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A New Method for Separating Peritoneal Lymphocytes from Macrophages

T. G. PRETLOW AND NEELAVATHY PUSHPARAJ

Rutgers Medical School, Department of Pathology, New Brunswick, New Jersey 08903, *U.S.A.*

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Summary. A simple method has been developed for separating sterile, viable mouse peritoneal lymphocytes from suspension of peritoneal cells using velocity sedimentation. Lymphocytes are obtained in 98·6–99·2 per cent purity; macrophages in 85·4–95·0 per cent purity.

INTRODUCTION

Boone, Harell and Bond (1968) suggested that a computer-generated, trapezoidal integration of the differential sedimentation equation might be useful in predicting the experimental conditions which would permit separation of cells differing with respect to density and/or diameter on gradients of Ficoll (polysucrose) in tissue culture medium; they demonstrated that cells exclude trypan blue and retain motility following separation on such gradients. It was subsequently shown that cell sedimentation is accurately described by the differential sedimentation equation (Pretlow and Boone, 1969a) and the technique for using the computer program in carrying out cell separation experiments under sterile conditions was described in detail. This technique has been used in the separation of malignant cells from disaggregated solid tumours (Pretlow *et al.*, 1970a), human leukaemia cells from peripheral blood (Abeloff, Mangi, Pretlow and Mardiney, 1970) and rat mast cells from each other according to degree of maturational differentiation (Pretlow and Cassady, 1970b). Cells have been grown in tissue culture, and tumour cells have been successfully transplanted into isogenic mice following cell separations on gradients of Ficoll in tissue culture medium (Pretlow *et al.*, 1968).

Normal peritoneal cells and peritoneal exudate cells from mice (Argyris, 1968), guinea-pigs (George and Vaughn, 1962), and rats (Koster and McGregor, 1970) are being used in the study of immune and inflammatory processes. The specific roles of individual cell types in normal cell physiology and in the pathogenesis of disease are being better defined as improved methods for studying individual types of cells are developed. We now report a method for obtaining sterile, viable purified lymphocytes from mouse peritoneal cell suspensions.

MATERIALS AND METHODS

Ficoll (polysucrose) was obtained from Pharmacia Fine Chemicals, Piscataway, N.J. Joklik's modification of minimum essential medium was purchased from Grand Island

Correspondence: Dr T. G. Pretlow, Departments of Pathology and Engineering Biophysics, University of Alabama in Birmingham, Birmingham, Alabama 35233, U.S.A.

Biological Company, Grand Island, New York. C57 Black mice were obtained from Jackson Laboratories, Bar Harbor, Maine.

Cell sample suspension. Two- to three-month-old, male, C57 black mice were killed by exanguination under ether anaesthesia. For each experiment, suspensions of $10–16 \times 10^6$ normal peritoneal cells were obtained by irrigating the peritoneal cavities of five mice with two successive 1 ml aliquots of Joklik tissue culture medium containing 10 per cent foetal calf serum and 1 unit/ml heparin. Peritoneal washings were kept in an ice bath while cell counts were performed. Differential counts were made counting 500 cells from the peritoneal washings used in each experiment.

Density gradients and centrifugation. Detailed accounts of the methods which are used (Pretlow *et al.*, 1969a) and the theory of gradient centrifugation as applied to mammalian cells (Boone *et al.*, 1968; Pretlow *et al.*, 1969a; Pretlow, Boone, Shrager and Weiss, 1969b) have been published previously and will therefore be omitted from this report.

Isopycnic centrifugation was performed by centrifuging at 600 ***g*** (measured at the sample-gradient interface 13·7 cm from the centre of rotation) for 2 hours at 4·0° using a 13 cm, 4–48 per cent (weight/volume), linear density gradient of Ficoll in tissue culture medium. Gradients for both isopycnic and rate-zonal experiments were contained in 100-ml polycarbonate centrifuge tubes (I.E.C. No. 2806). Optimal gradient design, speed of centrifugation, and duration of centrifugation for the rate-zonal purification of these cell types were determined as described previously (Pretlow *et al.*, 1969b). Rate zonal centrifugation was carried out by centrifuging at 55 ***g*** (measured at the sample-gradient interface 13·7 cm from the centre of rotation) for 30 minutes using a 13 cm, 2·7 to 5·5 per cent (weight/volume), linear density gradient of Ficoll in tissue culture medium.

Collection and counting of fractions. Gradients were collected in twenty-two 4-ml fractions. Cell counts were performed on all fractions using haemocytometer chambers. Refractive indices were measured for all fractions and were used to determine the density of each fraction (Pretlow *et al.*, 1969a). Microscope slides from all fractions were prepared using the cytocentrifuge (Shandon Scientific Company, Sewickley, Pennsylvania) and were stained with the Wright-Giemsa staining technique. Differential counts were made counting 500 cells from each gradient fraction. Viability was assessed using trypan blue exclusion counting 200 cells from each fraction.

RESULTS

Sample composition. From the two successive 1 ml aliquots of tissue culture medium which were used for each of five mice (total of 10 ml), 5·4–6·8 ml were recovered from their peritoneal cavities containing $10–16 \times 10^6$ cells. The cell suspensions comprised $35·0 \pm 2·63$ per cent lymphocytes, $62·7 \pm 2·82$ per cent macrophages, $1·0 \pm 0·1$ per cent mast cells, and $0·3 \pm 0·1$ per cent polymorphonuclear neutrophils (Fig. 1). Greater than 98 per cent of these cells excluded trypan blue. After using small aliquots of the pooled cell suspension for the preparation of microscope slides and haemocytometer counts, the sample volumes were made up to 7 ml and layered over the density gradients.

Isopycnic separation. Isopycnic centrifugation was not an effective method for purification. In isopycnic centrifugation experiments, 84–92 per cent of all cells were recovered in two successive, 4-ml gradient fractions from the 85-ml gradient (corresponding to a density of 1·095 to 1·117 g/ml). Neither lymphocytes nor macrophages were recovered in greater than 69·8 per cent purity following isopycnic centrifugation.

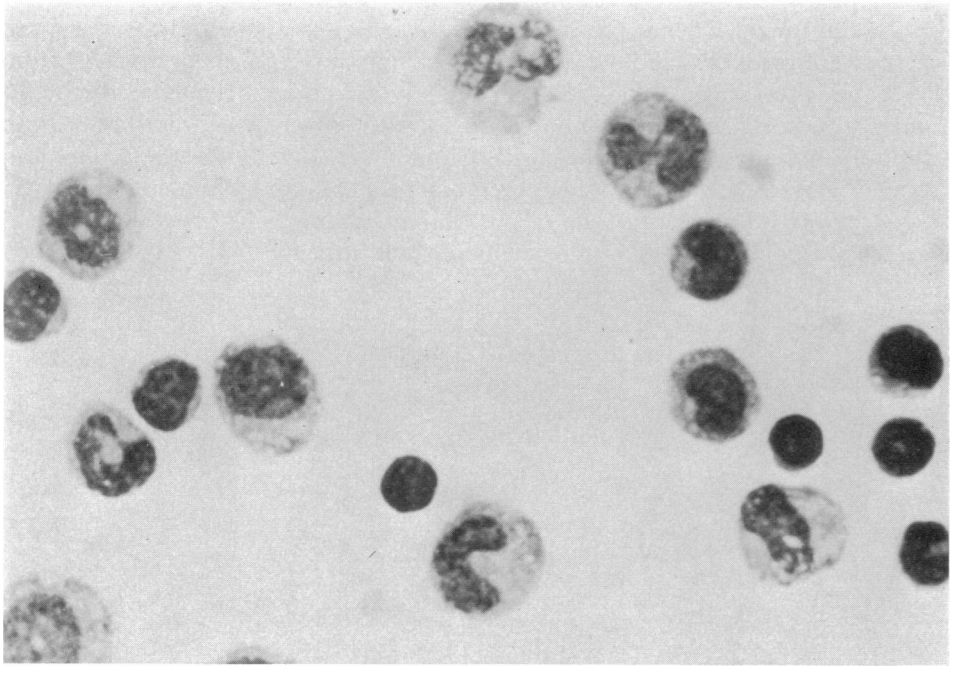

FIG. 1. Sample cell suspension which was layered over the gradient. Wright-Giemsa stain, × 360.

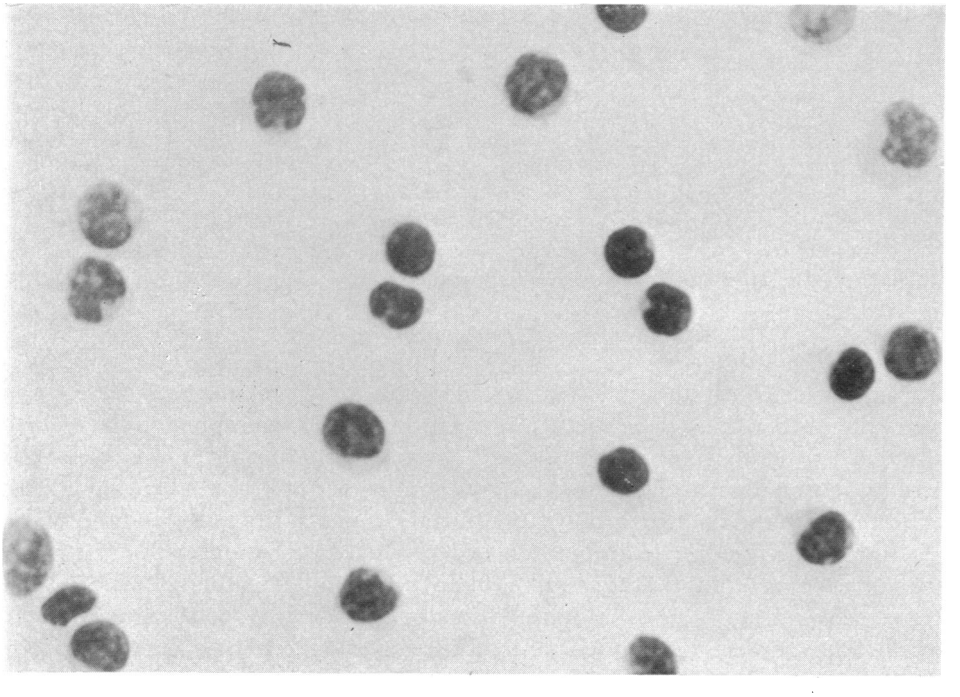

FIG. 2. Purified lymphocytes from rate-zonal separation, fraction 10. Wright-Giemsa stain, × 360.

Rate-zonal separation. Rate-zonal sedimentation proved to be a very effective method for separating macrophages from lymphocytes. In five repeated rate-zonal separations, lymphocytes were recovered in 98·6–99·2 per cent purity (Fig. 2). Maximum lymphocyte purity for each experiment (98·6–99·2 per cent lymphocytes) occurred in the 10th or 11th 4-ml fraction from the 85-ml, 13-cm, linear gradient. Fractions 10 and 11 always contained greater than 97·4 per cent lymphocytes (Fig. 2). Maximum macrophage purity ranged between 85·4 and 95·0 per cent macrophages and was located in fraction 18 (Fig. 3). More than 98 per cent of the cells in the purest fractions (fractions 10, 11, 17, 18 and 19) were viable as assessed by trypan blue exclusion.

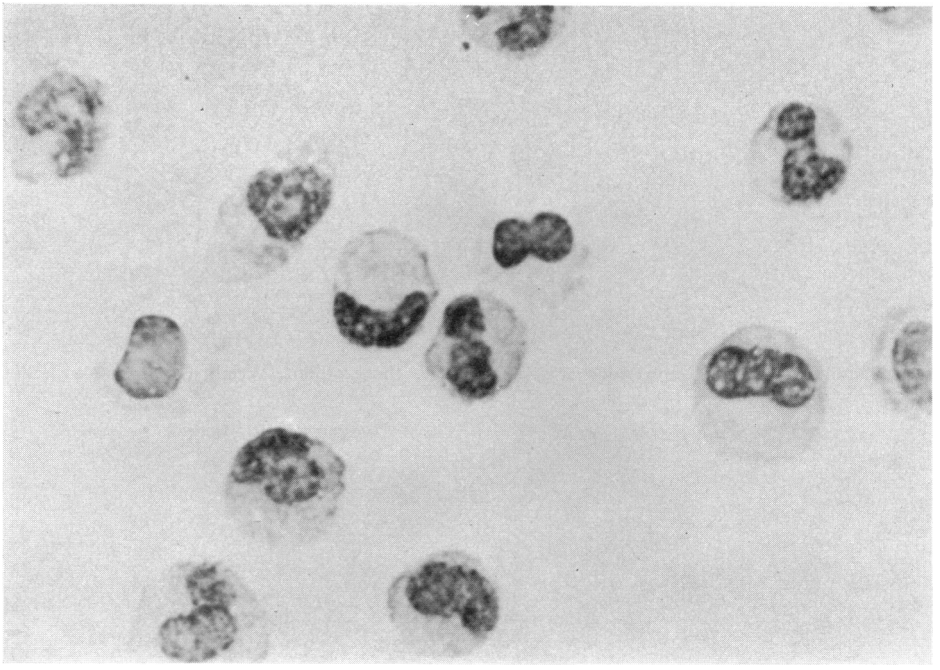

FIG. 3. Purified macrophages from rate-zonal separation, fraction 18. Wright-Giemsa stain, ×360.

DISCUSSION

Macrophages are commonly purified by exploiting their propensity to adhere to plastic and glass surfaces (Bloom and Bennett, 1966; Mosier, 1967). Lymphocytes are purified by the passage of leucocyte rich plasma through columns of nylon fibres (Cooper, 1968), the passage of bone marrow suspensions through columns of glass wool (Cudkowicz, Bennett and Shearer, 1964), and collection of lymph from the thoracic duct (Koster and McGregor, 1970). Antigen-coated columns are used in order to selectively remove immune cells from cell suspensions (Wigzell and Anderson, 1969; Abdou and Richter, 1969). Each of these separation techniques selects a subpopulation of the desired cell type by utilizing a specific cell characteristic, i.e. propensity to adhere to glass or plastic, immune status, etc.; none results in fractions of cells having 100 per cent purity. Sequential combinations of these techniques should allow the investigator to approach 100 per cent purity more

closely. The present report is, to our knowledge, the first reported use of a difference in diameter in the separation of purified, viable, sterile lymphocytes from peritoneal cells. The technique is recommended by the relatively innocuous centrifugal force (55 ***g***) employed, the capacity for maintaining a sterile procedure (Pretlow *et al.*, 1968), the use of a cell parameter (diameter) which selects for a different subpopulation of cells than is selected by other methods for cell purification, and the viability of several kinds of cells which have been grown in animals and tissue culture following rate-zonal sedimentation in Ficoll (Pretlow *et al.*, 1968). This technique should facilitate kinetic investigations of cell interactions during induction of antibody synthesis *in vitro* (Fishman, 1969) by permitting the investigator to recover pure lymphocytes from the mixture of cell types at specific times following the addition of antigen; it can be used to elucidate the roles of specific cell types in the transfer of immunity to spontaneous mammary carcinoma by peritoneal cells (Baldwin and Embleton, 1969).

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