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Discriminative Enumeration of Mixed Anisometric Cell Populations By an Electronic Counter.* (30283)

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During the course of quantitative studies designed to assess the immunological effect *in vitro* of lymphocytes on an established line of "target" tumor cells bearing homologous transplantation antigens, some method was required that would enable the enumeration of the larger target cells present in mixtures with the smaller lymphoid cells. The instrument of choice for this purpose seemed to be an electronic particle counter because of its convenience and simplicity of operation as well as the degree of precision possible(1,2). While the electronic counter has often been used for counting and sizing cells or other particulate matter from relatively homogeneous populations in suspension, including tissue culture cells(3,4), its use in the *discriminative* counting of one cell type when present in mixtures with another has been limited; moreover, the details of calibration of this instrument for this type of counting problem have not been published. This note, then, describes: a) the results obtained when an electronic counter was used to enumerate selectively, on the basis of cell volume, fibroblasts of larger volume from smaller sized lymphoid cells present together in suspension; and b) the procedures used to calibrate the counter for such discriminative counting. Particles having a known mean volume were also employed so as to provide a standard reference for establishing the absolute volumes of the cells counted.

Materials and methods. Enumeration. The Model B Coulter Counter‡ having a 100 μ aperture was employed. The aperture current (APC) and amplification (AMP) settings used depended on the particle being counted; selection of the appropriate settings was made according to the principles suggested by Brecher *et al*(2). Counts were performed on cells or particles suspended in a versene buffered counting fluid.§

Cells and particles employed. a) Lymphoid cells were obtained *via* standard techniques (5) from the axillary and brachial nodes of adult C57BL/6 female mice and from mature female DA rats. These cells were washed 3 times in Hanks' buffered salt solution and a sample taken for enumeration with a hemocytometer and the aid of a phase-contrast microscope.

b) Tissue cultured cells were of two types

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‡ Coulter Electronics Co., Hialeah, Fla. The authors are indebted to Dr. Leonard Hayflick for the use of the counter.

§ The counting fluid was made up as 1 l of a 10X stock solution containing NaCl 79.0 g; KCl 3.7 g; $Na_2EDTA \cdot 2H_2O$ 8.9 g; Na_4 EDTA 9.9 g; 4 ml 0.5% phenol red. One part of the stock was diluted with 9 parts distilled water and calf serum added to a final concentration of 0.2%.

grown as monolayers on glass: L cells‖ of C3H mouse origin and an established line (Le-1) of tumor cells derived from a kidney sarcoma induced with polyoma virus in Lewis rats.¶ Both these cell lines were maintained in 2 × Eagle's basal medium, 1 × Earle's balanced salt solution supplemented with 10% calf serum.** For use these cells were removed from glass with a trypsin versene mixture†† and clumps were dispersed into single cells by vigorous aspiration with a pipette. Examination of the suspensions with a microscope revealed no residual clumps of cells.

c) Suspensions of polystyrene latex particles and of ragweed pollen with known mean particle volumes were also employed.‡‡ The latex particles were supplied as a concentrated suspension; 0.5 ml of this suspension was directly diluted with 50 ml of counting fluid. A suspension of ragweed containing 10^4 particles/0.5 ml was obtained by wetting 5 mg of pollen with 1 ml of n-propanol and diluting to 50 ml with counting fluid.

Results. 1. *Calibration of the electronic counter with particles of known volume*: A virtue of the electronic counter is that it is possible to obtain volume-frequency distributions of particles in suspension. The volume distributions are recorded in terms of the midpoints of "window" settings which represent sequential counting intervals between upper and lower thresholds. These window settings can be converted to absolute units of volume by determining the window setting which corresponds to the median volume of particles of known size. Size distributions were obtained for ragweed pollen and polystyrene latex particles. The APC and AMP settings which proved to be suitable were 4 and 4 for the pollen and 1 and ½ for the latex particles. These frequency distributions were plotted as cumulative-per cent of total counts *vs* threshold interval midpoints on probability paper. These plots approximated straight lines over 70% of their length, thereby indicating that the populations of particles represented a normal distribution and, therefore, that the *median* and *mean* particle volumes were identical. On this graph, that point on the threshold scale corresponding to 50% of the counts was taken to be the proper A (lower) threshold corresponding to the median particle volume: 26 for the ragweed and 8 for polystyrene. Because the APC and AMP settings of the counter are linearly related, any combination of settings constitutes a threshold scale range; threshold settings on any range can be translated into corresponding settings on any other range by multiplication by an appropriate factor. Thus the median volume setting of 8 for the polystyrene particles on the APC/AMP = 1/½ range represented a setting of 0.25 on the APC/AMP = 4/4 range used for sizing the pollen. A plot of the 2 median volumes against median volume settings on the threshold scale was linear through the origin (Fig. 1) verifying the assumption of linearity on which the range technique of sizing was based, and allowing the conversion of any threshold setting to absolute volume units.

2. *Discriminative counting of fibroblasts in a suspension of fibroblasts and lymphocytes*: To determine the number of Le-1 or L cells present in mixtures with lymphoid cells, it is necessary to determine which lower threshold setting will exclude all lymphocytes from the count even when present in great excess. For this purpose frequency-size distributions were determined for Le-1 cells, DA rat lymph node cells, and mixtures of both as well as for L

‖ The L cells were obtained from Dr. Angus Graham's laboratory as suspension cultures and were subsequently adapted for growth on glass as monolayers.

¶ The Lewis tumors were obtained from Dr. Vittorio Defendi.

** Obtained from Baltimore Biological Laboratories, Baltimore, Md.; media were supplemented with the antibiotics penicillin (100 μ/ml) and streptomycin (100 μg/ml). The serum was preheated to 56°C for 30 minutes.

†† 0.25% trypsin (Flow Laboratories, Rockville, Md.) in a buffered salt solution containing 0.02% versene.

‡‡ The ragweed pollen (mean diameter 19.5 μ; mean particle volume 3880 μ^3) was obtained from Coulter Electronics Co.; polystyrene latex particles (mean diameter 3.49 μ; mean particle volume 22.26 μ^3) were kindly supplied by Mr. L. J. Lippie, Dow Chemical Co., Midland, Mich.

cells, C57BL/6 lymph node cells and mixtures of both. Single-cell suspensions of Le-1 or L cells were prepared at a concentration of 2×10^6 cells/100 ml counting fluid. Each suspension was divided into 2 50-ml aliquots, to one of which was added approximately 10^8 DA rat or (to the L cells) C57BL/6 mouse lymphoid cells in 1 ml. A third suspension was prepared consisting of 10^8 lymphoid cells alone diluted to 50 ml with counting fluid. Frequency distributions were then determined for these suspensions with the counter at 2, 5, or 10 unit "windows." The APC and AMP settings which proved to be suitable were 4 and 2 when counting the Le-1 cells, the L cells and the mixtures, and 4 and ½ when counting either the rat or mouse lymphoid cells. The means of duplicate counts over any window interval were then divided by the width of that interval and plotted against the interval midpoints on

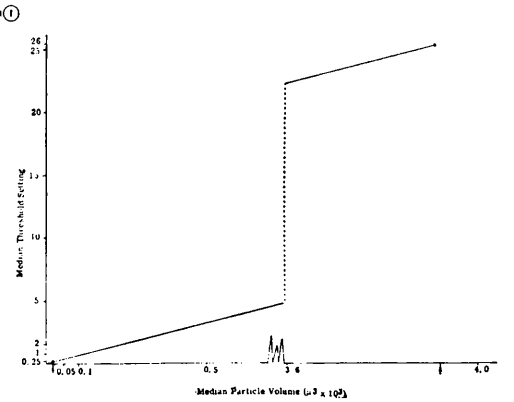

FIG. 1. Relationship between Median Particle Volume and Lower Threshold setting of the Model B, Coulter Counter (graph has been interrupted and a segment deleted to save space), showing colinearity at 2 determined points and the origin.

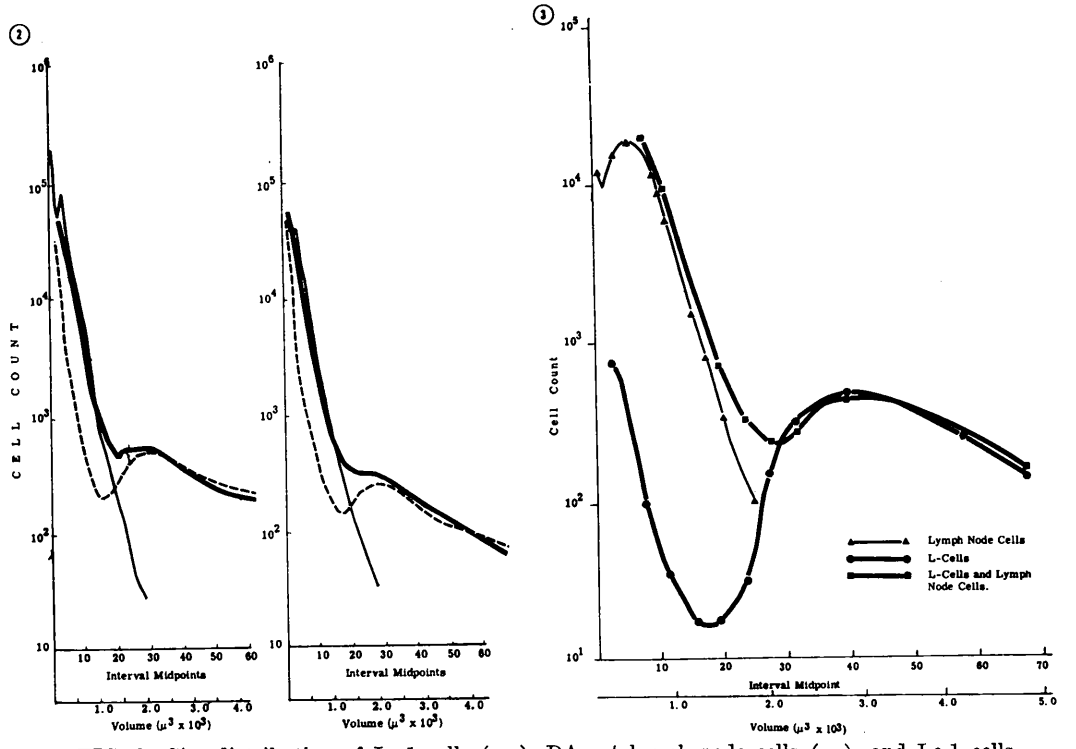

FIG. 2. Size distribution of Le-1 cells (– –), DA rat lymph node cells (—), and Le-1 cells + Lymphoid cells (—) based on electronic cell counts *vs* midpoints of various counting intervals on the A threshold of counter. From knowledge of the distribution of particles of known size it is possible to convert the midpoint interval settings into absolute units of volume—also expressed on the X-axis. With the A threshold of the counter set at 25, it is possible to exclude all lymphoid cells while counting 80% of Le-1 cells.

FIG. 3. Size distribution of L-cells (●—●), Mouse lymph node cells (▲—▲), and mixtures of L-cells and lymphoid cells (■—■) *vs* midpoints of various counting intervals on the A threshold of the electronic cell counter.

semi-logarithmic paper. Background counts on the counting fluid alone at the 2 AMP settings used were subtracted from the cell counts. In order to present the counts for the 3 suspensions—fibroblasts, lymphocytes, and mixtures—on the same graph, the midpoints on the axis for the lymphoid cell counts were divided by a factor of 4 to account for the 4-fold difference in the AMP settings. Examination of the size distributions of 3 independent plots (Fig. 2 and 3) shows that with the lower threshold set at 15, all the Le-1 or L cells are counted. When set at 25, the counter selects 80% of the Le-1 cells, and when set at 30 it counts 90% of the L cells while excluding all lymphoid cells even when present in a proportion of 100:1.

Discussion and conclusions. The results presented above show that the selective counting of one or the other of 2 cell types, differing with respect to volume present in the same suspension, is possible with the aid of an electronic counter. This is accomplished by electronically screening out smaller or larger cells from the desired counting interval. From knowledge of the proportion of cells expected to be present within the limits of that counting interval, the total number of cells of a particular type present can be computed. The applicability of this discriminative counting procedure depends in large part on sufficient volume differences among the cell populations present. In the event the cell volumes closely overlap when present as mixtures the counter would be unable to resolve them into distinct populations. In the present studies, a tissue culture line of Lewis rat tumor cells having a size distribution of 12.5-24 μ in diameter could be selectively counted when present in suspensions containing a high (100:1) multiplicity of lymphoid cells having a diameter of 6-15 μ. The same was true of L cells present (diameter = 14.0-24 μ) in mixtures with mouse lymph node cells (diameter = 6-15 μ).

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Hemoglobin, White Cell Count, Packed Cell Volume and Weight Gain in Turkeys Fed Certain Antibiotics.*† (30284)

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The growth promoting effect of orally administered antibiotics in young animals has been well established. Only a few investigations have concerned the effect of continued antibiotic ingestion on hematology. Lichtman *et al*(1) treated Addisonian pernicious anemia in human patients with 2 to 5 g of chlorotetracycline (Aureomycin) orally each day. A definite but submaximal increase in hematocrit and red cells resulted. Toon and Wangensteen(2) reported that macrocytic or normocytic anemia produced in the rat by formation of blind intestinal segments could

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