Chapter 5

5.1. Overview

Ultrastructural post-embedding immunogold methods allow the detection of granule products in properly prepared samples which contain mast cells or basophils. Moreover, the probe size is sufficiently flexible that small transport vesicles containing these products can be labeled and quantified. In addition to our studies of cytokine localizations [see Chapter 4] we used these methods to detect immunoreactive chymase (in RMCs), CLC protein (in HBs), and histamine (in RMCs). These methods and their results are reviewed here.

5.2. Chymase

Rat peritoneal mast cell chymase is a serine protease that cleaves peptide bonds in the carboxyl side of aromatic amino acid residues [176–182]. Early histochemical evidence suggested that this activity was localized to cytoplasmic granules [183], a concept supported by a large amount of biochemical and cell fractionation data [176–182].

We defined the ultrastructural localization of chymase in rat peritoneal mast cells (fig. 56) using standard aldehyde fixation and a microwave fixation method [184] that provided excellent preservation of subcellular details and retention of chymase that was visualized with a post-embedding immunogold
Fig. 56. Rat peritoneal mast cell, fixed for 7 s by microwave-assisted aldehyde fixation and prepared for the immunogold localization of chymase. Numerous gold particles are present over the granules. Cytoplasm and mitochondria (M) are not labeled. Bar = 0.2 μm. [From 152, with permission.]
procedure [152, 185]. Thin sections were exposed first to goat IgG anti-rat chymase and second to gold-conjugated rabbit Ig directed against goat IgG. By transmission electron microscopy, gold particles labeled the matrix of cytoplasmic granules (fig. 56).

Control sections treated with non-immune sera or by omission of the specific primary antibody did not exhibit labeling of mast cells. Thin sections treated simultaneously with purified RMC chymase and anti-chymase antibody showed marked reduction in granule staining.

5.3. Charcot-Leyden Crystal Protein

CLCs were initially described in 1853 by Charcot and Robin [186] in the tissues of a patient with leukemia. In 1872, Leyden [187] also identified these distinctive bipyramidal hexagonal crystals in the sputum of asthmatics. Since their initial description, CLCs have been associated with eosinophils and are considered a hallmark of eosinophil-associated diseases [188]. The CLC protein, which is the sole protein constituent of CLCs [189, 190], has been purified to homogeneity, characterized biochemically and immunochemically [189–192] and the cDNA has been cloned [193]. The CLC protein is also a prominent constituent of HBs, which form intracellular CLC under hypotonic conditions [194–196]. Basophil CLC protein is immunochemically indistinguishable from eosinophil CLC protein [194]. CLC protein is abundant in both granulocytes, with eosinophils containing approximately 8 pg/cell [192] and basophils containing approximately 3 pg/cell [194].

We used an immunogold post-embedding ultrastructural procedure to stain the CLC protein (fig. 8–10, 57, 58A–C) [151] as follows: 70-nm sections were placed on gold grids and floated on 50-μl drops of reagent at 25°C. The following sequence of reagents was used: (a) 4% sodium metaperiodate, 15 min; (b) 3 × wash, 10 min each in 0.2 m Millipore-filtered 20 mM Tris(hyroxymethyl)aminomethane buffer containing 0.9% saline, 0.1% globulin-free bovine serum albumin (BSA), pH 7.6 (TBS-BSA); (c) 5% normal goat serum in TBS-BSA, 1 h; (d) primary rabbit polyclonal affinity chromatography purified anti-CLC (150 μg/ml in TBS-BSA containing 1% Tween-20 and 1% normal goat serum, 2 h at 25°C; (e) 3 × wash, 10 min each in TBS-BSA; (f) secondary gold-labeled antibody (1:20 dilution of either 10-, 20-, or 30-nm colloidal gold conjugated to goat anti-rabbit IgG in TBS-BSA containing 0.1% Tween-20, 0.4% gelatin and 1% normal goat serum), 1 h; (g) 2 × wash, 10 min each, in TBS-BSA, and (h) 2 × wash, 10 min each, in distilled water.

Specificity controls included the following alterations of the standard sequence: (1) omission of specific primary antibody; (2) substitution of non-
Fig. 57. Mature human basophil developing in a 7-week cord blood culture supplemented with murine 3T3 fibroblast culture supernatant and prepared with immunogold to demonstrate CLC protein. Gold has labeled nuclear and cytoplasmic sites diffusely and almost all granules contain diffuse label as well. Some granules are partially empty, i.e., they have undergone PMD. Bar = 1 μm. [From 25, with permission.]
Fig. 58. A–C Human basophils prepared with an immunogold method to localize Charcot-Leyden crystal (CLC) protein. D–F Panels were prepared with an enzyme-affinity-gold method to label histamine [see Chapter 6]. Gold particles, indicating CLC protein in the central CLCs (C) within the particulate matrix of granules (G) and overlying the particulate matrix, are seen in A and B. Histamine, indicated by gold particles (D, E), resides in the particulate matrix and not in the homogeneous CLCs (C) within these granules (G). The non-particulate primary granules (G) are heavily labeled for CLC protein in C but not for histamine in F. A × 70,300. B × 56,100. C × 57,300. D × 57,000. E × 85,500. F × 66,500. [From 201, with permission.]
immune normal rabbit IgG (150 μl) for the specific primary antibody, and (3) substitution of solid-phase CLC protein-Sepharose-absorbed primary antibody for unabsorbed specific primary antibody.

Using this immunogold method, we defined the subcellular locations of the CLC protein in quiescent peripheral blood eosinophils [151] and basophils [197], and their activated counterparts in vivo [125, 198], ex vivo basophils [67, 199–201], and in IL-5-induced eosinophils which developed in suspension cultures of human CBC [25, 64, 65, 202]. In addition, we have described the endocytosis of CLC protein and its intralysosomal location in monocytes and macrophages in vivo and in vitro [64, 125, 203]. Although the cellular function(s) of this protein is still obscure, the presence of such large quantities in eosinophils and basophils (fig. 57) suggests an important role in normal or pathological events associated with these lineages. The cloning of the gene for the CLC protein [193] and expression of the protein product [204] aid our understanding of the biological properties of this unique basophil and eosinophil constituent.

HMCs, in contrast to basophils, do not stain for the CLC protein by light microscopy [205]. Since we noted an admixture of lineages in suspension cultures of cord blood supplemented with c-kit ligand-containing additives [see Chapter 2], we examined these cells for CLC protein using an immunogold method to detect this protein. We found that mature basophils that differentiated in these long-term cultures contained CLC protein (fig. 57) [25] and noted the subcellular distribution of this protein in relationship to activated phenotypes [see Chapter 2] which the mature basophils assumed over 3–14 weeks of culture. In addition to the previously identified locations (i.e., intragranular, cytoplasmic, nuclear, vesicular, and formed CLCs within granules, cytoplasm, and nucleus) (fig. 8, 9, 57, 58A–C), CLC protein was also present in Golgi-associated smooth membrane-bound vesicles. In contrast, none of the cells in the mast cell lineage contained CLC protein (fig. 29), thereby providing an excellent immunomorphologic difference for their distinction from basophils arising in the same cultures.

The presence of CLC protein (and its absence in mast cells) is a consistent finding regardless of the source of growth factors used or the individual cord sample from which starting cells were secured or the time in culture [see Chapter 2]. CLCs have been described in HBs before [19, 194, 197, 206]. By ultrastructural analysis, we have noted CLCs within particle granules and free in the cytoplasm or nucleus of basophils [197]. Our initial immunogold studies of the distribution of CLC protein in HBs showed labeled, formed CLCs within granules and rare cytoplasmic vesicles that contained CLC protein [197]. These studies were extended to show that CLC protein is contained within cytoplasmic and nuclear CLCs of activated peripheral blood basophils [67, 199,
and during release and recovery experiments of isolated, purified peripheral blood basophils, the CLC protein was not released extracellularly [207] but was instead redistributed to cytoplasmic, nuclear, plasma membrane, and vesicular locations [67, 199, 200]. In particular, basophils that displayed the morphological phenotype of PMD had increased diffuse labeling of cytoplasm and nucleus, as well as labeled plasma membranes [67]. Later, after recovery, labeling of these locations diminished and, again, the granular compartment became predominantly labeled (fig. 58A–C) [67; see Chapter 9]. We also noted the appearance of a heavily labeled, newly evident, homogeneously dense granule population (fig. 58C) [67] that resembled the CLC protein-containing primary granules of eosinophils [151].

The distribution of CLC protein in the basophils that differentiate in c-kit ligand-supplemented suspension cultures of cord blood [25] resembled that described before in peripheral blood basophils [197]. As in the stimulated secretory model described above [67, 200, 206], we found increased amounts of diffuse cytoplasmic, nuclear, and plasma membrane label in cells expressing the PMD phenotype [25]. At later times in cultures in which some basophils had recovered granule contents, we noted increased numbers of CLC protein-positive particle granules, intragranular CLCs, and CLCs free in the cytoplasm and nucleus [25]. Also noted were increased numbers of homogeneously dense, heavily labeled granules [25]. Occasionally, we found Golgi area vesicles that were positively labeled for CLC protein.

5.4. Histamine

Histamine released by mast cells plays an important role in immediate hypersensitivity reactions and in other inflammatory processes [reviewed in 208]. Several lines of evidence, including differential centrifugation [178, 209], biochemical determinations [208, 210], and autoradiographic [211] or immunohistochemical [212, 213] studies, indicate that histamine is associated with mast cell secretory granules.

We used a post-embedding immunogold approach to demonstrate the fine structural localization of histamine in the granules of unstimulated rat peritoneal mast cells that were fixed either by standard aldehyde fixation or by a microwave-aldehyde fixation method [152, 153, 184].

For histamine detection, post-embedding, immunogold staining methods were done as follows: 70-nm sections of unstimulated cells on nickel grids were floated section side down on 30-µl drops of reagents at 20°C. The following reagents were used in sequence: (a) 10% sodium metaperiodate for 0, 5, 15, 30, or 60 min to solubilize osmium and unmask antigens; (b) wash in 20 mM
Tris buffer containing 0.9% saline, 0.2% non-immune goat IgG, pH 7.6 [TBS-Ig]; (c) 5% non-immune goat IgG for 20 min; (d) primary antibody [guinea pig anti-histamine-BSA antiserum (Peninsula Labs, Belmont, Calif., USA), or rabbit anti-histamine-BSA antiserum (Milab, Malmo, Sweden, and Chemicon, El Segundo, Calif., USA), used neat or diluted up to 1:100, in TBS-Ig, 0.1% Tween-20, 1% normal goat serum] for 1 h at 20°C or for 24 h at 4°C; (e) wash 3 ×, 10 min each, in TBS-Ig; (f) secondary 5- or 10-nm gold-conjugated goat IgG directed against guinea pig or rabbit IgG (diluted 1:20–1:80 in TBS-Ig, 0.1% Tween-20, 1% normal goat serum) for 1 h at 20°C, and (g) wash 2 × in TBS-Ig, and wash 2 × in distilled water. Membrane contrast was enhanced with 0.25% lead citrate for 3 min.

Controls included the following: (a) omission of primary antibody and (b) substitution of normal guinea pig IgG for the specific guinea pig anti-histamine-BSA antiserum.

The specificity of the antibody-antigen reaction was further established by doing absorption and blocking controls. We absorbed the guinea pig anti-histamine-BSA antiserum with histamine bound to agarose (160–320 μl/ml) or to agarose alone for 1–3 h at 20°C. After centrifugation in a microfuge for 1 min at 1,000 rpm at 20°C, the supernatant was used in place of the primary antibody in the labeling experiments. For a blocking control, purified histamine hydrochloride (1–100 mg/ml) was incubated with guinea pig anti-histamine-BSA antiserum (final dilution 1:50 in TBA-Ig-Tween) for 1 h at 20°C and then the sections on the grids were added to the droplet for simultaneous incubation with the histamine hydrochloride and the primary antibody for 1 h at 20°C.

By transmission electron microscopy, gold particles indicating the presence of histamine were localized to the matrix of cytoplasmic granules (fig. 59). Control sections, including omission of the primary antibody, substitution of normal guinea pig IgG for the specific antibody, and exposure of the anti-histamine antiserum to either purified histamine or histamine bound to agarose were negative, or, in the histamine absorption, showed a significant reduction (p < 0.005) in granule staining [153].
Fig. 59. Post-embedding immunogold labeling for histamine in rat mast cell granule. Bar = 0.2 μm.