Storage Pool Deficiency in Cattle With the Chédiak-Higashi Syndrome Results From an Absence of Dense Granule Precursors in Their Megakaryocytes

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Platelets from cattle with the Chédiak-Higashi syndrome (CHS) have a storage pool deficiency and virtual absence of platelet dense granules. Megakaryocytes (MKs) from five control (n = 135) and five CHS (n = 133) cattle were evaluated using standard transmission electron microscopy. Osmophilic dense granules were not observed in control or CHS MKs. In MKs from normal cattle, clear vesicles of 200–650-nm diameter bounded by a sharp membrane were observed. They were easily differentiated from the demarcation membrane system, endoplasmic reticulum, and α granules. The clear vesicles were virtually absent in MKs from CHS cattle at all stages of maturation. MKs in bone marrow samples from two control (n = 91) and two CHS (n = 61) cattle that had been processed for the uranaffin reaction were also evaluated. The clear vesicles were replaced by uranaffin-positive granules in MKs from control cattle, but positive uranaffin granules were not observed in CHS MKs. These findings indicate that the platelet dense granule storage pool deficiency in CHS cattle results from an anatomic absence of dense granule precursors in maturing and mature CHS MKs.

An ultrastructural evaluation of MKs from control and CHS cattle was undertaken using standard fixation for transmission electron microscopy and the uranaffin cytochemical reaction. This is the first in-depth ultrastructural evaluation of MKs in CHS or other platelet SPDs and should increase our understanding of CHS, MK dense granule formation, and the pathogenesis of SPD in general. This article focuses on evaluation of dense granule precursors; the ultrastructure of other organelles in control and CHS bovine MK will be described in a separate publication.

MATERIALS AND METHODS

Processing for standard transmission electron microscopy. One hundred thirty-five MKs from five normal and 133 from five CHS cattle were examined. Ages ranged from one week to adult in both groups. MKs were classified as immature, maturing, and mature according to standard criteria.

Bone marrow specimens, obtained by surgical resection of 3 to 6 inches of the 12th rib at the costochondral junction, were rapidly immersed in a 2.0% glutaraldehyde/0.1 mol/L phosphate buffer/1% sucrose in 0.5% dimethylsulfoxide (DMSO) or a 2.0% glutaraldehyde/0.1 mol/L sodium cacodylate buffer solution, pH 7.4. The ribs were split lengthwise, and the marrow cells were harvested by gentle pipetting of fixative into the marrow space. To obtain larger specimens, some calves were killed (T 6 1, American Hoechst Corporation, Somerville, NJ, 0.1 mL/kg body weight), and the sternum was rapidly resected. Each sternebra was split perpendicularly to the sternum long axis. Medullary bone marrow was removed with bone gouges and dropped directly in the fixative within 15 minutes of death.

Single-cell suspensions were obtained by gentle flushing of the bone marrow pieces with fixative using plastic transfer pipettes. The bone marrow cell suspensions were then filtered through a 150-μm screen and fixed for two hours at room temperature. The bone marrow cells were washed three times in 0.1 mol/L sodium cacodylate buffer with 4.0% sucrose solution at pH 7.4 and postfixed for one hour with a solution of 1.0% osmium tetroxide, 0.1 mol/L sodium cacodylate buffer and 4.0% sucrose at pH 7.4. Some specimens were stained en bloc with a 1.0% calcium-enriched glutaraldehyde. Dense granules are also shown by electron microscopy of unfixed and unstained MK whole-mount preparations. Dense granule precursors can be demonstrated in MKs at all stages of maturation by means of the uranaffin reaction, in which the uranyl ions react with the phosphate groups of the 5′-phosphonucleotides stored in these precursors.

aqueous uranyl acetate solution for two hours at room temperature after being washed three times with double-distilled water. The specimens were pelleted in a 1.0% noble agar solution, and the solidified pellet was sectioned in 1- to 2-mm pieces in 0.1 mol/L sodium cacodylate buffer. Dehydration was done in ascending series of ethanol and propylene oxide, and the specimens were embedded in an epon-araldite mixture. Thin sections were obtained with a Sorval MT2-B ultramicrotome with a diamond knife and stained with uranyl acetate and lead citrate. The sections were examined on a Hitachi-S-600 electron microscope.

**Processing for the uranaffin reaction.** Ninety-one MKs from two normal cattle and 61 from two CHS cattle aged one and four weeks were examined. Bone marrow specimens were obtained as described above and processed with the technique reported by Richards and Da Prada. Incubation in 4% aqueous uranyl acetate for 30 hours was done to increase the sensitivity for detection of dense granule precursors.

**RESULTS**

Large numbers of well-preserved MKs were obtained with the procedures described. Most of the MKs were maturing or mature. Fewer than five immature MKs were identified for each normal cow and CHS cattle.

Osmiophilic platelet-like dense granules were absent in normal and CHS MKs fixed by standard procedures. Vesicles with diameters of 250 to 650 nm, with a clear content and bounded by a sharp membrane, were found early in the maturation process of MKs from normal cattle and increased in number during maturation (Fig 1). These vesicles were differentiated from cross-sections of the demarcation membrane system (dms) channels by their larger diameter, sharper membrane, and the absence of fine fibrillar material coating the internal side of their membrane. They were differentiated from dilated segments of rough endoplasmic reticulum by their larger size, their clear contents, sharp membrane, and the absence of ribosomes attached to their membrane. Almost all the clear vesicles were replaced by uranaffin-positive granules with diameters of 100 to 700 nm, with strong staining of the membrane, and/or of a dense core attached to the membrane (Fig 2).

A single, small membrane projection attached to the membrane on the luminal side of the clear vesicles was commonly observed and may represent the site where the dense core was located with the uranaffin reaction (Fig 3).

Clear vesicles/uranaffin granules and α granules were present in all the MKs studied. They were scarce in immature MKs and increased in number during maturation. Clear vesicles and α granules were counted for 20 maturing and mature MK prints from negatives taken at low magnifications to include all the cytoplasmic areas (2,000 to 5,000×). There was a mean of one clear vesicle/8.8 ± 3.6 α granules (range one clear vesicle/3.8 to 16.1 α granules). Uranaffin granules and α granules were counted for 20 maturing and mature MK prints from negatives taken at low magnifications. There was a mean of one uranaffin granule/6.5 ± 2.6 α granules (range one uranaffin granule/3.1 to 14.5 α granules). The discrepancy in the number and diameter of the clear vesicles and the uranaffin-positive granules resulted from the presence of small, uranaffin-positive granules (Fig 2). These small vesicles were not counted in MKs with standard fixation since they were not bound by a sharp membrane and could not be differentiated from cross-sections of the demarcation membrane system because of their small diameter.

The most prominent feature of CHS MKs processed with standard fixation was a virtual absence of clear vesicles at all stages of maturation (Figs 4 and 5). There were no positive uranaffin granules in CHS MKs (Fig 6). All other organelles of CHS MKs were negative for the uranaffin reaction. Enlarged vesicles were observed in only 3 of 133 MKs with standard processing, and these were associated with or in close proximity to the Golgi complex (Fig 7). Enlarged uranaffin-positive vesicles were not observed.

**DISCUSSION**

Platelet-like osmiophilic dense granules are generally absent in MKs processed for conventional electron microscopy. The clear vesicles in MKs from normal cattle are considered the precursors of platelet dense granules for the following reasons. First, they were completely replaced by uranaffin granules of similar sizes, which cytchemical reaction has been shown to be specific for adenine nucleotide-storing organelles in platelets and megakaryocytes. Second, they were easily differentiated from cross-sections of the dms and endoplasmic reticulum because of their larger diameter, clear content, and sharp membrane. Third, clear vesicles and uranaffin granules were absent in MKs from CHS cattle. Clear vesicles with diameters of 150 to 300 nm have also been described by Daimon and David in rat MKs using standard fixation and were suggested to be the precursors of platelet dense granules. The clear vesicles were larger in bovine MKs than in rat MKs; this is probably a reflection of the large size of bovine platelet granules as compared with platelet granules from other species.

The dense granule precursors and α granules appeared to be synthesized simultaneously since both types of granules increased during maturation, and immature MKs with mainly one type of granule were not observed. The range in the ratio of dense granule to α granules was wide and was independent of MK maturation. Why some MKs isolated from the same marrow have many dense granules and others have few dense granules is not clear. This observation suggests that dense granule formation is a regulated process, as has been suggested for α-granule proteins.

The origin of the dense granule precursors has not been established. They have been suggested to originate from α granules, the endoplasmic reticulum, and the Golgi complex. The bulk of the nucleotides stored in the dense granules are synthesized within the MKs. Once released into the circulation, platelets take up serotonin produced by the enterochromaffin cells through two distinct transport systems, an imipramine-sensitive transporter in the plasma membrane, and a reserpine-sensitive transporter in the dense granule membrane. An ATP-dependent proton gradient with acidic granule interior is the major force for the dense granule accumulation of serotonin. Within the granule, serotonin is complexed with the nucleotides and divalent cations. The formation of the storage complex provides osmotic stability to the dense granules and enhances the
Fig 1. Mature normal bovine MKs processed for standard transmission electron microscopy. Many clear vesicles (cv) bounded by a sharp membrane are present and evenly distributed between the α granules (α). The dms, α granules, and small mitochondria (m) compose most of the other cytoplasmic organelles with little rough endoplasmic reticulum and Golgi complex (g) present. (A: Original magnification ×7000. B: Original magnification ×10,300.)
Fig 2. Mature normal MK processed for the uranaffin cytochemical reaction. Numerous uranaffin granules (ug) are evident. They show a strong staining of the membrane and/or a dense core attached to the granule membrane and replaced most of the clear vesicles observed with standard fixation. Small uranaffin granules (arrowheads) are also observed and were not counted as clear vesicles with standard fixation since they could not be differentiated from cross-sections of the dms. Holes in the uranaffin granules represent dense cores lost during processing. N, nucleus; rer, rough endoplasmic reticulum; α, α granules. (Original magnification ×10,800.)

Fig 3. Clear vesicles from normal bovine MKs with standard processing often contain small membrane projections attached to the luminal side of the membrane (a through d). These small projections appear to be the site where the uranaffin-positive dense cores are attached to the membrane of the uranaffin granules (e through h). (A, ×25,000; B, ×39,800; C, ×17,500; D, ×32,000; E, ×33,000; F, ×60,000; G, ×22,000; H, ×41,800.)
efficiency of serotonin uptake. A study of serotonin processes in CHS bovine platelets showed that serotonin could be taken up by CHS platelets, that the amine was protected from degradation for short periods of time, and that serotonin was accumulated by a pH-dependent process within platelet granules which were not dense granules. In addition, the reserpine-sensitive amine uptake process considered unique to dense granules could not be demonstrated. The absence of dense granule-related amine uptake processes coupled with the virtual absence of stored adenine nucleotides and the absence of recognizable dense granule precursors within MKs suggest that the platelet SPD associated with bovine CHS results from an anatomic deficiency of dense granules rather than a functional alteration in which the dense granule is present but cannot perform processes required to obtain an amine/nucleotide/divalent storage complex.

The absence of uranaffin-positive granules and recognizable dense granule precursors in CHS MKs represents an unusual expression of the CHS trait. CHS is characterized by enlarged cytoplasmic granules in most granule-forming cells. This abnormality is generally attributed to abnormal granule fusion. Production of larger unit granules by the Golgi complex has also been postulated. The few enlarged vacuoles observed in CHS MKs associated with the Golgi complex may represent enhanced fusion of dense granule precursors. However, the numbers of these structures were insufficient to explain the marked reduction of dense granule precursors. These enlarged vacuoles were not stained with the uranaffin reaction and could also represent abnormal lysosomes. Enhanced fusion of dense granule precursors with other organelles is also a possibility. However, no other organelle abnormality was observed in MKs from CHS cattle other than the absence of dense granule precursors.

Several biochemical abnormalities have been described in
Fig 5. Mature CHS MKs with standard fixation. Clear vesicles are absent. The dms and α granules (α) are fully developed. Mitochondria (m), Golgi (g), rough endoplasmic reticulum, and ribosomes are scarce. The lumen of the dms is opening in b. N, nucleus; MT, microtubule. (A: Original magnification ×9000. B: Original magnification ×14,800.)
CHS. Cellular abnormalities including changes in membrane fluidity,39 microtubule assembly,40 cyclic nucleotides metabolism,41,42 and membrane–microtubule interaction43 have been documented. Whether these abnormalities are primary defects of CHS or secondary epiphenomena and how they may lead to platelet SPD and absence of platelet dense granule precursors in bovine CHS MKs has not yet been resolved.

There is heterogeneity in the expression of the platelet dense granule deficiency in human CHS patients. Some CHS patients do not have a platelet SPD.44 Biochemical and/or ultrastructural evidence for a platelet SPD has been reported for most CHS patients and all the animal models of CHS.2–15 However, use of different dense granule markers for anatomic determination of the granule’s presence is controversial. Dense granules are virtually absent in CHS platelets processed for conventional electron microscopy. Richard and Da Prada reported an absence of uranaffin granules in platelets and MKs from beige CHS mice.17 On the other hand, granules in platelets from beige CHS mice accumulate mepacrine. This antimalarial basic tricyclic amine with fluorescence properties accumulates in the dense granules of normal platelets owing to its high affinity for the ATP stored in these organelles.45 The number of mepacrine-positive granules in platelets and MKs from CHS mice is normal or slightly decreased, with reduced total mepacrine uptake by these granules and greatly diminished flashing phenomenon.47,48 There are at least two plausible explanations for the accumulation of mepacrine in platelets from beige CHS mice. One, platelets from CHS mice have a virtual absence of dense granule precursors and mepacrine is being accumulated by other types of granules. Mepacrine is a base that could be ion trapped within acidic vesicles such as lysosomes. Studies with serotonin accumulation in bovine CHS platelets49 indicate that acidic granules other than dense granules are present and can accumulate amines. If the normal storage site for mepacrine uptake is absent, the concentration of mepacrine in the platelet cytoplasm might elevate and increase the probability of mepacrine accumulation within other granules in amounts allowing visualization with fluorescence microscopy but not in amounts sufficient for flashing. Changes in leukocyte and erythrocyte membrane lipid composition and increased membrane fluidity and permeability have been described in CHS40–43 and may contribute to accumulation of mepacrine within other organelles such as lysosomes in CHS platelets. A second alternative is that the dense granule precursors are present in MKs and platelets from CHS beige mice and the difference between the mice
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and bovine CHS, which lack dense granule precursors, represents heterogeneity in animal models of CHS. Recently, Penner and Prieur\(^2\) reported that there is heterogeneity in animal models of CHS, as there is in human CHS patients.

In conclusion, dense granule precursors in normal bovine MKs appeared as clear vesicles with standard fixation, and these vesicles were replaced by uranaffin-positive granules with the uranaffin cytochemical reaction. The dense granule SPD in CHS cow platelets is caused by a virtual absence of dense granule precursors in their bone marrow MKs; this cannot be explained by enhanced granule fusion but appears to result from a synthesis defect.

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