. Infectious mononucleosis patients were selected from the University Student Health Service on the basis of guinea pig kidney-absorbed heterophile titers higher than 1:64, and greater than 20% atypical lymphocytes in the peripheral blood.

Freshly drawn, heparinized blood was allowed to sediment by gravity for 3 hr during which the leukocyte-rich plasma was collected at approximately 30-min intervals. The leukocyte-rich plasma was incubated on columns of glass beads as described by Rabinowitz (7) except the columns were washed with phosphate buffer at pH 7.4, followed by 20% calf serum in Hanks' balanced salt solution prior to application of the cell suspensions for incubation. These measures were adopted to avoid possible selective cell loss reported for some leukemic lymphocytes during cell separation (5, 9), and to achieve higher yields of lymphocytes.

Efficiency of cell separation was monitored by Wright-stained films of the plasma-cell suspension and column eluate (5). Erythrocytes and leukocytes appeared to be distributed randomly when spread on glass slides. Two hundred nucleated cells were counted to determine the number of lymphocytes and erythrocytes per 100 leukocytes. Estimation of platelet numbers was erratic because many adhered to the slide at the point of application. Accordingly, platelet counts were performed in 10 oil immersion fields at 5-mm intervals across the length of the slide, and reported as the mean number.

Lymphocyte-rich eluates were diluted in appropriate volumes of particle-free, sterile physiologic saline to obtain electronic cell counts of less than 12,000/mm^3. Cell enumeration, volume distribution and mean cell volume (MCV) were measured by the Coulter model B particle counter, automatic recording size distribution plotter and mean cell volume computer. Aperture size was 100 μ. The instrument was calibrated with pollen particles of known mean corpuscular volume suspended in a relatively particle-free, sterile saline solution. It was then adjusted to a base scale of 15 μ^3 increments for each excursion of the recording pen by appropriately setting the amplification and aperture current. The principle of the Coulter counter has been described by Brecher *et al.* (10). The MCV computer was calibrated to measure

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TABLE I. Results Following Glass Bead Column Separation of Normal, Leukemic, and Infectious Mononucleosis Lymphocytes.

Cell source	No. of subjects	Adjusted mean cell count of sample (mean ± SD)	Lymphocytes[a] (%; mean ± 1 SD)	Erythrocytes/100 nucleated cells (mean; range)	Platelets/10 oil immersion fields (mean; range)
Normal donors	10	8375 ± 2304	99.8 ± 0.5	102 (7–370)	77 (4–250)
Chronic lymphocytic leukemia	10	7776 ± 3585	99.7 ± 0.2	26 (3–91)	43 (6–199)
Acute lymphocytic leukemia	7	7443 ± 2573	99.4 ± 0.5[b]	43 (3–123)	18 (1–59)
Infectious mononucleosis	8	7979 ± 3096	99.8 ± 0.4	33 (8–111)	100 (7–307)

[a] Lymphocytes per number of nucleated cells.
[b] Includes prolymphocytes and lymphoblasts.

the mean cell volume of particles between 75 and 375 μ^3. Accuracy at these settings is ± 10 μ^3.

Results. The results of glass-bead column elution of leukocyte-rich plasma from normal subjects and patients with leukemia and infectious mononucleosis are listed in Table I. Lymphocyte purity of column effluent plasma was comparable in all groups when the precursor cells present in acute leukemia were included. Red cell and platelet contamination varied widely between individuals and groups of patients.

The mean volume distribution of lymphocytes from normal subjects and patients with IM and CLL is shown in Fig. 1A. Table II lists the pertinent values derived from analysis of the curves and associated measurements. Log probability analysis of a normal subject is presented in Fig. 1B. The original analysis plotted on the left shows a biphasic slope. When the values for the cells larger than 480 μ^3 are analyzed independently and plotted on the same scale, a straight line results. The modal volume of the second gaussian distribution is about 530 μ^3. Log probability analysis of data from patients with CLL yields a single gaussian distribution of small cells while that from IM patients appears multiphasic (Fig. 1C).

Acute leukemia patients were grouped in two categories on the basis of size distribution analysis. Since these groups correlated approximately with clinical status, the terms "relapse" and "remission" were arbitrarily chosen to designate them. For the purpose of this study, the classification of "relapse" was applied to all patients having a lymphocyte MCV greater than 280 μ^3. Of the seven acute leukemia patients studied, four were classified in "relapse" and three in "remission." Pertinent information on these seven patients is shown in Table III. Three of the four patients listed under "relapse" have obvious

TABLE II. Lymphocyte Volume Measurements.

	No. of subjects	Modal vol (μ^3)	Mean cell vol (μ^3 ± 1 SD)	99% Range (μ^3)	Curve characteristics shown in Fig. 1
Normal subjects	10	225–240	250 ± 20	150–660	Unimodal, skewed to right
Chronic lymphocytic leukemia	10	210–225	260 ± 20	105–370	Unimodal, gaussian?
Infectious mononucleosis	8	240–255	320 ± 40	110–600	Multiphasic, skewed to right, broad peak

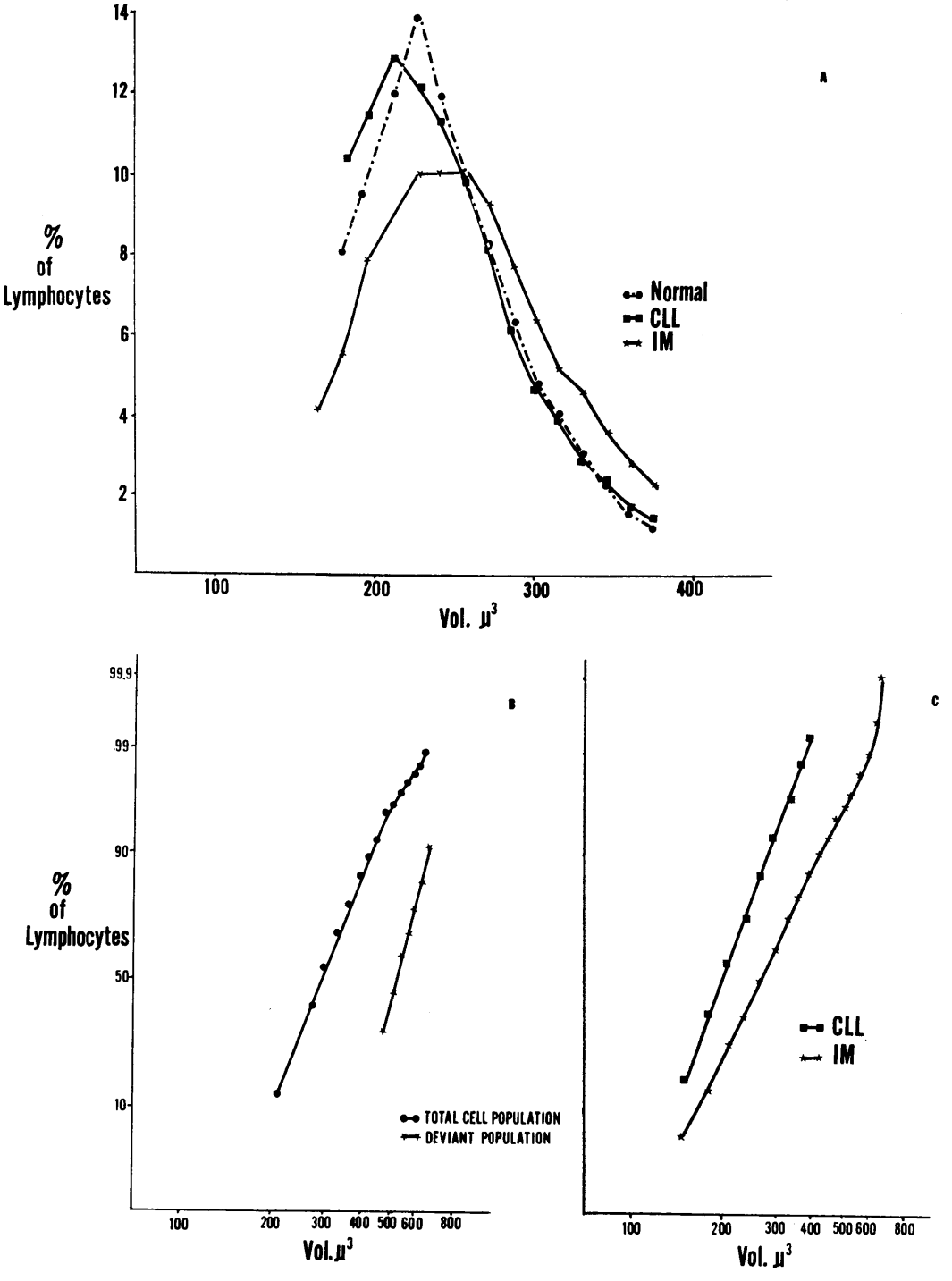

FIG. 1. Lymphocyte volume distribution: (A), lymphocytes from healthy subjects and patients with chronic lymphocytic leukemia (CLL) and infectious mononucleosis (IM); (B), log probability size distribution of lymphocytes from healthy subjects. "Deviant Population" includes cells larger than 480 μ^3 analyzed independently. (C), lymphocytes from representative patients with chronic lymphocytic leukemia (CLL) and infectious mononucleosis (IM).

TABLE III. Lymphocyte Measurements from Patients with Acute Lymphocytic Leukemia.

Patient	Chemo-therapy	Whole blood			Column eluate			
		WBC	Lympho-cytes (%)	Blasts (%)	Lympho-cytes (%)	Blasts (%)	Mean cell vol (μ^3)	Modal vol(s) (μ^3)
Relapse								
J.L.	No	13,400	9	83	45	55	480	205, 395
B.D.	Yes	4900	67	2	82	17	330	205, 440
S.S.	No	16,000	32	48	79	21	630	225, 575
W.S.	No	3900	51	16	94	5	370	255, 475
Remission								
G.D.	Yes	650	87	10	100	0	280	195
L.Z.	Yes	5400	66	0	100	0	270	210
J.M.	Yes	5000	26	0	100	0	280	210

bimodal lymphocyte volume distribution. Patient B.D. had no obvious bimodality, but did have an increased number of large cells with a possible second mode at about 440 μ^3. This patient was the only one in this category receiving chemotherapy. All four had immature cells in the peripheral blood and column effluent.

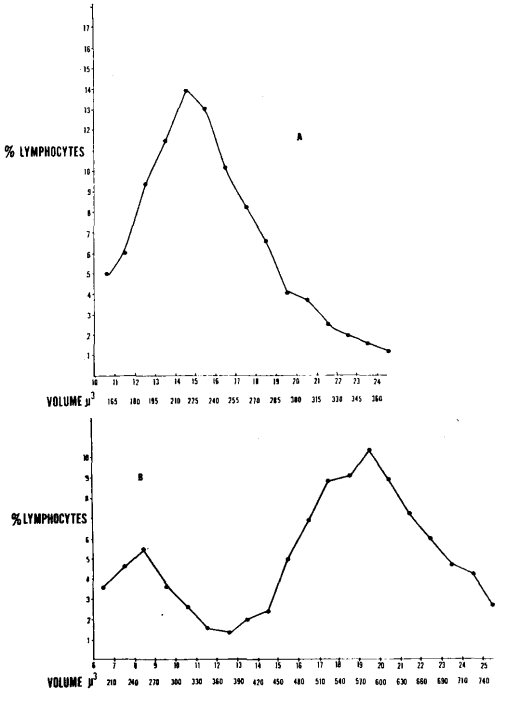

FIG. 2. Volume distribution of lymphocytes from patients with acute lymphocytic leukemia: (A), mean values of three "remission" patients; (B), bimodal distribution of patient S. S., an untreated child with typical blood and bone marrow findings of acute lymphocytic leukemia.

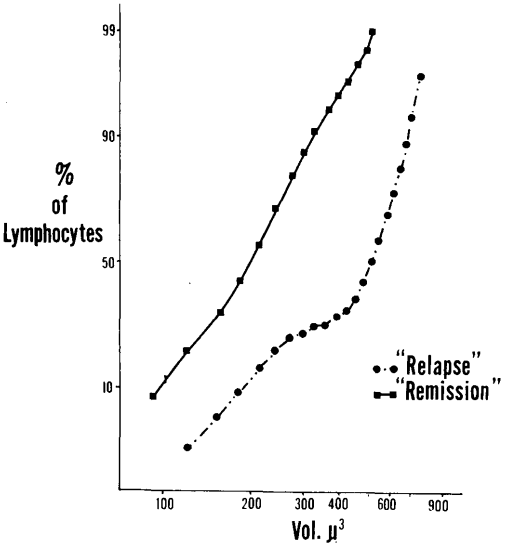

FIG. 3. Log probability graph of typical acute leukemia (ALL) patients in "remission" and "relapse" (see text).

In all three patients classified in "remission" no immature cells were seen when 200 cells of the column eluate were studied, although patient G.D. appeared to have 10% blasts in the peripheral blood. All "remission" patients were receiving chemotherapy and exhibited degrees of clinical response from no improvement to complete (bone marrow) remission.

Figure 2 shows the two types of lymphocyte volume distributions from patients with ALL. In Fig. 2A the values for the three

TABLE IV. Lymphocyte Diameter Calculated from Volume Measurements.[a]

Group	Modal vol ($\mu^3 \pm 1$ SD)	Mean diam (μ)	Modal diam (μ)	99% Range (μ)
Healthy subjects	230	7.8	7.4	6.6–11.0
Chronic lymphocytic leukemia	260	7.9	7.2	5.8–9.0
Acute leukemia				
Remission	215	8.0	7.6	4.8–9.2
Relapse	498[b]	9.8	—[c]	—[c]
Infectious mononucleosis	320	8.5	7.7	5.8–10.6

[a] $D = 2(V/\frac{4}{3}\pi)^{1/3}$, where V = cell volume (μ^3).
[b] Large population only.
[c] Wide individual variation.

patients in "remission" are plotted as the mean of the values for each volume increment. The unequivocal data from patient S. S. (Fig. 2B) was selected as an example of the bimodality exhibited by patients in "relapse." The data from Fig. 2B was further analyzed on log probability paper (Fig. 3) along with data from a single patient in "remission" selected for the best gaussian fit. The patient in "remission" reveals a multiphasic curved line. The patient in "relapse" demonstrates a definite biphasic slope. The population of small cells shows apparent gaussian characteristics. The larger population of cells with increased volume reveals bilateral skewing around a modal volume of 540 μ^3 when analyzed independently (not shown).

Discussion. Until recently, lymphocyte size was determined by visual measurement of fixed and stained cells spread on glass slides. Normal human lymphocyte diameters are between 6 and 20 μ when measured by this method (11). The use of new techniques which provide efficient cell separation and accurate volume determinations permit comparison with previous linear values. Table IV lists mean diameters of lymphocytes from healthy and diseased subjects calculated from electronic volume measurements. The smaller diameters derived from these calcuations are anticipated, since linear values include distortion from spreading of the cells on glass slides. The surprisingly narrow limits of cell size, however, suggest that cell distortion plays an important part in the morphology of large lymphocytes. For example, infectious mononucleosis patients in this study all had 20 or more large, atypical lymphocytes per 100 leukocytes, yet less than 1% of these cells have volumes approaching 4187 μ^3, the volume of a sphere with a diameter of 20μ. Hence, the atypical lymphocyte of infectious mononucleosis is an attenuated variant, not a large one.

Studies of the effect of anticoagulants on normal human leukocytes demonstrated that mean cell volume increases with time in the presence of heparin. Leukemic cells do not exhibit this response to heparin (5). It was found that removing the plasma-cell suspension from the sedimenting blood at frequent intervals eliminated these variations. The volume distributions reported here agree with those of freshly drawn blood treated with saponin less than 6 min before analysis.

Other methods of determination of human lymphocyte volume have produced higher modal values. Gauthier and co-workers (4), using unseparated cell samples, report the lymphocyte modal volume of a CLL patient to be about 300 μ^3. These cells were prepared without anticoagulant and measured electronically a minimum of 2 hr and 50 min after venipuncture. Van Dilla *et al.* (6), using an electronic cell separator, found the lymphocyte mode of a normal subject to be about 260 μ^3. These authors used blood collected in heparin, presumably measured within 1 hr and exposed to saponin for 10 min.

A growing body of evidence suggests that two or more functional lymphocyte populations circulate in the peripheral blood. Peterson and Good (12) demonstrated lymphocyte volume distribution differences between thymus and bursa of Fabricius cells from young chickens, while others (13–15) employed volume distribution analysis of animal lymphocytes to demonstrate oncogenesis of the lymph organ. The bimodal distribution of lymphocytes from patients with acute leukemia in relapse (Fig. 2B) presumably represents pathological duality. On the other hand, graphs of normal subjects reveal persistent skewness on the right (Fig. 1). Sipe and co-workers (16) have suggested the presence of an obscured population of large lymphocytes which may be responsible for the identical distribution asymmetry they have observed in calf lymphocytes.

Logarithmic probability analysis may be applied to frequency distribution data to determine the presence and nature of skewness. Values from these experiments reveal two curves which invite interpretation. Data from a healthy subject selected for the best gaussian fit yield two slopes which appear to be straight lines (Fig. 1B). Other normal subjects provide similar patterns. Patients with CLL, on the other hand, produce a single straight line (Fig. 1C). It is possible to speculate from these curves that certain healthy individuals have two species of lymphocytes detectable by cell volume differences, while patients with CLL have a single volume population with characteristics which resemble the subgroup of small normal cells. Despite morphologic similarities, however, it is well known that CLL lymphocytes function uniquely (11). Hence, it is unlikely that these are identical cells by any other criteria than size and appearance under the light microscope.

Finally, attempts to interpret functional or other homogeneity by distribution analysis of biological cell populations must be undertaken with caution. It is quite possible that no measurement of a homogenous sample will fit gaussian distribution when increments on the abscissa are small (Mainlander, personal communication). Conversely, nongaussian frequency distribution is not necessarily indicative of sample heterogeniety. Demonstration of functional heterogeniety among human peripheral blood lymphocytes untimately will rest on functional measurements in isolated cells or cell groups.

Summary. Human lymphocytes were separated on glass bead columns and measured electronically for cell volume distribution. Cells from healthy subjects have a modal volume of about 250 μ^3, and a mean cell volume around 250 μ^3. Skewness of the right limb of the volume distribution curve suggests a second population of large cells. Acute lymphocytic leukemia patients in relapse demonstrate true bimodality, while those in remission do not. Data are presented to suggest that lymphocytes which appear to be large on stained slide preparations may be merely more distensible cells of average volume.

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ERRATA

Vol. **130** (1969) page 848, the name of the first author of the paper "Absence of Synergism between Thymus and Bone Marrow in Graft-versus-Host Reactions" should read: "O. Stutman."

Vol. **131** (1969) in the article, "Persistence of Group A Streptococci Labeled with Fluorescein Isothiocyanate in Inflammatory Sites in the Heart and Muscle of Mice and Rabbits," by N. Rickles, Z. Zilberstein, S. Kraus, G. Arad, M. Kaufstein, and I. Ginsburg, pp. 525-530:

Page 526, column 1, under heading, *"Fluorescent microscopy,"* line 4 should read: "The pass filter was a 3-mm UG1 . . ."