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**A CYTOCHEMICAL SCANNING ELECTRON MICROSCOPY STUDY OF NON-SPECIFIC
ACID ESTERASE AND ACID PHOSPHATASE ACTIVITIES
IN HUMAN PERIPHERAL BLOOD LYMPHOCYTES¹**

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Abstract

We analyzed the distribution patterns of non-specific acid esterase and acid phosphatase activities with cytochemistry-scanning electron microscopy in backscattered and secondary electron imaging modes in isolated normal human peripheral blood lymphocytes. The analysis of non-specific acid esterase activity in the backscattered electron imaging mode showed, in some cells, focal distribution with a well-defined, homogenous deposit. Two patterns of acid phosphatase activity were evident with the backscattered electron imaging mode, i.e., focal and granular. Peripheral blood lymphocytes showing focal activity of both enzymes presented a smooth surface with few scattered microvilli as seen with the secondary electron imaging mode; while lymphocytes with a granular pattern of acid phosphatase activity had abundant microvilli. The correlation between patterns of enzymatic activity as seen in backscattering electron imaging mode, and surface morphological features as seen with secondary electron imaging mode, distinguished a subpopulation of lymphocytes of T lineage in human peripheral blood.

¹Dedicated to Professor J. Gómez on his 70th birthday.

Key words: peripheral blood lymphocytes, enzyme-scanning electron microscopy, secondary electron imaging, backscattered electron imaging, non-specific acid esterase, acid phosphatase.

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Introduction

Human peripheral blood lymphocytes (PBL) have been studied from a variety of approaches in order to determine whether different types of enzymatic activity may be correlated with specific immunological subsets (Pangalis et al., 1978; Basso et al., 1980; Catovsky et al., 1981; Hayhoe and Quaglino, 1988). Studies with light and transmission electron microscopy (TEM) have demonstrated different patterns of non-specific acid esterase (NSE) and acid phosphatase (AcP) activity, described in terms of distribution and subcellular location (Ferrarini et al., 1980; Grossi et al., 1980; Zicca et al., 1981; Grossi et al., 1982; Boesen, 1984; Robinson et al., 1984). These findings have been related to certain immunological types of lymphocytes (Bevan et al., 1980; Armitage et al., 1982; Crockard and Catovsky, 1983; de Waele et al., 1983; Clement et al., 1984; Hirt et al., 1984; Landay et al., 1984; Armitage and Cawley, 1985).

Studies of normal and pathological PBL with scanning electron microscopy (SEM) have thus far not allowed clearcut correlations to be established between specific morphological surface features and given immunological subsets (Polliack et al., 1973; Alexander and Wetzel, 1975; Polliack et al., 1975; Newell et al., 1976; Roath et al., 1978; Gamliel and Polliack, 1983). The problem has been compounded by the technical procedures required to prepare blood cells for SEM (Lichtman et al., 1976; de Harven et al., 1984). The use of the secondary electron imaging (SEI) and back-scattered electron imaging (BEI) modes provides reliable criteria with which to associate morphological surface features with different cytochemical activities (Becker and De Bruyn, 1976; Albrecht et al., 1978; Becker and Sogard, 1979; Soligo and de Harven, 1981; Soligo et al., 1981). These patterns of enzymatic activity are revealed in the BEI mode after the addition of high atomic number elements to the specimen in the course of processing for SEM-cytochemistry (Soligo et al., 1983).

We set out to establish the possible correlation between morphological surface features on human mature PBL as observed with SEI mode, and patterns of NSE and AcP activity in these cells as detected with BEI mode. These enzymes were used because previous

studies have shown their effectiveness in distinguishing specific types of human T lymphocytes (Grossi et al., 1978; Bevan et al., 1980; Ferrarini et al., 1980; Zicca et al., 1981; Armitage et al., 1982; Robinson et al., 1984).

Materials and Methods

Preparation of cell suspensions

Venous blood from healthy young males was collected and diluted (1:2 v/v) in Hank's balanced salt solution (HBSS, Gibco Laboratories, UK). Mononuclear cell (MC) suspensions were prepared by Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden) density gradient (d: 1.077 g/ml) centrifugation for 30 minutes at 400 g (Boyum, 1968). The MC thus obtained were washed three times in HBSS (5 minutes, 300 g, room temperature) and resuspended at a final concentration of 3.5×10^6 cells/ml. Monocytes were partially depleted by incubation for 60 minutes at 37°C in cell culture flasks (Nunc, Nunclon, Denmark).

SEM-cytochemistry

The nonadherent MC - peripheral blood lymphocytes - were prefixed in 0.1% glutaraldehyde (EM grade, Merck, FRG) in 0.1M cacodylate buffer, pH 7.2, for 10 minutes at room temperature.

NSE activity was demonstrated by a modification of the method proposed by Hanker et al. (1972). The incubation medium contained 12 mg 2-naphthylthiol acetate (Polysciences, Warrington, USA) dissolved in 0.2 ml acetone. To this was added 10 ml of a solution made up of 20 mg Fast Blue BB (Sigma, St. Louis, USA) as the diazonium salt, dissolved in 0.05M acetate buffer solution, pH 5.6, to constitute the final medium. The cells were incubated for 2 hours at 37°C, then washed in 0.05M acetate buffer (3 times, 300 g, 5 minutes) and postincubated in 1% osmium tetroxide in 0.1M phosphate buffer, pH 7.2, for 5 minutes at room temperature. Acid phosphatase activity was revealed with a modification of Barka and Anderson's method (1962). Briefly, after fixation the cells were incubated in a medium containing 13.9 mM sodium β -glycerophosphate (Sigma, Saint Louis, USA), 1mM Pb(NO₃)₂, 0.08% CaCl₂ and 0.5% sucrose in 0.05M acetate buffer, pH 5.0. After cytochemical incubation, the cells were fixed overnight in 2.5% glutaraldehyde in 0.1M cacodylate buffer pH 7.2, at 4°C.

Scanning electron microscopy

After fixation, the cells were washed in 0.1M cacodylate buffer, pH 7.2, (3 times, 300 g, 5 minutes), dropped onto poly-L-lysine (Sigma, Saint Louis, USA) coated coverslips, dehydrated in an increasing series of acetones and critical point dried. The samples were carbon sputter coated for BEI mode visualization, and examined with a Zeiss DSM 950 SEM operating at 30 kV.

Results

Backscattered electron imaging of NSE reaction patterns showed, in some human PBL, a focal pattern characterized by a single, dense, well-defined circular or oval body ranging in diameter from 0.5 to 0.7 μ m (Fig. 1). With secondary electron imaging, these lymphocytes appeared spherical (4-4.5 μ m in diameter) and displayed a smooth surface with few, short, scattered microvilli (Fig. 2). Lymphocytes with no NSE activity appeared covered with microvilli in the SEI (Figs. 3, 4).

Two well defined patterns of acid phosphatase activity were observed, in consonance with the patterns described in an earlier publication (Fernández-Segura et al., 1990). A focal pattern was formed by a cluster of 1-3 closely packed bodies, each measuring 0.4-0.7 μ m in diameter (Fig. 5). In SEI mode these cells had a smooth surface (Fig. 6). A granular pattern featured scattered granules of variable size (Fig. 7). In SEI mode these cells had abundant microvilli (Fig. 8). Cells which showed no AcP activity also presented with abundant microvilli.

Discussion

A number of SEM studies have attempted to establish a classification of different lymphocyte types, based on surface morphology in normal and leukemic cells and transformed leukocytes (Polliack et al., 1973; Alexander and Wetzel, 1975; Newell et al., 1976; Roath et al., 1978; Gamliel and Polliack, 1983; Dantchev and Mathé, 1984). However, Soligo et al. (1987) have aptly pointed out that although the main classes of normal leukocytes generally show distinct surface architectures, it remains very difficult to distinguish between their different subpopulations. With particular regard to human lymphocytes, these cells have usually been catalogued on the basis of morphological and immunological criteria; most workers agreeing that cell surface morphology is an expression of different stages of maturation, functionality, and metabolism (Arancia and Donnelly, 1988).

Under light microscopy and TEM, contrasting patterns of hydrolytic enzymatic activity have been related with two morphological types of T lymphocytes, one showing granular enzymatic activity and the other showing focal activity. These two types have been convincingly demonstrated in lymph nodes, fetal and mature thymocytes, human peripheral blood lymphocytes (Basso et al., 1980; Boesen et al., 1984; Hayhoe and Quaglino, 1988) and in various neoplastic cells of T lineage (Pangalis et al., 1978; Boesen, 1984). The simultaneous use of the BEI and SEI modes in SEM, which has now become more widespread in the last ten years, has made it possible to tie together observations of surface morphology features and findings obtained with SEM cytochemical techniques (Soligo and de Harven, 1981; Soligo et al., 1981; Soligo et al., 1983; Soligo et al., 1984; de Harven, 1987). We studied normal human lymphocytes processed with NSE and AcP in order to exploit the advantages of both approaches in order to catalogue these lymphocytes in terms that would be

equally meaningful for cell biologists and possibly clinical hematologists.

To demonstrate NSE activity we used a modification of the method proposed by Hanker et al. (1972), employing histochemical reagents with diazothio-ether groups (Payne et al., 1980). As far as we know, esterase determinations in BEI mode are usually performed at neutral pH, whereas with light microscopy techniques, esterase determinations in lymphocytes require an acid pH (Knowles et al., 1978). Our modification of the technique makes it possible to use acetate buffer instead of phosphate buffer, thus assuring that the BEI mode determination for esterase will be performed at pH 5.6. To demonstrate AcP activity we followed Soligo's guidelines using a modification of Barka and Anderson's medium (Soligo et al., 1981). The use of pre- and post fixation with glutaraldehyde, the latter after the incubation step, gives us an excellent picture of the cytochemical reactivity patterns in human lymphocytes while preserving the surface architecture of these cells. Postfixation has not to our knowledge been used in cytochemical methods in BEI mode.

Non-specific esterase activity in our material was present focally in a subset of lymphocytes. This pattern has been described under light microscope as a single, dense, paranuclear globule in lymphocytes with standard fixation and paraffin or plastic embedding (Beckstead and Bainton, 1980; Ranki et al., 1980). In cytochemical smears and also with light microscopy Ferrarini et al. (1980) and Zicca et al. (1981) distinguished two patterns, a single unifocal cytoplasmic localization seen in most of the lymphocytes and another paranuclear and/or granular pattern observed in a small group of lymphocytes. With TEM, NSE activity was located specifically in Gall bodies (Bozdech and Bainton, 1981) or cytoplasmic vesicle clusters (Monahan et al., 1981; Zicca et al., 1981) which are at the times intimately associated.

The focal character of NSE in human mature PBL, which we observed under SEM in BEI mode, are compatible with the above mentioned structures seen with TEM. Similarly, the focal pattern of AcP activity in association with Gall bodies has also been documented in some subpopulations with both light microscopy and TEM (Zicca et al., 1981). With SEM in BEI and SEI modes we have observed focal AcP activity in cells which show surface features identical to those of the lymphocytes which also present focal NSE activity. We consider this as clearcut evidence of a specific lymphocyte subset which can be identified by its well characterized surface features - smooth surface, small scattered microvilli - together with focal activity of both hydrolytic acid enzymes.

Another lymphocyte subset in our study, characterized under SEI mode by abundant surface microvilli, only displayed a granular pattern of acid phosphatase activity, and no NSE activity as revealed with the BEI mode. What is the actual nature of the relationships between the cell subtypes identified with SEM (BEI and SEI modes) and immunological subsets?

A focal pattern of NSE has been related with most human T cells (Armitage et al., 1982; De Waele et al., 1983), a subset of true helper cells (Armitage and Cawley, 1985), T cells presenting a CD4 phenotype (Armitage et al., 1982; Boesen, 1984) and T cells bearing receptor for the Fc portion of IgM - T_M cells - (Grossi et al., 1978; Bevan et al., 1980). The two latter cell types have also been shown to present focal activity with AcP (Robinson et al., 1984). In contrast, a granular pattern of activity for NSE and AcP has been related with CD8 cells (Clement et al., 1984; Robinson et al., 1984) and T cells bearing receptor for the Fc portion of IgG - T_G cells - (Ferrarini et al., 1980). Taken together, SEM surface features of human mature PBL, in association with acid hydrolase cytochemical observations, not only suggest a reasonable system for cataloguing different T lymphocyte subsets, but in addition provide us with a reliable method to distinguish those lymphocytes lacking both acid hydrolase activities - possibly B cells - which under SEM present a cell surface characterized by abundant microvilli.

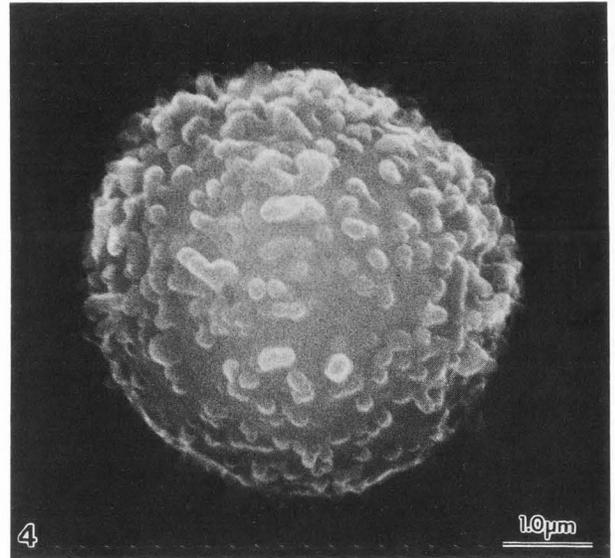
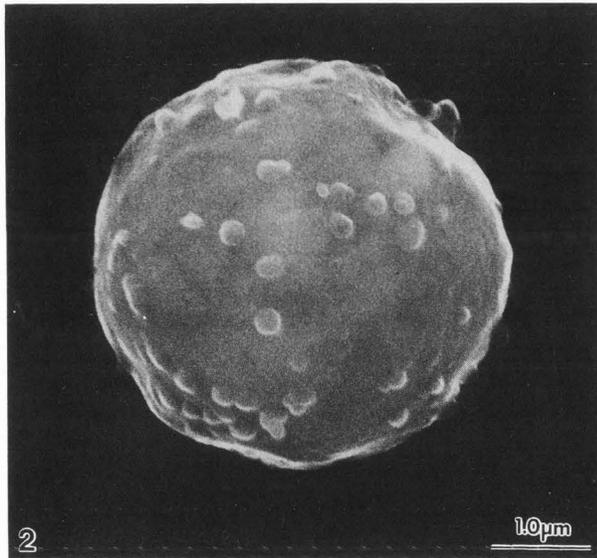
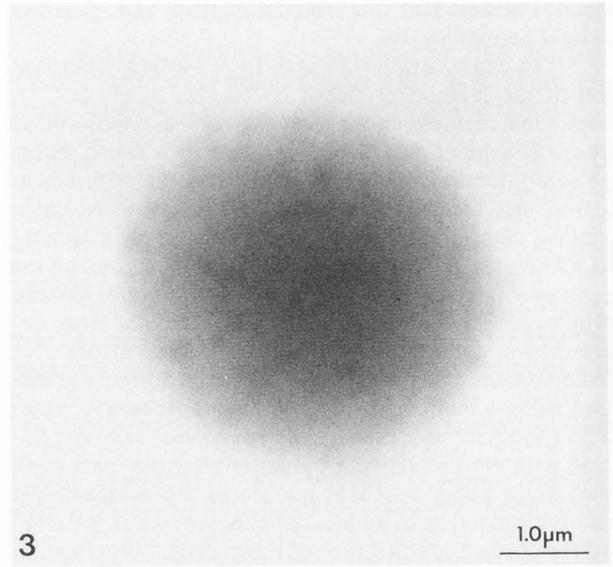
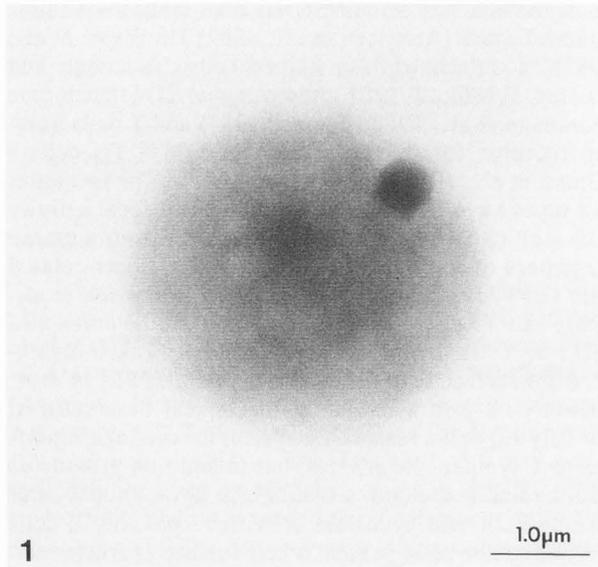
The collation of observations obtained with SEM in the SEI mode, and with enzymochemistry and immunogold labeling in the BEI mode (Soligo and de Harven, 1987), may well smooth the way towards a more accurate classification of different human peripheral blood lymphocytes. This may have the further effect of facilitating the flow of information between cell biologists, immunologists and clinical hematologists.

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Figures 1 and 2. SEM-cytochemical results in a peripheral blood lymphocyte after incubation in Hanker's medium to demonstrate NSE.

Figure 1. Focal distribution pattern - large, strongly esterase positive body - of NSE activity. **BEI mode.**

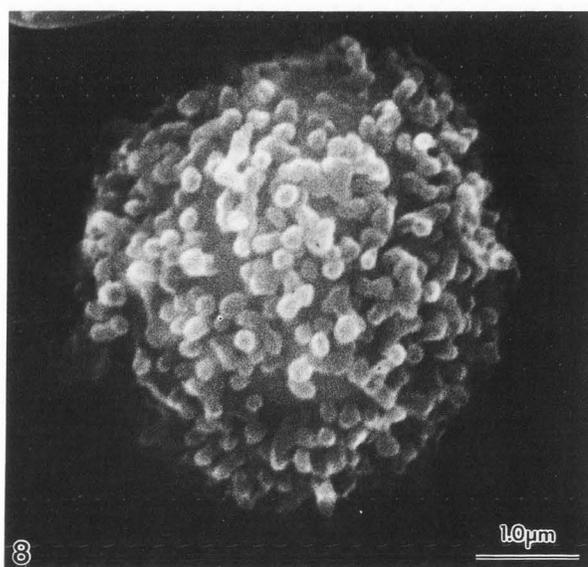
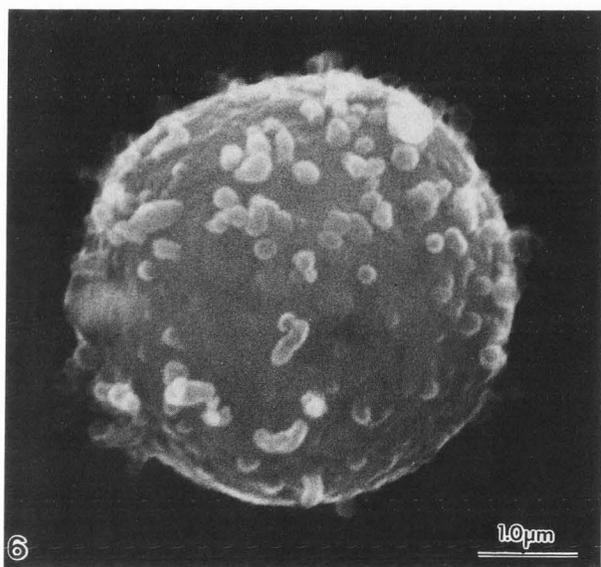
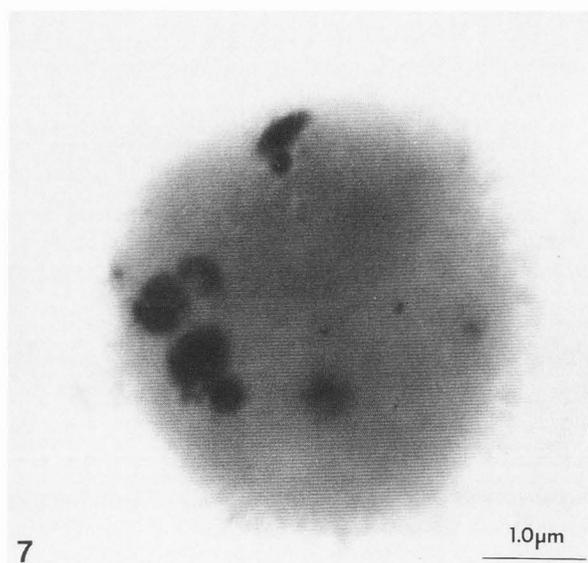
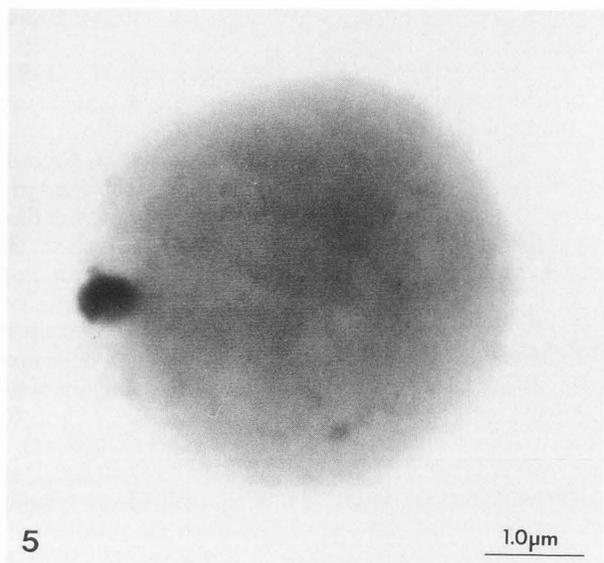
Figure 2. Cell surface morphology of the same cell as Figure 1, presenting a smooth surface with few short microvilli. **SEI mode.**

Figures 3 and 4. SEM-cytochemical results in a peripheral blood lymphocyte after incubation in Hanker's medium to demonstrate NSE.

Figure 3. Absence of NSE activity. **BEI mode.**

Figure 4. Cell surface morphology of the same cell as in Figure 3 presenting surface microvilli. **SEI mode.**

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Figures 5 and 6. SEM-cytochemical results showing AcP activity in a peripheral blood lymphocyte.

Figure 5. Focal distribution pattern. **BEI mode.**

Figure 6. **SEI mode** micrograph of the same cell as in Figure 5, presenting a smooth surface with few short microvilli.

Figures 7 and 8. SEM-cytochemical results showing AcP activity in a peripheral blood lymphocyte.

Figure 7. Granular distribution pattern. **BEI mode.**

Figure 8. Cell surface morphology of the same cell as in Figure 7 presenting surface microvilli. **SEI mode.**

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Discussion with Reviewers

D. Soligo: Looking at your pictures I don't see great differences between what you call "focal" and "granular" distribution pattern. Don't you think that "multifocal" would be a more appropriate term? In my experience acid phosphatase reaction products and, in particular, all lead depositions are slightly different, at the BEI, from what you show in Fig. 7. In general, due to the high Z of lead, I have already seen in my samples well defined small granules with high contrast and not large homogeneous, round granules with a "fuzzy" outline. How would you explain these differences?

Authors: In our opinion the terminology we use facilitates comparisons with cytochemical light microscopy observations. In light microscopy studies "focal" and "granular" are commonly used to characterize the deposits of cytochemical activity. A clearer definition of these terms was published in Fernández-Segura et al., 1990. The "fuzzy" outline of our granules may have resulted from pre- and postfixation, the latter of which effectively achieved considerable improvement in the preservation of surface features, possibly at the expense of some modification in the appearance of the granules.

D. Soligo: Do you know how many NSE and AcP positive cells were present in the same sample by light microscopy and SEM? This should give you an idea of the sensitivity of SEI method, which I suspect to be below that of LM.

Authors: In our experience, by light microscopy 65% of the cells were AcP positive and 55% NSE positive. With SEM in BEI mode our preliminary data indicate that 48% of the cells are AcP positive and 35% NSE positive. We agree that the sensitivity with SEM is probably lower but nevertheless permits a better correlation with the surface morphology.

Reviewer II: CD4 positive T cells with focal AcP; T cells positive for the IgM Fc receptor with focal AcP; T cells positive for the IgG receptor (T_G cells) with granular NSE and AcP, and CD8 positive cells with granular NSE and AcP have been reported in the literature. Did you find granular NSE or AcP in smooth cells with a few short microvilli? You found a villous cell with granular AcP. How would you have classified it? Do

you have any surface marker information on your cell populations? We did not find a granular NSE or AcP pattern in smooth cells with few short microvilli.

Authors: The short villous cell with a granular AcP pattern was provisionally classified as a CD8 T cell or a T_G cell. Recent studies have shown that ADCC and NK cells are included in the subset of T_G cells. Thus far we have only investigated CD16 in peripheral blood leukocytes with immunogold labeling with SEM in BEI mode. A subset of cells which are positive for CD16 are similar to the villous cells with granular AcP described in this paper (Proceedings of the XIIth International Congress for Electron Microscopy, Vol. 3, 866-867, 1990).