Lysosomes and melanin granules of the retinal pigment epithelium in a mouse model of the Chediak-Higashi syndrome.

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The origin of giant granules in the retinal pigment epithelium of the beige mouse was investigated with electron microscopy and ultrastructural histochemistry. These granules were found to contain melanin and acid phosphatase. Apparently they arise from fusions of primary lysosomes with melanin granules which are already enlarged from multiple fusions among melanosomes. Therefore, the giant granules are not primary lysosomes, nor are they simply enlarged melanin granules as suspected from light microscopic studies. A deficiency of primary lysosomes in the pigment epithelium results, suggesting a defect in intracellular digestion similar to that found in the leukocytes of Chediak-Higashi patients and several animal models. Affected humans probably have defective digestion in their retinal pigment epithelium also, which could impair the renewal process for rod outer segments. Thus, Chediak-Higashi patients may show an increased susceptibility to light damage due not only to hypopigmentation, but to defective intracellular digestion, as well.

The Chediak-Higashi syndrome is a rare human disorder of recessive inheritance which results in decreased oculocutaneous pigmentation, photosensitivity, nystagmus, and recurrent pyogenic infections. The increased susceptibility to infection is attributed mainly to a block in intracellular digestion, though neutropenia and lowered chemotaxis of leukocytes are contributing factors. Phagocytic leukocytes contain abnormally large granules which represent multiply fused lysosomes. Cells with such enlarged lysosomes fail to degranulate properly and are unable to carry out normal digestion. The pigment epithelium also contains enlarged granules, but these were reported to be abnormal melanin deposits, and their possible relation to lysosomes has not been investigated.

The beige mutant of the C57 black mouse is a good animal model for the Chediak-Higashi syndrome. We have studied its retinal pigment epithelium using electron microscopy and acid phosphatase histochemistry to determine the relations of the abnormally large granules to melanin granules and to lysosomes.

Materials and methods. Eyes were obtained from three black mice (C57BL/6 NH-/+ + ) and three beige mutants (C57BL/6 NH- bg/bg) ages 25 to 33 days. The central retina and choroid were excised in fixative and carefully sliced with a sharp razor blade into pieces that were 200 to 400 μm wide. These tissue sections were fixed for 180 minutes at 4° C. in 2.5 per cent glutaraldehyde and 6 per cent sucrose buffered to pH 7.2 with 50 mM sodium cacodylate. Some of the tissue sections were processed directly for ultrastructural studies. Others were washed overnight in 50 mM sodium cacodylate (pH 7.2) with 8 per cent sucrose at 4° C., rinsed in 200 mM Tris maleate at pH 5 for 60 minutes, and then were incubated at 37° C. for 60 minutes in one of the following media for acid phosphatase histochemistry.

- The incubation medium for the demonstration of acid phosphatase contained 5 ml H2O, 5 ml 50 mM MnCl2, 10 ml 200 mM Tris maleate (pH 5.0), 10 ml 25 mM cytidine 5'-monophosphate, 3 Cm. sucrose, and 20 ml 0.2 per cent lead nitrate.
- Control tissues were treated by one of the following methods: (1) heated at 60° C. for 30 minutes prior to incubation; (2) incubated in medium with 10 mM sodium fluoride added; (3) incubated in medium without cytidine 5'-monophosphate or other substrate; and (4) left unincubated and not postfixed.

Following incubation the tissues were rinsed briefly in 200 mM Tris maleate (pH 5.0) at 37° C. and then in 120 mM cacodylate buffer (pH 7.2) at room temperature. They were postfixed and embedded for electron microscopy. Only the first 100 μm of each piece was sectioned in order to avoid artifacts related to limited penetration by the histochemical media. Sections were examined and photographed with a JEM 100B electron microscope, unstained or after uranyl acetate and lead citrate staining.

Results. Comparative morphology. The retinal pigment epithelium of the beige mouse contains giant granules of irregular shapes which may approximate the sizes of nuclei (Fig. 1). These granules may be as large as 3 μm wide and 11 μm long. They are especially conspicuous for their high density (light and electron opacity). They contain material which is structurally identifiable as melanin, but they differ from typical melanin granules in that their content is not homogeneous, and their position in the cell is usually central rather than apical. The smaller (melanin) granules which are present abundantly in the apical portions of normal retinal pigment epithelium are absent.
Fig. 1. Retinal pigment epithelium and choroid of a beige mouse which is a mutant of the C57 mouse, and an animal model for the Chediak-Higashi syndrome. Compare the giant intracellular granules with the melanin granules of the C57 black mouse, as seen in the inset of the same magnification. (x12,900.)
The giant granules of the beige mouse often exhibit melanin in different stages of aggregation within the same granule. Some granules appear to be in the process of fusing, and many have concentric rings of varied densities, suggesting that additions to their size were acquired in discrete steps. They also contain material of low density in their peripheral regions which does not resemble melanin.

The giant granules do not have the structure of primary lysosomes, nor are they similar to any typical cell organelle.

The primary lysosomes in the retinal pigment epithelium of the beige mouse are decreased in number, even though their size range is normal (0.2 μm to 0.9 μm). The phagosomes (0.6 μm to 2 μm) and secondary lysosomes (0.3 μm to 2 μm) are also normal in size, but they are more abundant than normal and a greater proportion of them is localized basally in the pigment epithelial cells. The nuclei are morphologically normal with dimensions of approximately 3.6 μm by 8 μm. There appears to be no change in the number or morphology of mitochondria.

The melanocyte in the choroid of the beige mouse has giant granules also (Fig. 1). These are somewhat more regular in shape than those of the pigment epithelium, and may be as large as 2 μm by 4 μm.

The C57 black mouse has typical melanin granules (Fig. 1, inset). Some of the granules in the retinal pigment epithelium are spherical, with diameters near 1 μm, but most are oblong and may be as large as 0.9 μm on their short axis and 1.6 μm on their long axis. The choroid of the black mouse has melanin granules which are mainly spheroid and have diameters up to 0.7 μm.

**EM histochemistry.** Acid phosphatase, a common lysosomal enzyme, served as a marker for distinguishing lysosomes from organelles of similar structure. The pigment epithelium of both black and beige mice incubated for the demonstration of acid phosphatase activity (Figs. 2 and 3) showed significant amounts of lead precipitate in the primary and secondary lysosomes, and in the smooth endoplasmic reticulum adjacent to the Golgi. The melanin granules of the pigment epithelium in C57 black mice often showed small amounts of precipitate, perhaps just above background levels. Some of the giant granules of the beige mice exhibited a similar low level of reactivity also; however, many of these giant granules (Chediak-Higashi granules) exhibited high levels, equivalent to those found in lysosomes (Fig. 4). Such was never observed in the melanin granules of the black mice, even with extended incubation times.

We found in beige mice many lysosome-like bodies which appeared to have fused with the...
membranes of the giant granules (Fig. 4). In such instances the contents of the granules, at the periphery, and the contents of the “fused bodies” exhibited acid phosphatase activity. These enzyme-rich bodies which fused with the giant granules appear to be primary lysosomes. Their fusion with the giant granules shows an affinity normally reserved to reaction with phagosomes.

Beige mice showed many bodies which had the appearance of secondary lysosomes, but showed no lead precipitate (Fig. 3). Often these were located basally. They were interpreted to be phagosomes of long standing which had received insufficient acid phosphatase for digestion of their contents. In contrast, essentially all structurally distinct secondary lysosomes in the C57 black mouse demonstrated acid phosphatase activity.

No lead precipitate was observed in control tissues incubated without substrate. Some of the heat-treated tissues and those incubated with the addition of sodium fluoride showed a little precipitate, which appeared to be nonspecific. This was believed to result from incomplete inhibition or spontaneous precipitation, and was considered to be insignificant.

**Discussion.** The retinal pigment epithelium of the beige mouse contains giant granules which are highly variable in size and shape. Many of them exhibit acid phosphatase activity as well as structural evidence of melanin content. Our observations suggest that their enzyme activity derives from fusion of primary lysosomes with enlarged melanin granules. Such fusions appear to be common, and may lead to the observed deficiency in the number of primary lysosomes in the retinal pigment epithelium of the beige mutant. The digestion of engulfed outer segment membranes of the photoreceptor cells may be affected adversely. Such is consistent with our observation that there is an apparent accumulation of phagosomes in the retinal pigment epithelium of the beige mutant. We observed no extracellular accumulation of discarded rod outer segments.

Greatly enlarged cytoplasmic granules (inclusion bodies) and impaired intracellular digestion in leukocytes are said to be pathognomonic for the Chediak-Higashi syndrome in humanst, and for the beige mutant in mice. Granulocytes and other phagocytic cells appear to engulf bacteria normally, but the contact of digestive enzymes with ingested material is greatly delayed or absent. Apparently, the large cytoplasmic granules derive from multiple fusions of primary lysosomes, and the sequestering of their enzymes into one large lysosome (the Chediak-Higashi granule). Such bizarre lysosomes seldom fuse with phagosomes and, therefore, do not form secondary lysosomes in which substrate and enzymes are brought together.

**Fig. 3.** Retinal pigment epithelium of a beige mouse showing a secondary lysosome (above) which reacted positively for acid phosphatase activity, and a phagosome (below) which reacted negatively. (×23,000.)
Fig. 4. Retinal pigment epithelium of a beige mouse reacted for acid phosphatase activity. Chediak-Higashi granules as well as primary lysosomes are reacted positively. Often lysosomes (arrows) appear to fuse with the granules. (×37,000.)

Probably the retinal pigment epithelium of Chediak-Higashi patients, like that of the beige mouse, contains giant granules with melanin and acid phosphatase instead of typical pigment granules and normal numbers of primary lysosomes. Defective intracellular digestion in the pigment epithelium is suggested, and this may be accompanied by a delayed renewal of rod outer segments and perhaps by an increased susceptibility of the patient to irreversible light damage.

Preliminary evidence to be reported subsequently suggests an abnormal susceptibility of the beige mouse to photic damage. Probably defective digestion of rod outer segments is an important factor, but hypopigmentation must be contributory. The ocular tissues of beige mice and Chediak-Higashi patients that would normally provide protection from photons are lightly pigmented. Unlike normal tissues they concentrate what may be a typical amount of melanin in a few giant granules rather than disperse it into many small granules. The net effect is lighter pigmentation and less filtering capacity.

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REFERENCES
Many experimenters have observed infrequent saccadic pairs with smaller intersaccadic intervals. There are reports of saccades with intersaccadic intervals as small as 100 ms, 120 ms, 100 ms, 75 ms, 70 ms, 50 ms, 40 ms, 10 ms, and 0 ms. To elicit closely spaced saccades, some experimenters used pulse-step stimuli—for example, the target could jump five degrees right, pause for 50 ms, then jump another three degrees right. Other experimenters recorded more natural saccades during reading, fixation, or in response to a step change in target position. A variety of other effects can evoke closely spaced saccades: for instance, fatigue, flickering illumination, voluntary pauses, and abrupt decelerations of a moving target. In all of these reports, the second saccade was usually smaller, and in the same direction as the first saccade. One report emphasized that the second saccades of his pairs were abnormal, while the others made no mention of normality. Our present findings indicate that no refractory period is necessary in order for the subsequent saccadic eye movement to be normal.

Methods. The infrared photodiode method of eye position measurement was used to record the saccades of this report. The photodiodes were mounted on a pair of spectacle frames worn by the subject. The subject's head was stabilized with a head rest and a bite bar covered with dental impression compound. Saccades as small as three minutes of arc have been measured with this equipment. The bandwidth of the complete system, including photodiodes, direct current (DC) amplifiers, computerized velocity algorithm, computerized slow-down plotting routine, and the X-Y plotter was in excess of 1,000 Hz. The data of Fig. 3 are for five normal unfatigued subjects.

The saccades of Fig. 1 were made while tracking a spot of light that jumped periodically, with a frequency of 0.33 Hz, between various predetermined pairs of points. The oblique saccadic eye movements of Fig. 4 were made while repetitively saccading between two continuously observable targets. We have also recorded closely spaced saccades using electro-oculography (EOG). Corneal reflection, contact lens, photodiode, and psychophysical techniques have been used by others to record closely spaced saccades.

Results. Fig. 1 shows naturally occurring, normal, human saccades with successively smaller intersaccadic intervals (ISI). ISI is defined as the time between saccadic eye movements; more specifically, the time between the end of the first saccade, until the beginning of the next saccade. This definition of ISI is illustrated in the idealized drawing of closely spaced saccades near the vertical axis of Fig. 3. We have recorded many saccadic pairs with small intersaccadic intervals;