Histol Histopathol (1996) 11: 711-728

Histology and Histopathology

Invited Review

Human basophil recovery from secretion. A review emphasizing the distribution of Charcot-Leyden crystal protein in cells stained with the postfixation electron-dense tracer, cationized ferritin

A.M. Dvorak

Departments of Pathology, Beth Israel Hospital and Harvard Medical School, Boston, Massachusetts, USA

Summary. Basophils of two species, guinea pigs and humans, have been shown by ultrastructural analyses to recover from noncytotoxic secretory processes by conservation and synthetic mechanisms. In human basophils, an electron-dense tracer (cationized ferritin) used after fixation labels membranes in continuity with plasma membranes at planes of section out of view, thereby indicating internalization of previously externalized granule membranes. Conservation of previously emptied granule containers that remain in the cytoplasm after secretion was associated with reaccumulation of electron-dense particles and condensation of these dense materials therein. Granule- and vesicle-poor, previously stimulated cells developed large numbers of cytoplasmic vesicles beneath plasma membranes, in expanding Golgi areas and in peri-granular areas of the cytoplasm. Cytochemical labeling techniques to localize histamine and Charcot-Leyden crystal protein revealed reaccumulation of these two granule proteins in recovering human basophil granules. The mechanism(s) of their recovery likely involves both synthesis of new proteins and conservation by internalization of secreted proteins bound to cell surfaces.

Key words: Basophil, Secretion, Recovery, CLC protein, Histamine

Introduction

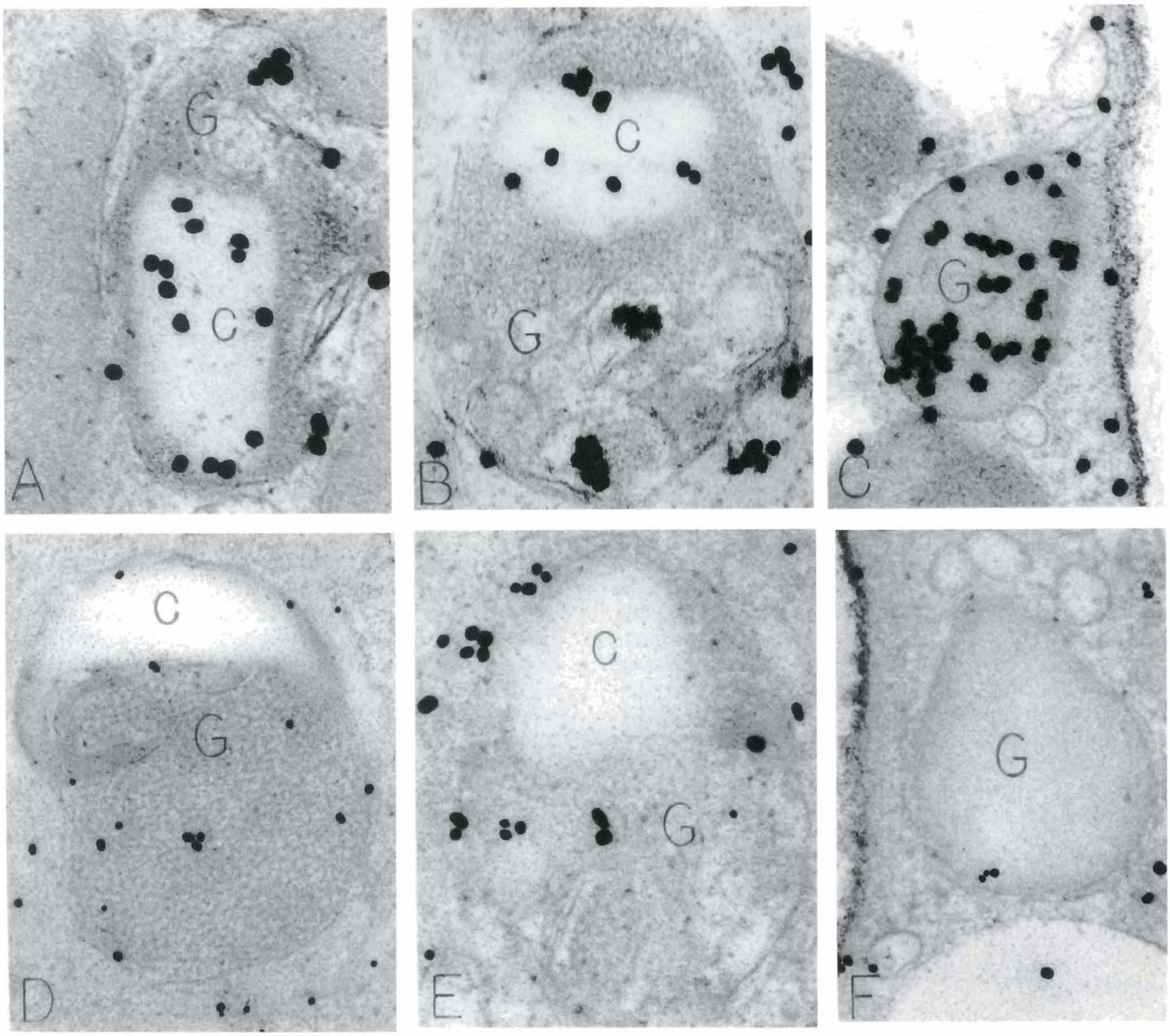
Granulocytes are generally viewed as short-lived endstage cells with little or no potential for recovery following secretion of stored granule proteins. Basophilic leukocytes (one of the granulocyte lineages) have, however, been shown to recover from regulated secretion in two species-guinea pigs (Dvorak et al., 1982, 1985a, 1987) and humans (Dvorak et al., 1995a, 1996c). The studies in these two species were made possible by methods devised to purify mature basophils from blood samples and to maintain them in short-term cultures (Dvorak et al., 1974; Galli et al., 1976; MacGlashan et al., 1982). Initially, routine ultrastructural analysis of the changing morphology of previously stimulated cells during recovery intervals established that the cells did not die, were not injured, maintained a mature phenotype, and reconstituted secretory granules by a mixture of synthetic and conservation mechanics (Dvorak et al., 1982, 1996c). These morphologic events were probed more extensively with a number of ultrastructural methods designed to examine the distribution of basophil-specific surface antigen(s) (localized with an immunoferritin technique [Dvorak et al., 1985a]), of non-specific esterase(s) (stained with a cytochemical method [Dvorak et al., 1987]), of a serine protease (localized by autoradiography of an isotope-labeled inhibitor of serine protease(s) [Dvorak et al., 1987]) in guinea pig basophils and with a postfixation electron-dense tracer, cationized ferritin (CF) (Dvorak et al., 1995a, 1996c), an enzyme-affinity-gold cytochemical technique to label histamine (Dvorak et al., 1993, 1995a) and an immunogold method to detect the basophil granule protein, Charcot-Leyden crystal (CLC) protein (Dvorak et al., 1988, 1990a,b, 1991a, 1992, 1994a,b; Dvorak and Ackerman, 1989; Zhou et al., 1992; Dvorak and Ishizaka, 1994) in human basophils. We review here these studies documenting the recovery potential of stimulated basophils with particular emphasis on human basophils. The postfixation electron-dense tracer, cationized ferritin (Dvorak et al., 1995a, 1996c) an enzyme-gold affinity-labeled histamine (Dvorak et al., 1995a) and immunogold-labeled CLC protein provide new information regarding membrane shifts and secretion and re-uptake of granule proteins during recovery from stimulated secretion of human basophils.

Offprint requests to: Dr. Ann. M. Dvorak, M.D., Department of Pathology, Beth Israel Hospital, 330 Brookline Avenue, Boston, Massachusetts 02215, USA

Methods

Isolation, purification, stimulation and histamine release assay of human basophils

Buffy coat cells, obtained from normal donors undergoing hemapheresis, were partially purified by countercurrent centrifugal elutriation (de Boer and Roos, 1986), and then placed on Percoll density gradients (1.075 and 1.066 g/ml). Purified basophils were recovered from the interface between the two Percoll layers (Warner et al., 1987). Final preparations contained



All figures are of human basophils prepared with an immunogold method to localize Charcot-Leyden crystal protein, except Fig. 1D-F, which were prepared with an enzyme-affinity-gold method to label histamine. All figures are of cells incubated in cationized ferritin after fixation and before further processing for electron microscopy. Fig. 1A,B are of basophils stimulated for 20 seconds with FMLP; Fig. 1C-F, 2-18 are of FMLP-stimulated basophils recovered from short-term cultures at 10, 20, 30 minutes, 1, 3 or 6 hours thereafter.

Fig. 1. Charcot-Leyden crystal protein (CLC protein) panels (**A-C**) and histamine (panels **D-F**) localization in basophil granules. 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 panels A and B. Histamine, indicated by gold particles (panels D and 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 panel C but not for histamine in panel F. A, x 82,000; B, x 66,000; C, x 67,500; D, x 66,500; E, x 100,000; F, x 78,000

32% basophils.

Histamine release assay

Cells were suspended in PAGCM (PIPES (piperazine n,n'-bis-2-ethane sulfonic acid)-albumin-glucose supplemented with 1mM $CaCl_2$ and 1mM $MgCl_2$) and stimulated with 1 µM f-Met peptide (FMLP) (formyl-methionyl-leucyl-phenylalanine) at 37 °C for various periods of time (0-6 hours) in a final

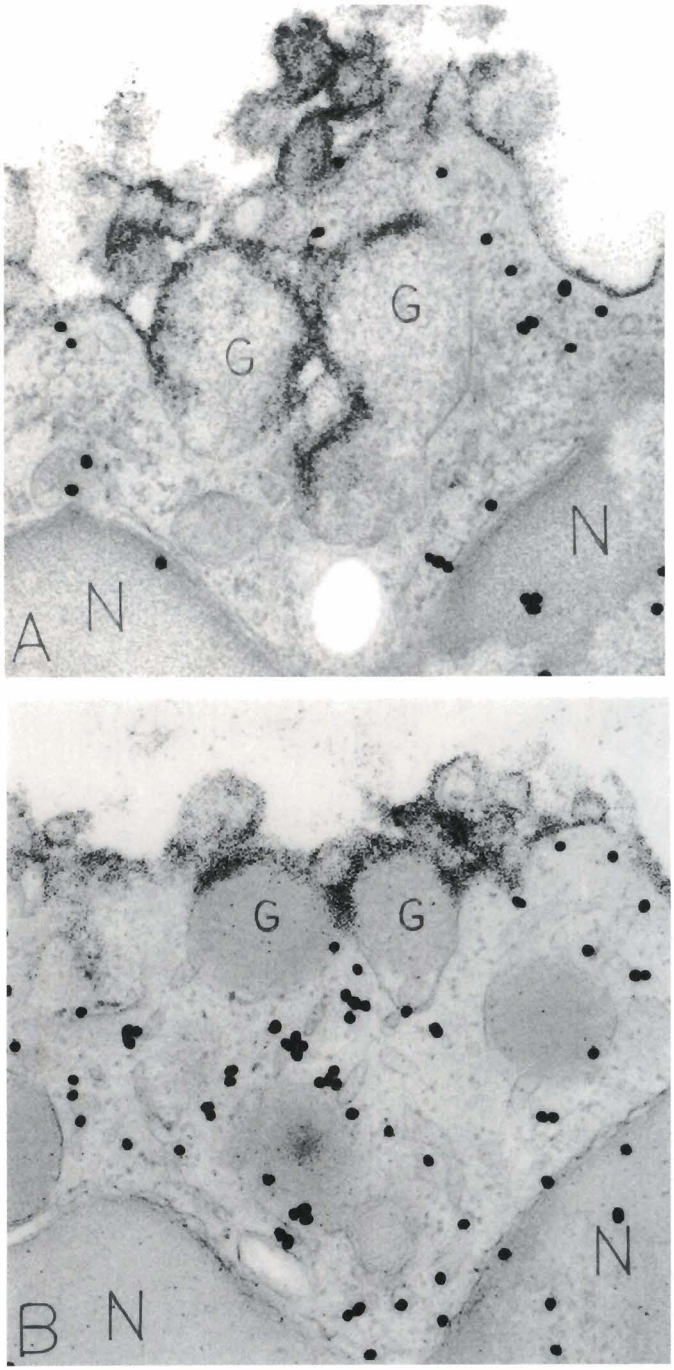


Fig. 2. Membrane-free, extruded basophil granules (G) reside in deep invaginations of the cell surface. An electron-dense layer of cationized ferritin coats the plasma membrane and outer surfaces of extruded basophil granules. Gold label, indicating CLC protein, is located in the cytoplasm and nucleus (N) but not in the secreted particle granules. A, x 44,000; B, x 45,000

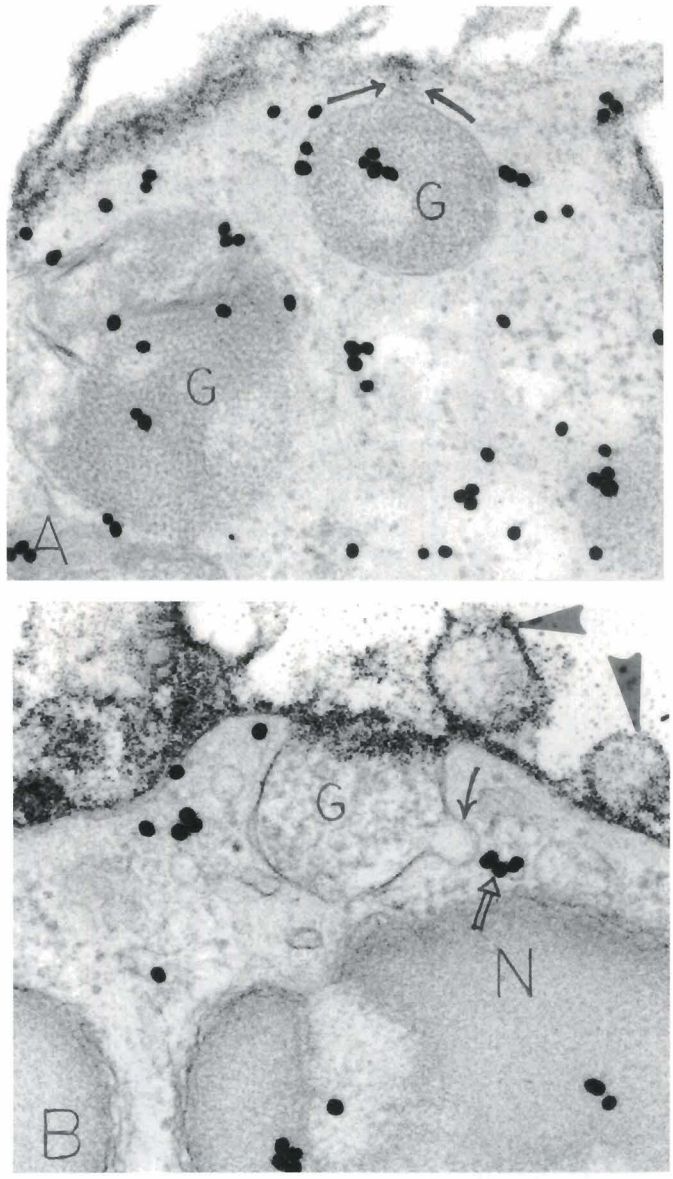


Fig. 3. A previously released basophil granule (G) shows a narrow pore filled with CF (arrows) which connects to the CF-positive cell surface in panel **A**. Narrow cytoplasmic processes virtually envelop this CLC protein-positive granule, indicating re-uptake of the granule into the cell. An adjacent granule contains label for CLC protein. Diffuse label is also present in the cytoplasm. At higher magnification in panel **B**, the loosely structured granules (G) is in continuity with the extracellular space. Note the prominent CF layer bound to the plasma membrane, to the outer surface of the granule (G) being re-internalized, and to all surfaces of two extruded granules (arrowheads). A single vesicle is attached to the granule undergoing internalization (closed arrow). Similar cytoplasmic vesicles contain CLC protein (open arrow) as does the nucleus (N). A, x 45,000; B, x 65,000

volume of 100 microliters containing ~1 million basophils, the supernatant removed and assayed for histamine release using the automated fluorometric technique of Siraganian (1974). Cells not stimulated with FMLP were also examined, and spontaneous histamine release was subtracted to give final stimulated histamine release values. Histamine release was 30% at 10 minutes and 36% at 30 minutes.

Fixation and preparation for routine electron microscopy

Basophils were fixed and prepared for electron microscopy as previously described (Dvorak et al., 1980). Briefly, cell suspensions were fixed by diluting them in a 10-fold excess of 2% paraformaldehyde, 2.5% glutaraldehyde and 0.025% $CaCl_2$ in 0.1M sodium cacodylate buffer, pH 7.4. Cells were fixed for 1 hour at room temperature, washed and resuspended in 0.1M sodium cacodylate buffer, pH 7.4, 4 ºC. Cell samples were suspended in warm 2% agar, rapidly centrifuged to form agar pellets containing cells and postfixed for 2 hours at 4 ºC in 2% aqueous osmium tetroxide and 1.5% potassium ferrocyanide in 0.1M sodium phosphate buffer, pH 6.0. Cell pellets were then dehydrated in a graded series of alcohols and embedded in a propylene oxide-Epon sequence. Thin sections were stained with lead citrate.

Postfixation electron-dense cationized ferritin tracer method (Danon et al., 1972; Dvorak et al., 1980, 1995a, 1996c)

Cells from some fixed samples were resuspended in Hanks' balanced salt solution, containing 150 microliters (0.5 mg) cationized ferritin (Miles Laboratories, Inc.) on a rotary shaker set at low speed for 30 minutes at room temperature. Further processing and embedding was done as in the above paragraph (routine electron microscopy).

Diamine oxidase (DAO)-gold enzyme affinity method to detect histamine (Dvorak et al., 1993, 1995a)

A colloidal suspension was prepared according to the method of Frens (1973). Four ml of an aqueous 1% solution of sodium citrate was added to a boiling aqueous solution of 100 ml 0.01% tetrachloroauric acid and allowed to boil for 5 minutes before cooling on ice. The pH of the colloidal gold suspension so produced was adjusted to 7 with 0.2M potassium carbonate. Preparation of the DAO-gold complex was according to the method of Bendayan (1984). Three mg of DAO was dissolved in distilled water and placed in polycarbonate ultrafuge tube with 10 ml of the gold suspension. The mixture was centrifuged at 25,000 rpm for 30 minutes, 4 ºC, in a Beckman ultracentrifuge with a #50.2 Ti rotor.

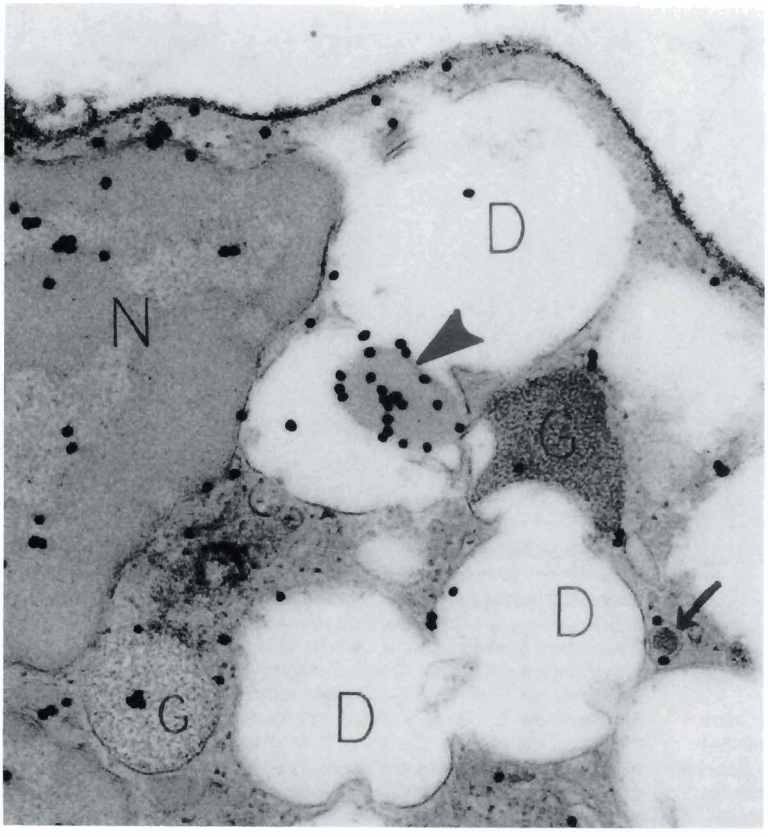


Fig. 4. Intracytoplasmic degranulation (D) structures persist in this recovering basophil. They are electron-lucent and do not stain with CF (in contrast to the plasma membrane) and, therefore, are not open to the exterior in this cell at different planes of section. CLC protein is present in the CLC (arrowhead) within a degranulation chamber, in the particulate matrix of granules (G) in the subplasma membrane cytoplasm, the nuclear (N) matrix and perinuclear membrane, and in a particle-filled cytoplasmic vesicle (arrow). x 36,000

The DAO-gold complex formed a red sediment that was carefully recovered and resuspended in 3 ml 0.1M PBS containing 0.02% polyethylene glycol, pH 7.6 (final concentration 1 mg DAO/ml).

For cytochemical labelling, section-containing grids were inverted and floated on PBS drops for 5 minutes, followed by incubation on a drop of DAO-gold at 37 °C for 60 minutes. The grids were vigorously washed in distilled water and stained with dilute lead citrate for 10 minutes.

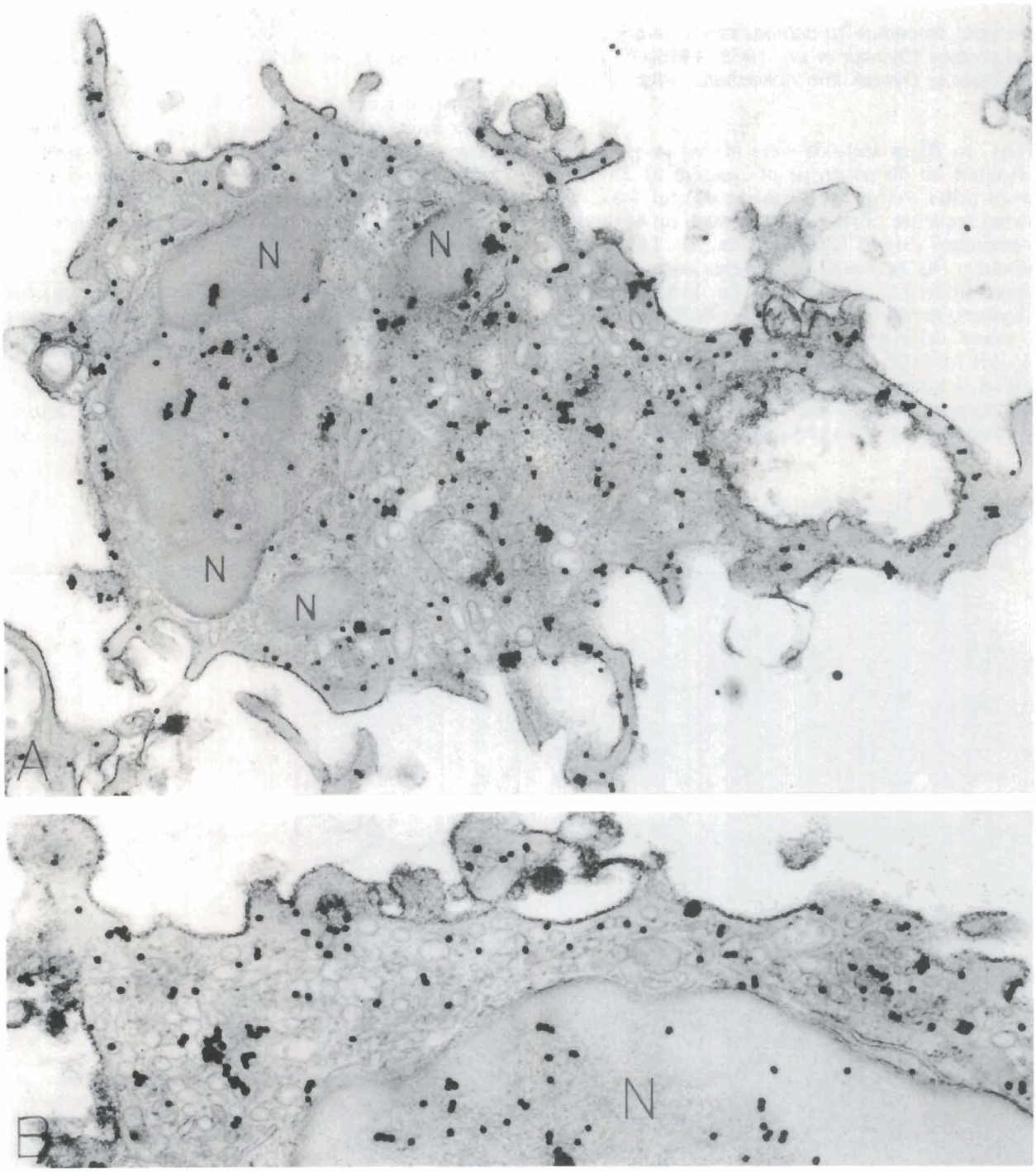


Fig. 5. Completely degranulated basophils (CDB) retain a polylobed nucleus (N), are undamaged, and express large numbers of cytoplasm vesicles during recovery from FMLP-stimulated secretion. CLC protein is labeled (panels **A** and **B**) in cytoplasmic vesicles, in the cytoplasm beneath the plasma membrane, and in the nuclear matrix. A, x 29,000; B, x 31,000

Specificity controls for the DAO-gold technique included: (a) incubation of grids with colloidal gold reagent only; (b) labeling of grids with DAO-gold that had been absorbed with histamine-agarose beads; (c) incubation of grids for 60 minutes at 37 °C in DAO, washing, and labeling with DAO-gold.

Immunogold procedure to demonstrate Charcot-Leyden crystal protein (Dvorak et al., 1988, 1990a,b, 1991a, 1992, 1994a,b; Dvorak and Ackerman, 1989; Zhou et al., 1992)

Fifty- to 70nm sections were placed on gold grids and floated on 50 µl drops of reagent at 25 °C on covered petri dishes containing dental wax. The following sequence of reagents was used: (a) 4% sodium meta-periodate (Sigma Chemical Co., St.. Louis, MO), 15 minutes; (b) 3x wash, 10 minutes each in 0.2 µM Millipore-filtered (Fischer Scientific, Indiana) 20 mM tris (hydroxymethyl)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 hour; (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 hours at 25 °C; (e) 3x wash, 10 minutes each in TBS-BSA; (f) secondary gold-labeled antibody (1:20 dilution of either 10-nm (Janssen, Life Sciences Products, Bromma, Sweden), 20-nm (EY Laboratories, San Mateo, CA), or 30-nm (Janssen) 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 hour; (g) 2x wash, 10 minutes each, in TBS-BSA; (h) 2x wash, 10 minutes each, in distilled water.

Specificity controls included the following alterations of the standard sequence: 1) omission of primary antibody; 2) substitution of non-immune normal rabbit IgG (150 µg) for the specific primary antibody; 3) substitution of solid-phase Charcot-Leyden crystal protein-Sepharose-absorbed primary antibody for unabsorbed specific primary antibody.

Ultrastructural analysis reveals morphologic mechanisms for human basophil granule reconstitution during recovery after stimulation with FMLP

Guinea pig and human basophils undergo exocytosis of membrane-free secretory granules when appropriately

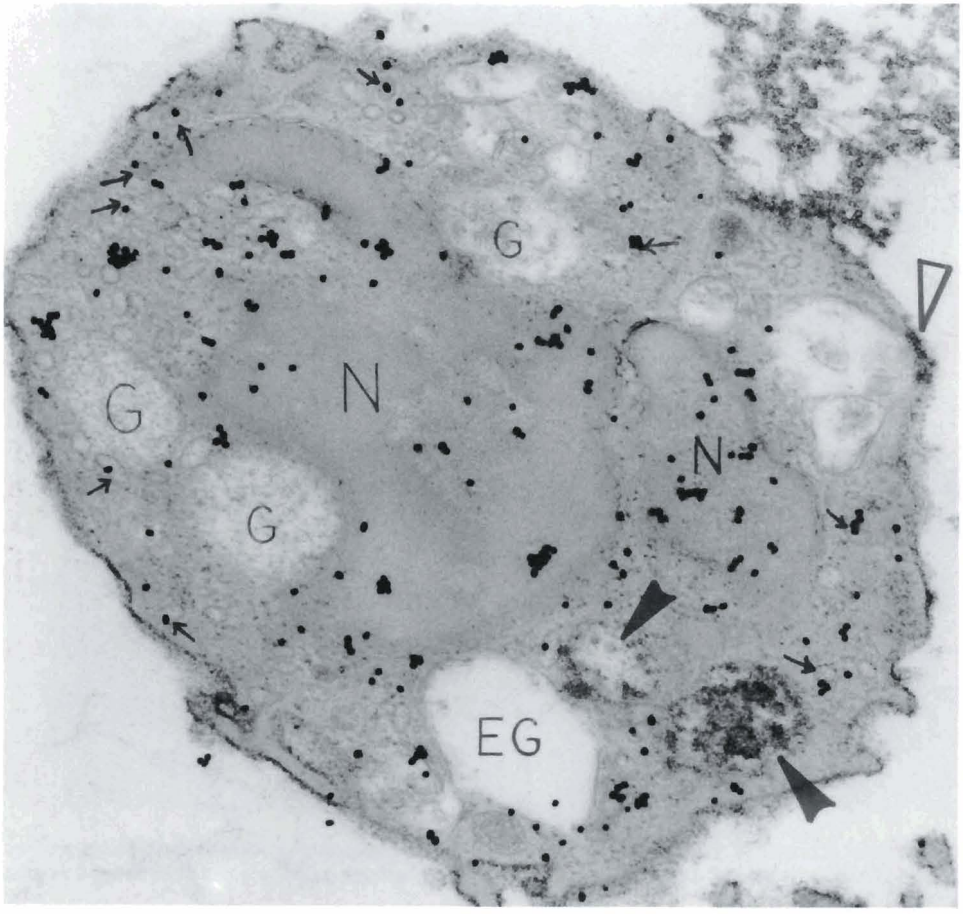


Fig. 6. A recovering basophil has gold label for CLC protein in the nucleus (N) and cytoplasm; much of the label is associated with the membranes and lumens of small vesicles (arrows). Reforming granules (G) with loosely textured contents are present. Some of the granule-sized structures are empty and lack CF (EG), are connected to an overlying CF-positive plasma membrane (open arrowhead), or are completely filled with CF (closed arrowheads). In the latter case, the CF-filled granule is in continuity with the surface at a different section plane. x 28,500

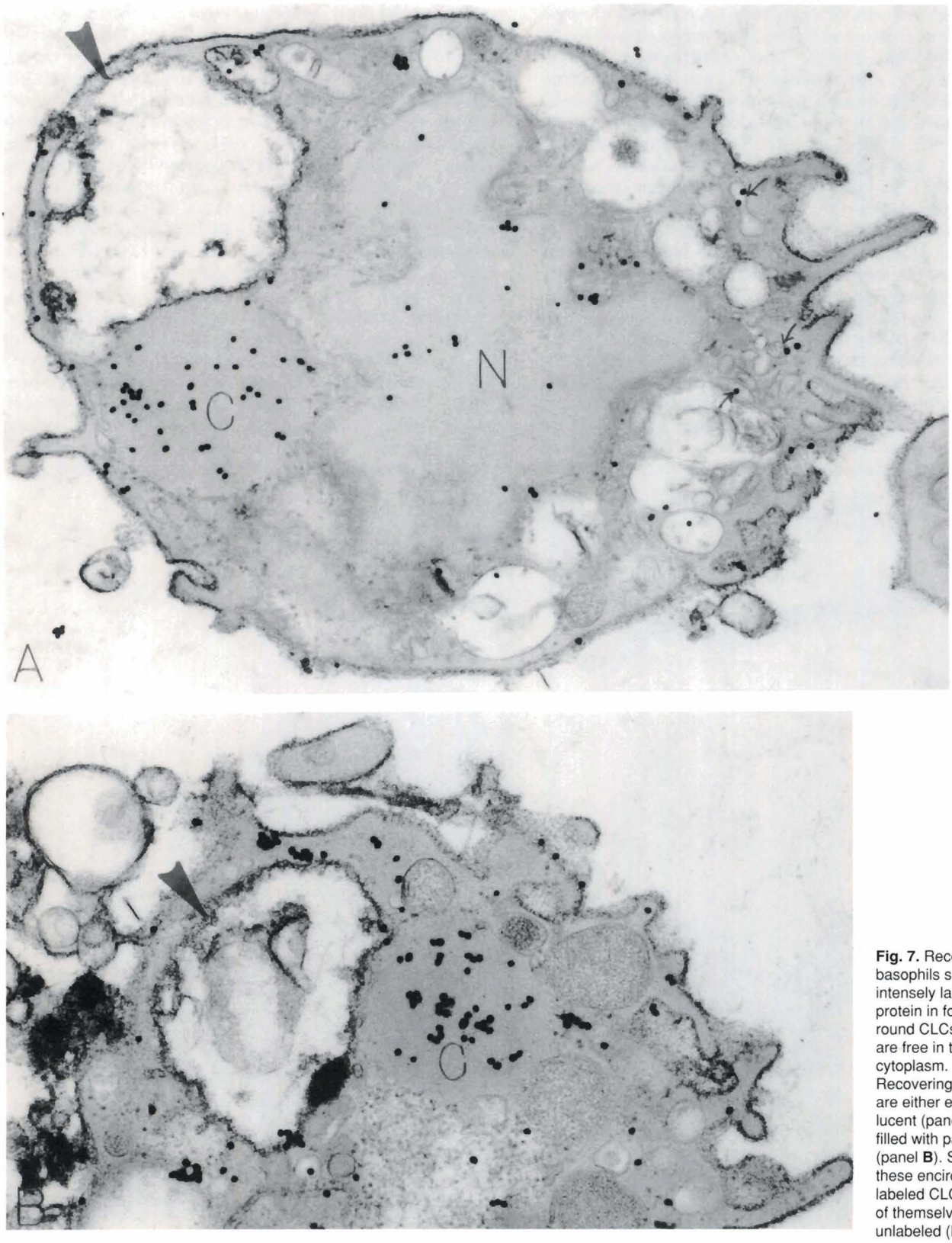


Fig. 7. Recovering basophils show intensely labeled CLC protein in formed, round CLCs (C) that are free in the cytoplasm. Recovering granules are either electron-lucent (panel **A**) or filled with particles (panel **B**). Some of these encircle the labeled CLCs but are of themselves unlabeled (B). Cytoplasmic vesicles (arrows) and nucleus (N) contain CLC protein. These recovering cells have CF-positive spaces of variable size in continuity with the cell surface (arrowheads). Membrane-bound CLC protein is attached to the CF-positive membranes of these structures. A, x 29,000; B, x 34,000

stimulated, a form of regulated secretion analogous to secretion from granulated secretory cells of many types (Dvorak, 1991). The secretagogue FMLP stimulates a morphological continuum during secretion from human basophils that encompasses the morphology of piecemeal degranulation (Dvorak, 1991, 1992, 1993), at early times post-stimulus (seconds), and anaphylactic degranulation (Dvorak, 1991, 1993), at later times post-stimulus (minutes) (Dvorak et al, 1991b). Morphological evidence of recovery from these secretory processes exists for guinea pig (Dvorak et al., 1982) and human basophils (Dvorak et al., 1996c), the latter beginning as quickly as 5-10 minutes post-stimulus (Dvorak et al., 1991b). Despite the different time frames for recovery in basophils of these two species, a time frame that probably reflects the variable speed of secretion, the morphology involved bears considerable similarities. Thus, basophils of each species recover from stimulated secretion by processes including conservation and synthesis (Dvorak et al., 1982, 1996c). In each instance, cell injury and death are absent, and viable cells renew their granules while retaining typical polylobed nuclei of mature granulocytes. The studies of human basophils employed an electron-dense post-fixation tracer, cationized ferritin, which stained the plasma membrane and the membranes of granule containers and cytoplasmic degranulation chambers in continuity with the cell surface out of the plane of section; those not in continuity failed to bind CF. For example, control, unstimulated basophils that did not undergo secretion during the recovery intervals examined did not develop the morphologies of recovery nor did they have CF-stained interior membranes (Dvorak et al., 1996c). The morphologic endpoint of secretion from human basophils (occurring in seconds to minutes) ultimately produced a completely degranulated basophil (CDB). CDBs are characteristically granule-free, vesicle-poor, viable, mature cells retaining polylobed nuclei (Dvorak et al., 1991b, 1996a). These cells, as well as cells with differing morphologies occurring earlier in the secretory spectrum (Dvorak et al., 1991b, 1996a), renewed granules by Golgi expansion and cytoplasmic

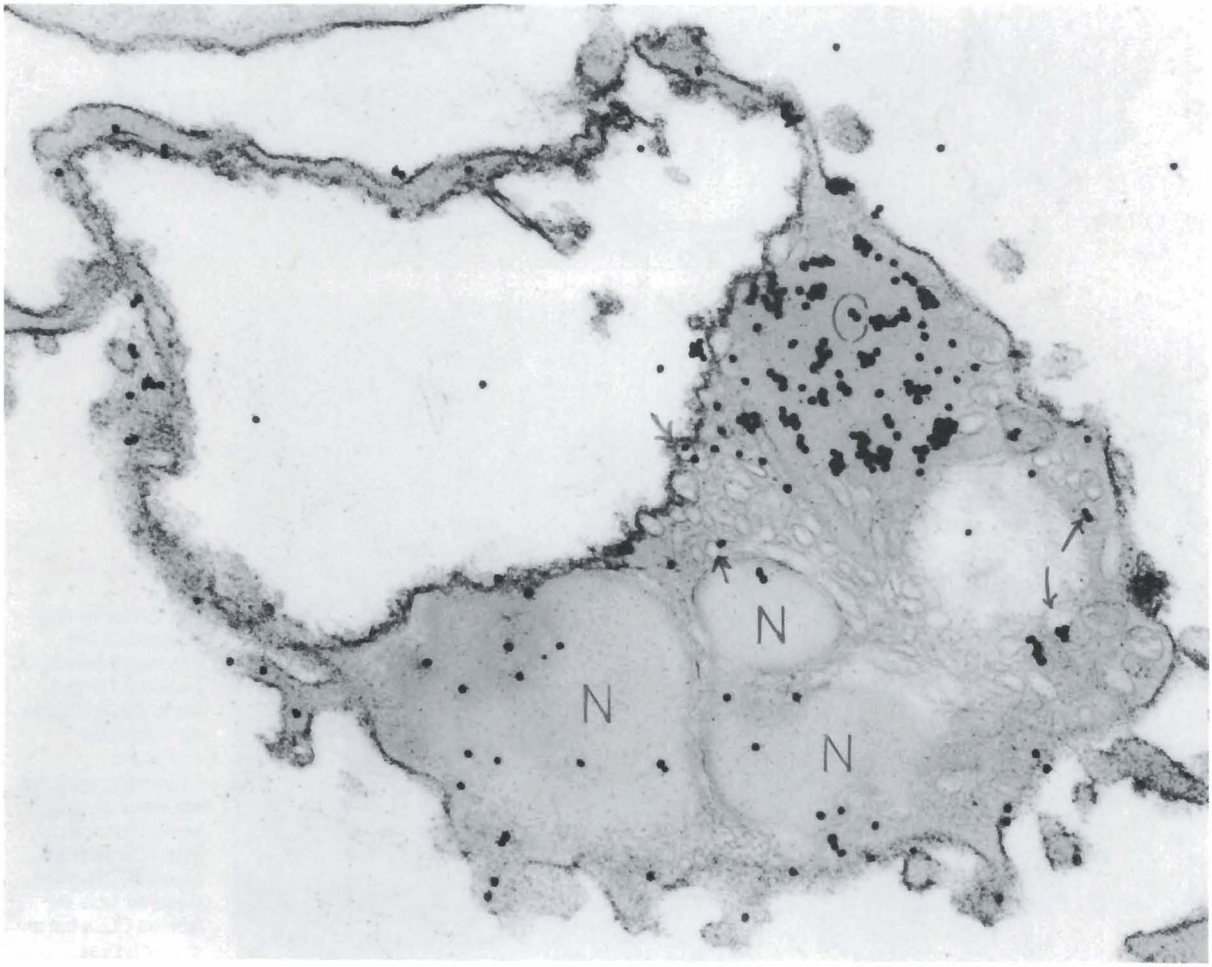


Fig. 8. This recovering basophil shows label, indicating CLC protein, concentrated in the round, cytoplasmic, nonmembrane-bound CLC (C). Cytoplasmic vesicles (arrows) and each lobe of the polymorphonucleus of this mature, granule-free cell are also labeled for CLC protein. The plasma membrane and a large, cytoplasmic electron-lucent space have CF bound to their surfaces, indicating continuity. x 31,000

vesicles increases (processes analogous to those in actively synthetic secretory cells) as well as by interiorization of membranes, vesicles, granules and granule contents, most of which could be the consequence of prior expansion of the plasma membrane and cell surface-attached materials resulting from stimulation of secretion. Thus, conservation of membranes and materials appears to be an integral part of basophil recovery from secretion.

Histamine-rich granules are reconstituted in human basophils after stimulated secretion

A new ultrastructural enzyme-affinity technique to localize histamine is based on the binding of diamine oxidase (DAO)-gold to its substrate, histamine (Dvorak et al., 1993). We used this method to determine whether the major granule population, which contains electron-dense particles, does indeed contain histamine (Dvorak

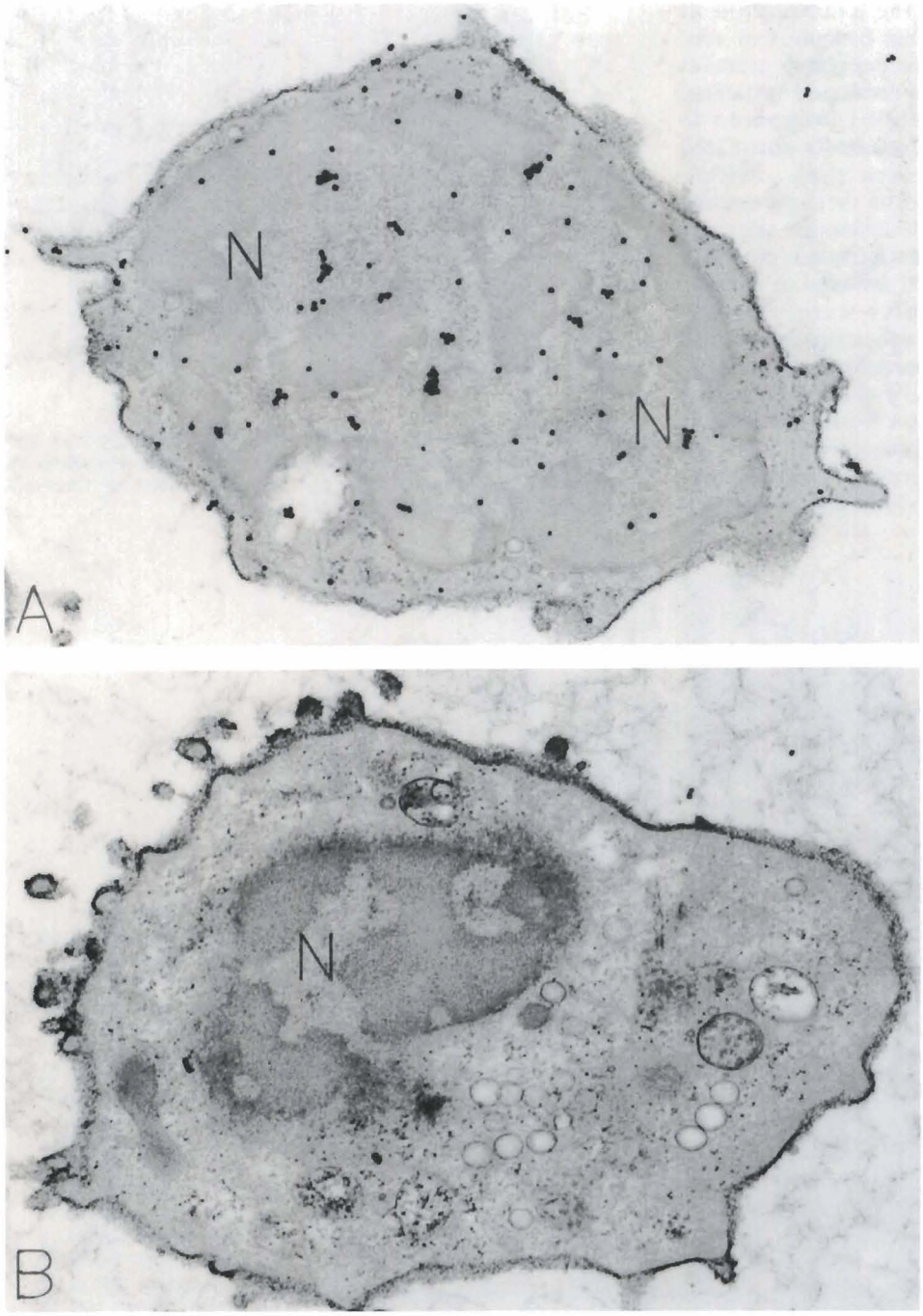


Fig. 9. A completely granule-free, recovering basophil (panel **A**) and a small lymphocyte (panel **B**), present in the same culture, are shown. The nucleus (N) and narrow cytoplasmic rim contain CLC protein in the small, granule-free basophil (panel A), allowing one to reliably distinguish this cell from the small lymphocyte (panel B) which contains no nuclear (N) or cytoplasmic CLC protein. The plasma membranes of both cells bind CF. A, x 22,000; B, x 21,000

et al., 1995a,b). While this might seem to be an obvious finding, these ultrastructural studies are among the first to actually demonstrate the granule location of histamine in basophils and mast cells at the ultrastructural level (Login et al., 1992; Dvorak et al., 1993, 1994c, 1995b). We also used this method to determine that small cytoplasmic vesicles also contained histamine in FMLP-stimulated, histamine-secreting, degranulating cells (Dvorak et al., 1995b). Thus, we proposed that these carrier vesicles were participating in secretion of histamine from stimulated human basophils. Application of the DAO-gold technique to the examination of degranulating and recovering human basophils *ex vivo* revealed that unaltered cytoplasmic secretory granules filled with electron-dense particles contained histamine (30.77 gold particles/μm^2; $p<0.001$ compared to background) and that specificity controls abrogated granule label for histamine (Dvorak et al., 1995a). Altered granules that were devoid of their particulate matrix in cells stimulated to release histamine were not labeled for histamine compared to background (p= N.S.). During recovery times spanning 10 minutes to 6 hours after FMLP stimulation, granules were partially reconstituted, again appearing electron-dense and containing histamine (33.49 gold particles/μm^2; p= N.S. compared to unaltered granules, and $p<0.05$ compared to altered granules at early stimulation times) (Dvorak et al., 1995a). Subcellular histamine-rich sites in recovering basophils included granules undergoing condensation of electron-dense products and collections

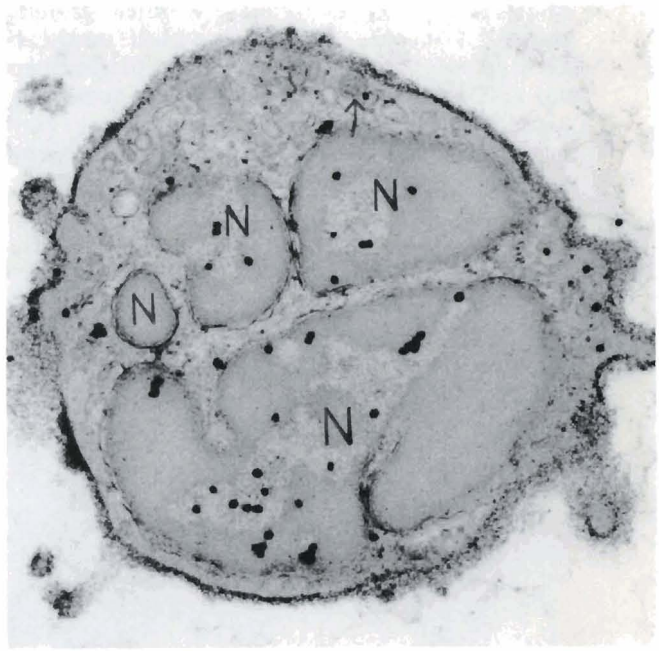


Fig. 10. This recovering basophil has no cytoplasmic granules and retains a mature, polylobed nucleus (N) with heavily condensed chromatin. The nucleus and one particle-filled vesicle in the peripheral cytoplasm (arrow) contain CLC protein. x 29,000

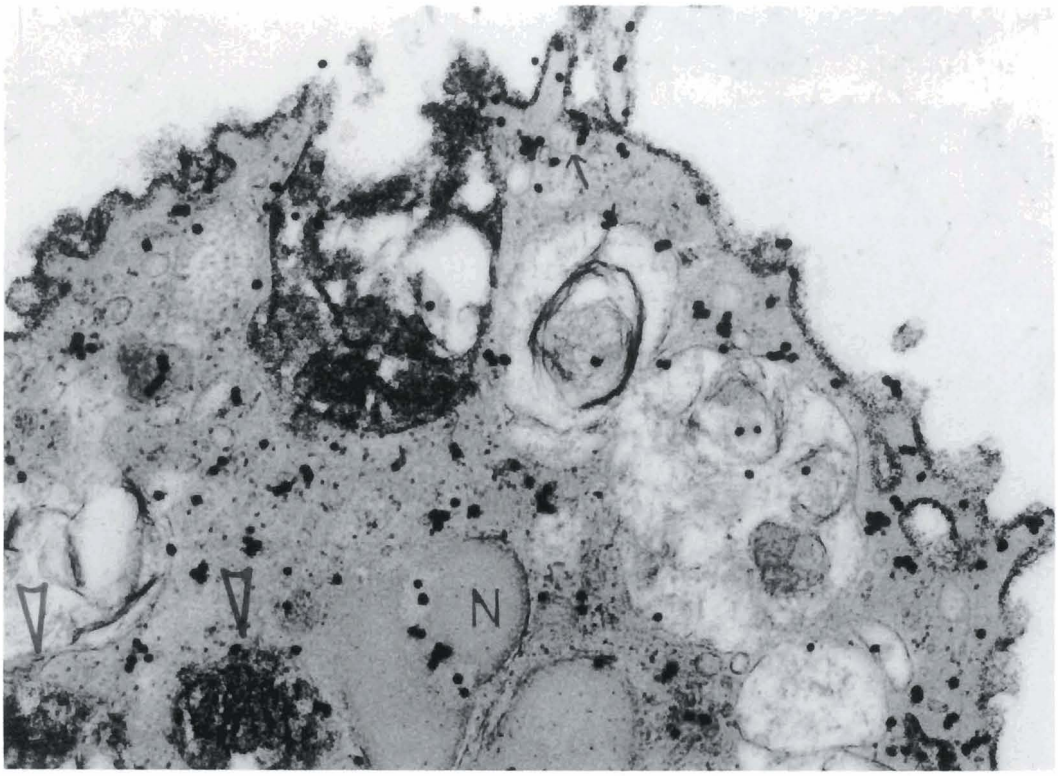


Fig. 11. A recovering basophil shows a CF-filled granule container in continuity with the CF-stained cell surface, several CF-free cytoplasmic granule containers with multiple dense membranes and CLC protein label, and several CF-filled cytoplasmic granule spaces (arrowheads). The nucleus (N), cytoplasm, plasma membrane, and cytoplasmic vesicles also contain gold particles. One of these is open to the surface, as indicated by both gold and CF labels (arrow). x 35,000

of cytoplasmic vesicles in three locations - beneath the plasma membrane, adjacent to granules, and in the Golgi region. Altogether, the enzyme-affinity method to detect histamine showed that unaltered granules of actively releasing human basophils, as well as similar granules that are reconstituted after FMLP-stimulated degranulation, contain histamine, but that altered granules in stimulated cells undergoing degranulation are devoid of histamine before (or whether or not) their membranes are also extruded. Reconstitution of histamine-rich granules is associated with DAO-gold-positive cytoplasmic vesicles, suggesting transport of histamine to granules that are derived from either new synthesis, re-uptake of released histamine, or both (Dvorak et al., 1995a).

Charcot-Leyden crystal protein is reconstituted in human basophils after stimulated secretion

An immunogold ultrastructural detection method for CLC protein (Dvorak et al., 1988) was applied to investigations of human basophils (Dvorak and Ackerman, 1989; Dvorak et al., 1994b). Human basophils have been shown to contain and form CLCs and the CLC protein (a lysophospholipase [Weller et al., 1980, 1982]) in morphological and biochemical studies (Ackerman et al., 1982; Dvorak, 1988, Dvorak et al., 1991b; Golightly et al., 1992). Ultrastructural localization studies of CLC protein in human basophils directly isolated from peripheral blood without exposure to time in buffers revealed this protein to reside in the major granule population (Dvorak and Ackerman, 1989). Similar cells after purification procedures and incubation in buffers over time had CLC protein in the nucleus, cytoplasm and granules (Dvorak et al., 1996a,b). Complex temporal and spatial related changes in the distribution of this granule protein during FMLP-stimulated secretion have been recorded (Dvorak et al., 1996a,b). CLC protein was found and quantitated in the following cellular compartments: nucleus, cytoplasm, granule, cytoplasmic vesicles, formed CLCs, degranulation channel, degranulation channel membrane, plasma membrane, and a newly evident granule population similar to primary granules in eosinophils. This combined analysis determined that CLC protein was transported to the cell surface in vesicles and by extrusion of intragranular CLCs (Dvorak et al., 1996a,b).

The reconstitution of CLC protein in human basophils recovering from FMLP (10 minutes to 6 hours) has also been examined. Ultimately, and as is the case in unstimulated cells, gold label indicating the presence of CLC protein in granules is evident in highest concentration within the homogeneous, central formed CLCs residing within the particulate matrix compartment of these granules (Fig. 1A,B). CLC protein labeled with gold is also evident in the particulate granule matrix (Fig. 1A,B). Recovering basophils,

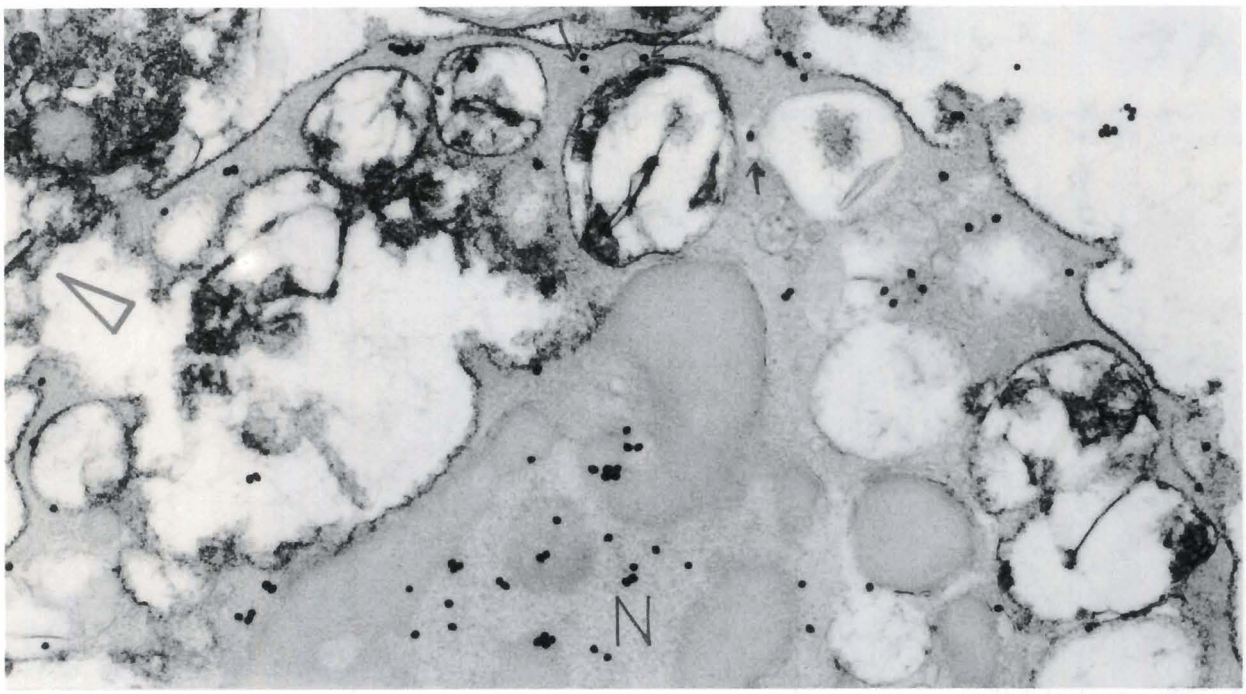


Fig. 12. Virtually all of the empty granules in this recovering basophil have CF-stained membranes, indicating continuity with the CF-stained plasma membrane in other planes of section. One large degranulation structure is open to the cell surface (arrowhead). Some partially full and empty granules do not have CF-stained membranes and, thus, are closed to the exterior. CLC protein label is visible in the nuclear (N) matrix and in vesicles near empty granules (arrows). x 30,000

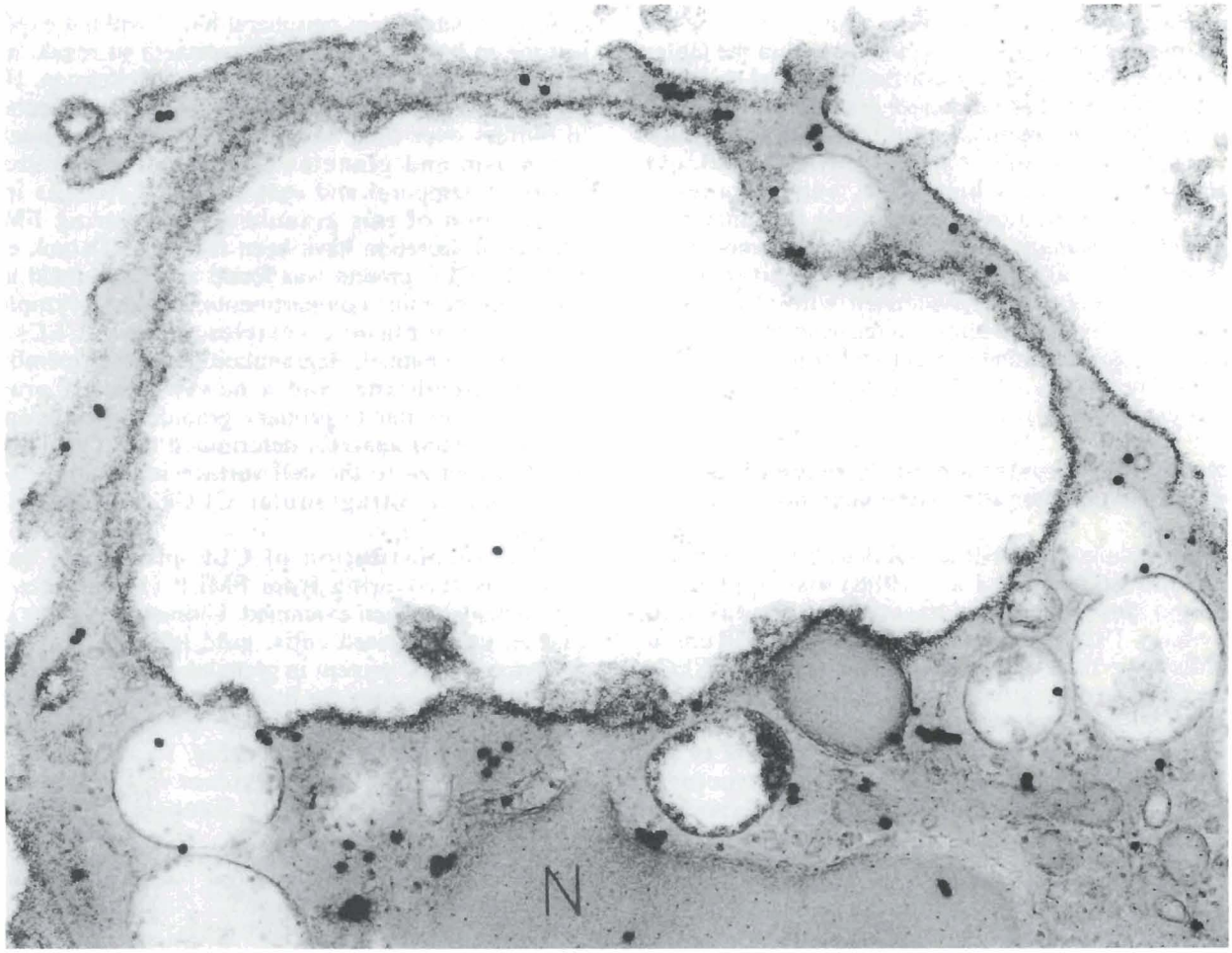


Fig. 13. A large, CF-stained enclosure fills the cytoplasm of this recovering basophil. CLC protein label is visible in the plasma membrane, cytoplasm and nucleus (N). x 39,000

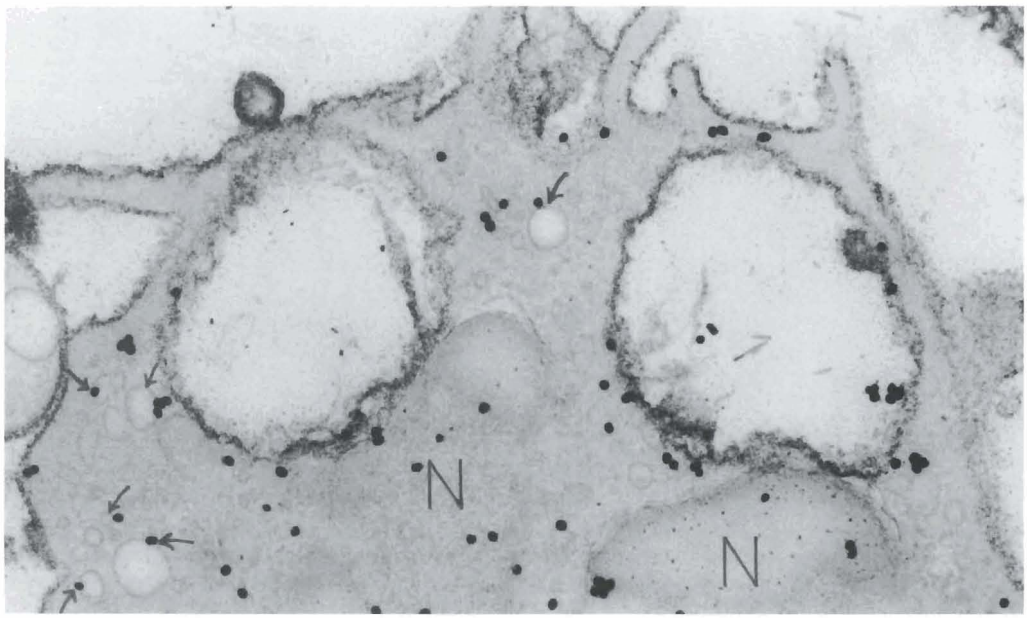


Fig. 14. A similar preparation of this recovering basophil shows enclosure of two CF-stained spaces in the granule-free cytoplasm. Gold particles label cytoplasm, nucleus (N) and the membranes of cytoplasmic electron-lucent vesicles (arrows). x 38,000

probed for histamine, show gold label in the particulate matrix but not in the formed CLC subcompartment of these granules (Fig. 1D,E). The newly evident granules (Dvorak et al., 1996a) which resemble eosinophil CLC protein-rich primary granules (Dvorak et al., 1988) in recovering basophils are homogeneously dense (Fig. 1C,F). That is, they do not contain formed CLCs or electron-dense particles and do not resemble the small paranuclear minor granule type described by Hastie (1974) (Dvorak, 1988). As shown in Fig. 1C, the newly evident granule type is heavily labeled for CLC protein providing further similarity to the human eosinophil primary granule (Dvorak et al., 1988; Dvorak and Ishizaka, 1994) but does not contain histamine (Fig. 1F). We have also noted increased numbers of these CLC protein-rich primary granules in human basophils that develop *de novo* from human cord blood cells cultured in suspension supplemented with the c-kit ligand (also called stem cell factor) (Dvorak et al., 1994b).

Particulate granules that have been exocytosed by

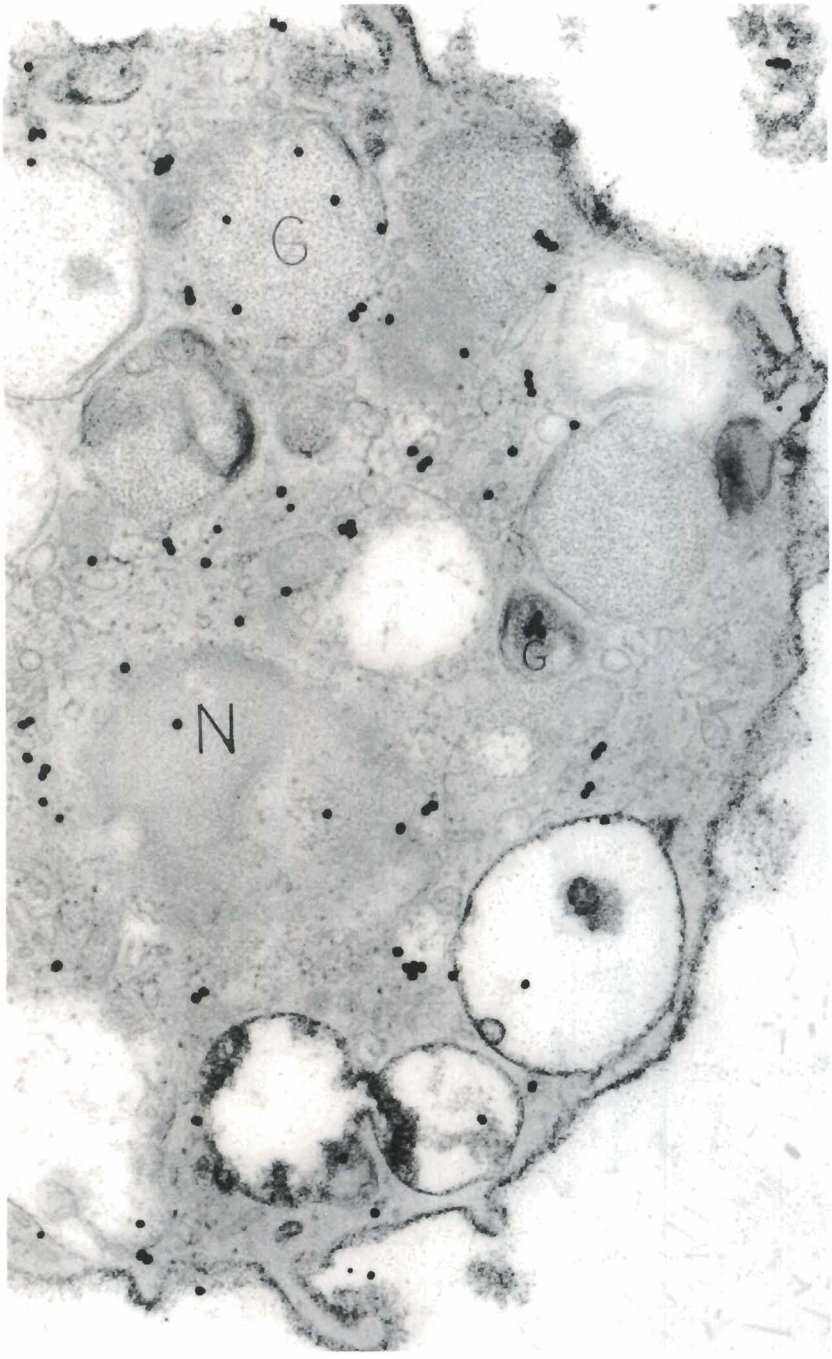


Fig. 15. This recovering basophil contains three CF-stained, empty granule spaces and several granules with variable particle packing. Most of the label for CLC protein is either free in the cytoplasm or in particle-containing granules (G). The CF-stained plasma membrane is virtually devoid of CLC protein label. N: nucleus. x 35,000

extrusion from FMLP-stimulated human basophils can remain visible attached to cell surfaces following their secretion (Dvorak et al., 1991b). Such well-formed granules, which persist in recovery intervals, show progressive re-internalization into basophils (Figs. 2, 3) (Dvorak et al., 1996c). In general, exteriorized particle granules persisting in open cul-de-sacs on the cell surface (Fig. 2) are devoid of CLC protein but regain label for CLC protein as they are re-incorporated into the cytoplasm (Fig. 3A). An additional feature of conservation during recovery is illustrated in Fig. 3B where budding of an endocytotic vesicle from the plasma membrane surrounding the basal aspect of an extruded granule is seen. Similar cytoplasmic vesicles nearby are labeled for CLC protein. Closed degranulation chambers (previously formed from the fusion of granule membranes) also persist in recovery intervals of FMLP-stimulated basophils (Fig. 4). While generally devoid of granule particles, formed CLCs (analogous to intragranular CLCs) can be present within these chambers; these are heavily labeled for the presence of CLC protein (Fig. 4). When formed, intragranular CLCs are extruded within seconds to minutes after FMLP-stimulation, they also contain CLC protein (Dvorak et al., 1996b). In contrast to the persistence of particulate granules attached to cell surfaces and re-uptake of these structures during recovery, we generally did not find labeled (or unlabeled) CLCs on the cell surface during recovery intervals.

The CDB is readily detected in these recovering samples (Fig. 5). First, the preparations were initially partially purified so that relatively few cells of other lineages were present (Dvorak et al., 1991b); second, these cells retained their typical condensed, polylobed granulocytes nucleus (and displayed no granules typical for the other granulocyte lineages - viz., neutrophils and eosinophils); and third, retention of CF-stained intracellular membranes, indicating membrane conservation during recovery was noted (Figs. 5A, 6-8). In addition, CLC protein was labeled with immunogold in various granule-free subcellular compartments (Fig. 5). These compartments were primarily nuclear, cytoplasmic and membranous in agranular recovering basophils (Fig. 5). In the nucleus, gold particles were primarily found in the nuclear matrix, with less label

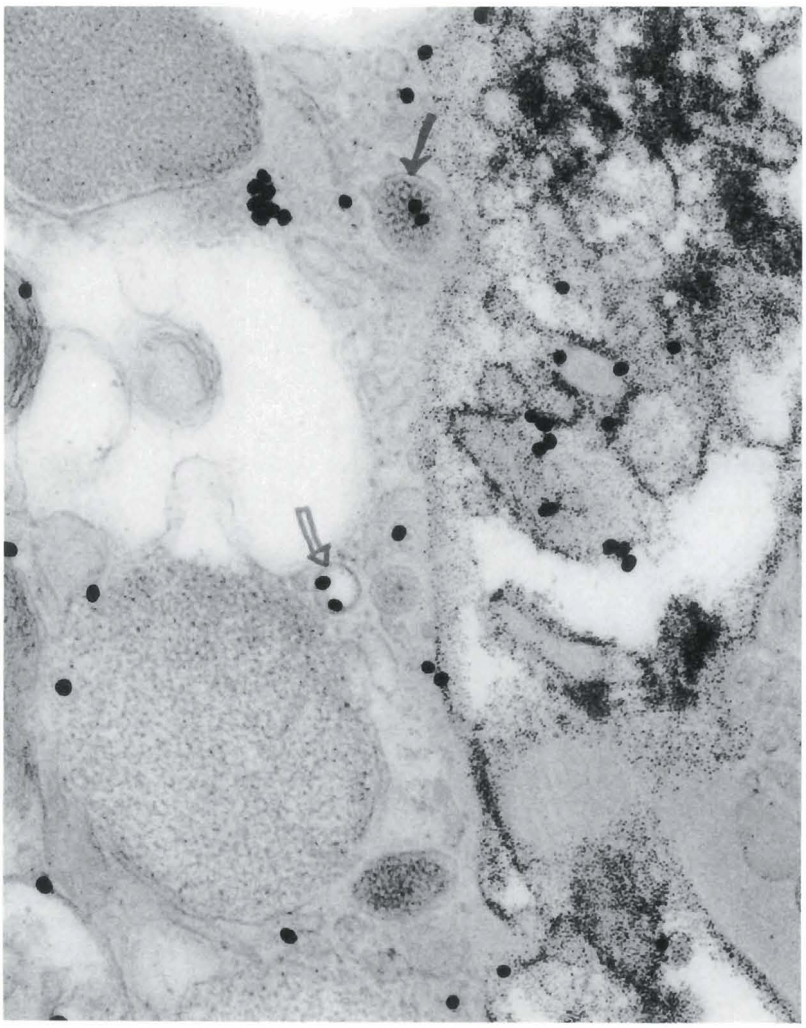


Fig. 16. This higher magnification view of a recovering basophil shows CLC protein label in a particle-filled vesicle (closed arrow) and in an electron-lucent vesicle (open arrow). Note that the full vesicle label is in the vesicle lumen and the empty vesicle label is attached to the vesicle membrane. CF stains the plasma membrane and the extensive associated surface structures and processes. Several of these are labeled with gold as well, indicating the presence of CLC protein. x 57,000

overlying condensed chromatin; some gold particles were adjacent to nuclear pores (Fig. 5A). Gold label in the granule-free cytoplasm was often attached to membrane of small vesicles, within lumina of small vesicles, and spread diffusely in the cytoplasm (Fig. 5). Gold label of the membranous compartment included the plasma membrane, vesicle membranes and perinuclear membranes (Figs. 5, 6). Some recovering, granule-poor cells showed large, round, nonmembrane-bound formed CLCs free in the cytoplasm, adjacent to CF-stained recycling plasma membrane (Figs. 7, 8). The large cytoplasmic formed CLCs were heavily labeled with gold, indicating the presence of CLC protein; such recovering cells displayed less diffuse cytoplasmic gold label but retained gold label attached to and within cytoplasmic vesicles (Figs. 7, 8). Some cells with gold-labeled formed cytoplasmic CLCs and CF-stained recycling plasma membranes had many newly developing, small, particle-filled granules adjacent to the labeled CLCs (Fig. 7B). Other, similar cells showed expanded, gold-labeled Golgi vesicles, indicating synthetic potential for CLC protein by basophils (Fig. 8). Since the plasma membrane of such recovering basophils still contained CLC protein (Fig. 8), some of the Golgi vesicles that were similarly labeled could represent Golgi traffic of endocytosed, previously secreted CLC protein (Dvorak et al., 1985b, 1996a,b). These experiments cannot completely distinguish between these possibilities. Indeed, replenishment of granule stores of CLC protein by recovering basophils may involve a mixture of endocytosis and synthetic sources.

Some CDBs persist without extensive evidence of morphologic recovery at early times examined after stimulated secretion. When these cells are sectioned through one nuclear lobe only (Fig. 9A), their distinction from lymphocytes (Fig. 9B), which are also present, is confounded. CF uniformly stains the plasma membrane of both cells, providing no means for distinction, since recycling membranes of conserved degranulation

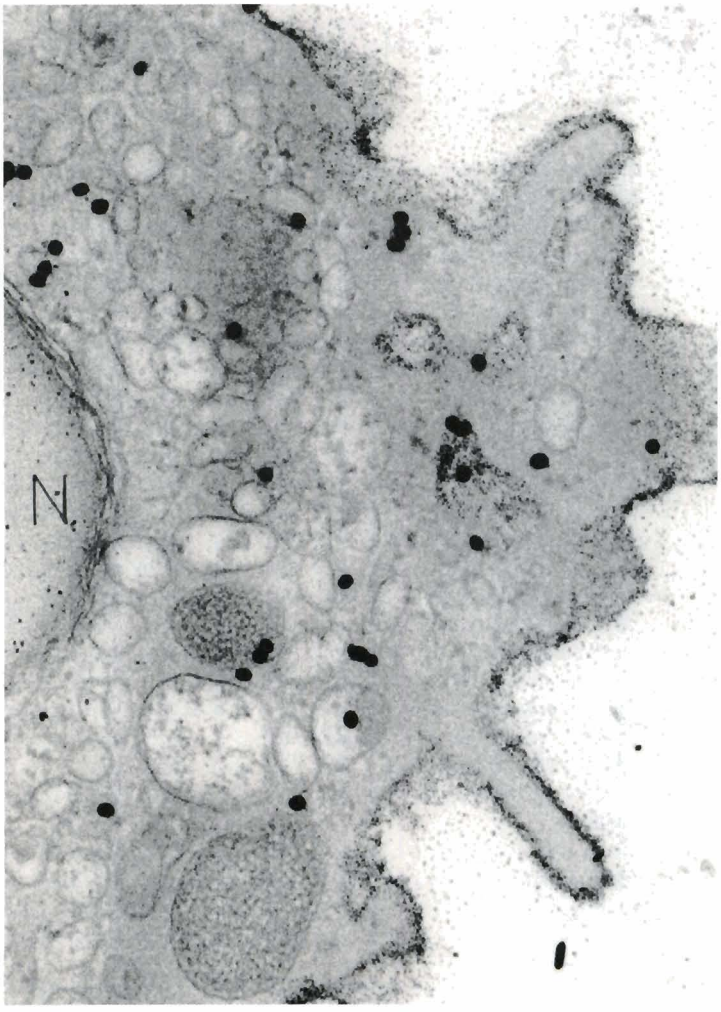


Fig. 17. Another recovering basophil imaged at higher magnification shows a vesicle-rich area of cytoplasm which spans the cell from nucleus (N) to the CF-stained plasma membrane. Gold particles, indicating CLC protein, are attached to many electron-lucent vesicles and one particle-filled vesicle; several CLC protein-labeled vesicles also contain CF and, thus, are open and in the process of internalization from the CF-positive plasma membrane. x 60,000

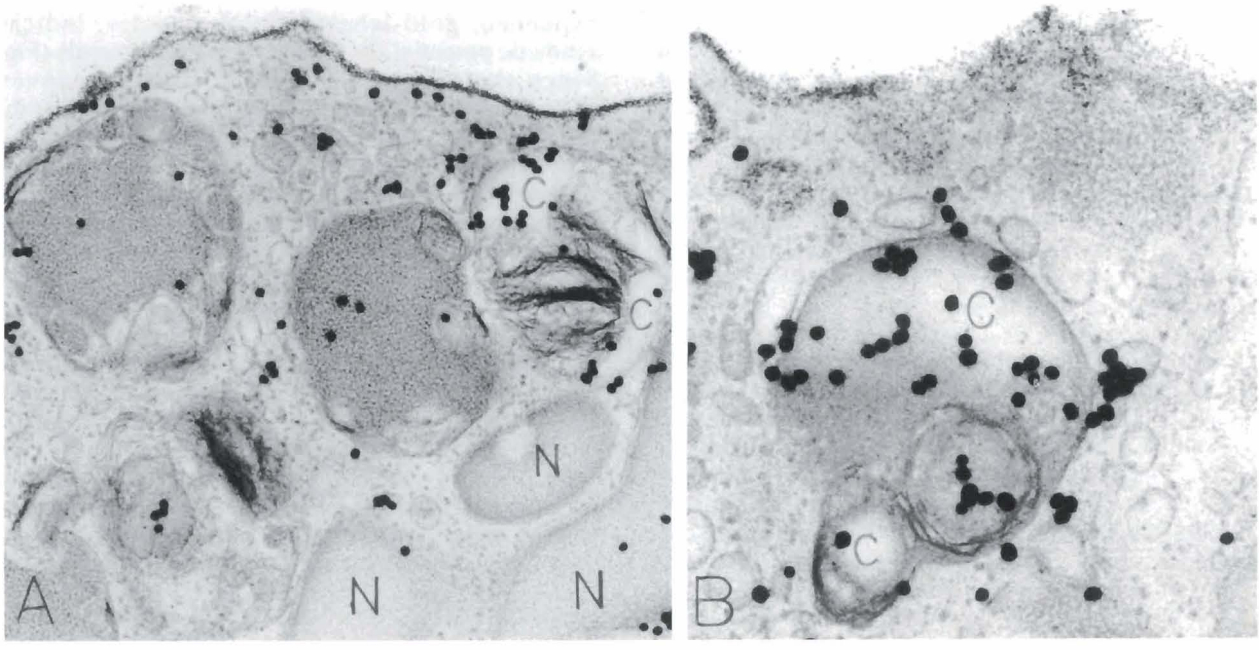


Fig. 18. Recovered basophils show reconstituted secretory granules. Granules filled with electron-dense particles (panel A) and/or homogeneous CLCs (C) and dense concentric or parallel membranes (panels A and B) contain CLC protein. Neither cell has gold particles attached to its CF-stained plasma membrane; a prominent array of gold particles is noted in the subplasma membrane cytoplasm (panel A). Cytoplasmic (panel A) and perigranular (panel B) vesicles are also labeled with gold. N: nucleus. A, x 34,000; B, x 58,000

containers are not present in the basophil (Fig. 9A). Staining with the immunogold method to detect CLC protein does allow identification of the previously degranulated basophil. For example, the nuclear, cytoplasmic and plasma membrane compartments of the CDB all label for CLC protein (Fig. 9A) but do not do so in the lymphocyte (Fig. 9B). Another CDB sectioned through multiple nuclear lobes clearly shows nuclear, cytoplasmic and membranous sites of CLC protein label (Fig. 10).

Higher magnification views of recovering human basophils localize recycled CF-stained membranes (Figs. 11-15) and CLC gold-labeled vesicles (Figs. 12, 14, 16, 17). The plasma membrane of such cells displays a uniform layer of CF particles (Figs. 11-15) and often contains gold label for CLC protein as well (Figs. 11-13). Large clusters of shed CF-stained membranes are adherent to surfaces of recovering basophils (Fig. 16) and are present within open granule spaces devoid of granule matrix (Fig. 11). Note other granules not similarly stained with CF in the same cell (Fig.11), suggesting the presence of recovered, dense concentric membranes in granules which no longer open to the cell's exterior surfaces. Recovering cells with mixtures of CF-stained (i.e., open) granule spaces and unstained spaces occurred in recovering samples (Figs. 12, 13); early condensation of particulate materials in some of the closed containers was visible in some cells (Fig. 12) and extensive in others (Fig. 15). Some of the newly filled (or re-internalized) particle-filled granules contained CLC protein (Fig. 15).

The number of cytoplasmic vesicles present in recovering basophils (Figs. 5, 16, 17) far exceeded the number present in the cytoplasm of cells at the peak of anaphylactic degranulation. These small vesicles typically were smooth membrane-bound and were either electron-lucent or filled with electron-dense particles (Fig. 16). Many were prominent in the peripheral cytoplasmic area beneath the plasma membrane (Fig. 16) and were labeled with gold, indicating the presence of CLC protein. Ultimately, recovered basophils with reconstituted, particle and formed CLC-containing granules prevailed (Fig. 18). Extensive gold label for CLC protein was evident in the formed intragranular CLCs and overlying the particulate matrix as well (Fig. 18). Perigranular collections of particle-filled and electron-lucent cytoplasmic vesicles carrying CLC protein (Fig. 18) provide documentation of vesicular transport to granules as a mechanism for increasing granule stores of this lysophospholipase (Weller et al., 1980, 1982; Zhou et al., 1992) as an integral part of the recovery processes of which human basophils are capable.

Acknowledgements. Supported by the Lillian Gong Memorial Research Fund and by U.S.P.H.S. Grant AI 33372. We thank ms. Linda Letourneau, Kathryn M. Pyne and Ellen S. Morgan for technical assistance.

References

Ackerman S.J., Weil G.J. and Gleich G.J. (1982). Formation of Charcot-Leyden crystals by human basophils. J. Exp. Med. 155, 1597-1609.

Bendayan M. (1984). Enzyme-gold electron microscopic cytochemistry: a new affinity approach for the ultrastructural localization of macromolecules. J. Electr. Microsc. Tech. 1, 349-372.

Danon D., Goldstein L., Marikovsky Y. and Skutelsky E. (1972). Use of cationized ferritin as a label of negative changes on cell surface. J. Ultrastruct. Res. 38, 500-510.

de Boer M. and Roos D. (1986). Metabolic comparison between basophils and other leukocytes from human blood. J. Immunol. 136, 3447-3454.

Dvorak A.M. (1988). Morphologic and immunologic characterization of human basophils, 1879 to 1985. Riv. Immunol. Immunofarmacol. 8, 50-83.

Dvorak A.M. (1991). Blood cell biochemistry. In: Basophil and mast cell degranulation and recovery. Dvorak A.M. (ed). Vol. 4. Harris J.R. (series ed). Plenum Press. New York.

Dvorak A.M. (1992). Basophils and mast cells: Piecemeal degranulation in situ and ex vivo: A possible mechanism for cytokine-induced function in disease. In: Granulocyte responses to cytokines. Basic and clinical research. Coffey R.G. (ed). Marcel Dekker. Inc. New York. pp 169-271.

Dvorak A.M. (1993). Ultrastructural analysis of anaphylactic and piecemeal degranulation of human mast cells and basophils. In: Immunopharmacology of mast cells and basophils. Foreman J.C. (vol ed). (in the series, The handbook of immunopharmacology; Page c. [series ed)]. Academic Press. London. pp 89-113.

Dvorak A.M. and Ackerman S.J. (1989). Ultrastructural localization of the Charcot-Leyden crystal protein (lysophospholipase) to granules and intragranular crystals in mature human basophils. Lab. Invest. 60, 557-567.

Dvorak A.M. and Ishizaka T. (1994). Human eosinophils in vitro. An ultrastructural morphology primer. Histol. Histopathol. 9, 339-374.

Dvorak A.M., Newball H.H., Dvorak H.F. and Lichtenstein L.M. (1980). Antigen-induced IgE-mediated degranulation of human basophils. Lab. Invest. 43, 126-139.

Dvorak A.M., Galli S.J., Morgan E., Galli A.S., Hammond M.E. and Dvorak H.F. (1982). Anaphylactic degranulation of guinea pig basophilic leukocytes. II. Evidence for regranulation of mature basophils during recovery from degranulation in vitro. Lab. Invest. 46, 461-475.

Dvorak A.M., Colvin R.B. and Monahan R.A. (1985a). Immunoferritin electron microscopic studies with antibasophil serum of guinea pig basophil degranulation and regranulation *in vitro*. Clin. Immunol. Immunopathol. 37, 63-76.

Dvorak A.M., Dvorak H.F. and Galli S.J. (1985b). Surface membrane traffic in guinea pig basophils exposed to cationic ferritin. Int. Arch. Allergy Appl. Immunol. 77, 267-273.

Dvorak A.M., Monahan-Earley R.A., Dvorak H.F. and Galli S.J. (1987). Ultrastructural cytochemical and autoradiographic demonstration of nonspecific esterase(s) in guinea pig basophils. J. Histochem. Cytochem. 36, 351-360.

Dvorak A.M., Letourneau L., Login G.R., Weller P.F. and Ackerman S.J. (1988). Ultrastructural localization of the Charcot-Leyden crystal protein (lysophospholipase) to a distinct crystalloid-free granule population in mature human eosinophils. Blood 72, 150-158.

Dvorak A.M., Letourneau L., Weller P.F. and Ackerman S.J. (1990a). Ultrastructural localization of Charcot-Leyden crystal protein (lysophospholipase) to intracytoplasmic crystals in tumor cells of primary solid and papillary neoplasm of the pancreas. Lab. Invest. 62, 608-615.

Dvorak A.M., Weller P.F., Monahan-Earley R.A., Letourneau L. and Ackerman S.J. (1990b). Ultrastructural localization of Charcot-Leyden crystal protein (lysophospholipase) and peroxidase in macrophages, eosinophils and extracellular matrix of the skin in the hypereosinophilic syndrome. Lab. Invest. 62, 590-607.

Dvorak A.M., Furitsu T., Letourneau L., Ishizaka T. and Ackerman S.J. (1991a). Mature eosinophils stimulated to develop in human cord blood mononuclear cell cultures supplemented with recombinant human interleukin-5. I. Piecemeal degranulation of specific granules and distribution of Charcot-Leyden crystal protein. Am. J. Pathol. 138, 69-82.

Dvorak A.M., Warner J.A., Kissell S., Lichtenstein L.M. and McGlashan Jr. D.W. (1991b). F-Met peptide-induced degranulation of human basophils. Lab. Invest. 64, 234-253.

Dvorak A.M., Ackerman S.J., Furitsu T., Estrella P., Letourneau L. and Ishizaka T. (1992). Mature eosinophils stimulated to develop in human cord blood mononuclear cell cultures supplemented with recombinant human interleukin-5. II. Vesicular transport of specific granule matrix peroxidase, a mechanism for effecting piecemeal degranulation. Am. J. Pathol. 140, 795-807.

Dvorak A.M., Morgan E.S., Schleimer R.P. and Lichtenstein L.M. (1993). Diamine oxidase-gold labels histamine in human mast-cell granules: a new enzyme-affinity ultrastructural method. J. Histochem. Cytochem. 41, 787-800.

Dvorak A.M., Furitsu T., Estrella P., Letourneau L., Ishizaka T. and Ackerman S.J. (1994a). Ultrastructural localization of major basic protein in the human eosinophil lineage in vitro. J. Histochem. Cytochem. 42, 1443-1451.

Dvorak A.M., Ishizaka T., Letourneau L., Albee E.A., Mitsui H. and Ackerman S.J. (1994b). Charcot-Leyden crystal protein distribution in basophils and its absence in mast cells that differentiate from human umbilical cord blood precursor cells cultured in murine fibroblast culture supernatants or in recombinant human c-kit ligand. J. Histochem. Cytochem. 42, 251-263.

Dvorak A.M., Tepper R.I., Weller P.F., Morgan E.S., Estrella P., Monahan-Earley R.A. and Galli S.J. (1994c). Piecemeal degranulation of mast cells in the inflammatory eyelid lesions of interleukin-4 transgenic mice. Evidence of mast cell histamine release in vivo by diamine oxidase-gold enzyme-affinity ultrastructural cytochemistry. Blood 83, 3600-3612.

Dvorak A.M., MacGlashan Jr. D.W., Morgan E.S. and Lichtenstein L.M. (1995a). Histamine distribution in human basophil secretory granules undergoing FMLP-stimulated secretion and recovery. Blood 86, 3560-3566.

Dvorak A.M., Morgan E.S., Monahan-Earley R.A., Estrella P., Schleimer R.P., Weller P.F., Tepper R.I., Lichtenstein L.M. and Galli S.J. (1995b). Analysis of mast cell activation using diamine oxidase-gold enzyme-affinity ultrastructural cytochemistry. Int. Arch. Allergy Immunol. 107, 87-89.

Dvorak A.M., MacGlashan Jr. D.W., Warner J.A., Letourneau L., Morgan E.S., Lichtenstein L.M, and Ackerman S.J. (1996a). Temporal and spatial expression of Charcot-Leyden crystal protein during f-Met peptide-induced degranulation of human basophils. I. Morphologic phenotypes of releasing and recovering basophils.

Dvorak A.M., MacGlashan Jr. D.W., Warner J.A., Letourneau L.,

Morgan E.S., Lichtenstein L.M. and Ackerman S.J. (1996b). Temporal and spatial expression of Charcot-Leyden crystal protein during f-Met peptide-induced degranulation of human baosphils. II. Kinetic analysis and role of vesicular transport in releasing and recovering basophils.

Dvorak A.M., Warner J.A., Fox P., Lichtenstein L.M. and MacGlashan Jr D.W. (1996c). Recovery of human basophils after FMLP-stimulated secretion. Clin. Exp. Allergy 26, 281-294.

Dvorak H.F., Selvaggio S.S., Dvorak A.M., Colvin R.B., Lean D.B. and Rypysc J. (1974). Purification of basophilic leukocytes from guinea pig blood and bone marrow. J. Immunol. 113, 1694-1702.

Frens G. (1973). Controlled nucleation for the regulation of the particle size in monodisperse gold suspensions. Nature Phys. Sci. 241, 20-22.

Galli S.J., Galli A.S., Dvorak A.M. and Dvorak H.F. (1976). Metabolic studies of guinea pig basophilic leukocytes in short-term tissue culture. I. Measurement of histamine-synthesizing capacity by using an isotopic-thin layer chromotographic assay. J. Immunol. 117, 1085-1092.

Golightly L.M., Thomas L.L., Dvorak A.M. and Ackerman S.J. (1992). Charcot-Leyden crystal protein in the degranulation and recovery of activated basophils. J. Leukoc. Biol. 51, 386-392.

Hastie R. (1974). A study of the ultrastructure of human basophil leukocytes. Lab. Invest. 31, 223-231.

Login G.R., Galli S.J. and Dvorak A.M. (1992). Immunocytochemical localization of histamine in secretory granules of rat peritoneal mast cells with conventional or rapid microwave fixation and an ultrastructural post-embedding immunogold technique. J. Histochem. Cytochem. 40, 1247-1256.

MacGlashan Jr. D.W., Lichtenstein L.M., Galli S.J., Dvorak A.M. and Dvorak H.F. (1982). Purification of basophilic leukocytes from guinea pig and human blood and from guinea pig bone marrow. In: Cell separation: Methods and selected applications. Vol. 1 Pretlow IInd T.G. and Pretlow T.P. (eds). Academic Press. Inc. New York. pp 301-320.

Siraganian R.P. (1974). An automated continuous-flow system for the extraction and fluorometric analysis of histamine. Anal. Biochem. 57, 383-394.

Warner J.A., Reshef A. and MacGlashan Jr. D.W. (1987). A rapid Percoll technique for the purification of human basophils. J. Immunol. Methods 105, 107-110.

Weller P.F., Goetzl E.J. and Austen K.F. (1980). Identification of human eosinophil lysophospholipase as the constituent of Charcot-Leyden crystals. Proc. Natl. Acad. Sci. USA 77, 7440-7443.

Weller P.F., Bach D. and Austen K.F. (1982). Human eosinophil lysophospholipase: the sole protein component of Charcot-Leyden crystals. J. Immunol. 128, 1346-1349.

Zhou Z.-Q., Tenen D.G., Dvorak A.M. and Ackerman S.J. (1992). The gene for human eosinophil Charcot-Leyden crystal protein directs expression of lysophospholipase activity and spontaneous crystallization in transiently transfected COS cells. J. Leukoc. Biol. 52, 588-595.