Microtubule Organization of Unstimulated and Stimulated Adherent Human Neutrophils in Chediak-Higashi Syndrome

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The numbers and length of centriole-associated microtubules of two patients with Chediak-Higashi syndrome (CHS) were examined. Detergent-extracted whole-mount preparations of adherent cells were studied by stereo high-voltage electron microscopy. Under conditions of random migration, neutrophils from both patients had a microtubule organization similar to that of the control; microtubule numbers (28 ± 3) and length (7.0 ± 2.8 μm) were within normal range. When cells were treated with phorbol myristate acetate (PMA), differences in the response of the two patients were noted. Neutrophils from patient No. 2 and the control showed a significant rise in numbers (38 ± 5) and length (9.5 ± 3.6 μm) of microtubules. In contrast, neutrophils from patient No. 1 were unresponsive to PMA treatment. Because vitamin C is used therapeutically in CHS patients and has been shown to correct microtubule-related cell function, neutrophils were exposed to ascorbic acid. A significant increase in microtubule numbers (35 ± 6) was observed in cells from the control and patient No. 2 after ascorbate treatment; neutrophils from patient No. 1 showed no increase in microtubule numbers. While ascorbic acid did not affect microtubule length in the control cells, it caused a significant increase in microtubule length in neutrophils from both patients. Results suggest that adherent CHS neutrophils contain centriole-associated microtubules which are normal in number and length. However, differences between patients are observed regarding neutrophil responsiveness to stimuli which induce microtubule polymerization.

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The Chediak-Higashi syndrome (CHS) in humans is characterized by the presence of abnormal giant granules in all granule-containing cells. Because of the high susceptibility of CHS patients to infection, the neutrophil has been the cell type most extensively studied. In the neutrophil, the giant granules are thought to resemble secondary lysosomes, which appear to be formed during granulopoiesis by fusion of azurophil and specific granules. These abnormal granules probably contribute to defective cellular function, including poor mobilization of neutrophils from the bone marrow, deficient chemotaxis, and depressed degranulation and bactericidal activity.

The basic defect in CHS is not known, but experimental evidence suggests that altered membrane, elevated cyclic adenosine monophosphate levels, microtubule assembly, and microtubule membrane interactions may be important. Poor cellular function has been attributed to a defect in the assembly of microtubules. For example, concanavalin A (con A)-induced capping takes place spontaneously on CHS neutrophils, whereas microtubule inhibitors are required to induce con A capping on normal cells.

The defect in microtubule assembly is corrected by treating cells with agents that elevate cellular levels of cyclic guanosine monophosphate. Furthermore, ascorbic acid has been shown both in vitro and in vivo to increase the number of centriole-associated microtubules and to improve microtubule-dependent leukocyte function.

Because significant advancements have been introduced in ultrastructural studies with the advent of stereo high-voltage electron microscopy (HVEM) of unsectioned cells, this study was undertaken to re-evaluate microtubule functions in CHS neutrophils. Inconsistent with the concept of a defective microtubule system in neutrophils are the findings that CHS lymphocytes, monocytes, platelets, and fibroblasts contain normal numbers of assembled microtubules. It would seem improbable that the neutrophil is the only blood cell with an abnormal microtubule system. Therefore, we have re-examined the ability of CHS neutrophils to assemble microtubules in whole-mount preparations of detergent-extracted neutrophils by HVEM. Unlike previous investigations in which microtubules are counted in a thin-section cut through the centriole, whole-mount preparations allow for evaluation of the entire microtubule system. Thus, both numbers of centriole-associated microtubules as well as length of microtubules can be examined.

Neutrophils from two CHS patients were stimulated with ascorbic acid or phorbol myristate acetate (PMA), a potent co-carcinogen that stimulates an increase in microtubule numbers and length in normal neutrophils. The results suggest that adherent CHS neutrophils have normal numbers and length of centriole-associated microtubules. However, the two patients differ in their ability to polymerize tubulin after PMA stimulation and differ with the control in response to ascorbate stimulation.

MATERIALS AND METHODS

Case description. Neutrophils from two patients with CHS have been studied. The clinical features of one of the children have been described previously. Both patients were advised of procedures and attendant risks, in accordance with institutional guidelines, and gave informed consent. In spite of ascorbic acid, which had previously been shown to normalize granulocyte bactericidal activity, the patient entered the accelerated phase. At the time of the study, patient No. 1, who had entered the accelerated phase of the disorder six months previously, was in remission following therapy with prednisone and antithymocyte globulin. She had no evidence of
hepatosplenomegaly and her absolute peripheral neutrophil count exceeded 1,000 cells per cubic millimeter. She had not received therapy for one month.

Patient No. 2, age 7 years, did not suffer excessively from bacterial infection, but her neutrophils showed typical impaired chemotaxis and degranulation when analyzed in vitro prior to ascorbate therapy. She has been receiving ascorbic acid (20 mg/kg/d) for six years and has remained well. At the time of the study, she had not been receiving therapy for one month.

Isolation of neutrophils. Neutrophils were isolated from 50 mL of heparinized venous blood by Ficoll-Hypaque density centrifugation as previously described. The cell pellets contained >94% neutrophilic polymorphonuclear leukocytes. Viability was >98%, as determined by trypan blue dye exclusion.

The cells were resuspended to 5 × 10⁶ cells per milliliter in Gey’s tissue culture medium supplemented with 10% human type AB serum and 0.1 mL of the cells were allowed to adhere to formvar-coated gold grids attached to 11 × 22-mm glass coverslips. Cells were incubated for 45 minutes at 37 °C in a 5% CO₂ incubator. Unattached cells were removed and the monolayer was layered with 1 mL of Gey’s medium containing 10% type AB serum and placed at 37 °C.

PMA and ascorbic acid treatment. PMA (Sigma Chemical Co, St Louis) was dissolved in dimethyl sulfoxide (Sigma) at a concentration of 1 mg PMA per milliliter and further diluted in Gey’s medium containing 10% type AB serum to 10 ng PMA per milliliter. The amount of dimethyl sulfoxide (0.001%) present in the incubation media did not alter cellular function. Cells were incubated with 10 ng PMA per milliliter for 30 minutes at 37 °C in a 5% CO₂ chamber.

Ascorbic acid (5 mmol/L) was dissolved in Gey’s medium containing 10% type AB serum and was incubated with cells for 30 minutes at 37 °C in a 5% CO₂ chamber.

Cell extraction and fixation. Following exposure to PMA or ascorbic acid, the monolayer was washed quickly with Gey’s balanced salt solution (without Ca and serum), fixed for 30 minutes with 1.5% glutaraldehyde in Gey’s balanced salt solution with 10 mmol/L EGTA, 2 mmol/L MgCl₂, pH 7.0 (fixation buffer), washed three times with fixation buffer, and post-fixed with 1% OsO₄ in water for two minutes. Controls included no exposure to the drugs.

For cytoskeleton examination, cells were washed quickly with 60 mmol/L 1,4-piperazaine diethylsulfonic acid (PIPES), 25 mmol/L N-2-hydroxyethylpiperazine N-1-2-ethanesulfonic acid (HEPES), 10 mmol/L EGTA, and 2 mmol/L MgCl₂, pH 6.9 (PHEM buffer) at 37 °C and then lysed with 0.2% Triton X-100 (Sigma Chemical Co, St Louis) in PHEM buffer for one minute, fixed with 1.5% glutaraldehyde in PHEM buffer supplemented with 0.2% tannic acid for ten minutes, washed three times in buffer, and post-fixed with 0.5% OsO₄ in water for one minute. The lysis and fixation were performed at room temperature.

Specimen preparation for HVEM. Preparations of fixed cells attached to gold grids were dehydrated in ethanol and critical point-dried from CO₂. Following rotary evaporation of a thin carbon coating.
film, grids were viewed in an AEI or JEOL high-voltage electron microscope at 1,000 kV. Stereo pairs were generated at tilt angles of 12 to 16°, depending on the final magnification.

Cells were transported in fixation buffer from Indianapolis to Madison, Wis, where cells were post-fixed with OsO₄, dehydrated, critical point-dried, and viewed on a high-voltage electron microscope.

**Microtubule counts.** Microtubules were counted at a final magnification of 25,000. The contour length of microtubules was determined with a Zeiss Videoplan computer image analyzer (Carl Zeiss, West Germany). Only centriole-associated microtubules were traced, since free microtubules were rare.

**RESULTS**

**Ultrastructure of whole-mount preparations of CHS neutrophils.** An unsectioned CHS neutrophil fixed under conditions of random locomotion and viewed by HVEM is shown in Fig 1. This cell, as with most other cells in the preparation, is well-spread on the substrate. It is characterized by a bilobed nucleus, numerous large abnormal granules, smaller less dense vesicles or granules, and secondary lysosomes. The giant granules vary in number, from two to as many as 56 per cell. Variations are also present in granule shape and size; granule size ranges from 0.4 to 2.5 μm in diameter. The interior of the granules is difficult to evaluate in these whole-mount preparations due to their density. Secondary lysosomes are frequent and appear to contain a network of fibers and a few intact granules. Few normal-appearing granules were observed. All organelles are contained within an extensive three-dimensional network of fine filaments composing the microtrabecular lattice.31,32

Triton X-100 extraction of whole-mount preparations of neutrophils (Fig 2) dissolved the plasma membrane and most of the microtrabecular strands. The large granules are more resistant to Triton X-100. Microtubules are not clearly identifiable in unextracted neutrophils (Fig 1); however, after Triton X-100 extraction, a more open appearance of the cytoplasm is evident, and the organization of the microtubule system can be visualized (Fig 2). In these cells, microtubules can be traced from the centrosome to the cell margin. A high-magnification stereo image of a centrosomal region of a CHS neutrophil is shown in Fig 3. Analysis of extracted neutrophils from the control and the patients with CHS reveals that the majority of microtubules originate near the centriole, from which they radiate toward the cell periphery.

**Microtubule numbers.** The numbers of centriole-associated microtubules were counted in neutrophils under conditions of random locomotion. The cells were extracted with Triton X-100 to visualize the cytoskeletal system, as illustrated in Figs 2 and 3.

Control cells had an average of 28 ± 3 microtubules per...
cell (Table 1). The average number of microtubules was within the normal range for one patient (patient No. 2), and slightly higher, but statistically significant, for the other patient (patient No. 1). There was no significant difference in microtubule numbers in the two patients when compared to each other.

Microtubule polymerization was tested with PMA, a potent co-carcinogen which has been shown to stimulate an increase in microtubule numbers and length in neutrophils. Control and CHS neutrophils demonstrated numerous cytoplasmic vacuoles after incubation with PMA. A significant increase in microtubule numbers was observed in the control and in one of the patients (patient No. 2) after incubation with PMA (Table 1). While neutrophils from patient No. 1 demonstrated typical vacuolization after PMA, microtubule numbers were not affected. As described previously, PMA induced the centrosome to split into two distinct centrioles, each with its own aster of microtubules. Splitting of centrioles was observed in neutrophils of both the control and the patients.

Since ascorbate corrects leukocyte function and enhances microtubule assembly in CHS, neutrophils were incubated with ascorbic acid to determine whether it affects microtubule numbers. Similar to PMA, incubation of cells with ascorbic acid (Table 1) caused a significant increase in microtubule numbers of the control and patient No. 2. Ascorbic acid had no effect on microtubule numbers in the neutrophils of patient No. 1. However, the total number of microtubules in both patients did not significantly differ from control cells that were exposed to ascorbic acid.

**Length of microtubules.** Microtubule length from the centrosome to the cell margin was investigated in normal and CHS neutrophils under conditions of random locomotion and after stimulation with PMA or ascorbic acid (Table 2). Under conditions of random locomotion, the average length of microtubules of both patients did not significantly differ from the control (7 μm). However, after stimulation with PMA, a significant increase in microtubule length was observed in the control and in patient No. 2; cells from patient No. 1 were unresponsive to PMA.

There was a significant increase in microtubule length in both patients after ascorbic acid treatment. In contrast, no increase in microtubule length was observed in control cells after ascorbate exposure.

**DISCUSSION**

Stereo HVEM of whole-cell preparations enables examination of the three-dimensional organization of neutrophils. Examination of numerous unextracted CHS cells reveals that, similar to observations made with thin sections, a striking feature of these cells is the presence of giant inclusions that vary in size and shape. There are few normal-appearing granules. Secondary lysosomes are easily identified in these whole-cell preparations and appear to contain microtubular strands and intact granules. Similar to normal neutrophils, all organelles are dispersed within an extensive three-dimensional network of fine filaments, or microtubufibrils. This microtrabecular lattice (MTL) extends throughout the cytoplasm from the cell margin to the nuclear lobes. Until extensive studies are conducted with normal neutrophils, it is difficult to evaluate whether or not the MTL is normal in CHS neutrophils. The MTL is thought to mediate granule translocation along microtubules in chro-

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**Table 1. Number of Microtubules in Chediak-Higashi Neutrophils After Treatment With PMA or Ascorbic Acid**

<table>
<thead>
<tr>
<th></th>
<th>Random Locomotion</th>
<th>PMA (Split Centrosome)</th>
<th>Ascorbic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Centriole 1</td>
<td>Centriole 2</td>
<td>C1 + C2*</td>
</tr>
<tr>
<td>Control</td>
<td>28 ± 3(8)</td>
<td>25 ± 3</td>
<td>14 ± 3</td>
</tr>
<tr>
<td>Patient No. 1</td>
<td>32 ± 4‡(12)</td>
<td>22 ± 5</td>
<td>11 ± 3</td>
</tr>
<tr>
<td>Patient No. 2</td>
<td>29 ± 6(11)</td>
<td>28 ± 7</td>
<td>13 ± 4</td>
</tr>
</tbody>
</table>

Values are means ± SD. Significance was determined at P < .05 by the Student's t test. The number of cells measured is indicated in parentheses.

Neutrophils were incubated with 10 ng PMA per milliliter or 5 mmol/L ascorbic acid for 30 minutes.

*Sum of the microtubules associated with centriole 1 and centriole 2.

†Mean is significant when compared to that of untreated cells within the subject.

‡Mean is significant when compared to the control mean.
during random locomotion. These data suggest that microtubules were slightly higher, but statistically significant, than in the control or in patient No. 2. In fact, the number of microtubules in patient No. 1 was unchanged. The observation that microtubules from patient No. 1 is not understood. The cells from both patients demonstrated typical vacuolization after PMA. It appears that one patient is unresponsive to the signal that stimulates tubulin assembly.

The entire microtubule system of neutrophils is visualized using a combination of detergent extraction and stereo HVEM. Thus, it is possible not only to count numbers of centrosome-associated microtubules, but also to determine their length. It is suggested that the number of centrosome-associated microtubules represents the number of microtubule-nucleating elements, where each nucleating element nucleates the assembly of one microtubule. Elongation, or assembly of tubulin, is determined by measuring the length of each centrosome-associated microtubule.

Under conditions of random locomotion, microtubule numbers and length of CHS neutrophils were within normal range. It is therefore improbable that Triton X-100 extraction is more deleterious to CHS neutrophils than control cells. In fact, the number of microtubules in patient No. 1 was slightly higher, but statistically significant, than in the control or in patient No. 2. These data suggest that microtubule assembly of adherent CHS neutrophils is not defective during random locomotion.

The average number of centrosome-associated microtubules in CHS neutrophils is 31. It is difficult to compare the number of microtubules reported here with values reported in the literature, mainly for two reasons: first, published microtubule numbers in CHS neutrophils are based upon counts of microtubule profiles in thin sections passing through the centrosome. Because of the variable number of microtubule profiles in a given micrograph, the values obtained may vary. For example, for unstimulated CHS neutrophils, the mean number of centrosome-associated microtubules reported is 0.5 and 3.3; the values for normal neutrophils is 11.9 and 22.3. Second, these counts are based upon cells fixed in suspension, whereas, here, adherent cells are examined. Since surface perturbation also induces microtubule assembly, randomly migrating cells have to be regarded as activated. Therefore, the greater number of microtubules reported in this study, when compared to other studies, is probably because we investigated unsectioned adherent cells.

We have observed that soluble factors known to induce microtubule numbers in cell suspensions induce microtubule numbers in adherent cells by 25%, and that this stimulation is accompanied by an almost twofold increase in length of microtubules. Thus, by stimulating adherent cells, it is possible to evaluate microtubule assembly in terms of numbers and elongation. PMA stimulated an increase in microtubule length and numbers in neutrophils of the control and patient No. 2. Assembly of microtubules of CHS neutrophils from patient No. 1 was unchanged. The observation that PMA did not stimulate microtubule assembly in neutrophils from patient No. 1 is not understood. The cells from both patients demonstrated typical vacuolization after PMA. It appears that one patient is unresponsive to the signal that stimulates tubulin assembly.

Similar to control cells, PMA caused the centrosome of CHS neutrophils to split into two distinct centrioles, each with its own aster of microtubules. In addition to PMA, centrosome splitting has also been observed after stimulation of normal neutrophils with the chemotactic peptide f-Met-Leu-Phe and zymosan-activated serum. Centrosome splitting is dependent upon a cytochalasin D-sensitive cytoskeletal system.

Because ascorbate therapy appears to improve microtubule-dependent function in CHS neutrophils, cells were exposed to ascorbic acid in order to evaluate microtubule assembly. In this study, we confirm that ascorbate promotes microtubule assembly of CHS neutrophils. Similar to PMA, an increase in microtubule numbers was observed in the control and in patient No. 2; the numbers of centrosome-associated microtubules of neutrophils from patient No. 1 were unaffected by ascorbate. It should be noted that there was no significant difference in microtubule numbers of ascorbate-treated cells from patient No. 1 with the control. However, ascorbate promoted an elongation of microtubules in neutrophils from both patients. Unlike CHS neutrophils, control cells exhibited no response in microtubule elongation after ascorbate treatment. Other studies have shown that ascorbate has no effect or increases microtubule numbers in control neutrophils. In vitro studies suggest that ascorbate maintains tubulin in a polymerizable form, and that organic acids stabilize the colchicine-binding activity of tubulin. The greater sensitivity of microtubules of CHS neutrophils to elongate after ascorbate requires further investigation.

It is perplexing that microtubules of patient No. 1 elongated after ascorbate and not after PMA stimulation. Since neutrophils from patient No. 1 are capable of polymerizing tubulin, the defect probably lies in failure to respond to a surface signal. A similar hypothesis has been postulated based on the response of CHS neutrophils to con A; microtubule assembly is observed with con A and ascorbate, but not with con A alone.

In summary, it appears that CHS neutrophils contain centriole-associated microtubules that are normal in number and length. However, microtubule assembly in CHS neutrophils is variable among patients. The defect probably resides in the inability of neutrophils from some patients with CHS to respond to certain signals generated during surface stimulation.
ACKNOWLEDGMENT

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REFERENCES

Microtubule organization of unstimulated and stimulated adherent human neutrophils in Chediak-Higashi syndrome

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