PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 152, 330-333 (1976)

Quantitation of Human T-Lymphocytes in Blood: Use of Automatic Smearing Instrument for Preparing Fixed Slides of E-Rosettes[1,2] (39390)

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The presence of functionally distinct populations of lymphocytes (B and T lymphocytes) in the peripheral blood is generally recognized (1). Human T-lymphocytes are commonly identified as the lymphocytes that form rosettes with SRBC (E-rosettes) when incubated under appropriate physical conditions. Quantitation of E-rosettes is commonly performed in cell suspension using a hemocytometer (2). The disadvantages of this method are lack of permanent preparation, inability to precisely identify the morphology of the rosette-forming cells, and the necessity of counting the rosettes within a short period of time due to their fragile nature. To obviate the above limitations, smears of E-rosettes were prepared by an automatic smearer which yielded very satisfactory preparations with no significant destruction of the rosettes.

Materials and Methods. Ten normal volunteers, three patients with chronic lymphocytic leukemia, and one patient with Sézary syndrome were studied (Table I). Venous blood was obtained in heparinized plastic syringes. Lymphocytes, isolated by Ficoll-Isopaque method (3), were washed twice with phosphate-buffered saline (PBS) with 0.1% glucose and suspended at a concentration of 40×10^6/ml. Sheep red cells (SRBC) suspended in Alsever's solution (Grand Island Biological, New York) were washed twice in PBS and resuspended in PBS at a concentration of 3000×10^6/ml. Fetal calf serum (FCS) (Grand Island Biological, New York) was absorbed twice with SRBC at 37° for 30 min (800×10^6 SRBC/ml FCS) and heat-inactivated at 56° for 1 hr.

For hemocytometer assay, 0.5 ml of the lymphocyte suspension readjusted with PBS to 15×10^6/ml was mixed with 0.5 ml of washed SRBC diluted to 300×10^6/ml and 0.01 ml of absorbed and inactivated FCS in a round bottom 75 × 15-mm plastic tube. Following gentle agitation the mixture was centrifuged at 200 *g* for 10 min at room temperature prior to incubation at 4° for 16 to 20 hr. The incubation tube was gently rotated by hand to resuspend the cells. A Neubauer chamber was gently filled with the cell suspension using a Pasteur pipet and allowed to stand at 10° for 10 min. Five-hundred lymphocytes were counted under phase contrast at a magnification of 400.

For automatic smearing method 0.05 ml of lymphocyte suspension (2×10^6 cells) was mixed with 2.5 ml of medium 199 with 25 m*M* HEPES buffer (Grand Island Biological, New York) in a round-bottom 75 × 15-mm plastic tube. Following centrifugation at room temperature for 10 min at 500 *g*, the supernatant was drained off. To the cell pellet, 0.01 ml of the SRBC suspension (30×10^6 cells) and 0.01 ml of the absorbed, inactivated FCS were added. Following gentle agitation the mixture was incubated at 37° for 15 min, at 4° for 16 to 20 hr, and finally at room temperature for 1 to 2 hr. The incubation mixture was gently rotated by hand until no clumps were visible. About 0.01 ml of the incubation mixture was placed on a glass slide and smeared with the automatic smearer, Hemaprep (Geometric Data Corp., Wayne, Pa.), adjusted to the thickest smear setting. This instrument makes smear by pulling the slide

[1] Research supported by the U.S. Energy Research and Development Administration, and in parts by NIH Grant No. 5RO1 CA 14742, American Cancer Society Grant No. NT-5, and the National Leukemia Association.

[2] Written informed consent, as required by our Institutional Review Committee, was obtained from normal volunteers and patients.

TABLE I. PERCENTAGE ROSETTE-FORMING T-LYMPHOCYTES IN BLOOD.

Samples	SRBC's/lymphocyte ≥1 Wet	SRBC's/lymphocyte ≥1 Automatic smearer	SRBC's/lymphocyte ≥2 Wet	SRBC's/lymphocyte ≥2 Automatic smearer	E-Rosettes[a] Wet	E-Rosettes[a] Automatic smearer
Normal volunteers						
1	55.0	56.2	44.0	43.3	42.0	40.6
2	67.5	65.7	59.9	58.6	56.4	51.7
3	50.7	62.2	44.2	54.6	41.9	50.1
4	58.3	76.7	47.3	61.9	45.1	55.8
5	74.6	62.1	67.6	56.8	65.0	52.7
6	73.2	71.8	67.0	63.4	65.4	59.6
7	71.6	71.4	59.6	62.4	57.3	58.8
8	84.4	78.7	81.0	69.9	79.2	66.9
9	81.6	76.6	77.6	72.0	72.8	68.0
10	62.9	64.0	58.1	61.0	54.3	58.8
Mean	68.0	68.5	60.6	60.4	57.9	56.3
±1 SD	11.2	7.6	13.0	8.1	12.8	8.1
SE	3.5	2.4	4.1	2.5	4.0	2.6
Patients						
MW (CLL)[b]	36.0	26.9	28.0	22.3	24.0	18.0
CS (CLL)	11.0	11.4	NA	6.4	2.0	4.7
CR (CLL)	28.2	28.1	21.2	25.7	19.5	24.9
AL (SS)[c]	72.4	72.8	66.4	66.0	64.0	59.6

[a] E-Rosettes were defined as lymphocytes with 3 or more SRBC.
[b] CLL, Chronic Lymphocytic Leukemia.
[c] SS, Sézary syndrome; NA, not available.

containing the drop of blood at a constant preselected speed against a glass spreader positioned at a fixed angle. Air-dried smears were fixed in absolute methanol for 5 min and stained with Wright Giemsa. Five-hundred lymphocytes were counted under light microscope.

In some instances, permanently fixed slides of E-rosettes were prepared according to the manual smearing technique (4).

The results were recorded as follows: lymphocytes without SRBC and with 1, 2, and ≥ 3 SRBC attached. Lymphocytes with 3 or more SRBC attached were considered to be E-rosettes.

Results. In normal volunteers E-rosettes ranged from 42 to 79% by hemocytometer and from 40 to 68% by Hemaprep method (Table I). The range of E-rosettes in patients with CLL was 11 to 36% by hemocytometer and 11.4 to 28% by Hemaprep method (Table I). The E-rosettes from the patient with Sézary syndrome as obtained by automatic smearing technique (59.6%) are shown in Fig. 1 (magnification 186) and Fig. 2 (750×).

The rosettes from manually prepared slides were not enumerated as there was considerable disruption of rosettes.

Discussion. Quantitation of B- and T-lymphocytes has proven to be of value in the study of immune diseases (5). E-Rosettes are commonly determined in the cell suspension using a hemocytometer. E-Rosettes in the normal human peripheral blood have ranged from 5 to 80% as reported by various investigators (2, 6, 7). Fragile nature of E-rosettes is one of the factors responsible for this wide range of difference. These nonimmune rosettes are known to be very sensitive to physical changes such as temperature, pH of the medium, incubation period, and trauma of manipulation during the preparation for counting (8–11).

A manual smearing technique has been described to prepare fixed and stained preparations of E-rosettes (4). In our hands considerable disruption of rosettes occurred during manual smearing, resulting in reduced number of E-rosettes as compared to the generally accepted hemocytometer method. The difference in the individual manual

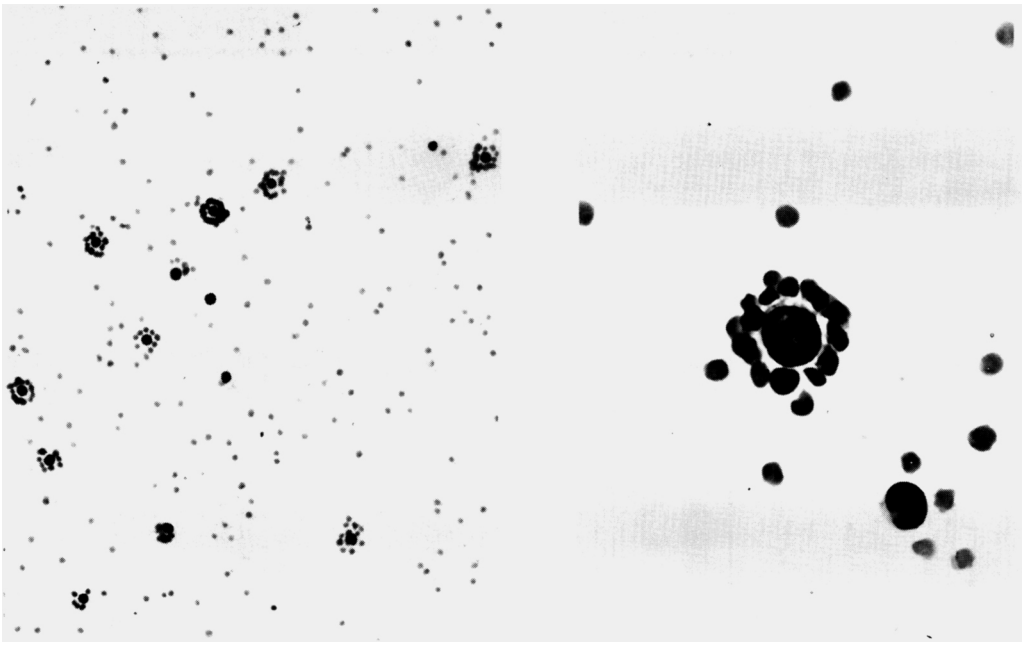

FIG. 1. This photograph shows rosette-forming and nonrosette-forming lymphocytes from a smear made by automatic smearer. This preparation is from the blood lymphocytes of a patient with Sézary syndrome. (186×)

FIG. 2. Rosette-forming cell from the same preparation as shown in Fig. 1 at higher magnification (750×). Typical convoluted nucleus of Sézary cell is clearly identified.

smearing technique, angulation of the smearing slide, and the size of the drop of the suspension containing E-rosettes could each be contributing to the destruction of the rosettes. Cytocentrifugation, an alternate to manual smearing, may also produce changes in the apparent proportion of E-rosettes and was not recommended by a recent report from a World Health Organization sponsored workshop (12).

In order to eliminate the individual variations of manual smearing and the disruptive changes produced by manual or cytocentrifugation techniques, we investigated the use of Hemaprep for preparation of fixed smears of E-rosettes. The results obtained were reproducible and comparable to the hemocytometer method. The smears made with the automatic smearer showed no disruption of E-rosettes and the morphological features of the rosettes-forming cells could be clearly identified (Figs. 1 and 2).

Summary. Human T-lymphocytes were enumerated in 10 normal volunteers and 4 patients by a new method utilizing an automatic smearing instrument to prepare permanent fixed slides of E-rosettes. The results were compared to the commonly employed hemocytometer method.

Results obtained smearing instrument were similar to those obtained by the hemocytometer method with the added advantages of permanent record, convenience of quantitation, and precise morphological identification of rosette-forming cells. As opposed to well-preserved E-rosettes obtained by the automatic smearing instrument, hand-made smears showed disruption of E-rosettes.

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Received January 7, 1976. P.S.E.B.M. 1976, Vol. 152.