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A method for isolation of rat lymphocyte-rich mononuclear cells from lung tissue useful for determination of nucleoside triphosphate diphosphohydrolase activity

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ABSTRACT

Methods for the isolation of peripheral blood mononuclear cells (PBMCs) and human lung mononuclear cells (LMCs) have been proposed previously. This study describes a method that allows the separation of lymphocyte-rich LMCs from rats. Trypan blue was applied to determine cell viability. White blood cell and differential cell counts were also performed. Relationships between nucleoside triphosphate diphosphohydrolase (NTPDase, EC 3.6.1.5) activities expressed in milligrams of protein, millions of cells, and millions of viable cells were examined as linear correlations. The lung tissue yielded 82.46% lymphocytes, 8.6% macrophages, 2.20% monocytes, and 1.27% polymorphonuclear cells (PMNs). In LMCs, a very strong correlation was observed as follows: between NTPDase activity, as determined using ATP or ADP as a substrate, expressed in milligrams of protein and that expressed in millions of cells ($r \geqslant 0.91$), between that expressed in milligrams of protein and that expressed in millions of viable cells ($r \geqslant 0.91$), and between that expressed in millions of cells and that expressed in millions of viable cells ($r \geqslant 0.98$). Based on our results, we affirm that NTPDase activity could be expressed in millions of viable cells, millions of cells, or milligrams of protein.

Airways are often exposed to pathogens, dust particles, sulfur dioxide, and cigarette smoke. Despite adaptations, such as pseudostratified columnar ciliated epithelium with goblet cells, some antigens also get into the small airways. The lung cells involved in innate immunity are activated neutrophils (polymorphonuclear cells [PMNs]),[1] alveolar macrophages (AMs), and eosinophils (Eos), but during the establishment of a chronic infection, acquired immunity, which is characterized by recruited lymphocytes, is activated [1].

Lymphocytes have an important role in the development of lung diseases, such as emphysema and chronic obstructive pulmonary disease (COPD) [2,3], and are responsible for preventing infection from spreading [4]. One of the ways in which lymphocyte function is regulated is through a family of enzymes called ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases), which catalyze the sequential dephosphorylation of nucleoside triphosphates to nucleoside monophosphates (ATP → ADP → AMP) [5,6]. These nucleotides (ATP, ADP, and AMP) are involved in the regulation of immune defenses and serve as a mean of communication among immune cells in lymphoid organs; lymphocytes are a well-known recipient of nucleotide signaling [5,7]. Some authors have demonstrated the inherent role of ecto-nucleotidases in airway diseases [6] as well as the involvement of lymphocytes in some immune system diseases and host–pathogen interactions [8–11].

Because lymphocytes are involved in the management of pathologies and in triggering the immune response [12–18], the development of a rapid and adequate method that allows the separation of a rich lymphocyte phase from lung tissue is essential. For this purpose, using a combination of approaches [19,20]

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[1] *Abbreviations used:* PMN, polymorphonuclear cell; AM, alveolar macrophage; Eo, eosinophil; COPD, chronic obstructive pulmonary disease; E-NTPDase, *ecto*-nucleoside triphosphate diphosphohydrolase; WBC, white blood cell; FS, physiological solution; EDTA, ethylenediaminetetraacetic acid; PBMC, peripheral blood mononuclear cell; P_i, inorganic phosphate; TCA, trichloroacetic acid; LMC, lung mononuclear cell.

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and experiments, we have developed a novel method for isolating rat lung lymphocytes. To verify the efficacy of this new assay in maintaining viable cells and achieving purity, cell integrity, white blood cell (WBC) counts, and differential cell counts were assessed.

Materials and methods

Chemicals

Nucleotides, Coomassie Brilliant Blue G, and trypan blue solution were obtained from Sigma Chemical (St. Louis, MO, USA). Ficoll–Hypaque (Lymphoprep) was purchased from Nycomed Pharma (Oslo, Norway). Physiological solution (0.9 g NaCl/100 ml distilled water) was obtained from Fresenius KABI. All other reagents used in the experiments were of analytical grade and the highest purity.

Animals

Male Wistar rats of 200–300 g bodyweight were used for all of the experiments, which were performed in accordance with the guidelines of the Committee on Care and Use of Experimental Animal Resources and in accordance with international guidelines.

Lung tissue preparation and procedures

A total of 10 rats were used to isolate lung lymphocytes. Lungs from 2 rats were pooled (4 g) to obtain a sufficient amount of cells and to attain a final suspension of 0.1–0.2 mg of protein. After excision, the specimens were washed with physiological solution (FS), transferred to Petri dishes with 12 ml of FS at 0 °C, and then chopped using scissors. The cell suspension was then filtered through a 1.5-mm nylon strainer. The resulting filtrate was centrifuged (Sigma Laborzentrifugen 4K15) for 10 min at 252.45*g*, and the supernatant was discarded. The pellet was suspended with FS to obtain a final volume of 6 ml. To purify the suspension, the homogenate was carefully layered over 4 ml of a standard Ficoll–Hypaque gradient in another tube and immediately centrifuged for 30 min at 417.31*g*. The cell interface layer, which was composed of mononuclear cells, was carefully harvested and transferred to another tube. A 10-ml volume of FS was pipetted over this sample, which was then homogenized and centrifuged for 10 min at 289.8*g*. The supernatant was discarded, and the pellet was resuspended with 5 ml of hemolytic buffer–ethylenediaminetetraacetic acid (EDTA)–ammonium chloride and centrifuged for 10 min at 128.8*g*. The supernatant was discarded, and the pellet was resuspended with FS to a final protein concentration of 0.1–0.2 mg/ml.

Isolation of mononuclear cells from blood

Peripheral blood mononuclear cells (PBMCs) were isolated from rat peripheral blood collected with EDTA and separated on Ficoll–Hypaque density gradients as described previously [19].

Cell integrity

Cell membrane integrity was determined by trypan blue dye exclusion [21]. In sum, 1 part of 0.4% trypan blue and 1 part cell suspension were mixed and allowed to incubate for approximately 3 min at room temperature. A binocular microscope (Olympus CX40 RF200) was used to count the unstained (viable) and stained (unviable) cells.

WBC count and differential cell count

WBC counts were performed using a microscope counting chamber, the Neubauer hemocytometer (Brand, Wertheim, Germany). Differential cell counts of Giemsa-stained cytocentrifuge (FANEM 216) preparations were performed using overall morphological criteria, including differences in cell size and nuclear shape.

NTPDase activity assay

After the isolation of lung lymphocytes and other mononuclear cells, NTPDase activity was determined as described previously by our group [22], measuring the amount of liberated inorganic phosphate (P_i) using a colorimetric assay. The reaction medium contained 0.5 mM $CaCl_2$, 120 mM NaCl, 5 mM KCl, 60 mM glucose, and 50 mM Tris–HCl buffer (pH 8.0) in a final volume of 200 μl. Then 20 μl of intact mononuclear cells suspended in saline solution was added to the reaction medium (2–4 μg protein) and preincubated for 10 min at 37 °C. The reaction was started by adding the substrate (ATP or ADP) at a final concentration of 2 mM and was stopped with 200 μl of 10% trichloroacetic acid (TCA) to provide a final concentration of 5%. The samples were chilled on ice for 10 min before assaying the release of P_i as described previously [23], using malachite green as a colorimetric reagent and KH_2PO_4 as a standard. Light absorbance was measured at 630 nm in a spectrophotometer (Biospectro SP-22). Control reactions were performed by adding the enzyme preparation after the addition of TCA to correct for nonenzymatic nucleotide hydrolysis. All samples were run in triplicate, and the specific activity is reported as nanomoles of P_i released per minute per milligram of protein.

Protein determination

Protein was measured by the Coomassie blue method as described previously [24]. Briefly, a solution of Coomassie (117 μM Coomassie, 0.85 M ethyl alcohol, and 1.46 M ortho-phosphoric acid) was prepared. A standard curve with bovine serum albumin varying from 0.1 to 0.5 mg of protein per milliliter was performed. To quantify the protein content, 50 μl of sample was added to 2.5 ml of Coomassie solution and, after 5 min, the absorbance was read in a spectrophotometer (Biospectro SP-22) at 595 nm.

Statistical analysis

The relationships between NTPDase activities expressed in milligrams of protein, millions of cells, and millions of viable cells were examined as linear correlations using Pearson's correlation coefficient.

Results

Cell integrity

Trypan blue staining showed that between 89.5 and 98.0% of the blood mononuclear cells were viable and that between 77.0 and 98.5% of the lung mononuclear cells (LMCs) were viable (Table 1).

WBC count and differential

After the isolation of mononuclear cells, we found that the WBC count ranged from 2.60 to 7.27 $\times$ 10^6 cells in PBMCs and from 1.80 to 5.05 $\times$ 10^6 cells in LMCs (Table 1). For the differential cell counts of Giemsa-stained cytocentrifuged preparations (Fig. 1), we observed percentages of 93.0% lymphocytes, 4.2% monocytes, and