The Chediak-Higashi Syndrome: Cytoplasmic Sequestration in Circulating Leukocytes

By James G. White

A rare familial disorder characterized cytologically by enormous particles in all varieties of leukocytes has been reported from various parts of the world and is known in this country as the Chediak-Higashi (C.H.) syndrome. The variable clinical manifestations of patients with this disease have included defective pigmentation, neuropathies, lymphadenopathy, hepatosplenomegaly, leukopenia, thrombocytopenia, marked susceptibility to recurrent infection, and early death due often to lymphoma.

Recent investigations utilizing electron microscopy and ultrastructural cytochemistry demonstrated that the characteristic massive leukocyte granulations are giant lysosomes. The ease with which substrates, capture ions, and fluorescent dyes entered the large particles, and their tendency to undergo internal degeneration, indicated that C.H. granules may be abnormal lysosomes. Virus-like particles present in degenerating massive granules of leukocytes from two patients with C.H. syndrome suggested further that the massive lysosomes may be functionally abnormal and may conceivably be involved in the pathogenesis of recurrent infections afflicting these patients.

In the course of these studies into the nature of the C.H. granulation anomaly, another unusual process was observed in peripheral leukocytes of three patients with the disorder. Small, electron-dense particles, identical to normal leukocyte lysosomal granules, were frequently observed in circular arrangements around areas of C.H. cell cytoplasm. Union of the membranes of individual granules yielded partial or complete circular profiles enclosing cytoplasmic elements. The double-walled organelles formed in this unusual manner appeared to be sequestration vacuoles. Selective staining of the rings of fused particles by the enzyme reaction product of acid phosphatase activity, and numerous examples in which the enclosed contents appeared to be in various stages of degeneration, have suggested that sequestration leads to digestion.

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of focal areas of C.H. cell cytoplasm. Although isolation and destruction of portions of cytoplasm have been noted in cells of many other tissues,\textsuperscript{12-16} the process has not previously been reported in white blood cells. Its occurrence in leukocytes with massive abnormal lysosomes may indicate a relationship between sequestration and the giant C.H. granulation anomaly.

**Materials and Methods**

Blood samples for this study were obtained from three children with the clinical and laboratory stigmata of the C.H. syndrome. Two of the patients were described in previous reports,\textsuperscript{8,9} and all three will be part of a more extensive future communication.\textsuperscript{17} Clinical manifestations varied in the three children, but all have the characteristic giant granules in their circulating leukocytes.

The blood was processed for electron microscopic evaluation according to methods previously recorded from this laboratory.\textsuperscript{9,11,18} After separation of white cell and platelet-rich plasma from whole blood, the samples were recentrifuged in the cold (4 C.) to obtain a button. Supernatant plasma was discarded. The preparations for direct electron microscopy were washed twice in cold phosphate buffered saline, and then fixed in cold (4 C.) 1 per cent veronal buffered osmic acid, pH 7.3 for 2 hours. Dehydration was accomplished with increasing concentrations of acetone, and Vestopal-W was used as the embedding media.

Cell samples for ultrastructural cytochemistry were washed six times in .08 M cacodylate buffer with .18 M sucrose, and fixed for 2 hours in 3 per cent glutaraldehyde in .05 M cacodylate buffer with 1 per cent sucrose, pH 7.3. The glutaraldehyde-fixed cells were again washed six times in cacodylate buffer, then incubated for 90 minutes at 37 C. in a modified Gomori media containing sodium β-glycerolphosphate and lead nitrate.\textsuperscript{19} Following incubation, the samples were washed three times in cacodylate buffer, refixed in 1 per cent osmic acid for 1½ hours, dehydrated and embedded in Vestopal.

Thin sections of the cell-rich preparations were examined in the Phillips 200 electron microscope. Uranyl acetate and lead citrate were used to enhance contrast of the thin sections, after localization of enzyme activity had been ascertained on unstained material.

**Results**

Leukocytes from the peripheral blood of patients with the C.H. syndrome appear similar to normal white cells except for the presence of the massive cytoplasmic granules and their degenerating residues (Fig. 1A). Small lysosomal granules, mitochondria, vesicles and vacuoles, and other cytoplasmic elements are present in their usual numbers and distribution. Preparations of C.H. leukocytes stained for acid phosphatase by an ultrastructural cytochemical technic\textsuperscript{19} reveal specific deposits of enzyme reaction product in the enormous granules (Fig. 1B) and in the membrane-bound masses of debris produced by breakdown of the large particles (Fig. 1C).

In the course of examining white cell preparations from three C.H. patients, an unusual process was noted which had not been observed in leukocytes from normal individuals or patients with other diseases. The phenomenon involved alterations in the small, normal-sized lysosomal granules present in their usual abundance in C.H. neutrophils and monocytic cells. Areas of leukocyte cytoplasm were noted in which small granules appeared to line up in an oriented pattern, rather than in the random distribution which they ordinarily assume (Fig. 2A, B). The granules occasionally formed a relatively straight line, like beads on a rosary. More commonly, circular or simicircular
Fig. 1.—Neutrophils from circulating blood of patients with the Chediak-Higashi syndrome.

Fig. 1A.—This cell has the typical ultrastructural appearance of a normal osmic acid fixed neutrophil except for the presence of a number of massive particles in its cytoplasm. The giant granules are the characteristic cytologic anomaly of the C.H. syndrome. Several of the large particles in this cell show signs of internal breakdown. Large numbers of small, specific neutrophil lysosomes are distributed throughout the cell cytoplasm in a random pattern. Mag. × 18,600.

Fig. 1B.—Neutrophil from a sample fixed initially in glutaraldehyde and incubated in Gomori media for acid phosphatase activity. Normal neutrophil granules appear pale against the background cytoplasm of the cell, and do not ordinarily stain for acid phosphatase. Enzyme reaction product is specifically localized within the huge C.H. granule in the cytoplasm. Mag. × 11,850.

Fig. 1C.—A C.H. neutrophil from a leukocyte sample prepared in the same manner as the cell shown in Fig. 1B. In this example the reaction product of acid phosphatase activity is specifically deposited in areas of cytoplasm occupied by giant degenerating C.H. granules. Mag. × 16,000.
Fig. 2.—Cytoplasmic sequestration in a C.H. leukocyte. The extent of this process in a single cell is indicated in two views of the cytoplasm of one C.H. white cell.
granule arrangements were observed (Fig. 3A, B, C). The closely associated granules appeared to become joined together, resulting in formation of circular channels of granular material (†). The granules at the ends of the wall often retain their oval shape, while a portion joined to the double-walled vacuole appears elongated (‡). Lysosomal granules in the surrounding cytoplasm are similar to the particles taking part in formation of the sequestration vacuoles. Mag.: A × 33,000, B × 39,500, C × 43,250.

Fig. 3.—Several C.H. leukocytes manifesting areas of cytoplasm sequestered by concentric rings of granules or channels of granular material (†). The granules at the ends of the wall often retain their oval shape, while a portion joined to the double-walled vacuole appears elongated (‡). Lysosomal granules in the surrounding cytoplasm are similar to the particles taking part in formation of the sequestration vacuoles. Mag.: A × 33,000, B × 39,500, C × 43,250.

Fig. 2A.—Instead of assuming the random distribution pattern evident in normal leukocytes, small membrane-bound lysosomal granules with dense internal substance have formed unusual structural arrangements in this cell. The particles appear to line up in circular or semicircular arrangements resembling beads on a rosary (†). The small granules in close proximity (‡) appear to fuse with one another resulting in formation of double-walled circular channels of fused granular material. Mag. × 44,100.

Fig. 2B.—Another area of cytoplasm in this C.H. cell reveals the extent of this unusual process. The formation of circular chains of granules and their apparent fusion to form double-walled vacuoles has not been noted in normal cells. The granules forming the concentric rings of particles are identical to small lysosomal granules in the adjacent cytoplasm. Enclosed cytoplasmic contents do not differ from the matrix of the cell outside the sequestration vacuole. Mag. × 46,000.
Fig. 4.—Examples of sequestration organelles at various stages of formation in C.H. white cells (1). The membrane to membrane contact of the granules forming the wall apparently precedes their incorporation (1). The matrix of the wall has the same density as the granule substance, and the inner and outer unit membranes appear to derive from the surfaces of the granules. Mag.: A × 36,800, B × 37,70 C × 37,700, D × 63,800.

The “doughnut-shaped” organelle appeared to derive from the surface membranes of the fused particles, and the enclosed matrix was identical in appearance to the substructure of other granules in the surrounding cytoplasm. Enclosed cytoplasm and cytoplasmic elements did not differ from similar cell components outside the organelle.

Serial sections through sequestration vacuoles of C.H. leukocytes indicated that the observed process was not due to fortuitous sectioning of circles of granules at a single level of the cell. Multiple sections in the same plane were required to reach the upper and lower limits of the double-walled organelles. In one example seven serial sections were still within the confines of a single sequestration vacuole. The organelles, therefore, appear
to be double-walled spheres or discs derived from an intimate association of cytoplasmic granules in three dimensions.

Many leukocyte samples were studied in an attempt to determine the fate of the cytoplasmic elements enclosed within the unusual sequestration organelles. Vacuoles were observed in which the contents appeared to be in various stages of degeneration, or had disappeared, leaving residues or clear spaces (Fig. 6A, B, C, D). In the organelles manifesting destruction of sequestered cytoplasm, disappearance of the matrix between the two unit membranes of the vacuole wall and breaks in the inner membrane of the wall were often observed.

The preparations of leukocytes incubated for acid phosphatase activity yielded evidence pertinent to the formation of the unusual sequestration vacuoles. Small, normal-sized leukocyte lysosomes do not ordinarily appear acid-phosphatase positive by this technic.9 Frequently, however, partially completed or complete circles of joined granules were found which were acid-phosphatase positive (Fig. 7A, B, C). Inner and outer unit membranes were evident in these vacuoles despite the obscuring deposits of lead phosphate.
Fig. 6.—Digestion of cytoplasmic components enclosed within the sequestration vacuoles.

Fig. 6A.—The residual membranous structure in this organelle was probably a mitochondrial. The matrix of the wall has disappeared, and its two unit membranes are in close apposition. Mag. × 67,600.

Fig. 6B.—The matrix between the two membranes forming the double wall of this sequestration vacuole is gone. Inside the organelle, recognizable cytoplasmic structures are absent. Only a nonspecific residue remains in the organelle. Mag. × 64,500.

Fig. 6C.—A giant C.H. granule at lower left (†) in this cell is close to a sequestration vacuole (†). A few small vesicles, dense residues, and membranous remains are present in the organelle. Mag. × 34,000.

Fig. 6D.—Another sequestration vacuole (†) in close proximity to a giant C.H. particle (†). The matrix between the two unit membranes of the vacuole wall is gone. Internally the contents appear largely digested. Mag. × 52,500.

(Fig. 8A, B, C). Cytoplasmic components isolated within the acid-phosphatase positive wall of the vacuoles could be recognized. Examples were observed in which enzyme reaction product appeared within the vacule, associated with the enclosed elements of partially digested cytoplasm (Fig. 9A, B, C).

The close relationship of sequestration vacuoles to the giant C.H. granules is suggested in Figure 6C and D and in the final illustration (Fig. 10A, B,
C, D). Two massive C.H. granules are in close apposition, and both are acid-phosphatase positive, although the upper particle is more heavily stained. Small granules in the surrounding cytoplasm do not contain enzyme reaction product. In the second section (Fig. 10B) a double-walled vacuole appears in close proximity to the enormous C.H. granule. The matrix between the two unit membranes of the wall contains acid phosphatase activity. The inner surface of the wall is not complete, and two small lipid residues are evident in the otherwise clear vacuole. The third micrograph (Fig. 10C) reveals a different aspect of the same vacuole, and the last area photographed (Fig. 10D) is beyond the plane of the vacuole.

**DISCUSSION**

The unusual cytoplasmic organelles of C.H. leukocytes described in this report are distinct from the enormous granules which characterize the disease. Small, normal-sized lysosomal particles present in abundance in the C.H. cells appear to provide the fundamental units for construction of walls surrounding areas of cytoplasm. That is, portions of cytoplasmic substance indistinguishable from the rest of the cell matrix become enveloped by circular
Fig. 8.—Serial sections through two large sequestration organelles, and two small vacuoles. Lead phosphate, the reaction product of acid-phosphatase activity is deposited in the wall of each sequestration organelle. Mag.: A × 33,100, B × 32,200, C × 32,800.
arrangements of electron-dense granules identical to the randomly distributed lysosomes of C.II. leukocytes. Apparently the individual particles of the ring become joined to one another resulting in formation of circular or semicircular tubes or channels of fused granular material. Elements of cytoplasm enclosed by the concentric chains of granules have been observed in various stages of degeneration, suggesting that digestion occurs within the double-walled organelles. The cytoplasmic sequestration observed in C.II. leukocytes appears to result in formation of autophagic vacuoles, similar in many respects to the process of autophagy described in cells of many other tissues.\textsuperscript{12-14}

The circular arrangements of small leukocyte granules leading to formation of sequestration vacuoles in C.II. cells have not been noted in normal leukocytes or white cells from patients with a variety of diseases. This experience includes over 100 normal samples and leukocyte preparations from patients with myelogenous leukemia, Wiskott-Aldrich syndrome, rubella, rheumatoid arthritis, Fabray’s syndrome, ceroid storage disease, glycogen storage disease, fatal granulomatous disease, and several other disorders in which cell function may be compromised. On the basis of this experience the phenomenon of
Fig. 10.—Four sections through the cytoplasm of a C.H. leukocyte from a sample incubated for acid phosphatase activity. Two massive C.H. particles in close apposition are evident in this cell (Fig. 10A). Both giant granules are stained by enzyme reaction product. Normal-sized lysosomal particles in the surrounding cytoplasm are unstained.

In Figure 10B a circular profile of granular material containing acid phosphatase activity appears in close apposition to the massive C.H. particles. Tiny bits of cytoplasm and two lipid residues remain inside the sequestration vacuole. The organelle is transected again in Figure 10C (†). At this level the vacuole is slightly removed from the giant particles and is devoid of cytoplasmic elements. The fourth section (Fig. 10D) is beyond the plane of the sequestration vacuole, but still within the substance of the massive particles. Mag. (A, B, C, D) × 27,300.

cytoplasmic sequestration appears limited to C.H. white cells, but it is likely that future studies will reveal a similar process in leukocytes due to toxic substances or other disease states.

Although the lysosomal particles of normal white blood cells appear uniformly distributed in the cytoplasmic milieu, phase microscopic studies of living cells have demonstrated that granules maintain their relative positions despite active cell movements and pseudopod formation. Intimate contact
between individual granules is minimized, and only with cell death or serious injury does random motion of the particles become evident. Fusion of individual granules with each other and with phagocytic vacuoles occurs normally during bacterial phagocytosis, but in the unaltered white cell union of lysosomes is extremely rare. Thus the rosettes of small granules apparent in C.H. leukocytes represents a significant variation from the usual distribution pattern of particles in white cells; their apparent fusion to form double-walled vacuoles suggests a basic alteration in the individual granules permitting union with one another.

The fusion of granules leading to development of sequestration vacuoles in C.H. leukocytes appears to be an active phenomenon occurring in the living cells. Since the electron microscope peruses static sections of fixed leukocytes, the dynamic process of particle union cannot be ascertained directly. However, the matrix within the circular channels resulting from union of small particles is identical to that of adjacent granules, and some granules are in membrane to membrane contact with the developing organelles. In some examples a bulbous swelling at the extremity of an incomplete sequestration vacuole retained the oval shape of an individual cytoplasmic particle, while the portion joined to the organelle appeared identical to the vacuole wall. The presence of reaction product of acid-phosphatase activity in the matrix between the unit membranes of the sequestration vacuoles strongly indicates a relationship of the double-walled organelles to cytoplasmic granules which are known to contain this enzyme.

An appearance similar to a complete or partially complete vacuole wall would be produced by fortuitous sectioning through a giant C.H. granule deeply indented by cytoplasm. Massive particles with sufficient surface depression to suggest the presence of cytoplasmic elements within the body of the granule have been observed, although they occur with extreme rarity. The walls of such indented C.H. granules are extremely thick, and there is no difficulty distinguishing such particles from sequestration vacuoles. In addition, serial sections of the unusual double-walled vacuoles surrounding areas of cytoplasm have demonstrated that they are not part of giant granules.

The concept that the unusual sequestration vacuoles of C.H. leukocytes represent a form of autophagic vacuole was suggested by the degenerative changes observed in the cytoplasmic elements enclosed by the double-walled organelles. The alterations include breakdown of the normal appearance of enveloped structures, such as mitochondria, development of membranous configurations and lipid residues in the vacuoles, and complete clearing of the interior of some of the unusual organelles. Breaks in the inner membrane of the double-walled vacuole were frequently noted in examples manifesting destructive changes in the enclosed cytoplasm. The presence of acid-phosphatase reaction product in the wall of the organelle and associated with the dissolving contents suggested that lysosomal enzymes were involved in the digestion process. The hydrolytic enzymes most likely enter the sequestration vacuoles through breaks in the inner membrane, but may in part originate from Golgi vesicles which are frequently noted in the organelles.
Serial sections through multiple levels of cell cytoplasm indicate that the sequestration organelles are complete vacuoles of spherical or discoid shape. This sealing off of areas of cytoplasm from the rest of the cell in membrane-bound vacuoles and subsequent digestion of enclosed contents by lysosomal enzymes is markedly reminiscent of the process of autophagocytosis.

The ability of cells in some tissues to isolate and digest areas of their own cytoplasm inside membrane-bound vacuoles without the rest of the cell suffering irreparable damage is well established. Recognition of this phenomenon stemmed from the work of Clark,22 who described mitochondria within vacuoles of the proximal convoluted tubule in the kidney of newborn rats. The organelle containing cytoplasmic remnants and protected from the rest of the cell by a membrane was termed a "cytolosome" originally,13 and more recently an "autophagic vacuole."10 Combined electron microscopic and histochemical techniques have established the presence of acid phosphatase activity within autophagic vacuoles,27 indicating that they are a form of lysosome.13

The double wall of the sequestration vacuoles in Chediak leukocytes differs from the single membrane limiting the autophagic vacuoles in other types of cells. Ashford and Porter12 observed autophagy in glucagon-perfused rat liver cells and considered the wall of the vacuole to arise spontaneously. Behnke15 recognized the process in the cells of developing rat gut where accretion of Golgi vesicles appeared to cause enlargement of the vacuole wall. Novikoff16,28 has postulated that the wall sequestering an area of cytoplasm may arise from existing endoplasmic reticulum. Golgi membranes have also been cited as a possible source for the walls of autophagic vacuoles.27 In these several studies the wall of the autophagic vacuole appeared to be a single unit membrane.29

The wall of the sequestration vacuole in C.H. leukocytes is sheathed by both inner and outer unit membranes, separated by the matrix of fused granules. This structural arrangement of vacuole walls may be peculiar to circulating leukocytes which do not have a well-developed endoplasmic reticulum to supply membranes for vacuole formation. The surface coat of each of the numerous leukocyte granules represents a vast potential reservoir of unit membranes for the formation of walls of sequestration vacuoles, just as the granule membranes can fuse with and increase the surface of phagocytic vacuoles.22 Novikoff16 has pointed out that cytoplasmic sequestration may arise in different ways in different cells, and the origin of the isolating wall may reflect the variation in building materials available for autophagic vacuole formation in different cell types.

Containment and destruction of areas of cytoplasm by C.H. leukocytes may be intimately related to the giant granules which characterize the disorder. Previous studies have shown that the membranes surrounding massive C.H. granules are unusually permeable to substrates and fluorescent stains.6,9,10 This defect in the "structure-linked latency" of giant C.H. lysosomes may allow leakage of injurious substances from the massive granules or their degenerating remnants to the surrounding cytoplasm. Resultant injury to cytoplasmic constituents may act as the stimulus for the process of autophagy. That such a damage control mechanism may be insufficient to prevent cell destruction
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is suggested by the high frequency of leukopenia and granulocytopenia found in C.H. patients.6 Disintegration of leukocytes is frequently noted in C.H. bone marrow. Up to 25 per cent of the granulocytic elements may appear damaged or destroyed. It is unlikely that the two rare phenomena, giant C.H. granules and cytoplasmic sequestration occurring simultaneously in the same leukocyte population, are unrelated. The definitive relationship, however, will require further investigation since leakage of injurious substances from massive C.H. particles has not been demonstrated, although recent evidence suggests that giant granules release hydrolytic enzymes more readily than normal lysosomes.30

The possible involvement of the giant C.H. granules in cytoplasmic injury may not be limited to cells of the leukocyte series. Thrombocytopenia is a frequent finding in C.H. patients, and giant granules occur in their blood platelets.31 Neurologic disorders, particularly peripheral neuropathy, often accompany other manifestations of the C.H. syndrome. Light microscopic studies have shown degenerative changes in the nervous system,6 and we have recently identified the presence of massive C.H. particles in the Schwann cells of peripheral nerve.32 The results of the several studies undertaken in this laboratory indicate that the C.H. syndrome is not only characterized by giant abnormal lysosomes in a variety of cells and tissues, but in addition the massive particles appear inextricably bound to definitive pathologic features of the disease.

SUMMARY

In the course of investigations into the nature of the giant granulation anomaly of leukocytes from patients with the Chediak-Higashi syndrome, an unusual process was observed in white cells from three patients with the disease. Small, normal-sized, lysosomal granules present in abundance in C.H. leukocytes were observed in the concentric arrangements around areas of cytoplasm. Apparent union of the small granules resulted in development of “doughnut-shaped” channels of fused granular material. Serial sections have indicated that the organelles formed in this manner are complete spheres or discs. Cytoplasmic constituents enclosed by the double-walled vacuoles were frequently observed in various stages of destruction. Ultrastructural cytochemical staining for acid phosphatase defined the presence of this lysosomal enzyme in the matrix between the unit membranes of the double-walled vacuoles, and associated with fragments of enclosed cytoplasm undergoing digestion. Sequestration and destruction of areas of C.H. cell cytoplasm within membrane-bound vacuoles is strikingly similar to the process of autophagy reported in other tissues. The possible relationship of the process of cytoplasmic sequestration to the giant lysosomes which characterize the leukocytes of patients with the C.H. syndrome is discussed.

SUMMARIO IN INTERLINGUA

In le curso de investigationes del natura del anormalitate de granulation gigante in le leucocytos ab patientes con le syndrome de Chediak-Higashi,
un proceso inusual esseva observate in le leucocytos ab tres patientes con le morbo. Micre granulos sysosomal (de dimensiones normal), presente in abundantia in leucocytos Chediak-Higashi, esseva observate in un disposition concentric circum areas de cytoplasma. Un apparente fusion de micre granulos resultava in le disveloppamento de “pistas” de fusionate material granular. Sectiones serial ha revelate que le organellas formate in iste maniera es spheras o discos complete. Constituentes cytoplasmatic includite per le vacuolos a duple pariete esseva frequentemente observate in varie stadios de destruction. Tincturation con methodos de cytochimia ultrastructural visante a deteger phosphatase acide ha servite a definir le presentia de iste enzyma lysosomal in le matrice inter le membranas individual del vacuolos a duple pariete. Esse trovat, in plus, que le enzyma esseva assoicate con fragmentos de includite cytoplasma currentemente sub digestion. Le sequestration e le destruction de areas de cytoplasma intra le membranate vacuolos de cellulas Chediak-Higashi es frappantemente simile al processo de autophagia reportate in altere tissus. Le relation possibile inter le processo de sequestration cytoplasmatic e le lysosomas gigante que characterisa le leucocytos de patientes con le syndrome de Chediak-Higashi es commentate.

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