

# Correction of Characteristic Abnormalities of Microtubule Function and Granule Morphology in Chediak-Higashi Syndrome with Cholinergic Agonists

STUDIES IN VITRO IN MAN AND IN VIVO IN THE BEIGE MOUSE

JANET M. OLIVER and ROBERT B. ZURIER

*From the Departments of Physiology and Pathology and Division of Rheumatic Diseases, Department of Medicine, University of Connecticut Health Center, Farmington, Connecticut 06032*

**ABSTRACT** Chediak-Higashi (CH) syndrome is a genetic disorder of children and certain animal species including the beige mouse. We have previously described a membrane abnormality in CH mouse polymorphonuclear leukocytes (PMN). Whereas normal mouse PMN do not form surface caps with concanavalin A except after treatment with agents such as colchicine that inhibit microtubule assembly, CH mouse PMN show spontaneous cap formation. This capping is inhibited by 3',5' cyclic guanosine monophosphate and by the cholinergic agonists carbamylcholine and carbamyl  $\beta$ -methylcholine that increase 3',5' cyclic guanosine monophosphate generation. These data suggested that microtubule function may be impaired in CH syndrome perhaps secondary to an abnormality in 3',5' cyclic guanosine monophosphate generation. The cholinergic agonists were also shown to prevent development of the giant granules that are pathognomonic of CH syndrome in embryonic fibroblasts isolated from CH mice and cultured in vitro.

In this report it is shown that an extreme degree of spontaneous concanavalin A cap formation is also characteristic of peripheral blood PMN from two patients with CH syndrome. This indicates an abnormality of microtubule function in CH syndrome in man. 3',5' cyclic guanosine monophosphate, carbamylcholine, and carbamyl  $\beta$ -methylcholine reduce spontaneous capping in CH cells. In addition, it is shown that monocytes isolated from the patients' blood and incubated in tissue culture generate a large complement of abnormal gran-

ules. When the same cells mature in vitro in the presence of carbamylcholine or carbamyl  $\beta$ -methylcholine, the proportion of cells containing morphologically normal granules is significantly increased.

These responses can be reproduced in vivo in the beige (CH) mouse. Animals treated for 3 wk and longer with carbamylcholine or carbamyl  $\beta$ -methylcholine show normal granule morphology and a normal degree of concanavalin A cap formation in peripheral blood PMN leukocytes.

## INTRODUCTION

The beige mouse, a spontaneous mutant of the C57 black mouse, is considered a homologue of Chediak-Higashi (CH)<sup>1</sup> syndrome of man (1-3).

We recently described an abnormality of concanavalin A (Con A) distribution on polymorphonuclear leukocytes (PMN) from CH mice that suggested a defect in microtubule assembly in these cells (4). In lymphocytes (5), virus-transformed 3T3 fibroblasts (SV3T3) (6) and other cells, the disassembly of microtubules by treatment with agents such as colchicine and vinblastine that interact with microtubules (7), favors the aggregation of Con A into a surface cap (reviewed in 8). Consistent with this, PMN from normal black mice showed a random surface distribution of Con A except after

<sup>1</sup>*Abbreviations used in this paper:* Bethanechol, carbamyl  $\beta$ -methylcholine; carbachol, carbamylcholine; cGMP, 3',5' cyclic guanosine monophosphate; CH, Chediak-Higashi; Con A, Concanavalin A; FITC, fluorescein isothiocyanate; FITC-Con A, Fluorescein isothiocyanate conjugated Con A; PMN, polymorphonuclear leukocytes.

*Received for publication 9 May 1975 and in revised form 22 December 1975.*

incubation with colchicine which permits cap formation. By contrast, Con A was capped spontaneously on PMN from the beige or CH mouse to the same degree as on colchicine-treated normal cells. In addition, it was found that cyclic 3',5' guanosine monophosphate (cGMP) and the cholinergic agonists carbamylcholine (carbachol) and carbamyl  $\beta$ -methylcholine (bethanechol) that are capable of increasing cellular cGMP levels (9) normalized the surface distribution of Con A on CH PMN and antagonized the colchicine effect on normal PMN. This suggested that defective cGMP generation was linked with impaired microtubule function in CH PMN. Studies by Zurier et al. and Weissmann and co-workers (10, 11) have previously indicated that elevation of cGMP and assembly of microtubules may be related processes in human PMN.

Further studies showed that the continued presence of cholinergic agonists could also prevent generation of abnormal lysosomal granules in CH fibroblasts. Primary embryonic fibroblasts isolated from CH mice and maintained in tissue culture in the absence of drugs, developed the giant granules that are characteristic of CH syndrome in more than 60% of cells at confluency. However, when CH fibroblasts were plated at low cell density and maintained to confluence (10–14 days) in medium supplemented with carbachol (10  $\mu$ M) only 9% of cells contained one or more giant granules (12).

We report here the extension to man of these in vitro observations in mouse and indicate the potential therapeutic value of cholinergic agonists by studies of mice in vivo.

## METHODS

The patients with the CH syndrome were 5 and 32 yr old males, the former brought to our attention by Dr. Thomas Stossel and the latter by Dr. George Buchanan, both of Children's Hospital Medical Center, Boston, Mass.

We were able to study the child's leukocytes through the cooperation of his personal physician, Dr. William Collins, New Bedford, Mass., his consulting hematologist, Dr. George Buchanan, and Dr. Stossel. For some studies, the personnel in the Department of Pathology, St. Luke's Hospital, New Bedford, Mass., obtained blood samples from the patient and allowed us use of their facilities for cell separation procedures. Signed informed consent was obtained from the patient's parents before studies were begun. The patient was in the accelerated phase of the disease and was being treated with alternate day corticosteroids (10–60 mg prednisone q.o.d.). Blood was obtained just before a scheduled dose of prednisone or on the alternate nontreatment day and only at a time when the patient's blood was drawn for studies necessary to the clinical management of his disease. The second patient has photophobia, pale irides, marked horizontal nystagmus, and a peripheral neuropathy. Giant granules were readily apparent in Wright stained preparations of peripheral blood leukocytes from both patients.

*Peripheral blood mononuclear leukocytes* from human subjects were obtained by means of Hypaque-Ficoll gradients

(13) and resuspended in phosphate buffered saline (PBS). In some experiments the fractions were stored on ice for 3 h (during transportation) before use. Alternatively, whole blood was stored on ice, and the cells were isolated within 3 h in our laboratory. No difference was observed between cells handled by these two techniques. The suspension of mononuclear cells was diluted with Dulbecco's modified Eagle's medium containing penicillin and streptomycin (Grand Island Biological Co., Grand Island, N. Y.), and 2-ml portions were plated into 3.5-cm plastic petri dishes containing three 15-mm glass cover slips. After 2 h, non-adherent cells were removed, and the medium was replaced with fresh Dulbecco's modified Eagle's medium containing 40% fetal calf serum with and without added carbachol (10  $\mu$ M) or bethanechol (0.1 mM). The medium was changed daily. Cell numbers remained approximately constant between the two groups between days 2 and 7 of culture.

*Human peripheral blood PMN* were obtained by centrifugation of whole blood (stored on ice during transportation) at 400 g for 10 min at 4°C in a Sorvall RC5 centrifuge, (Ivan Sorvall, Inc., Norwalk, Conn.) followed by collection of the buffy coat. The cells were pelleted by centrifugation for 3 s in an Eppendorf microfuge, contaminating erythrocytes were removed by osmotic lysis, and the cell suspension, containing approximately 80% PMN and 20% mononuclear cells, was filtered through nylon and resuspended in Dulbecco's PBS (approximately 10<sup>6</sup> cells/ml). Mouse PMN were isolated as previously described from peripheral blood collected by heart puncture (4).

*Granule morphology* was monitored by incubating cells adherent to cover slips for 3 min with acridine orange (1:10<sup>6</sup>) in PBS. The cells were observed with a Zeiss photomicroscope III with an epi-illuminator, fluorescein isothiocyanate (FITC) interference filter, 50 dichroic mirror, 53 barrier filter and a  $\times 40$  planapochromatic objective (Carl Zeiss, Inc., New York). Acridine orange stains lysosomes an intense orange color against a background of green cytoplasm and nucleus (14, 15). The use of acridine dyes for characterization of leukocyte lysosomal granules, including those in CH leukocytes, has been established by previous investigators (15, 16).

*The distribution of Con A on human PMN* was observed by a modification of the method used previously (4) for mouse PMN. Portions (0.5 ml) of the cell suspensions were incubated in 2-ml plastic tubes at 37°C in the presence of 5  $\mu$ g/ml fluorescein isothiocyanate conjugated Con A (FITC-Con A) for 5 min. 0.5 ml 2% paraformaldehyde was added to fix the cells during a further 5–10-min incubation at room temperature, after which the labeled cells were collected by centrifugation, washed once with PBS, and resuspended in 20–50  $\mu$ l PBS. 10- $\mu$ l portions were sealed under glass cover slips, and the distribution of fluorescence was observed by combined phase-fluorescence using a Zeiss Universal fluorescence microscope equipped as above but using a  $\times 100$  neofluar phase objective. 100 PMN were identified by phase-contrast microscopy and scored for the presence or absence of surface caps. The distribution of Con A was classified into three categories: A cell was considered (a) capped when all of the fluorescence was present in a knob or projection at one pole of the cell; (b) random, when fluorescence was seen as a discrete ring around the cell periphery, and (c) patched, when fluorescence was distributed in intracellular vesicles either around the cell periphery, under a capped area of the cell surface, or in aggregates near the center of the cell. Intracellular Con A was identified by its resistance to removal by postincubation of cells with the competing hapten sugar,  $\alpha$ -methyl-D-mannoside.

All Con A could be eluted with the competing sugar from randomly-labeled and capped cells.

*The distribution of Con A on mouse PMN* was observed using cell monolayers as previously described (4). Monolayers formed during a 30-min incubation of mouse peripheral blood leukocytes on glass cover slips followed by vigorous rinsing in PBS contained 80–85% PMN and 15–20% mononuclear cells. These cells were labeled with FITC-Con A (5  $\mu\text{g}/\text{ml}$ ) for 10 min, rinsed, wet mounted, and examined by combined phase-fluorescence microscopy as described above. The same monolayer technique was used for initial experiments with human PMN.

*In Vivo experiments.* In initial studies, two normal black (C576J +/+) and two CH (C576J bg/bg) mice (The Jackson Laboratory, Bar Harbor, Maine) were injected subcutaneously with 5  $\mu\text{g}$  carbachol or 10  $\mu\text{g}$  bethanechol twice daily. Rapid onset (2 min) of transient (5 min) lacrimation and rhinorrhea was observed during the 1st wk after each carbachol injection in CH but not normal mice. Bethanechol did not produce any apparent side effects. Treatment was continued for at least 3 wk before sacrifice. More recently, carbachol and bethanechol have been administered orally to groups of up to 30 mice. No apparent adverse effects occur in CH mice given relatively high doses of drug orally for up to 6 mo: 200  $\mu\text{g}/\text{ml}$  carbachol, or 400  $\mu\text{g}/\text{ml}$  bethanechol ad lib. in the water bottles. Each mouse (approximate weight, 25 g) ingests between 0.5 and 1.0 mg carbachol or between 1.0 and 2.0 mg bethanechol daily.

## RESULTS

*Distribution of Con A on human PMN.* In experiments with the first patient the distribution of FITC-Con A on CH PMN was compared with the distribution on normal human PMN using cell monolayers formed on glass cover slips at 37°C as previously used for mouse PMN (4). In experiments with the second patient the distribution of FITC-Con A was determined using the new suspension assay described above. The results were qualitatively similar, but the suspension assay was found to be considerably more suitable than the monolayer assay for human cells.

Monolayers of normal human PMN showed surface caps on 3–7% of cells, with the remainder showing a rim of peripheral (surface) fluorescence. Intracellular vesicles, identified by their persistence in cells post-treated with  $\alpha$ -methyl-D-mannoside, were also present in 50–70% of cells. Preincubation of these monolayers with colchicine (1  $\mu\text{M}$ ) increased the proportion of cells that appeared to be capped with Con A to 32–45% in five experiments, with intracellular lectin being apparent in both the capped and noncapped cells. PMN from the first CH patient exhibited spontaneous cap formation on a substantial portion of cells (31 and 46% in two experiments). This high degree of capping was not increased by colchicine but was completely antagonized by 10  $\mu\text{M}$  carbachol. In two experiments only 7 and 9% of cells were capped with Con A after carbachol treatment. However, intracellular vesicles were again seen in most cells.

A procedure was then developed that would allow

further examination of the redistribution of Con A on the cell surface without the complication of simultaneous internalization of lectin. This internalization obscured the surface distribution of Con A on some cells. Further, the possibility that the apparent reduction in capping in cells treated with cholinergic agonists was due to an increased rate of internalization of caps could not be eliminated. It was first found that labeling of human PMN in suspension rather than on monolayers resulted in a lower rate of internalization. We then discovered that very little internalization occurred in PMN isolated rapidly from buffy coat, whereas PMN isolated by conventional procedures, by dextran sedimentation or centrifugation through bovine serum albumin or Hypaque-Ficoll, showed a significant rate of pinocytotic internalization of lectin. While this apparent activation of pinocytosis is of interest, the procedure that essentially eliminated pinocytosis was adopted for subsequent capping studies.

The results obtained when control PMN and PMN from the second patient were labeled using the suspension assay are shown in Table I. In normal preparations only 6% of cells formed caps with Con A, and the majority of cells showed a completely random distribution of fluorescence, as illustrated in Fig. 1A. Colchicine caused a dramatic increase in capping to 82% of cells (Fig. 1B). CH PMN formed caps spontaneously on 75% of cells (Fig. 1C), and no further increase followed colchicine treatment. Spontaneous capping was restricted to PMN and was not observed on lymphocytes or monocytes present in the cell suspensions. Colchicine-induced capping on lymphocytes but not monocytes was observed in both normal and CH human cells. In both colchicine-treated normal PMN and untreated CH PMN, cGMP and cholinergic agonists, in the presence of imidazole to inhibit phosphodiesterase activity, reduced capping (Fig. 1D). However, capping was not completely inhibited as previously observed with cell monolayers of mouse PMN (4) or CH PMN from the first patient (described above). The proportion of cells showing intracellular fluorescence (patched cells) was consistently low in all cell preparations.

*Granule morphology in CH human monocytes.* When peripheral blood monocytes are incubated on surfaces in medium supplemented with serum, they mature into typical macrophages within 5–10 days, developing long cell processes and generating a large complement of lysosomal granules. This process has been extensively studied and described by Bennet and Cohn (17).

Fig. 2A shows the morphology of acridine orange-stained blood monocytes from a normal adult human after 6 days in culture. The cell contains many small granules dispersed throughout the cytoplasm. By contrast, the monocyte from the first CH patient after 6 days in culture (Fig. 2B) contains fewer granules, and

TABLE I  
Distribution of Con A on Human PMN

| Preincubation<br>(30 min)            | Distribution of fluorescence |         |        |        |         |        |
|--------------------------------------|------------------------------|---------|--------|--------|---------|--------|
|                                      | Normal                       |         |        | CH     |         |        |
|                                      | Random                       | Patched | Capped | Random | Patched | Capped |
| PBS                                  | 86                           | 8       | 6      | 18     | 7       | 75     |
| Colchicine (1 $\mu$ M)               | 17                           | 1       | 82     | 19     | 8       | 73     |
| Imidazole (1 mM)                     | 80                           | 10      | 10     | 32     | 6       | 62     |
| cGMP (1 mM) + imidazole              | 86                           | 9       | 5      | 49     | 9       | 42     |
| Carbachol (10 $\mu$ M) + imidazole   | 79                           | 14      | 7      | 43     | 6       | 51     |
| Bethanechol (0.1 mM) + imidazole     | 90                           | 9       | 1      | 45     | 9       | 46     |
| cGMP + imidazole + colchicine        | 65                           | 0       | 35     | —      | —       | —      |
| Carbachol + imidazole + colchicine   | 50                           | 0       | 50     | —      | —       | —      |
| Bethanechol + imidazole + colchicine | 59                           | 6       | 35     | —      | —       | —      |

Leukocytes were isolated from 10 ml heparinized blood from normal donors and from the second CH patient. Both blood samples were held on ice for 3 h before isolation of leukocytes and labeling of cell suspensions with FITC-Con A. Normal PMN showed identical labeling patterns when cells were separated immediately after withdrawal of blood or after the 3-h period of storage of blood on ice. Results are the average of four separate determinations with leukocytes from three normal donors and of two determinations with leukocytes from the second CH patient. Example of a random and capped distribution of FITC-Con A are shown in Fig. 1. A patched distribution results from internalization of FITC-Con A by pinocytosis during the labeling period.

these granules are distinctly larger than normal. Fig. 2C shows a cell with apparently normal granule morphology. This cell was obtained from a 6-day culture of CH cells incubated in the continuous presence of 0.1 mM bethanechol.

The proportion of cells with giant granules was quantified by scoring 100 cells from each of three cover slips for the presence or absence of abnormal acridine-orange stained inclusions. Monocytes from normal blood developed no giant granules. In two experiments with

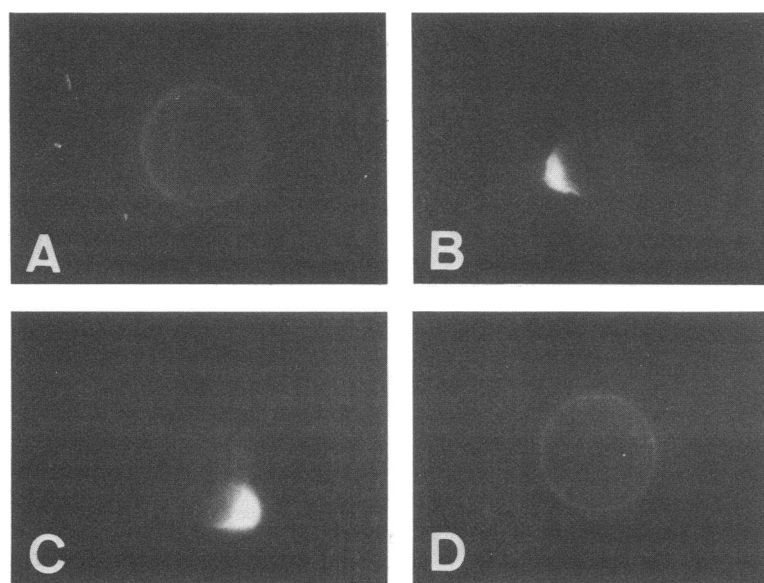
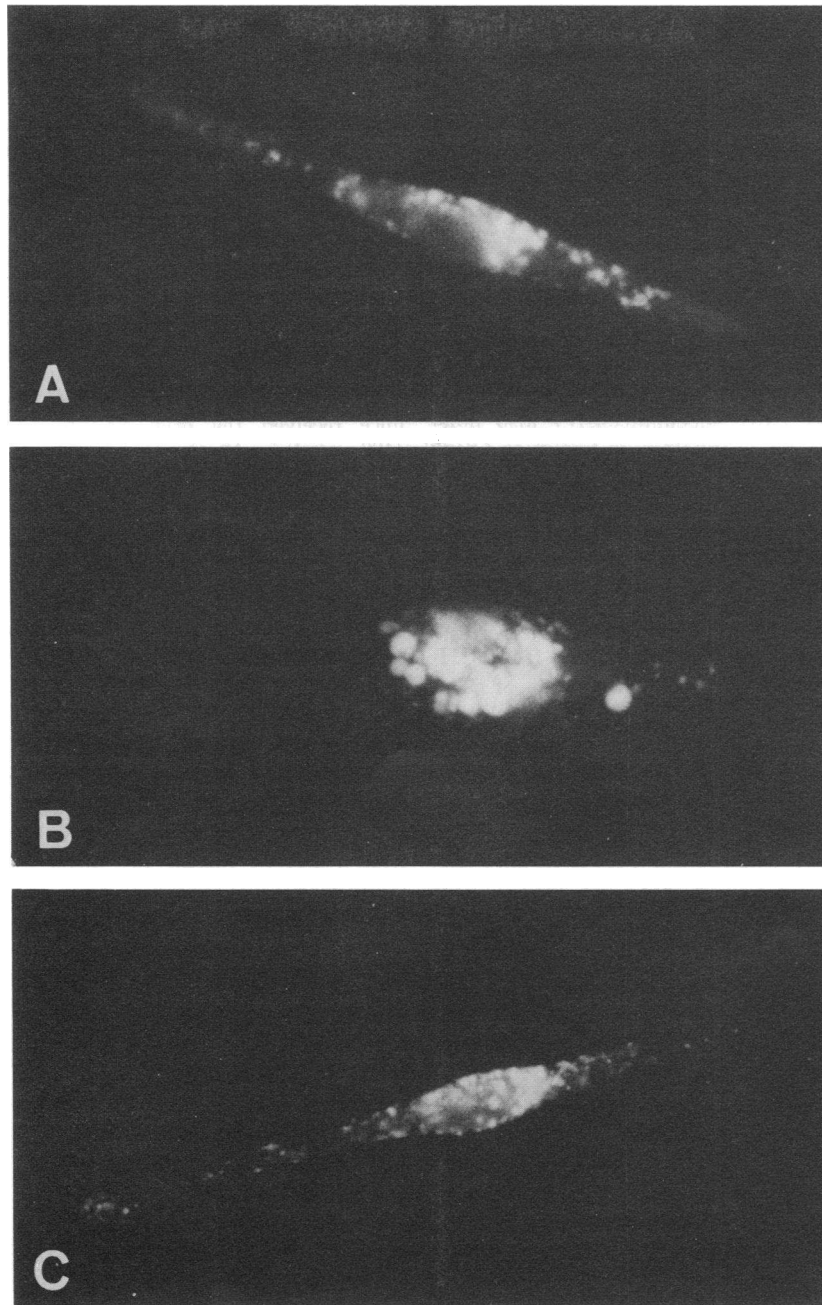


FIGURE 1 Distribution of Con A on normal and CH PMN. Cell suspensions were incubated for 30 min at 37°C in PBS with or without addition of drugs and then labeled with FITC-Con A (5  $\mu$ g/ml) for 5 min. Untreated normal cells (A) showed a random distribution of fluorescence, and most cells were capped after treatment with  $\mu$ M colchicine (B). By contrast CH PMN capped spontaneously with Con A (C). The proportion of CH cells that capped was reduced by treatment with cGMP or cholinergic agonists (D). Initial magnification  $\times$  1,000.



**FIGURE 2** Granule morphology in normal and CH peripheral blood monocytes after 6 days of in vitro culture. Normal cells (A) showed a large number of small granules after vital staining with acridine orange. CH cells showed large granules in the majority (about 66%) of cells (B). When carbachol ( $10 \mu\text{M}$ ) or bethanechol ( $0.1 \text{ mM}$ ) were included in the culture medium, granule morphology was normalized in about 65% of cells (C). Initial magnification  $\times 500$ .

cells from the first CH patient, an average of 68% of cells contained giant granules after incubation for 6 days in medium alone. However, cells cultured in the presence of  $10 \mu\text{M}$  carbachol or  $0.1 \text{ mM}$  bethanechol showed

giant granules in only 34 and 24%, respectively, of cells after 6 days of in vitro culture. Similar data were obtained in one experiment with cells from the second patient. After 8 days in medium alone, 65% of cells

cultured (in medium alone) contained giant granules. This proportion was reduced to 48% in the presence of carbachol and to 39% with bethanechol.

*In vivo studies.* Granule morphology of peripheral blood leukocytes isolated from carbachol or bethanechol-treated CH mice was dramatically different from granule morphology in untreated CH mouse PMN. In leukocytes from CH mice (80% PMN, 20% lymphocytes and monocytes) giant granules were apparent in most cells (compare the granules in normal cells, Fig. 3A with those in CH cells, Fig. 3B). By contrast, very few giant granules were present in leukocytes from two carbachol-treated mice (5  $\mu\text{g}$  twice daily subcutaneously). Bethanechol (10  $\mu\text{g}$  twice daily subcutaneously) also normalized leukocyte granule morphology in two mice (Fig. 3C). In addition, oral administration of bethanechol (400  $\mu\text{g}/\text{ml}$  ad lib.) for 4–12 wk was as effective as subcutaneous administration in normalizing granule morphology in each of five mice.

Oral administration of bethanechol also caused a significant decrease in spontaneous Con A cap formation in PMN (Table II). Monolayers of PMN from CH mice show Con A capping on a high proportion of cells (56%). By contrast, only 22% of PMN from bethanechol-treated CH mice showed caps with Con A. This is not different from the proportion of cells from normal mice that cap (22%). The monolayer technique was retained for these capping studies because of its greater convenience with mouse PMN. First, excessive internalization of surface-bound Con A does not occur when

TABLE II  
*The Distribution of Con A on PMN from Untreated and Bethanechol-Treated Mice*

| Mouse                  | Distribution of fluorescence |         |        |
|------------------------|------------------------------|---------|--------|
|                        | Random                       | Patched | Capped |
| Normal (C57/6J black)  | 67                           | 11      | 22     |
| CH (C57/6J beige)      | 26                           | 18      | 56     |
| Bethanechol-treated CH | 64                           | 14      | 22     |

PMN were isolated and labeled on monolayers as before (4). Results are average of three separate determinations using mice of approximately 6 mo of age. The bethanechol-treated mice received the drug ad lib. in the drinking water (400  $\mu\text{g}/\text{ml}$ ) for 10 wk.

mouse cells are attached to a surface, as shown by the low proportion of patched cells in Table II. Second, suspensions of mouse peripheral blood leukocytes contain a much higher proportion of lymphocytes (about 50%) than similar preparations of human blood leukocytes (about 20%). These lymphocytes are rather similar in size to mouse PMN so that differentiation between lymphocytes and PMN in mouse leukocyte preparations by phase contrast microscopy is more difficult than with human leukocyte populations. Most lymphocytes do not adhere to surfaces and so can be conveniently separated from PMN by plating cell suspensions onto glass, followed by vigorous washing to remove nonadherent cells.

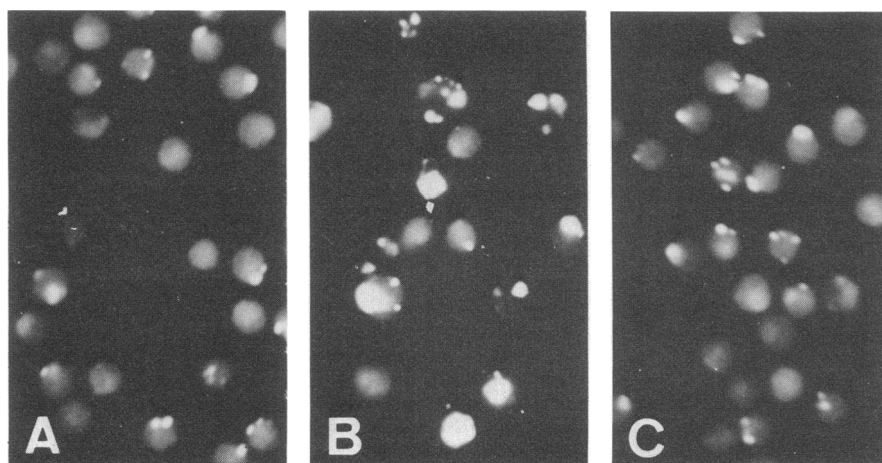


FIGURE 3 Granule morphology in mouse peripheral blood leukocytes. Monolayers of leukocytes (80% PMN, 20% mononuclear cells) isolated from peripheral blood from normal black mice, untreated CH and bethanechol-treated (3 wk, subcutaneous administration) CH mice were stained with acridine orange. The leukocytes from untreated CH mice (B) showed giant granules in most cells, while very few giant granules were seen in leukocytes from bethanechol-treated CH mice (C) or from normal black mice (A). Carbachol administered subcutaneously and bethanechol administered orally also normalized granule morphology in PMN. Initial magnification  $\times 625$ .

## DISCUSSION

CH syndrome in man is a rare autosomal recessive disorder characterized by partial oculocutaneous albinism, photophobia, nystagmus, frequent pyogenic infections, and the presence of giant irregularly shaped lysosomes in most granule containing cells (1, 2, 18). Mild to severe peripheral neuropathy has been observed in several CH patients. Febrile episodes are common and an accelerated (lymphoma-like) phase develops, during which patients have hepatosplenomegaly and lymphadenopathy due to lymphoid and histiocytic infiltrates in these organs (19). Death usually occurs in childhood, due to infection or hemorrhage (1). A similar disorder has been described in mink (20), cattle (21), and mice (3, 22) as well as in cats and in a killer whale (23, 24). Neutropenia, presumably due to intramedullary destruction of cells (25, 26) is common, and marrow granulocyte responses are decreased (26). Chemotactic responses of neutrophils from man (27) and mouse (3) are impaired, but particle uptake by CH neutrophils is normal. The complement of lysosomal enzymes is relatively normal (28) although reductions in myeloperoxidase and  $\beta$ -glucuronidase have been reported (29). These cells exhibit a delay in killing intracellular bacteria (30), perhaps as a result of a selective degranulation defect which has been detected in leukocytes from CH patients (28).

In this report, we establish that CH human PMN show the same surface abnormality previously described in the beige (CH) mouse (4). Exaggerated cap formation by Con A occurred with cells from the first patient who was in the accelerated phase of the disease and was receiving steroid therapy and from the second patient who was not chronically drug treated and whose disease has never progressed to the accelerated stage. Thus, we suppose that spontaneous surface Con A capping is an intrinsic manifestation of CH syndrome and is unrelated to the severity of disease or to steroid therapy.

Con A cap formation can be induced in normal human PMN by compounds that inhibit microtubule assembly. Three such agents are presently known. The plant alkaloids colchicine and vinblastine (and their derivatives such as Colcemid and vincristine) promote capping in a variety of cell types including human PMN (8). In addition, the new synthetic antimicrotubule agent methyl-5[2-thienyl-carbonyl-1H-benzimidazol-2-yl] carbamate (31) promotes Con A capping in normal human PMN (32). Although these agents may have sites of action besides inhibition of microtubule assembly or disruption of microtubules, their structural dissimilarity makes it unlikely that another common site exists that could account for enhancement of Con A capping. Thus, we consider that capping in normal human PMN after incubation with antimicrotubule drugs reflects the absence

of polymerized microtubules. By extension, spontaneous capping in CH PMN is most likely a direct consequence of the inability of these cells to assemble microtubules.

Electron microscopic studies that confirm this conclusion will be presented shortly. We have established that incubation of normal human PMN with Con A for 5 min is accompanied by extensive assembly of cytoplasmic microtubules. CH human PMN show a complete absence of microtubules under the same conditions.<sup>2</sup> Since CH cells divide normally and the amount of colchicine-binding protein (presumably tubulin) is the same in liver and lymphocytes from both normal and CH mice,<sup>3</sup> it is unlikely that the failure of polymerization in CH PMN is due to the absence of tubulin. Therefore, we suppose that a signal to microtubule assembly is activated in normal PMN but not in CH PMN by a surface event such as Con A binding.

Enhanced Con A capping is antagonized in CH human PMN as in CH mouse PMN by cGMP, carbachol, and bethanechol. This suggests that these agents may promote microtubule assembly in CH cells. It has previously been established that cGMP enhances microtubule assembly in normal human PMN (10, 11). The reduction in capping in human CH PMN by cGMP and cholinergic agonists measured by the suspension assay is less profound than that observed with human cells on monolayers and that previously reported with monolayers of mouse cells. This may reflect the difference in assay technique: perhaps contact with a surface per se facilitates microtubule assembly as suggested from the observation that colchicine induces less capping (30–40%) on normal PMN on monolayers as compared to the capping (75–90%) measured in suspension, or it may be that the optimal concentrations of cGMP and cholinergic agonists have not yet been tested in the suspension assay, due to the rather limited availability of cells.

The cholinergic agonists also modify granule morphology in peripheral blood monocytes obtained from the patients and stimulated to mature to macrophages *in vitro*. Without drugs, most of the cells (>65%) generate a population of very large granules which are morphologically quite aberrant when compared with the small granules that develop in normal blood monocytes. However, when the cells matured in the presence of cholinergic agonists, only 24–48% of cells contained giant granules, and the morphology of the granules in the remaining cells was not different from normal. As for the capping data, the granule response is less dramatic than that observed in mouse fibroblasts *in vitro* (12) and in mouse peripheral blood leukocytes *in vivo* shown

<sup>2</sup> Albertini, D. F., R. B. Zurier, and J. M. Oliver. Manuscript in preparation.

<sup>3</sup> Sherline, P., and R. B. Zurier. Unpublished results.

here. However, it should be noted that at least 20% of CH monocytes contained a small number of giant granules at the time of plating. Studies of granule turnover are required to find if these same granules are still present after a week in culture in addition to the new granules that develop in vitro.

The precise relationship between the surface abnormality (capping) and the granule abnormality in CH syndrome is not known. Since both characteristics respond to cholinergic agents and since the surface abnormality likely reflects a microtubule defect it is tempting to speculate that abnormal microtubule function may also be involved in generation of giant granules. The mechanism by which increased cGMP generation leads to increased microtubule assembly also remains to be established.

It is particularly encouraging that the cholinergic agonists are able to reverse several characteristic abnormalities of CH mice in vivo. Administration of carbachol or bethanechol to CH mice for 3 wk results in the appearance in these animals of a new population of peripheral blood leukocytes in which the granules do not differ from granules in leukocytes from normal mice. Since granule fusion to produce giant lysosomes in PMN occurs during maturation in the bone marrow (33) our results suggest that cholinergic agonists can gain access to this tissue. This is of potential clinical importance since in man the marrow is the site of abnormal granule formation and also of accelerated leukocyte destruction (25). Con A capping is also reduced to normal levels in PMN from mice treated with cholinergic agonists in vivo, just as previously described in experiments where isolated PMN were exposed to the drugs in vitro (4). It is possible that the therapeutically altered population of PMN may show improved lysosomal degranulation and chemotaxis since colchicine may depress and cGMP and carbachol enhance both these functions in normal PMN (9, 10, 34).

Each abnormal property of CH mouse or human cells tested thus far, whether in vivo or in vitro, has responded favorably to carbachol and/or bethanechol, although more dramatic responses were consistently observed in mice than in human cells. It is not known, of course, whether these or similar compounds might retard or halt the infiltrative, lymphoma-like phase of the disease that occurs in man. Vincristine has been the mainstay in efforts to control the accelerated phase of the disease (1). The drug, however, prevents microtubule assembly, and cells from these patients already show evidence of impaired microtubule function. The observations presented here support a rational alternative: treatment of CH syndrome with bethanechol. Prolonged oral administration of bethanechol is not without precedent. Children with familial dysautonomia have

been treated with up to 2 mg per Kg daily without serious toxic reactions (35).

#### ACKNOWLEDGMENTS

Ms. Joyce Krawiec and Ms. Donna Sayadoff provided excellent technical assistance.

This paper was supported by American Cancer Society grant BC-179, National Institutes of Health grants ES00106, AI 12225, AM17309, and a clinical center grant of the Arthritis Foundation.

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