Immunocytochemical Distinction Between Primary and Secondary Granule Formation in Developing Human Neutrophils: Correlations With Romanowsky Stains

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Electron-microscopic studies with peroxidase cytochemistry have shown that primary (azurophilic) granules in human neutrophils are synthesized in promyelocytes, while secondary (specific) granules are formed in myelocytes. However, these studies were limited by the lack of specific markers for secondary granules and by the inability to make direct comparisons of electron-microscopic morphology with the light-microscopic appearance of the same cell. Thus secondary granules cannot be identified reliably and the relevance of these findings to the light-microscopic interpretation of clinical bone marrow specimens cannot be evaluated. To circumvent these problems, we developed a method to permit immunofluorescent demonstration of primary and secondary granule markers in cells stained with Romanowsky agents. Normal human marrow cells were stained with May-Grünwald-Giemsa and photographed. The slides were decolorized in buffered glycerine and saline for 24 hr and then stained with fluorescein- and rhodamine-conjugated monospecific antisera to human granulocyte myeloperoxidase, cathepsin G, elastase, lysozyme, and lactoferrin. The same cells were then located and examined for conjugate binding. Double stains with fluorescein- and rhodamine-labeled antisera offered the additional advantage of simultaneous identification of primary and secondary granules. This approach confirmed the partition of primary and secondary granule proteins and related their appearance to the maturation of developing neutrophils in normal human bone marrow.

Human polymorphonuclear neutrophils (PMN) have two distinct types of cytoplasmic granules. Primary (azurophilic) granules are large, round, electron dense, and contain myeloperoxidase, elastase, lysozyme, cathepsin G, and many acid hydrolases. Secondary (specific) granules are smaller, electron lucent, and responsible for the characteristic color of the cell in Wright-stained preparations. In human PMN only lactoferrin and lysozyme have been definitively associated with these particles. Histochemical stains for peroxidase are used routinely as markers for primary granules, but there are no stains

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These terms are frequently used interchangeably, but in this paper we shall use primary and secondary to denote those granules defined by specific protein markers, and azurophilic and specific to denote only the granules of the appropriate color in Romanowsky-stained cells.
specific for lactoferrin; secondary granules have been identified by their morphologic appearance and myeloperoxidase negativity.1

Electron-microscopic studies1,4,7 have shown that primary granules are formed in promyelocytes, while secondary granules are synthesized in myelocytes. Unfortunately, these studies were limited by the lack of a marker specific for secondary granules and by the inability to relate the electron-microscopic morphology to the light-microscopic appearance of the same cell stained with Romanowsky agents. Thus the identification of secondary granules is unreliable, and the relevance of the results to the clinical interpretation of conventionally stained marrow specimens has not been determined. Mason et al.,8 using an immunoperoxidase technique, found that lactoferrin was present in metamyelocytes, bands, and mature PMN. However, they did not identify primary granules; thus the relationship between the two granule types was not determined.

To circumvent these difficulties, we studied granulopoiesis in human marrow with a triple-staining technique. Cytoplasmic granules were identified with simultaneous immunofluorescent stains using lactoferrin as a marker for secondary granules and myeloperoxidase, elastase, or cathepsin G for primary granules. This approach is specific and permits the simultaneous localization of two components within a single cell.9 In addition, the cells were stained with Romanowsky agents to correlate immunofluorescent results with light-microscopic appearance of the same cell. With this approach we traced neutrophil development in normal human bone marrow, identified the stage of cellular maturation at which primary and secondary granules are formed, and correlated these findings with the light-microscopic appearance of the same cells.

MATERIALS AND METHODS

Preparation of labeled antisera. High-titer monospecific antisera to human granulocyte myeloperoxidase, cathepsin G, elastase, lysozyme, and lactoferrin, raised in goats and rabbits, were conjugated with rhodamine or fluorescein. The purity of the antigen preparations used for immunization, the specificity of the resultant antisera, and the procedures used to label antisera have been previously described.10

Preparation of bone marrow cells and histochemical staining. Bone marrow was aspirated from patients undergoing evaluation for anemia or lymphoma and at sternotomy during cardiac surgery. Only those samples found to be free of evidence of disease were included. Marrow particles were forced through a 25-gauge needle and washed in 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) with 5% EDTA pH 7.4. The cells were suspended to 10^6/ml in the same solution and deposited on glass slides by cytocentrifugation. The slides were air dried and fixed for 1 min in buffered formalin acetone (20 mg disodium phosphate, 100 mg monopotassium phosphate, 30 ml distilled water, 25 ml formaldehyde [40% v/v, Fisher] and 45 ml acetone; pH 7.2). They were stained with May-Grünwald-Giemsa and photographed with Kodak Ektachrome professional film (ASA 50) under oil immersion without a coverslip, and the substage micrometer coordinates were recorded.

Immunocytochemical staining. The immersion oil was removed with xylene. The cells were decolorized with glycerol:PBS (9:1) for 30 min and washed in PBS pH 7.4 at 4°C for 24 hr.

Double stains for different protein markers were performed with two antisera separately conjugated with rhodamine and fluorescein, mixed together in appropriate dilutions. The decolorized slides were layered with the diluted conjugates, incubated at 4°C for 30 min, washed for 30 min in PBS, and mounted in glycerol:PBS. Some slides were also counterstained with 0.01% methyl green in PBS to enhance nuclear detail.

The slides were examined with a Leitz Orthoplan microscope using incident-light excitation with the Leitz Ploempak 2.1 and filter system H for fluorescein excitation and filter system N for rhodamine excitation with edge filter K480 to minimize quenching. Photographs were taken with the Leitz NPL.
Fluorator 100/1.32 objective and Orthomat-W automatic camera using Kodak Ektachrome 200 film push-processed to ASA 800.

**Controls.** Specificity of the conjugates and the triple staining method were determined with the following experiments:

1. To determine if the May-Grünwald-Giemsa stain produced autofluorescence, the decolorized cells were observed for fluorescence when excited at the fluorescein and rhodamine wavelengths.
2. To check for nonspecific binding of the antisera, immunoglobulins obtained from the same animals prior to immunization were conjugated and tested for staining of target cells.
3. To evaluate the immunologic specificity of the antisera, cells were preincubated with each unconjugated antiserum and were stained with the same conjugated antiserum.
4. To determine monospecificity of each reagent, the conjugates were absorbed with myeloperoxidase, cathepsin G, elastase, lysozyme, and lactoferrin. The reaction mixture was incubated for 30 min at 37°C, held overnight at 4°C, and centrifuged at 1500 g for 10 min, and the supernatant was tested for staining of target cells. The antisera to myeloperoxidase, lactoferrin, and lysozyme were also absorbed by affinity chromatography against their respective antigens.

Each experiment was controlled by staining the slides with conjugated preimmunization immunoglobulins as in (2) (above) and with cells that had not been stained with May-Grünwald-Giemsa.

**RESULTS**

**General comments.** In initial experiments combining Romanowsky and immunofluorescence stains, we stained with the immunofluorescent reagent first and stained the cells with May-Grünwald-Giemsa second. The results were disappointing, because the May-Grünwald-Giemsa stain failed to reveal cytoplasmic detail. The PBS wash during the fluorescent stain was responsible for the poor results, and this effect occurred regardless of fixation or stain (Wright, Giemsa, or May-Grünwald-Giemsa).

In subsequent experiments, we eliminated this problem by staining the slides with the Romanowsky agent first. The methanolic dyes were then extracted with buffered glycerin and prolonged washing in PBS. At completion, the cells were not visibly stained and the leukocytes did not autofluoresce. Slight residual red cell autofluorescence did not interfere with subsequent specific immunofluorescence.

Formalin acetone is not the optimal fixative for Romanowsky stains, since it causes cellular shrinkage and produces paranuclear clefts. However, all other fixatives investigated (absolute methanol, ethanol, 1% paraformaldehyde, formaldehyde vapor, and heat) were either no better or resulted in diffuse cytoplasmic staining with the fluorescent conjugates.

Fluorescent staining of cells was completely blocked by antisera absorbed by affinity chromatography or in liquid phase and by preincubation of the target cells with unconjugated antisera. Fluorescent staining was not blocked by incubation of the antisera with other granule proteins. The conjugated preimmunization immunoglobulins did not stain the cells. The May-Grünwald-Giemsa stain did not diminish or alter fluorescent binding.

Neutrophils were intensely stained with these reagents (Figs. 1A–1C). The conjugates were bound in a granular pattern, and no nuclear or membrane binding was observed. When the cells were stained simultaneously for two primary granule markers, the conjugates appeared to be bound by the same granules. However, combinations of a primary granule marker and lactoferrin produced dissimilar reaction patterns, and the two markers appeared to be present in separate granules. This distinction was most easily demonstrated in ruptured cells in which the individual granules were more dispersed (Figs. 1D and 1E).
Fig. 1. See legend facing page.
**NEUTROPHIL GRANULOPOIESIS**

*Triple staining of bone marrow cells.* Myeloid cells, monocytes, and reticulum cells were stained with one or more of these reagents. Monocytes were strongly positive for lysozyme and weakly positive for myeloperoxidase but were negative for elastase, cathepsin G, and lactoferrin. Reticulum cells were occasionally positive for lysozyme but negative for all other markers. The pigment in pigmented macrophages was strongly autofluorescent, as were eosinophilic granules. Other marrow elements did not stain (Figs. 1A–1C).

*Promyelocytes* were the earliest myeloid cells in which the markers could be demonstrated. They were positive for myeloperoxidase, elastase, and cathepsin G (Figs 1D and 1E). The immunofluorescent intensity appeared to correlate with azurophilic granules observed in the May-Grünwald-Giemsa stain. Occasionally, primary granule marker proteins were identified immunocytochemically before azurophilic granules were seen in the Romanowsky stains, but the reactions were faint and difficult to photograph due to fluorescent quenching occurring during the long exposure times required. Lysozyme was also present in promyelocytes but was not as intense as myeloperoxidase or cathepsin G and may have been present in a distinct granule. Lactoferrin was not present in promyelocytes (Figs. 1F–1H).

*Myelocytes* were distinguished in May-Grünwald-Giemsa stains by the characteristic “dawn of neutrophilia”; identification of lactoferrin was the immunofluorescent counterpart of this tinctorial change (Figs. 11–1K). In early myelocytes with predominantly basophilic cytoplasm, lactoferrin fluorescence was localized to a small perinuclear region, but in most cells it was distributed throughout the cytoplasm even though the Romanowsky stain showed that only a small portion of the cytoplasm was “neutrophilic.” Both lactoferrin and lysozyme appeared to increase as myelocytes matured, but myeloperoxidase, cathepsin G, and elastase remained the same or decreased in intensity (Figs. 1L–1N).

*Mature cells.* Metamyelocytes, bands, and PMN were positive for all markers and did not differ from circulating PMN.

**DISCUSSION**

This report demonstrates that immunocytochemical identification of specific protein markers can be successfully combined with Romanowsky staining of the same cells. The success of the method is dependent on the purity of the original antigens and the specificity of the antisera. The antisera used in this study were

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**Fig. 1.** Simultaneous immunofluorescent and Romanowsky stains of normal human bone marrow. (A–C) Bone marrow neutrophil reacted with May-Grünwald-Giemsa (A), fluorescein-labeled antmyeloperoxidase (B), and rhodamine-labeled antilactoferrin (C). Negative staining of other marrow elements by the conjugates demonstrates specificity. (D, E) Promyelocyte stained with fluorescein-labeled antmyeloperoxidase (D), rhodamine-labeled antielastase (E) and counterstained with methyl green. Rupture of the cytoplasmic membrane allowed dispersion of the granules and emphasizes the conjoint binding of both markers in identical granules. (F–H) Promyelocyte (P) and myelocyte (M) stained with May-Grünwald-Giemsa (F), fluorescein-labeled antmyeloperoxidase (G), and rhodamine-labeled antilactoferrin (H). Lactoferrin is not present in the promyelocyte but is strongly reactive in more mature cells in this field. Myeloperoxidase is present in all cells. (I–K) Myelocyte stained with May-Grünwald-Giemsa (I), fluorescein-labeled anti-cathepsin G (J), and rhodamine-labeled antilactoferrin (K) showing the appearance of a specific granule marker in the cell. (L–N) Three myelocytes and one band stained with May-Grünwald-Giemsa (L), fluorescein-labeled anticathepsin G (M), and rhodamine-labeled antilysozyme (N). Lysozyme appears more intense in the more mature cells (top and bottom), while an azurophilic granule marker decreased or remained the same. Similar results were seen with lactoferrin.
highly reactive and monospecific. They were tightly bound to their respective antigens, and conjugate binding by membrane-associated Fc receptors was not observed. This is not surprising, since binding due to unaggregated IgG is much weaker than binding due to aggregated IgG usually used to demonstrate Fc receptors. The blocking experiments and the demonstration that the reaction patterns were distinct for primary and secondary granule markers demonstrate the specificity of each antiserum. The lack of staining of lymphocytes and normoblasts and the limited staining of monocytes are consistent with biochemical data and are another demonstration of reagent specificity. Also, the antecedent May-Grünwald-Giemsa stains did not interfere with subsequent immunologic methods.

The results confirm that primary and secondary granules are synthesized at distinct stages of cellular maturation. In addition, we showed that promyelocytes, which contain azurophilic granules, are always positive for the primary granule markers myeloperoxidase, elastase, and cathepsin G and that myelocytes containing specific (neutrophilic) granules are always positive for the secondary granule marker lactoferrin. Thus Romanowsky-stained preparations reliably demonstrate primary and secondary granules and can yield valuable information concerning these particles.

However, primary granule markers were present in some immature cells in which azurophilic granules were not seen with the May-Grünwald-Giemsa stain. Whether these proteins were present in the endoplasmic reticulum and Golgi apparatus or in mature granules that were not stained with the Romanowsky agent cannot be determined. Nevertheless, it is clear that some cells that would be considered myeloblasts in conventionally stained preparations contain primary granule proteins. The results also establish that most primary granule proteins appear simultaneously in promyelocytes.

Lysozyme is unique because it is present in both classes of granules. Spitznagel et al. showed that primary granules can be separated into “slow” and “fast” sedimenting bands by careful cell fractionation techniques and that myeloperoxidase predominates in the slow fraction while lysozyme is a major constituent of the fast band. Accordingly, it was interesting to find that some early promyelocytes, strongly reactive for myeloperoxidase, contained minimal amounts of lysozyme. This suggests that slow granules are synthesized first. However, differences in the sensitivity of the antisera could also explain the finding, and further studies will be required to resolve this issue.

The finding that the primary granule markers became less intense while lactoferrin and lysozyme became more intense in developing myelocytes is undoubtedly due to continued synthesis of secondary granules in these cells, a result entirely consistent with a previous report by Bainton et al. The results also demonstrate that lactoferrin is an excellent marker for secondary granules and can provide an index of neutrophil maturation.

In other studies we have shown that the dual-labeled fluorescent antibody technique is useful in studying abnormal blood cells. The integration of this fluorescent technique with conventional Romanowsky stains now adds a further dimension to cellular identification and is a powerful technique anticipated to yield valuable data in other studies.
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