Large Granular Lymphocytes in Human Peripheral Blood: Ultrastructural and Cytochemical Characterization of the Granules

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Large granular lymphocytes (LGL) are defined as nonadherent mononuclear cells with cytoplasmic azurophilic granules, avid receptors for the Fc portion of IgG, and cytotoxic functions (NK or ADCC activities). In the present study, the granules of LGL isolated from human peripheral blood have been analyzed by enzyme cytochemistry and electron microscopy. It has been found that: (1) in the single cells, granules at different stages of maturation could be detected; in addition, packaging of the granules took place in the proximity of the Golgi apparatus, which is similar to that seen in secretory cell types. (2) Acid phosphatase (AP) was observed within the granules and the vesicles located in the Golgi area; the Golgi apparatus identified through its thiamine pyrophosphatase-positivity was consistently negative for AP. (3) Alpha naphthyl-acetate esterase (ANAE) activity was localized in the granules as well as on the membrane of LGL and monocytes. (4) The ANAE activity of LGL was of the monocytic and not of the lymphocytic type, as shown by NaF inhibition. (5) The LGL granules, although identifiable as primary lysosomes, were not involved in the process of phagocytosis, since LGL failed consistently to ingest latex particles or opsonized red cells.

IN THE PAST, studies on human peripheral blood mononuclear cells depleted of monocytes have led to the identification of a population of non-T, non-B cells bearing receptors with high avidity for the Fc portion of IgG. Unlike the large majority of T and B lymphocytes, these cells formed rosettes with human O+ red cells coated with anti-CD (Ripley) antibody and were provisionally named “third population cells” (TPC). More recently, it has been found that certain cells forming rosettes with sheep erythrocytes (E) (and therefore supposedly T cells) have high avidity Fc receptors and are capable of binding the Ripley reagent. These have been termed Tγ or Tδ cells.

The identical properties of the Fc receptors on both Tγ cells and TPC have suggested possible similarities between the two cell subsets. The finding that the great majority of both Tγ cells and TPC are large lymphoid cells characterized by the presence of azurophilic granules in their cytoplasm has given further support to this suggestion. Morphological studies both at the light and at the electron microscope level have failed to detect any characteristic feature distinguishing the granular cells in the Tγ fraction from those in the TPC fraction. Furthermore, the granular cells from both fractions were found to be positive for acid hydrolases with a characteristic cytochemical pattern of staining in the paranuclear area, where the granules are found. In addition, apart from their capacity to form E rosettes, no specific surface markers were detected that distinguished granular cells in the Tγ from those in the TPC fractions. Both Tγ cells and TPC have been found to be capable of mediating antibody-dependent cell cytotoxicity (ADCC) or natural killer (NK) activities and ultrastructural studies have identified the granular cells in both fractions as those responsible for the cytotoxic functions.

Although the azurophilic granule containing mononuclear cells with a “lymphocyte” morphology have been termed monocyctic lymphocytes in the past, the present preferred nomenclature is that of large granular lymphocytes (LGL). Some investigators also refer to LGL as NK cells because of their functional properties.

The present study was intended to characterize the granules of LGL, with particular emphasis on their ultrastructure, biogenesis, enzyme content, and possible function. Elucidation of the properties of these granules bears some relevance to the question of whether LGL belong to the lymphocytic lineages or to the mononuclear-phagocyte system.

MATERIALS AND METHODS

Preparation of Cell Suspensions

Mononuclear cell suspensions prepared from heparinized blood samples of adult healthy volunteers by Ficoll-Hypaque density gradient centrifugation were depleted of adherent cells by incubation on plastic Petri dishes for 1 hr and subsequently fractionated into T and non-T cells by rosetting with neuraminidase-treated sheep erythrocytes (Eα) followed by Ficoll-Hypaque density gradient centrifugation.

Both Tγ cells and TPC were used as a source of LGL. Tγ cells were prepared by rosetting T cells with ox erythrocytes coated with rabbit IgG antibody (EA) followed by Ficoll-Hypaque separation. This fraction contained >80% LGL. TPC were obtained from non-T-cells by enrichment of cells rosetting with Ripley-coated erythrocytes or depletion of surface immunoglobulin bearing cells. Of those cells rosetting with Ripley erythrocytes 60%-80% were clear cells with a “lymphocyte” morphology have been termed monocyctic lymphocytes in the past, the present preferred nomenclature is that of large granular lymphocytes (LGL). Some investigators also refer to LGL as NK cells because of their functional properties.

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identified as LGL, the residual percentage being mainly monocytes.\textsuperscript{19} Cells bearing surface immunoglobulin were removed from non-T preparations by rosetting with human erythrocytes coated with rabbit anti-human (F(ab')\textsubscript{2}) antibody by the chromium chloride technique. This method consistently yielded populations containing >70% LGL. Specificity controls and other details of the above methods have been published elsewhere.\textsuperscript{19}

The basic observations reported herein were made primarily on T\textsubscript{c} cells because they were easier to isolate, and their contamination with monocytes was much lower than that of TPC. However, further studies indicated that TPC were indistinguishable from T\textsubscript{c} cells. In addition, since the process of preparation of both T\textsubscript{c} and TPC involves a positive selection of cells (reacted with EA indicator cells), which in principle could induce morphological and/or cytochemical changes, the LGL present in unseparated T or non-T cell fractions were also examined.

Cells recovered from the plastic surfaces after 1 hr incubation of mononuclear cells were used as a source of monocytes. These preparations ("activated" monocytes) always contain >90% monocytes, as shown by cytochemical staining for acid hydrolases.\textsuperscript{18,17} In some experiments, monocytes were also separated by Percoll density gradient centrifugation, with the two step procedure described by Pertoff et al ("resting" monocytes).\textsuperscript{18}

**Electron Microscopy (EM)**

Cells were fixed with 1.25% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.6, for 30 min at room temperature. After washing in the same buffer, cells were postfixed in 1% osmium tetroxide in cacodylate buffer for 30 min at room temperature, dehydrated with ethanol, and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate and examined under a Siemens Elmiskop 101 transmission electron microscope.

**Cytochemical Localization of Enzyme Activities**

Fixation with 1.25% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.6, was employed preliminarily in all of the cytochemical procedures. For the light microscopy demonstration of enzyme activities, cells were cytocentrifuged onto slides following fixation and subsequently incubated with the substrate. For EM cytochemistry, fixed cells were incubated with the substrates in suspension, postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.6, and then treated for resin embedding.

 Peroxidase activity (PO) was detected at both the light and EM level by the Graham and Karnovsky technique,\textsuperscript{19} using 0.05% 3,3'-diaminobenzidine and 0.01% \( \text{H}_2\text{O}_2 \) in 0.05 \( M \) Tris-HCl buffer, pH 5, as substrate.

Two different techniques were employed for the demonstration of acid phosphatase (AP). One millimolar naphthol-AS-BI acetate and 6% hexazotized p-rosanilin were used as substrate\textsuperscript{20} in 0.15 \( M \) acetate buffer, pH 5, for light microscopy studies. Samples processed for EM as well were incubated in a medium containing 2.7 \( mM \) cytidine monophosphate as substrate\textsuperscript{21} in 0.05 \( M \) acetate buffer, pH 5.2, followed by treatment with hydrogen sulfide vapors. The latter treatment was omitted when cells were processed for EM.

Thiamine pyrophosphatase (TPP) was detected at both light and EM level by the method of Novikoff and Goldfisher,\textsuperscript{22} using 2.6 \( mM \) thiamine pyrophosphate in 0.2 \( M \) Tris-maleate buffer, pH 7.2, as substrate. Alpha naphthyl acetate esterase (ANAE) localization was studied at the light as well as at the EM level, using 1.3 \( mM \) alpha-naphthyl acetate as substrate and 6% hexazotized p-rosanilin in 0.067 \( M \) phosphate buffer, pH 5.8.\textsuperscript{23} However, for this enzyme activity, both cytocentrifuge preparations and cell suspensions were incubated in the substrate. Inhibition of ANAE activity with 10–50 \( mM \) NaF was carried out on parallel samples of the different cell preparations.\textsuperscript{14}

Specificity controls for the above cytochemical reactions always included incubation of the cells in media that did not contain the specific substrates.

**Criteria for LGL Identification**

Since previous studies had demonstrated that there was difficulty in distinguishing LGL from monocytes,\textsuperscript{16,11} strict criteria for the identification of monocytes had to be established. Cytochemical and ultrastructural observations on "resting" or "activated" monocyte suspensions permitted the identification of the following distinctive features: (1) unlike LGL, monocytes consistently displayed a strong, granular positivity for PO and the reaction product was confined to a variable proportion of the granules;\textsuperscript{15,20} (2) monocytes displayed an intense granular staining for both ANAE or AP (with the azo-dye technique), with a variable degree of cytoplasmic diffusion; (3) in contrast to LGL, monocytes contained secondary lysosomes and endocytosis vacuoles. These were detectable in 85%–90% of the "activated" monocytes and 20%–30% of the "resting" cells.

**Phagocytosis Assays**

Preparations enriched for LGL or monocytes were mixed with EA (1:50 ratio) or with 1 \( \mu \)m latex particles (1:100 ratio). EA or latex particles were shaken with LGL or monocytes (2 × 10\textsuperscript{6} cells/ml) in Hank’s balanced salt solution containing 20% fetal calf serum for 2 hr at 37°C.\textsuperscript{27} Following incubation, the cell suspensions were cleared of noningested red cells by osmotic lysis or of free latex particles by centrifugation through gradients of fetal calf serum. Cell suspensions were then incubated for the demonstration of PO activity as above and treated for resin embedding. Semithin sections were analyzed for phagocytosis and PO activity by light microscopy.

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Fig. 1. Ultrastructural features of LGL. (A and B) The heterogeneity of the granular inclusions of LGL. Several mitochondria can be distinguished from the granules in the cytoplasmic area shown in B. (C, D, and E) The relationship of the Golgi apparatus with maturing granules and vesicles. Arrow in C points to a coated vesicle. (A) ×10,000; (B) ×30,000; (C) ×35,000; (D) ×35,000; (E) ×35,000.
Results

Ultrastructural Heterogeneity of the LGL Granules

Several common features of the granules were recognized by ultrastructural analysis of numerous LGL from a number of different preparations (see Fig. 1). (1) The granules were occasionally scattered through the cytoplasm but, more often, appeared concentrated in a fairly restricted paranuclear area that also contained the Golgi apparatus and, sometimes, the centrioles; (2) the granules were not found directly adjacent to the arrays of Golgi tubules but were most common in the mature (trans) aspect of the Golgi apparatus; (3) the granules differed in size but were always bound by unit membranes; (4) the electron opacity of the granular matrix was variable. Whereas smaller granules contained a fairly translucent matrix, larger granules were more electron-opaque. A round, heavily electron-opaque inclusion was occasionally found within the granular matrix. This heterogeneity may be suggestive of a variable degree of maturity.

Numerous vesicles were seen in the cytoplasmic area between the Golgi cisternae and the granules (Fig. 1). Whereas most of these vesicles were bound by smooth membranes, a few of them showed the typical fuzzy appearance of the "coated" vesicles. Neither the smooth nor the coated vesicles appeared to contain electron-opaque material. The close proximity of the area rich in vesicles and the Golgi zone is suggestive of an active involvement of the Golgi apparatus in packaging of secretory materials into granules and coated vesicles.

Localization of AP in LGL

Identical values for the percentages of cells positive for AP activity were detected using two techniques for the light microscopic analysis of enzyme localization in LGL. The azo-dye reaction showed diffuse enzyme activity in a paranuclear area with some granular staining. The lead sulfide technique showed a pattern of scattered granular precipitates, suggesting that both granules and some ER vesicles were reactive (Fig. 2A). That the ER vesicles were positive for AP was supported by the observation that the number of lead sulfide precipitates was much larger than that of the azurophilic granules. These results might indicate that the azo-dye technique, although revealing basically the same structures, provides less precise cytochemical detail, possibly because of some diffusion of the reaction product.

The ultrastructural localization of AP, studied by
the lead salt technique supported the light microscopy findings in revealing a number of discrete precipitates in LGL. The precipitates were found within the matrix of the granules and in the lumen of some of the vesicles in the proximity of the granules (Fig. 2B and C). The vesicles were generally located near the parallel arrays of tubular Golgi cisternae, which appeared negative (Fig. 2D; see also Fig. 1 for ultrastructural details). In a minority of cells, the reaction product was also detected within the perinuclear cisterna and in the lumen of isolated profiles of the rough ER.

In order to obtain a more precise localization of the Golgi apparatus, cells were reacted for a specific enzyme marker of the Golgi TPP. As shown in Fig. 2E, the reaction product for TPP was detected in the majority of the Golgi cisternae in addition to the perinuclear cisterna and some isolated profiles of the rough ER. In contrast, the AP-positive vesicles and granules were consistently TPP-negative. AP activity was seen in the majority of the electron-dense granules and smooth vesicles of monocytes in purified suspensions as well as of those contaminating TPC preparations. In agreement with other published data, monocyte AP activity, unlike that seen in LGL, was not prominent within the perinuclear cisterna or the rough ER.

Localization of ANAE in LGL

Cells were prefixed, cytocentrifuged onto slides, and stained for ANAE. The enzyme was localized both in a paranuclear area (often located in the nuclear notch) and in the surrounding granules (Fig. 3A). Incubation with the substrate in the presence of 10–50 mM NaF inhibited both the staining of LGL (Fig. 3B) and of the monocytes contaminating the TPC fractions or purified as described above (Fig. 3E and F). In contrast, the T_M cells in the T_G-cell-deprived suspensions or those contaminating the T_G preparations, retained their characteristic dot-like pattern of ANAE positivity even in the presence of NaF (see Fig. 3B).

In another series of experiments, cells were fixed and stained in suspension for ANAE, prior to resin embedding for ultrastructural analysis. Following incubation, a portion of these cells were also cytocentrifuged and examined by light microscopy. The cytochemical pattern of staining by this procedure was different from that seen with direct ANAE staining on slides, since both monocytes and LGL displayed accumulations of the reaction product on the plasma membrane. Monocytes were also stained at the cytoplasmic level, whereas LGL showed only a weak granular positivity (Fig. 3C and G). NaF treatment completely abolished the cytoplasmic staining of both LGL and monocytes. The membrane reaction was decreased but still detectable in both cell types (Fig. 3D and H). At the EM level, only the membrane localization of ANAE was detected in both monocytes and LGL, and the staining was decreased by NaF treatment. No cytoplasmic localizations of ANAE activity were detected in monocytes or LGL.

LGL Do Not Possess Phagocytic Capacities

Preparations of T_G cells, TPC, or monocytes (purified by adherence to plastic) were incubated with latex

![Fig. 3. Cytochemical localization of ANAE activity in LGL (A–D) and monocytes (E–H). In A, B, E, and F, cytocentrifuged cells were stained for ANAE. In C, D, G, and H, cells were incubated in the substrate for ANAE prior to cytocentrifugation. The bottom row shows the pattern of reaction in the presence of NaF treatment. Note the predominant membrane staining of LGL in C. This staining is not completely inhibited by NaF, as shown in D. Monocytes in G display a strong membrane reaction beside the cytoplasmic staining. Such a membrane reaction is not seen in E and is not completely inhibited by NaF (H). Arrow in B indicates a T_M cell with NaF-resistant ANAE activity. (A–H) x800.](#)
ULTRASTRUCTURAL AND CYTOCHEMISTRY OF LGL

Fig. 4. LGL are not capable of phagocytosis. (A) LGL reacted for PO. A PO-positive monocyte (arrow) is seen among PO-negative LGL. (B) Two monocytes that have phagocytosed latex particles are seen beside two nonphagocytosing LGL. Light (C) and electron microscopy (D) of PO-positive monocytes that have ingested opsonized red cells, together with PO-negative, nonphagocytosing LGL. Staining with Toluidine blue does not permit a clear-cut identification of LGL granules in semithin sections. (A, B, C) ×800; (D) ×8000.

Human peripheral blood LGL are easily identifiable by both light and EM through the presence of azurophilic, electron-opaque granules. The aim of this study was to examine the structure and the contents of these granules in order to gain information on their possible functional significance. The unit membrane-bound granules appear heterogeneous in size and in the electron opacity of their matrix and are related to a cytoplasmic area rich in smooth and coated vesicles. This area is in close proximity to the Golgi cisternae and may represent the site where the granules are packaged. From the Golgi area, the granules are likely to be transported to other cytoplasmic sites where concentration of their contents and final maturation occurs. This is reminiscent of the secretory mechanism described in several exocrine or endocrine cell types and of the granulogenesis taking place in hemic cells, such as granulocytes, monocytes, and megakaryocytes.

The ultrastructural localization of AP has provided a further insight into the mechanisms of granule formation in LGL, since this enzyme seems to be packaged into granules in the vesicle-rich area described above. This area, although close to the Golgi apparatus, is distinct from the parallel arrays of smooth cisternae easily demonstrated by TPP staining and is often referred to as GERL. The GERL may be the site of origin of primary lysosomes. Thus, the above data suggest that the LGL granules are likely to represent primary lysosomes. This is further supported by light microscopy studies in which β-glucuronidase and ANAE activity were localized in a paranuclear zone, possibly corresponding to the vesicle- and granule-rich area detectable by EM. It is of note that ANAE could not be seen in the cytoplasm of LGL or monocytes by EM, although it was clearly detectable within granules at the light microscopy level. This was somewhat unexpected, since ANAE was detected in the course of previous EM studies on Tg cells. One possible explanation for this failure may reside in the insufficient quantity of electron-opaque material deposited within the granules following the cytochemical reaction.

AP activity was demonstrated within the granules and the coated vesicles localized both in the Golgi area and scattered in the cytoplasm of LGL. Therefore,
analogous with that observed in promonocytes and monocytes, LGL seem to possess two distinct types of primary lysosomes. However, in contrast to monocytes, LGL neither possess phagocytic capacity nor contain secondary lysosomes or endocytosis vacuoles. In this connection it is worth mentioning that controversial data on this issue have been reported previously. The monocyctotic nature of phagocytosing cells in both T and TPC preparations was established by the use of strict criteria for monocyte identification.

The function of LGL lysosomes is far remains obscure. Since LGL have well defined cytotoxic activity (ADCC and NK), it is possible that these granules might be involved in the mechanism of killing. Recent experiments on Chediak-Higashi patients and beige mice (regarded as an analogue of the human Chediak-Higashi disease), have shown a profound defect in LGL cytotoxicity, and marked alterations of the GERL (leading to defective lysosome formation) have been described, at least for beige mice.

The presence of AP activity in the perinuclear cisterna and/or in strands of the rough ER of a minority of LGL could suggest that these cells were less mature than the majority of LGL not displaying these cytochemical features. This finding, together with the active granulogenesis occurring in all of the LGL, might suggest that these cells are still maturing in the circulation.

The above aspects of LGL maturation are similar to those described in the bone marrow for maturing cells of the myelomonocytic lineage, from which LGL can be distinguished through the absence of PO activity.

Although several differences exist between LGL and monocytes, one should also discuss some relevant similarities. For example, the cytochemical localization of ANAE activity in LGL is identical to that described for monocytes, i.e., lysosomes and plasma membrane. Furthermore, in both the cell types the lysosomal activity and, to a lesser extent, the plasma membrane reaction are inhibited by NaF. Quantitative analysis of ANAE activity in lysates of purified cell subsets have confirmed the results obtained by cytochemical techniques (Franzi A.T. et al. manuscript in preparation). Ranki et al. have recently reported that the ANAE activity of NK cells is of the lymphocyte type since it is not inhibitabbe by NaF. The reason for this discrepancy is not clear.

Another similarity between LGL and monocytes is shown by the presence on both cell types of certain determinants that are recognized by monoclonal antibodies such as the OK M1 reagent. However, the presence of one or more common markers in two different cells does not necessarily indicate their common origin. The assignment of a cell to a particular lineage is more likely to be derived from ontogenic studies. The present work may be of importance in tracing the markers for maturing LGL.

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REFERENCES

14. Herberman RB, Djen JY, Kay D, Ortaldo JR, Riccardi C,
15. Ferrata A, Storti E: Le Malattie del Sangue. Milano, S.E.L. 1948
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