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Cytochemical Properties of Earthworm Coelomocytes Enriched by Percoll

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Abstract: Coelomocytes of *E. fetida* were separated by Percoll gradient and based on cytomorphology and cytochemistry, classified into four major categories: acidophils, basophils, chloragocytes cells and neutrophils. Basophils exhibited heterogeneity with respect to staining properties of granules. The enzyme acid phosphatase was present in all coelomocytes, but was especially abundant in basophils and neutrophils. Alkaline phosphatase was detected in basophils and acidophils and α-esterase was found in all types except neutrophils. Acidophils and basophils possessed the corresponding granules and neutrophils contained both; acidophilic granules are often excreted. Basophils and neutrophils were more active in killing the tumor target, K562, which partially reflects their role in the earthworm's immune system.

Key words: Earthworm, enzyme, Percoll, coelomocyte, immunity

INTRODUCTION

The earthworm's immune system is complex, composed of diverse cell types referred to as coelomocytes (leukocytes)[1]. These cells are suspended in Coelomic Fluid (CF), which contains humoral immune components (e.g. lysins, agglutinins) that are synthesized and secreted by coelomocytes. They display certain surface markers[2,3]. Coelomocytes have been separated into two major functional cell types by FACS basophils and lymphocytic coelomocytes[4-6]. Basophils, small in size and identified by light microscopy[7,8] are lymphocytic coelomocytes identified by electron microscopy and participate in graft rejection[9]. Transplant destruction is initiated by inflammatory type, large neutrophilic coelomocytes[10] that form granulomas *in vitro* after the tumor K562 by small basophils[6].

Despite these and other characteristics of coelomocytes, there have been no systematic attempts to develop methods for enriching and purifying any single coelomocyte type. Successful enrichment could then be used to analyze separate cell types with respect to their unique function as well as functional cell interactions. In this study we have combined three approaches that characterize coelomocytes: 1) Percoll separation; 2) cytochemistry; 3) ^{51}Cr-release assay (cytotoxicity) which confirms that two of the enriched cell types at certain stage are actively involved in the killing of K562 tumor target cells.

MATERIALS AND METHODS

Earthworms and husbandry: Adult *Eisenia foetida* were purchased from Carolina Biological Supply Company (Burlington, North Carolina) and maintained at a constant temperature of 15°C in plastic boxes. Three days prior to harvesting coelomocytes, earthworms were fasted and kept in plastic boxes on wet paper towels. After coelomocyte extrusion earthworms were further maintained in plastic boxes in a moist environment and fed oatmeal cereal.

Coelomocyte harvesting: Donor earthworms were first cleansed in distilled water and dried on paper towels. They were handled with care to prevent premature extrusion of coelomocytes, which results in lower yields. Several earthworms (~3) were then placed in small Petri dishes (60x15 mm) containing 3 mL of PBS with 0.25 mM EGTA to prevent coelomocyte aggregation. Extruded of coelomocytes was achieved via electrical stimulation (6 V)

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of earthworms and cells were transferred into conical glass centrifuge tubes previously "Sigmacote-Coated" (Sigma) to avoid cell adherence.

Coelomocyte separation by Percoll gradient: Percoll (Pharmacia) was used as a cell separation media and was diluted with 0.15 M NaCI to the following concentrations (55-45, 35-25 and 15-5%). Two (2) mL of each concentration (55- 45, 35-25 and 15-5%) was carefully layered into a test tube to build a six-step gradient. Coelomocyte suspensions (3 mL in PBS) were transferred onto the Percoll gradient and centrifuged for 20 min at 1500 rpm, yielding four separate coelomocyte bands. The cell free supernatant was removed and transferred from each gradient using Pasteur pipettes into fresh, Sigma coated test tubes. Coelomocytes obtained from the respective bands in the Percoll gradient were pooled since they contained the same cell type. Coelomocytes were then washed twice in PBS (10 min at 1500 rpm) before further use.

Cytocentrifugation and wright stain preparations: Percoll separated coelomocytes were placed on microscopic slide (Shadon cytoslides) for marking cytocentrifuged-stained preparations. Six to eight drops ($5x10^5$ coelomocytes/mL) of resuspended coelomocytes were added to the cytofunnels (Shadon), mixed with one drop of albumin, centrifuged for 5 min at 800 rpm and air-dried. After fixation in methanol for 1 min, the slides were transferred to a differential solution (Baxter B4 132-12) for 3 min, then rinsed in tap water for 10 min and stained with Giemsa for 5 min. Finally, they were rinsed by immersing them in tap water 10 times, air dried and mounted in Canada Balsam.

Cytochemical analyses to demonstrate enzymes

Acid phosphatase: A sigma assay kit, for demonstrating acid phosphatase in leukocytes was used. According to instructions (Sigma No. 387), coelomocytes on slides were incubated in a solution containing Naphthol AS-BI phosphoric acid and freshly diazotized in fast garnet GBC at 37°C for 45 min. Controls were done without adding substrate solution.

Alkaline phosphatase: Alkaline phosphatase was demonstrated by using a Sigma assay kit designed for leukocytes (Sigma procedure No. 86). To perform this assay, fixed coelomocytes on slides were incubated at 37°C for 45 min in a solution containing Naphthol AS-BI phosphate and freshly prepared fast blue BB salt at pH 9.5 with 2-amino-2-methyl-l, 3- propanediol (AMPD). Sites of phosphate activity appeared as blue granules. Controls were performed by adding PBS instead of substrate.

Naphthol AS-D chloroacetatesterase and alpha naphthyl acetate esterase: The presence of Naphthol AS-D chloroacetate esterase and alpha naphthyl acetate esterase were investigated using fixed coelomocytes on slides (Sigma kits). Enzyme detection was performed according to Sigma procedure 91 and controls without adding substrate.

Determination of cell and nuclear size: Cell size as well as nuclear diameter was measured with a calibrated ocular scale (10x), using a Zeiss microscope (40x) objective and a microcytometer (American Optical).

^{51}Cr Release Assay (Cytotoxicity): As one single assay, which indicates normal coelomocyte function, we measured the cytotoxic activity of coelomocyte effectors derived from Percoll gradients against K562 tumor cell targets in a classical 4 h ^{51}Cr release assay. Freshly collected coelomocytes from each Percoll band were resuspended at a concentration of $1x10^6$/mL in PBS and maintained in complete media (RPMI 1640 + 5% FBS +1% antibiotic and 1% anti mycotic). Fifty micro liter of ^{51}Cr was added to 100 µL of K562 ($1x10^6$) in complete media, incubated for 1 h at 37°C and washed three times with RPMI 1640. One hundred microliter of effector cells (coelomocytes) were added per well on microtiter plates to 100 µL of labeled K562 target cells. After effectors and targets were added to the wells, the microtiter plate was centrifuged (5 min, 200 rpm). Effector and targets were allowed to interact at 37°C for 4 h in an atmosphere of 5% $C0_2$ / 95% air. ^{51}Cr release was determined by centrifuging the plates at 1000 g for 5 min and harvesting 100 µL of the culture supernatant for later counting in a Gamma counter (Beckman G50). Spontaneous release was determined by adding 100 µL of labeled K562 in complete media and total release was determined by adding 100 µL of K562 plus triton. The percent ^{51}Cr release was determined from the experimental (R_c), spontaneous (R_s) and total (R_t) release by following the formula:

$$\%^{51}\text{Cr-release} = \frac{(R_c - R_s)}{(R_t - R_s)}$$

RESULTS

Cell separation by Percoll: Coelomocytes were separated on a Percoll gradient (55-45, 35-25 and 15-5%) by centrifugation (20 min, 1500 rpm) resulted in four separated bands visible at concentrations of 10, 25, 35 and 45% (Fig. 1). Acidophilic cells were present in the first band at 10%. The second band, at 25%, was comprised of mainly basophils and few chloragocytes, which were

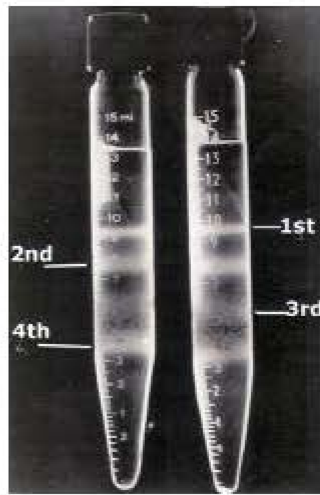

Fig. 1: Cells from the CF were separated on a Percoll gradient (55+45, 35-25 and 15-5%) by centrifugation (20 min, 1500 rpm). Four separated cell bands became visible at Percoll concentrations of 10, 25, 35 and 45%. The first bands at 10%: mainly acidophilic cells; the second (25%) mainly basophils and some chloragocytes; chloragocytes, 35% in the third band; fourth band (45%) acidophils, basophils and neutrophils. Neutrophils appeared enriched in the fourth band

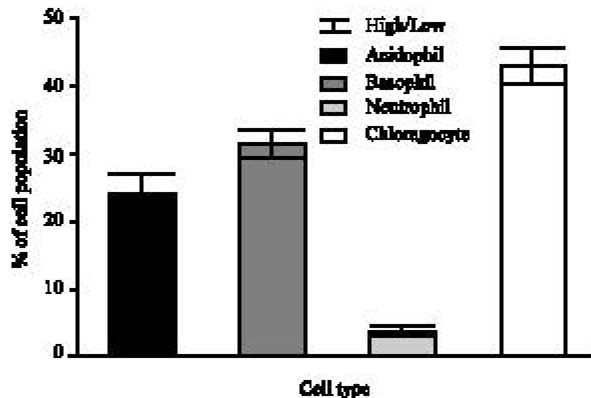

Fig. 2: Mean±SD of percentage values of each coelomocyte type. Chloragocytes show the highest, basophils second, followed by acidophils, with neutrophils showing the lowest

enriched at 35% in the third band. The fourth band, at 45%, was composed of acidophils and basophils as well as neutrophils, which displayed an enriched appearance (Fig. 1). Characteristics of coelomocytes without Percoll separation revealed varying percentages of cell and nuclear sizes for each coelomocyte type (Fig. 2-4). The percentage of coelomocytes and cell size of each type per band after Percoll separation also revealed variation (Fig. 5 and 6).

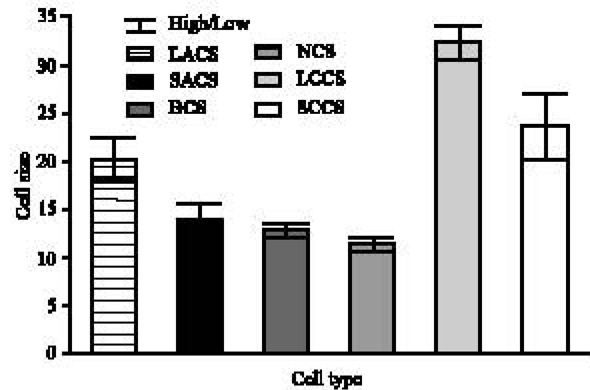

Fig. 3: Mean±SD of cell size of all coelomocytes. The two types of chloragocytes, large and small, have the largest cell size. Two acidophil cell types are next in cell size, followed by basophils and neutrophils, which are the smallest

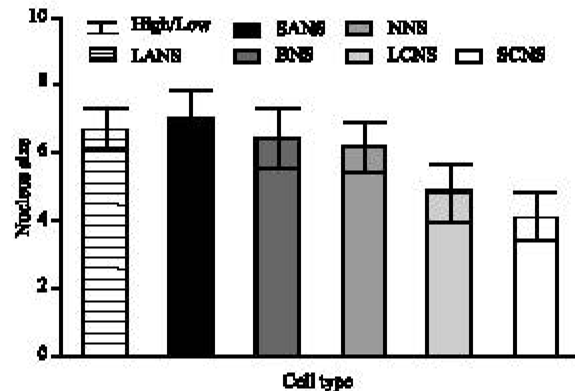

Fig. 4: Mean±SD of nuclear size of all coelomocytes. The nuclear size of both acidophil types, small and large, is larger than the nuclear size of the entire population; however, the small acidophils have larger nuclei than large acidophils. Basophils have the second largest nucleus, followed by neutrophils. The two types of chloragocytes (small and large) have more or less the same nuclear size, but they are also the smallest compared to all coelomocytes

Table 1: Acid phosphatase, alkaline phosphatase, specific and non-specific esterases activities of the general population of coelomocytes

	Types of cells			
Enzyme	Basophil	Acidophil	Neutrophil	Chloragocyte
Acid phosphatase	Intense (+++)+ve	Low to moderate (++)+ve	Moderate (++)+ve	Low (+)+ve
Alkaline phosphatase	Moderate e (++)+ve	Moderate (++)+ve	-ve	-ve
Specific esterase	-ve	-ve	-ve	-ve
Non-specific esterase (α-esterase)	Low to moderate e (++)+ve	Low to (++)+ve	-ve	Moderate (++)+ve

The acid phosphatase showed the highest intensity in basophil while the alkaline phosphatase was moderate in basophil and acidophil and the α-esterase was ranged from low to moderate in all types except neutrophil

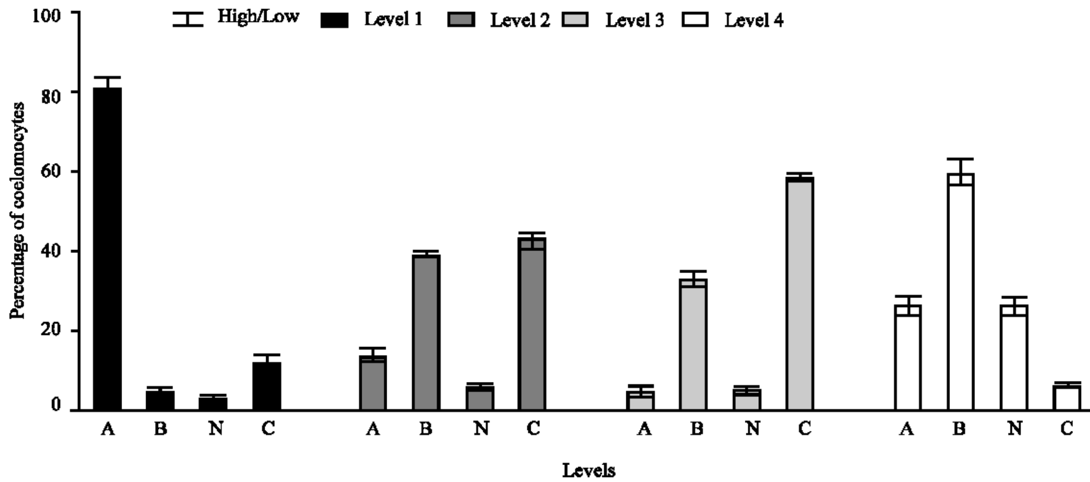

Fig. 5: Mean±SD of percentage of coelomocytes types in each fraction. The greatest percentage of acidophils was present in fraction 1, basophils in fraction 2 and 4 and neutrophils in fraction 4: most chloragocytes in fraction 3. A stands for acidophils; B stands for basophils; N stands for neutrophils; C stands for chloragocytes

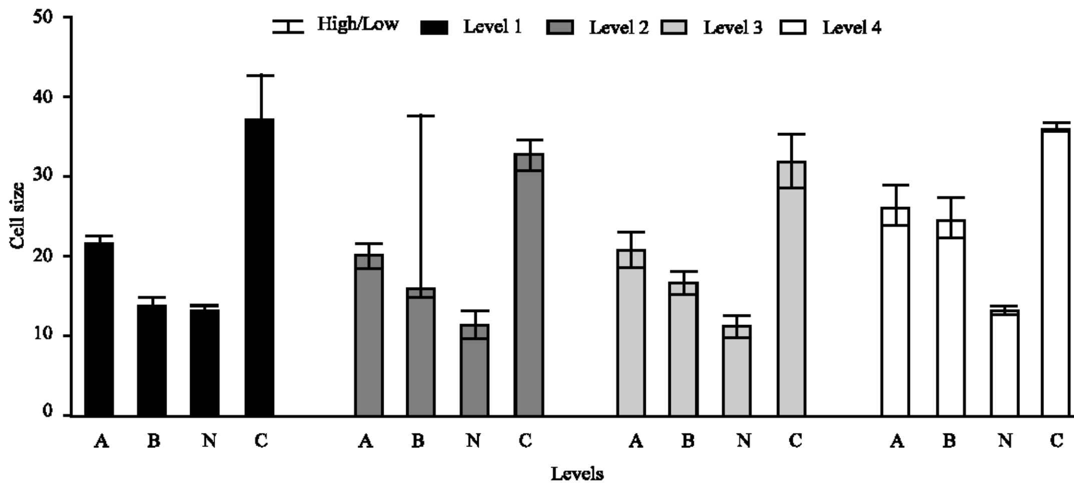

Fig. 6: Mean±SD of cell size of each fraction after Percoll-gradient separation of coelomocytes. The cell size of small acidophil was present in levels 1, 2 and 3, while the large size was in level 4. Small basophil was in level 1 and 2 and large basophil in level 3 and 4. Small neutrophil was in level 2 and 3 and the large neutrophil in level 1 and 4. Small chloragocyte was in level 2 and 3 and large chloragocyte was in level 1 and 4

Four cell types in the CF: In *E. fetida* four major coelomocyte types were differentiated: basophils, acidophils, neutrophils and chloragogen cells based on light microscopy and Wright staining (Fig. 7).

Basophils: Basophils were the most numerous coelomocyte types, staining strongly basophilic with occasional small, dark blue granules. The more abundant cytoplasm was lighter blue and clear vacuoles were frequently present. The smaller basophils, 12.8±0.8 μm, displayed a strong tendency to aggregate. With respect to the nucleus, we found it to be compact, about 6.4±0.9 μm in diameter and either centrally or peripherally located. Chromatin was condensed, stained dark blue violet and the nucleolus was not visible (Fig. 8 a-d). The cell size of large basophils was about ±21 μm and their nuclear size ±6.5 μm. We observed three enzymes in the cytoplasm: acid phosphatase was present in large amounts (Fig. 8b); alkaline phosphatase as deep blue granules of different sizes (Fig. 8g); α-esterase mainly as brown granules (Fig. 8b and Table 1).

Acidophils: Acidophils were usually granular cells with distinct outlines and usually occurred in two types based upon granules and cell size; both types stained pink to red. In type I, large cells, 20±2.3 μm, although there were few granules, still filled the cells completely (Fig. 8 e-h). The nucleus was always in an eccentric position and its

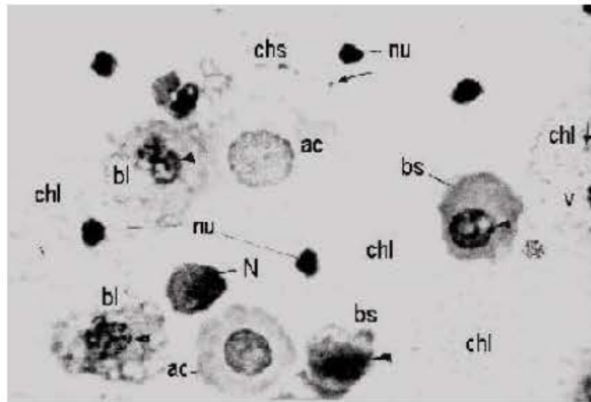

Fig. 7: All cell types obtained from *Eisenia foetida*. Nearly 50% of the whole cell population is chloragocyte, which were seen to be different sizes, small (chs) and large (chl), with a small nucleus (nu). The cytoplasm contains numerous vacuoles (v) and lipid inclusions (arrows). The next high number of coelomocytes is represented by basophilic cells, large basophil (bl) and small basophil (bs), with an eccentric large nucleus (head arrows). Neutrophils (N) appear in small numbers and size and the cytoplasm contains many granules. Notice the acidophil cell (ac). (methanol-Wright stain) (x, 400)

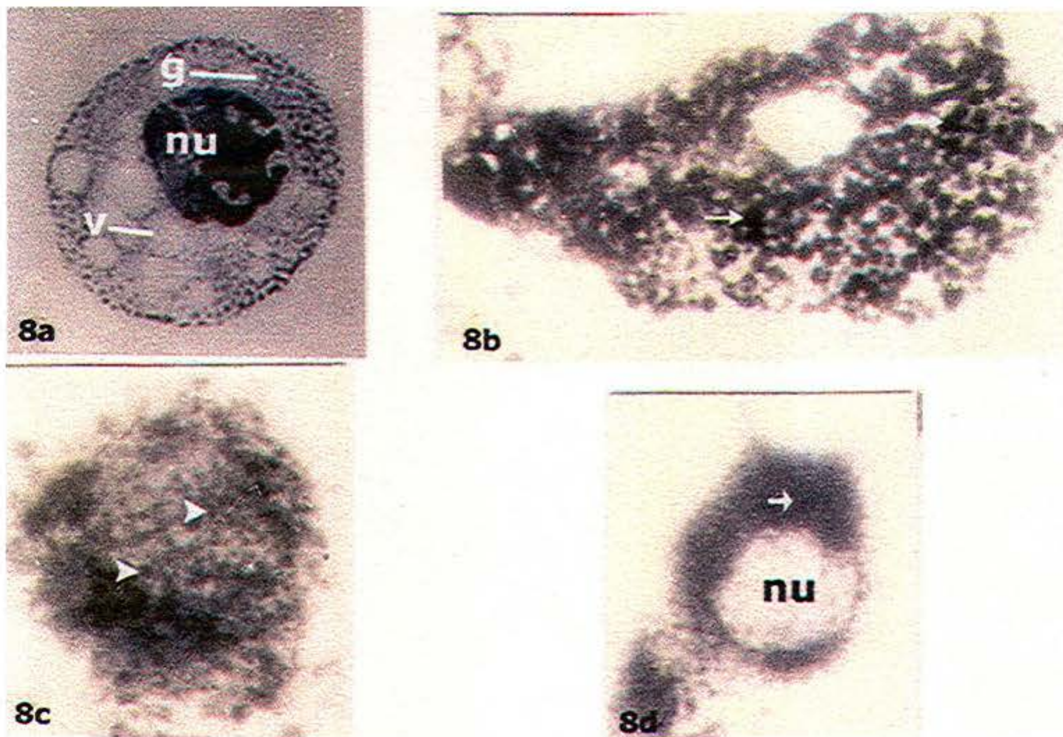

Fig. 8: a: Large basophil cell with eccentric compact nucleus (nu) with heterochromatin. The cytoplasm was strongly basophilic and a large number of granules (g) were recognized. Note the vacuole (v) (methanol-Wright stain).
b: A large deep intensely stained granules were obtained in the cytoplasm of basophil indicating an intense reaction of acid phosphatase, which was higher than the general population (arrows).
c: Alkaline phosphatase was detected as deep blue granules of different sizes (head arrows) in basophil cells.
d: A low to moderate α-esterase activity was obtained in the cytoplasm of basophil (arrow) while the nucleus (nu) was negative.

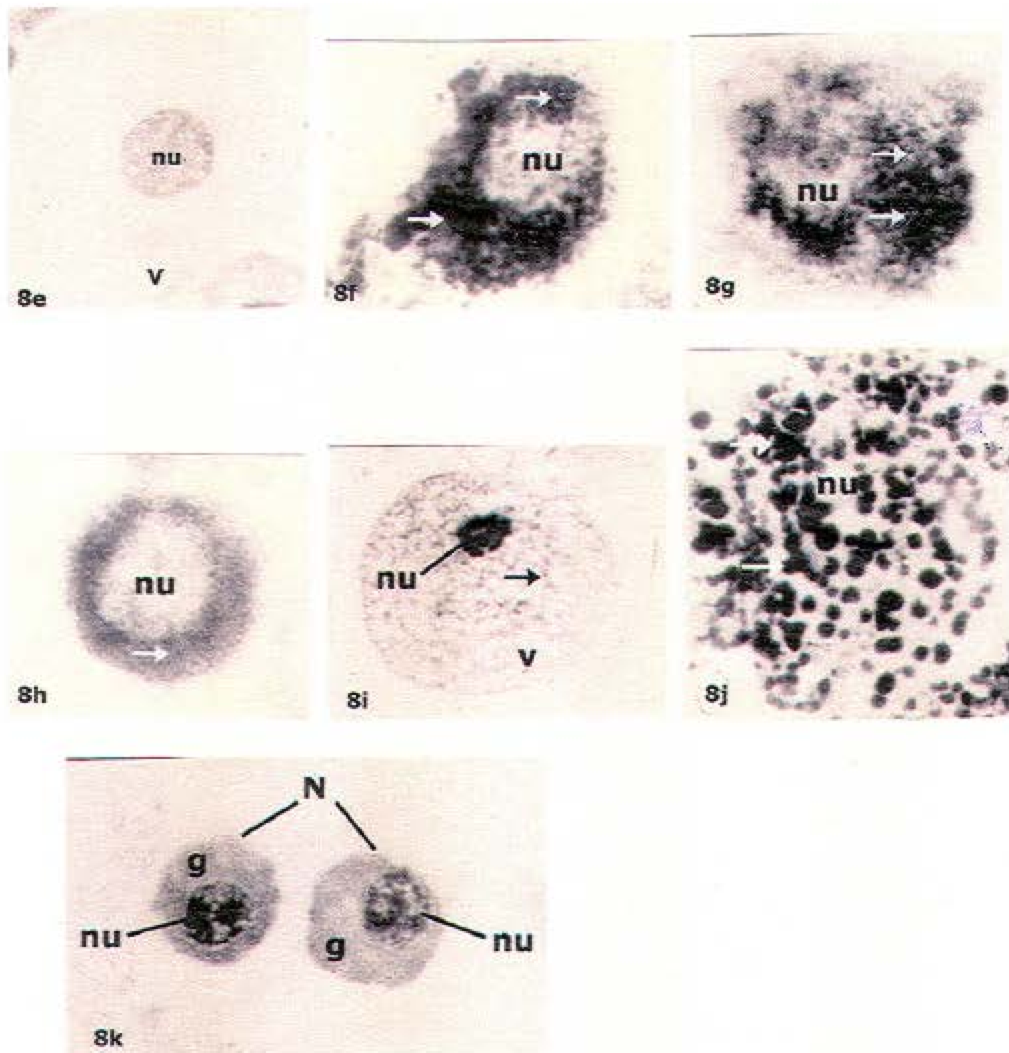

Fig. 8: e: A large acidophilic cell with a distinct cell membrane, notes the pale nucleus (nu) with eccentric position and the cytoplasm spreads with a number of vacuoles (v) (methanol-Wright stain).

f: Acid phosphatase was found as granules in low to moderate frequency in the cytoplasm (arrows), but never in large amount in acidophil cell. Notice, nucleus (nu).

g: Moderate alkaline phosphatase activity was obtained as dark blue granules in the cytoplasm (arrows), while eccentric nucleus (nu) was a negative reaction.

h: A non-specific esterase activity was obtained ranging from low to moderate in the cytoplasm of acidophil (arrow). Notice, nucleus (nu) gave no indication to enzyme activity.

I: A chloragocyte with a heterochromatin nucleus (nu) which in an eccentric position. The cytoplasm contains numerous vacuoles (v) and lipid inclusions (arrow) (methanol-Wright stain).

j: The amount of acid phosphatase in chloragocyte was much smaller than acidophil, basophil, neutrophil and was indicated as large granules (arrows) around the nucleus (nu).

k: A moderate α-esterase activity was shown in the chloragocytes as granules of different sizes (head arrows).

l: Neutrophilic cells (N) which are smaller than the other coelomocytes. They possess relatively less cytoplasm, which is characterized by numerous granules (g). The nucleus (nu) is more or less centric and relatively large compared to the cytoplasm (methanol –Wright stain). All figures x, 1000.

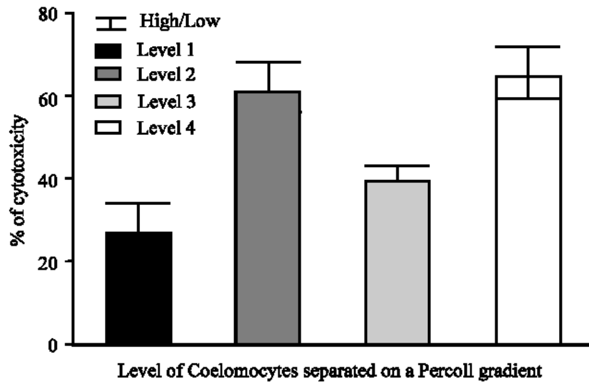

Fig. 9: Mean±SD of ^{51}Cr release assay using K562 targets and coelomocytes effectors. Coelomocytes were separated into 4 fractions using Percoll gradients. Cells from these individual fractions were then incubated together with K562 targets (4 h, 37°C) and the results were obtained with a gamma counter. Fraction 2 and 4, which were enriched mainly with basophil and neutrophil, displaced a very high percentage of cytotoxicity

size was about 6.7±0.6 μm. The cytoplasm of smaller cells (type II) I3.9±1.8 μm sometimes appeared homogeneous and without granulation whereas type II acidophils contained mostly large granules which also completely filled the cytoplasm. The nucleus (7±0.8 μm) was located either centrally or peripherally, appeared flattened. The nucleolus was not visible. Three enzymes were detected in acidophil cells: acid phosphatase in low to moderate frequency, distributed diffusely throughout the cytoplasm (Fig. 8f); alkaline phosphatase as deep blue cytoplasmic granules; the nucleus gave no indication of enzyme activity (Fig. 8g); a-esterase activity was different from low to moderate (Fig. 8h and Table 1).

Neutrophils: The most prominent characteristic of neutrophils, which were easily observed and contrasted to those in acidophils was the profusion of granules scattered throughout the cytoplasm (Fig. 8i). Neutrophils (11.3±2 μm) contained both basophilic and acidophilic granules of an intermediate color, neither red not blue. The nucleus measured 6.1±0.8 μm, stained medium to dark purple and displayed condensed chromatin. Although indication of acid phosphatase was significant, they stained less intensely. The percent of acid phosphatase in neutrophils was high and was exceeded only by that of basophils (Fig. 8f). In contrast to other coelomocytes, neutrophils contained neither alkaline phosphatase nor α-esterase (Table 1).

Chloragocytes: These cells occurred in two forms of different cell size and were sometimes arranged in clusters of four to six cells. Both types were larger than acidophils, basophils and neutrophils (Fig. 8j-l). Large chloragocytes were oblong with a cell size of 32±1.8 μm; the nucleus was 4.5±l.7 μm. The cytoplasmic granules, which were spheroid, stained bright blue with Wright-stain. Small chloragocytes 23.4±3.4 μm had circular nuclei which measured 4.1±0.7 μm in diameter. The nuclear shape appeared uneven in a peripheral position. The amount of acid phosphatase in chloragocytes (Fig. 8k) was less than in acidophils, basophils and neutrophils. There was no indication of alkaline phosphatase. α-esterase was moderately frequent in the cytoplasm as brown granules (Fig. 8 and Table 1).

Cell division: Mitotic divisions were not observed in any coelomocyte type and in all instances; the nuclei of acidophils and basophils were in the interphase stage.

^{51}Cr release assay: The effect of coelomocytes on K562 targets was observed using ^{51}Cr release assay. Coelomocytes were separated into 4 fractions using Percoll gradient and were incubated with K562 targets. Results showed an increase in ^{51}Cr release in fractions 2 and 4, which were enriched with basophils and neutrophils (Fig. 9).

DISCUSSION

We have separated earthworms' coelomocytes for the first time on Percoll gradient and revealed four bands at Percoll concentrations of 10, 20, 35 and 45% (Fig. 1). The second band was composed mainly of basophils and the fourth band at 45% Percoll appeared enriched with neutrophils. The separation procedure is rapid, reproducible and the inert nature of the Percoll along with its lack of toxicity makes it a useful medium. The lower viscosity of Percoll allows for more rapid cell isolation, without cell death, suggests that it may be superior for separating of particularly sensitive cell populations, or when for some reason cells must remain in the separation medium for long periods of time[11]. Density separations have been widely used for many immunological tests[12-19].

A method for separating of human blood monocytes and lymphocytes has been described[20]. Mononuclear leukocytes were centrifuged on a continuous gradient of colloidal silica particles (Percoll) in phosphate -buffered saline. This leads to formation of 4 bands: a layer containing dead material (if present) which did not enter the gradient; a layer near the bottom of the tube containing granulocytes and red cells and two other

bands in between, of which the upper one is enriched with monocytes (av. 78%). The final yields of these cell types were 73 and 79%, respectively and their viability is greater than 95%. No functional impairments could be detected by different functional assays including the ability of B lymphocytes to produce immunoglobulins when stimulated with pokeweed mitogen and the ability of monocytes to phagocytize opsonized red cells and latex particles. Another method for isolation of eosinophils from human peripheral blood using isomolar solution of polyvinylpyrrolidone-coated silica gel (Percoll) is described[21]. The purity ranged from 86 to 99% eosinophils in the final preparation and the recovery was 38-56%. The separation technique did not affect the viability or the metabolic capacities of the cells.

One important factor observed is the high degree of cell variability in invertebrate leukocytes. Such variability results from the high polymorphic nature of these cells and to a lesser degree, the presence of intermediate or transitional cell forms[8]. The range of variations encountered for each coelomocyte type is an important indication of the diverse functional capabilities of each cell type. All coelomocyte types display this variability to one degree or another. Basophils are occasionally heterogeneous and also contain notable amounts of cytoplasmic basophilia, which due primarily to their RNA content, in electron micrographs of basophils, large numbers of ribosomes are observed, both as free and membrane-associated forms[6,10].

In some Wright's-stained acidophils, the cells contain strongly acidophilic material, but lack observable granules[8]. This reflects a particular developmental stage in which much of the granular material has been synthesized but not yet "packaged" into discrete granules. In cytochemical preparation, acidophils of both types have been observed and appear to have unknown secretory activity. In other invertebrates, substances secreted by granular cells range from clotting factors in Limulus and several species of crustaceans to lysosomal enzymes in the mollusc *Mercenaria mercenaria*[22]. Although the coelomic fluid of Lumbricus is known to contain haemagglutinins, the specific cell responsible for synthesizing and secreting them has not been identified although specific agglutinins have been shown to be secreted in vitro in response to stimulate by rabbit erythrocytes in response to stimulation[23].

Neutrophils contain a large nucleus and less heterochromatic than those of other coelomocytes, which is a characteristic usually associated with relatively undifferentiated cells. Neutrophils are of particular interest to invertebrate immunologists. In addition to being highly phagocytic, they are responsible for the invasion and destruction of foreign tissue grafts[9]. Basophils have been found at the graft site, but they appear to play a secondary or scavenger role and are not the primary agent of graft rejection. In experiments involving chemotaxis toward foreign tissue or bacteria[24], it has been found that neutrophils, the major coelomocyte type, comprise 92-94% of the responding cells, although they comprise an average of only 18% of the total coelomocyte population[8].

Basophils and neutrophils also contain significant amounts of the enzyme acid phosphatase in discrete granules or vesicles, which are presumed to be lysosomes. Using electron micrographs, very large (4-6 μm) acid phosphatase-positive vacuoles are found in basophils and neutrophils and are thought to be phagosomes[10]. Acid phosphatase is not as abundant in acidophils as in basophils and neutrophils[8].

Chloragogen cells have been compared to the liver of vertebrates[7] and postulated to have a trophic function and two types of phospholipids. Our investigations have confirmed the presence of these substances. In electron micrographs of chloragogen cells, certain granules contain crystalline structures, which interpreted as hemoglobin[25].

Although no cell division was observed, newer findings in relation to coelomocyte multiplication have been observed during cytotoxic activity against the erythromyeloid human tumor cell line K562. In vitro cultures, two cell types (i.e. small and large coelomocytes) retained their morphological features, their FNA content was significantly less than that of human K562 and significant percentages of coelomocytes were found to be in S or G@/M phases of the cell cycle. When cultivated alone for up to three hours, coelomocytes formed no aggregates, but upon mixing with K562, coelomocytes spontaneously killed tumor cells and cytoxic reactivity was accompanied by the formation of multiple aggregates similar to granulomas. These results are described as non-specific "inflammatory" responses of earthworms in vitro against tumor cells[6].

Earthworm coelomocytes affect cytotoxicity at significantly high levels against the NK-sensitive, human tumor cell line, K562 and the NK-resistant targets (U937, BSM, OEM). Release of ^{51}Cr was weakly dependent on the effector/target ratios, decreasing from 80% lysis at 25:1 to 50% at 1:1 and the activity of earthworm coelomocytes was significantly higher than that of human PBE[5]. Using K562 as a target for earthworm effector coelomocytes suggests the possibility that, these cells kill by mechanisms akin to those of vertebrate NK cells, so that they are trivial to the immune system. Although NK cells can kill after they acquire Ig through Fc receptors on their cell surface, they also can kill cells spontaneously in the absence of Ig or any prior activation[4,26,27]. Present results

revealed that the cytotoxic activity was observed in all four levels after Percoll separation, but the highest values were obtained in levels 2 and 4, which were enriched with neutrophils and basophils.

Present results suggest that the gradients we described here demonstrate that the Percoll preparation was valuable in several types of cell separations and it was a rapid method for the separation of coelomocytes cell populations. This material is inexpensive and non-toxic for immunocyte function. Small numbers of cells with good yields has made this a routine tool in our laboratory.

In the present investigation, certain cytochemical properties of each of the different coelomocyte type separated by Percoll gradient have been described. Since these are the first cytochemical and ^{51}Cr- release observations to be made on Percoll separation of earthworm coelomocytes, a number of specific points remain unresolved: 1) the nature and the enzymatic changes that takes place in both small and large cells during phagocytosis and killing; 2) at What stage exactly, the cell start to be active in both two activities. The cytochemical information reported here should serve as a foundation for further studies those related to immune responses.

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