Impaired Microtubule Function Correctable by Cyclic GMP and Cholinergic Agonists in the Chediak-Higashi Syndrome

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The Chediak-Higashi (CH) syndrome of man and several animal species is characterized by the presence of abnormal giant granules in all granule-containing cells and by defects in chemotaxis and lysosomal degranulation during phagocytosis in polymorphonuclear leukocytes (PMNs). Since similar functional abnormalities have been reported in normal PMNs following exposure to colchicine and other agents that disrupt microtubules it was proposed that microtubule function may be impaired in the CH syndrome. The mobility of concanavalin A (con A)-receptor complexes on PMN membranes was used to test microtubule integrity. Normal PMNs showed a uniform distribution of membrane-bound con A. By contrast, con A was aggregated into surface caps on both colchicine-treated normal PMNs and untreated PMNs from mice and a patient with CH syndrome. This result is consistent with impaired microtubule function in the CH cells. The spontaneous capping response of CH PMNs was inhibited by cyclic GMP and by cholinergic agonists that can elevate cyclic GMP levels in neutrophils. This raised the possibility that the microtubule defect in CH cells may be correctable by treatments that increase cyclic GMP generation. Direct evidence for both the absence of microtubule assembly in con A-treated PMNs from the CH patient and for normal microtubule assembly in CH PMNs incubated with cyclic GMP and cholinergic agonists prior to con A treatment was obtained by electron microscopy. In addition, evidence for a direct relationship between the microtubule defect and the development of giant lysosomes in CH cells was obtained. Thus, CH fibroblasts grown *in vitro* developed abnormal lysosomes in the majority of cells. However, the same cells cultured in the presence of cholinergic agonists developed a majority of lysosomes that were morphologically normal at the level of the light microscope. Similarly, granule morphology appeared normal in peripheral blood leukocytes from mice treated chronically *in vivo* with cholinergic agonists. (Am J Pathol 85:395–418, 1976)

POLYMORPHONUCLEAR LEUKOCYTES (PMNs, neutrophils) provide the first line of host defense against infection. Thus, it is not surprising that a long list of clinical aberrations of the inflammatory response are linked to insufficient numbers of circulating neutrophils, to absence or defects in humoral factors required to promote chemotaxis and phagocytosis, and to absence of specific lysosomal and cytoplasmic enzymes involved in the intracellular killing and digestion of bacteria (reviewed

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recently by Stossel [1]). Several additional disorders of PMN function have been described that are unrelated to any known humoral insufficiency, enzyme defect, or gross neutropenia. In at least some of these cases it seems likely that the basic defect may lie in the subcellular structural elements—the microfilaments and microtubules—that are involved in PMN functions such as chemotaxis, phagocytosis, and lysosomal degranulation. Boxer and co-workers [2] have recently presented evidence for a defect in microfilament organization and function in a patient with severe impairment of chemotaxis and phagocytosis. I review here a series of experiments that are strongly suggestive of a microtubule defect in PMNs from mice and a patient with an inherited disorder of leukocyte function.

The Chediak-Higashi Syndrome

The Chediak-Higashi syndrome has been extensively characterized in man by Wolff and co-workers,[3,4] in mink and cattle by Padgett and co-workers,[5] and in the beige (CH) mouse by Gallin *et al.*[6] In all species it is characterized by partial albinism, the presence of giant granules in all granule-containing cells, and increased susceptibility to bacterial infection. In man, death usually occurs in childhood as a result of infection or hemorrhage and is often preceded by development of an accelerated or lymphoma-like phase of the disease.

Two major defects in PMN function in the Chediak-Higashi syndrome have been documented: the PMNs fail to show directed movement in response to chemotactic stimuli *in vivo* and *in vitro*,[6,7] and they exhibit a delay in killing of intracellular bacteria which appears to result from a reduced rate of lysosomal fusion with phagocytic vacuoles.[8] The similarity between these defects and the defects induced in normal PMNs by exposure to colchicine [9,10] led to the proposal that impaired microtubule function may underly the impaired inflammatory response in patients and animals with CH syndrome.

Assay of Microtubule Function in Intact Cells

During the past several years, our laboratory has been primarily concerned with analysis of the role of microtubules in the regulation of cell surface organization and function. In the course of these studies, two systems that test for the functional integrity of microtubules in PMN have been developed (reviewed in Oliver [11]).

The first system involves measurement of the partitioning of selected membrane proteins between the plasma membrane and the phagocytic vesicle membrane during the process of phagocytosis. It was established

that phagocytosis is normally accompanied by segregative movement of transport proteins [12] and glycoprotein receptors for plant lectins,[13] respectively, out of or into internalized portions of the plasma membrane. This directed movement of membrane proteins no longer occurs after colchicine treatment (10^{-6} M, 30 minutes).[13,14] These experiments provided the first evidence that colchicine-sensitive structures, presumably microtubules, interact with membranes to control the topographic organization of the PMN cell surface.

The second system involves determination of the translational mobility of glycoprotein receptors for plant lectins such as concanavalin A and *Ricinus communis* agglutinin (RCA) in membranes. Surface-bound lectins show an essentially homogeneous distribution on the membrane of normal mouse and human PMNs.[15-17] However, when normal cells are pretreated with colchicine followed by short exposure to con A, the con A–receptor complexes aggregate into surface caps (illustrated in Figures 1C and 2C). Similar observations were previously made with lymphocytes [18] and SV3T3 fibroblasts.[19]

Recent studies have strengthened our confidence that the presence or absence of con A capping on PMN reflects the presence or absence of tubulin that is competent to polymerize into microtubules. Thus, the earlier studies with PMNs and other cells relied almost exclusively on the use of colchicine to generate a microtubule-defective cell. Colchicine may have other nonmicrotubule sites of action that could account for changes in membrane protein mobility.[20,21] We have now established that another agent that is structurally unrelated to colchicine and yet shares with colchicine the ability to bind with tubulin and inhibit microtubule assembly also promotes con A cap formation on PMN.[17] This agent is methyl [5-(2-thienylcarbonyl)-1H-benzimidazol-2-yl] carbamate (R17934), a synthetic drug developed by Janssen Pharmaceutica and shown by DeBrabander and co-workers to mimic the action of colchicine in a variety of cells *in vivo* and *in vitro*.[22,23] In addition, we have shown that microtubule assembly in PMNs is rapidly inhibited by diazenedicarboxylic acid bis(*N N*-dimethylamide) (diamide), a specific glutathione (GSH)-oxidizing agent.[24,25] In this case, inhibition of polymerization is a consequence of oxidation of essential tubulin sulfhydryl groups. Microtubule disruption by lowering intracellular GSH levels, like microtubule disruption by binding of colchicine or R17934 to tubulin subunits, leads to extensive con A cap formation on human PMNs. Since impairment of microtubule integrity by these different pharmacologic methods leads to con A cap formation in all cases, we consider that demonstration of con A capping on PMNs provides a reliable index of impairment of microtubule function.

Microtubule Function in PMNs From Mice With Chediak-Higashi Syndrome

The relative ability of PMNs from normal and CH mice to form caps with con A is illustrated in Figure 1 and quantified in Table 1.[15] When monolayers of peripheral blood PMN from normal mice are incubated with fluorescein isothiocyanate-conjugated con A (F-con A, 10 μg/ml) for 10 minutes at 37 C, then rinsed, fixed, and examined by fluorescence microscopy, the majority of cells show a random distribution of lectins and very few form surface caps (Figure 1A and Table 1, Line 1). However, when normal cells are pretreated with 10^{-6} M colchicine for 30 minutes prior to labeling, there is a significant change in the distribution of con A, with a high proportion of cells now appearing in the capped configuration (Figure 1C and Table 1, Line 2).

By contrast with the normal cells, CH mouse PMNs form con A caps spontaneously on a high proportion of cells (Figure 1B and Table 1, Line 6) and colchicine does not further increase the proportion of capped cells (Table 1, Line 7). This result is consistent with the impairment of microtubule function predicted from the functional abnormalities of CH leukocytes.

Table 1—Distribution of F-Concanavalin A on Mouse PMNs

Preincubation (30 min)	Percent		
	Random	Patched	Capped
Normal PMNs			
1. Buffer	62	27	11
2. Colchicine (10^{-6}M)	23	22	55
3. Lumicolchicine (10^{-6}M)	73	16	11
4. cAMP (10^{-3}M) + theophylline (10^{-3}M)	67	25	8
5. cGMP (0.5×10^{-4}M)	70	22	8
Chediak-Higashi PMNs			
6. Buffer	33	23	44
7. Colchicine (10^{-6} M)	32	18	50
8. cAMP (10^{-3}M) + theophylline (10^{-3} M)	41	21	38
9. PGE_1 (2.8×10^{-6} M) + theophylline	48	20	32
10. cGMP (0.5×10^{-4} M)	76	14	10
11. Carbachol (10^{-5} M)	72	20	8
12. Bethanechol (10^{-5} M)	69	14	17
13. PMA (10 ng/ml)	69	25	6
14. Guanine (0.5×10^{-4} M)	25	29	46
15. Guanosine (0.5×10^{-4} M)	30	24	46
16. GMP (0.5×10^{-4} M)	35	18	47

Monolayers of peripheral blood PMNs were labeled with F-con A (10 μg/ml, 10 to 15 min) and the distribution of bound lectin examined by fluorescence microscopy as previously described.

Random = a uniform distribution of fluorescence, capped = fluorescence concentrated in a polar shell or knob, patched = fluorescence inside the cells in a patchy distribution due to endocytosis of lectin-receptor complexes.

Microtubule Function in PMNs From Humans With Chediak-Higashi Syndrome

The results of similar studies of the distribution of F-con A on normal and CH human PMNs are illustrated in Figure 2 and quantified in Table 2. The data with normal PMNs are averaged from a large number of experiments with cells from adult donors, both male and female. The data with Chediak-Higashi PMNs were obtained from studies with a 32-year-old male who has never advanced to the accelerated phase of the disorder and had not received drugs recently. His clinical features have been described.[16] Qualitatively similar data (not shown) were obtained with PMN from a 5-year-old patient under treatment for lymphoproliferative disease.[16]

Peripheral blood PMNs from normal human donors that are incubated in suspension with F-con A (5 μg/ml) for 5 minutes, then fixed, washed, and examined show a random distribution of con A on the majority of cells (Table 2, Line 1, and Figure 2A). Pretreatment of normal cells for 30 minutes with colchicine (10^{-6} M) or R17934 (10^{-6} M) or for 5 minutes with diamide (50 mμmoles/10^6 cells) leads to con A cap formation on most cells (Table 2, Lines 2–4, and Figure 2C).

PMNs from the CH patient form con A caps spontaneously on the majority of cells (Table 2, Line 6, and Figure 2B). Thus, the abnormality detected in CH mouse PMNs is present in PMNs from at least 1 patient with CH syndrome.

Table 2—Distribution of F-Concanavalin A on Human PMNs

	Percent		
Preincubation (30 min)	Random	Patched	Capped
Normal PMNs			
1. Buffer	75	16	9
2. Colchicine (10^{-6} M)	8	14	78
3. R17934 (10^{-6} M)	9	10	81
4. Diamide (50 mμmoles/10^6 cells)	10	3	87
5. cGMP (10^{-3} M) + imidazole (10^{-5} M)	81	12	7
CH PMNs			
6. Buffer	16	14	70
7. Colchicine	19	8	73
8. Imidazole (10^{-5} M)	15	22	63
9. Cyclic GMP (10^{-3} M) + imidazole (10^{-5} M)	48	16	36
10. Carbachol (10^{-5} M) + imidazole (10^{-5} M)	44	24	34
11. Bethanechol (10^{-4} M) + imidazole (10^{-5} M)	57	14	29

The data for normal cells are the average of five experiments. The data for CH cells are the average for two separate experiments in which parallel cell suspensions were prepared for electron microscopic examination.[26] Data from similar earlier experiments with cells from this and another patient have been published.[16]

It is apparent that the degree of spontaneous capping on the untreated CH human cells, as well as on colchicine-treated normal human PMNs, was double that measured with the corresponding mouse cells. We suppose that this difference may reflect differences in the capping assay *per se*. As noted, mouse cells were allowed to form monolayers on glass coverslips prior to labeling.[15] The major reason is that suspensions of mouse leukocytes contain approximately 50% lymphocytes. The majority of lymphocytes are nonadherent, and so one can obtain cell monolayers that are considerably enriched for PMNs (80% PMNs, 20% mononuclear cells). This simplifies the final process of identifying PMNs by phase contrast microscopy prior to observation of the distribution of F-con A. On the other hand, it is clear that the process of attachment to glass may itself induce microtubule assembly,[27] so that more microtubules may be initially present in these cells. By contrast, suspensions of human leukocytes isolated quickly (in 10 to 20 minutes) by rapid centrifugation already contain 80% PMNs, so that further purification via monolayer formation is considered unnecessary. More importantly, monolayers of human PMNs are not suitable for capping assays. Unlike mouse PMNs, human PMNs undergo a poorly understood process of activation when isolated by conventional methods (BSA or Hypaque–Ficoll gradient centrifugation, dextran sedimentation) or when incubated in suspension with serum or allowed to form monolayers by 30 minutes' incubation on glass coverslips. The consequence of this activation is enhanced pinocytic internalization of F-con A (patching) and decreased surface cap formation (discussed in Oliver and Zurier [16]). Thus the capping assays with human cells must be performed with cells isolated and labeled under conditions that minimize pinocytic activation. These conditions most likely minimize exposure to inducers of microtubule assembly and leave the process of con A binding as the sole inducer of assembly.

The Biochemical Basis of the Proposed Microtubule Defect

The simplest explanation of the capping data in CH PMNs would be a structural alteration in the cytoplasmic tubulin that prevents or impairs its polymerization to form microtubules. However, this explanation seems unlikely to be correct. We have described a fluorescence technique that allows study of the copolymerization of small amounts of tubulin from one source with large amounts of tubulin obtained from another source.[28] When rabbit brain tubulin was used as a "carrier" to look at polymerization of microtubule out of a fluorescein isothiocyanate–prelabeled mouse brain homogenate *in vitro*, we found no difference in copolymerization between normal black and CH mouse brain with rabbit tubulin. In

addition, Sherline and Zurier [29] have established that the amount of colchicine-binding protein (presumably tubulin) is at least normal and most likely higher than normal in granulocytes and other cells from beige mice.

Therefore, we considered an alternative hypothesis, that a factor or factors involved in controlling microtubule polymerization may be absent or defective in CH cells. Zurier and co-workers [30] have shown that cyclic nucleotides can either enhance (cyclic GMP) or depress (cyclic AMP) the colchicine-sensitive process of lysosomal degranulation during phagocytosis in PMNs. These data led us to test the effects of cyclic nucleotides on the distribution of con A on normal and CH PMNs.

The results obtained in the mouse are summarized in Table 1.

Cyclic AMP and prostaglandin E_1 (PGE_1) which raises cyclic AMP levels in PMN,[30] had no significant effect on capping on either normal (Table 1, Line 4) or CH (Table 1, Lines 8 and 9) PMNs.

However, cyclic GMP caused a dramatic reduction in con A capping in CH PMNs to the levels seen in normal cells (Table 1, Line 10). This effect is illustrated in Figure 1. The majority of cyclic GMP–treated CH PMNs (Figure 1D) appear identical to untreated normal PMNs (Figure 1A).

A number of agents that have been shown to increase cyclic GMP generation in PMNs also reduced con A capping in CH cells to normal levels. These include two analogs of acetylcholine, carbamylcholine (carbachol) and carbamyl-β-methylcholine (bethanechol) and the cocarcinogen phorbol myristate acetate (PMA) (Table 1, Lines 11–13).[30-32]

On the other hand, three potential degradation products of cyclic GMP, guanine, guanosine, and GMP, did not affect con A capping on CH cells (Table 1, Lines 14–16). Thus, the effect appears to be specific for the cyclic nucleotide.

Qualitatively similar data were obtained with PMNs from the CH patient. Whereas cyclic GMP and cholinergic agonists did not affect the distribution of F-con A on the surface of normal PMNs (Table 2, Line 5), these agents, in the presence of the cyclic GMP phosphodiesterase inhibitor imidazole,[33] reduced con A capping substantially on CH cells (Table 2, Lines 9–11). The reduction was somewhat less than seen on the CH mouse cells.

Microtubule Assembly in Normal and Chediak-Higashi PMNs

Although the capping data were strongly suggestive of impairment of microtubule assembly in CH cells and its correction by cyclic GMP, several alternative explanations could not be eliminated. First, it was possible that increased mobility of surface proteins in CH cells reflected

changes in the fluidity of the membrane *per se* rather than absence of microtubule assembly- and/or microtubule-membrane interactions. Kanfer *et al.*[34] have previously reported increased turnover of sphingomyelin in CH leukocytes which could lead to changes in membrane composition. It was also possible that reduction in capping by exogenous cyclic GMP and cholinergic agonists reflects direct effects on membrane components rather than on microtubules. Therefore, electron microscopic examination of microtubules in normal and CH cells was undertaken.[35] The CH cells were from the same 32-year-old male patient.

The cells were isolated and labeled with con A (100 μg/ml) exactly as for the capping assays.

After con A treatment, the cells were pelleted and resuspended in 1% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 30 minutes at room temperature followed by two washes in buffer alone. Cell pellets were postfixed in 2% osmium tetroxide in 0.1 M *s*-collidine buffer for 30 minutes, washed in distilled water, and stained *en bloc* in aqueous 2% uranyl acetate for 30 minutes at 60 C. The cells were then dehydrated through a graded series of cold ethanols and embedded in Epon. Silver sections were cut with a diamond knife on an MT-2 Porter-Blum ultramicrotome, picked up on 300-mesh copper grids, and stained lightly with uranyl acetate and lead before examination in a Philips 300 electron microscope.

Sections of human PMNs containing centrioles were examined for the presence or absence of microtubules. Two complementary methods were used for quantification. The numbers of microtubules located within a 2-μ square of a centriole were counted by direct observation in the electron microscope at a stage magnification of 55,000 ×. This enabled examination of a large number of cells in each group. However, only longitudinal sections of microtubules that coursed out of the centriolar area into the less electron-dense cytoplasm were visible. Shorter microtubules, as well as cross-sectional profiles of microtubules, were not easily discernible. Therefore, a smaller number of sections showing centrioles were chosen at random and photographed at a magnification of 9300 ×. Microtubules were not visible by direct examination in the microscope at this magnification, thus precluding samples bias. They were rendered visible by final magnification of photomicrographs to 56,000 ×. More microtubules were counted within a 2-μ square of centriole by this method, since longitudinal profiles of microtubules closely adjacent to centrioles could now be resolved and cross-sections of microtubules could be counted. *En bloc* staining, which enhances the bilayer appearance of membranes, facilitated differentiation between small Golgi vesicles and cross sections through microtubules.

The results obtained are illustrated in Figures 3 and 4 and summarized in Table 3.

Untreated normal PMNs contained a pair of centrally located centrioles typically surrounded by several microtubule profiles (Figure 3A). Centriole-associated microtubules measured 25 to 27 nm in diameter and, when seen in longitudinal sections, coursed for 40 to 90 nm. The mean numbers of microtubules in these cells were 1.1 (by direct examination) and 2.6 (from randomly sampled micrographs). These numbers are lower than numbers recently reported by Hoffstein *et al.* (17.8 microtubules in a 2-μ square centered on a centriole [36]). However, it should be remembered that our cells have been minimally stimulated, whereas Hoffstein and co-workers isolated cells by dextran sedimentation at 37 C. Cells may be exposed to serum factors that promote microtubule assembly during this procedure.

Concanavalin A treatment produced a proliferation of microtubules emanating from the centriole (Figure 3B). The mean numbers of microtubules determined from direct counting on the microscope (4.9) and from randomly sampled micrographs (17.7) represent increases of 4.4- and 6.8-fold, respectively, over untreated controls (Table 3). Cytoplasmic granules became regularly arrayed along microtubules that often extended into the cell cortex in con A–treated cells. Increases in both number and length of microtubules following con A treatment were also observed, but not quantified, in lymphocytes and monocytes present in the suspensions of normal leukocytes. Qualitatively similar data were reported by Hoffstein and co-workers:[36] a two- to fourfold increase to 43.3 microtubules in a 2-μ square centered on a centriole.

The centrioles in CH PMNs were normal in structure. However, in contrast with normal PMNs, virtually no microtubules were detectable in

Table 3—Mean Microtubule Numbers in Normal and Chediak-Higashi Human Neutrophils

Treatment	Direct counts (mean ± SE)	Micrograph counts (mean ± SE)
Normal untreated	1.1 ± 0.4 (52)	2.6 ± 0.4 (11)
Normal con A	4.9 ±0.3 (49)	17.7 ± 2.5 (17)
CH untreated	0.5 ± 0.2 (24)	1.0 ± 0.6 (5)
CH con A	1.3 ± 0.4 (44)	1.7 ± 1.2 (9)
CH con A cyclic GMP (10^{-3} M)	9 ± 0.7 (15)	26 ± 4.3 (4)
CH con A carbachol (10^{-5} M)	9 ± 1.0 (19)	28 ± 1.3 (6)
CH con A bethanechol (10^{-4} M)	7 ± 1.0 (16)	21 ± 3.1 (9)

Microtubules were counted by direct examination of centriolar regions in the electron microscope (first column) and by inspection of randomly selected micrographs of the centriolar area (second column). The number of cells examined is given in parenthesis. Imidazole (10^{-5} M) was included in incubations of CH PMN with cyclic GMP and cholinergic agonists.

the centriolar region of either untreated or con A-treated CH PMNs (Figure 3C). In addition, no changes in the distribution of lysosomal granules followed con A treatment in CH cells. As shown in Figure 4A, granules were haphazardly situated around the centriolar region. Most granules were not different in size from the lysosomal granules in normal PMN although thin sections of cells usually revealed the presence of between two and four giant granules often containing myelin-like material. A similar distribution of small and large granules is characteristic of CH PMNs from other patients.[37,38] The microtubule counts in con A-treated CH cells are essentially identical to recent counts with normal human PMNs pretreated with colchicine and diamide.[24]

Exposure of CH PMNs to cyclic GMP or the cholinergic agonists carbamylcholine (carbachol) and carbamyl-β-methylcholine (bethanechol) followed by con A treatment resulted in a marked increase in the numbers of centriole-associated microtubules (Figure 3D). By direct counting, a sevenfold increase from 1.3 to approximately 8 microtubules was measured, while for micrograph counts a 15-fold increase from 1.7 to approximately 25 microtubules was determined. In addition, the cytoplasmic granules became regularly aligned along microtubules radiating from the cell center (Figure 4B). This ordered granule distribution was similar to that seen in normal PMNs following treatment with con A alone.

These data provide direct confirmation of the hypothesis based on con A capping studies that microtubule assembly is defective in PMNs from the CH patient. The reversibility of the defect by cyclic GMP and cholinergic agonists confirms that the basic defect in CH PMNs is not absence or structural modification of the tubulin subunits, but failure of a signal to induce microtubule assembly. This signal most likely involves generation of cyclic GMP.

Relationship Between Microtubule Assembly and Granule Morphology

The development of abnormal granules resembling autophagic vacuoles and proliferation of annulate lamellae is characteristic of both untreated CH cells[39] and normal cells treated chronically with low doses of antimicrotubule agents.[23,40-42] Thus, we considered that the giant granules and other abnormal cytoplasmic inclusions in Chediak-Higashi PMNs may be another consequence of the microtubule defect. If this were so, agents that appear to promote microtubule assembly might normalize lysosome morphology in CH cells.

To test this, we isolated primary embryonic fibroblasts from CH and normal mice and grew the cells in tissue culture until they reached confluency.[43] The cells were stored frozen and were thawed at intervals to

give a supply of rapidly growing cells capable of steady division for about 3 to 4 weeks. The two cell lines grew at similar rates but differed in at least three respects.

First, the CH fibroblasts were highly susceptible to shape changes induced by colchicine: exposure to 10^{-6} M colchicine for 30 minutes caused these cells to become rounded up and to detach from the culture dishes. By contrast, cells from the black mouse showed no morphologic change during acute exposure to colchicine, suggesting a greater degree of microtubule stability. In this property, CH cells are similar to SV3T3 cells which also show shape changes and loss of adhesiveness during short exposure to colchicine.[11,19]

Second, the CH fibroblasts showed spontaneous surface cap formation with F-con A (25 μg/ml) on a high proportion of cells (48%). By contrast, normal fibroblasts showed some surface patching and pinocytic internalization of con A, but large aggregates or caps were seen on less than 10% of cells.[11] This property of CH fibroblasts is an exaggeration of an established property of SV3T3 fibroblasts: transformed cells, unlike their parental 3T3 line, form surface caps with con A after brief exposure to colchicine.[19]

Third, the CH cells developed giant lysosomal granules, as illustrated in Figure 5A and B. These abnormal structures are considerably larger and more prominent in confluent cultures than in growing cultures. At confluence, at least 60% of cells contain these giant granules, and the remainder of cells have granules that are larger than normal.

To test the effect of cyclic GMP on these properties, cells were plated sparsely and allowed to grow in Dulbecco's modified Eagle's Medium (DMEM) containing 10% fetal calf serum (FCS) plus carbachol (10^{-5} M) or PMA (10 ng/ml). The media were changed daily. These drugs were chosen for use rather than cyclic GMP itself because cyclic GMP would most likely be destroyed rapidly by enzymatic action.

Neither carbachol nor PMA affected the growth characteristics of the cells. The growth rate, determined by counting cells on petri dishes at daily intervals between plating and confluency, was essentially identical in treated and untreated dishes, and cell density at confluency was unchanged.[41] However, significant changes in colchicine sensitivity and lysosomal morphology were observed.

First, the cells were no longer rounded or detached by colchicine. Concanavalin A cap formation was also reduced in the drug-treated CH fibroblasts. After a 5-minute labeling period with F-con A (25 μg/ml), only 11% of PMA-treated cells and 19% of carbachol-treated cells formed caps, compared with 48% of untreated cells.[11]

When the same cells were stained with the lysosomotropic fluorescent

dye acridine orange for 3 minutes and then examined with the fluorescence microscope, further modifications were seen. Figure 5D shows the appearance of lysosomal granules in normal fibroblasts. The cells contained large numbers of small, randomly distributed granules that fluoresced a bright orange against a background of green cytoplasm and nucleus. Figure 5C shows the typical appearance of carbachol- or PMA-treated CH fibroblasts. Approximately 90% of cells had only one or two large granules. Thus, CH cells grown with these agents develop granules that are morphologically similar to those in normal fibroblasts, at least at the level of the light microscope.

We infer from these data that the microtubule defect is expressed in fibroblasts as well as in PMNs. In addition, we propose that granule morphology is determined at least in part by microtubules. No precise mechanism can be given at present. However, a plausible hypothesis is derived from some of our previous studies.

As noted earlier, we have shown that when cells phagocytize, there is selective removal of certain proteins and lipids from the cell surface into phagocytic vesicles and selective retention of other components in the plasma membrane.[11-24,44,45] Colchicine does not inhibit phagocytosis but abolishes this selective rearrangement of membrane components that normally accompanies phagocytosis. As a result, the membrane composition of phagocytic vesicles from colchicine-treated cells is different from that of normal cells. The membrane of secondary lysosomes must be derived at least in part,[46] and possibly in full,[47] from endocytic vesicle membrane. Thus, making the assumption that the composition of pinocytic vesicle membranes is abnormal in a partially microtubule-defective cell, we predict that the membrane composition of secondary lysosomes may be different between CH and normal cells. In the continued presence of carbachol or PMA, the internalized membrane may have the proper composition so that secondary lysosomal granules develop a normal morphology. In support of this, it is clear that in a number of cell types the pinocytic rate increases dramatically at confluence.[48] As noted, giant granules also become most prominent at confluence in CH cells.

In Vivo Studies of the Effects of Cholinergic Agonists on Chediak-Higashi Mouse Leukocyte Granule Morphology

Davis *et al.*[49] have shown that the huge granules characteristic of CH mink PMNs appear at the intermediate and late stages of cell maturation in bone marrow and are considerably less prominent in early developmental stages. We tested the possibility that the process of granule enlargement described by Davis and co-workers might be inhibited *in*

vivo by cholinergic agonists by comparative studies of the size of acridine orange–stained granules in peripheral blood PMNs from normal black mice, untreated CH mice, and CH mice treated chronically with carbachol and bethanechol. The drugs were administered by two routes: by twice daily subcutaneous injection with 5 μg carbachol or with 10 μg bethanechol over a 3- to 5-week period, and by oral administration of bethanechol (400 μg/ml *ad libitum* in the water bottles) over periods of 6 weeks to 6 months.[16]

Normal peripheral blood PMNs (Figure 6A) showed a small number of orange-red granules dispersed throughout the cytoplasm, Lymphocytes rarely showed any fluorescent granules. Most CH PMNs characteristically contained one or more extremely large fluorescent granules, as well as a few relatively normal sized granules (Figure 6B). Occasionally large stained granules were also present in lymphocytes. PMNs from the drug-treated mice with Chediak-Higashi syndrome showed a fluorescence staining pattern that was very similar to that of normal cells. The numbers of stained granules per cell were rather low (two to four per PMN), and these granules did not appear to be larger than in control PMNs in the majority of CH cells (Figure 6C). By contrast, large granules persisted in small numbers in peritoneal macrophages, perhaps reflecting the slow turnover of the latter cells.

We originally supposed that the granule normalization resulted from modification of the genesis of primary lysosomes during granulocyte maturation in bone marrow. However, an alternative explanation is offered here. It is clear that relatively few granules per cell are stained with acridine orange in suspensions of freshly isolated leukocytes from both normal and CH mice. Many more granules of a smaller size were previously demonstrated by Bennett *et al.*[50] in fixed preparations of normal and CH mouse PMN stained with Sudan black and peroxidase. The possibility exists that most or all of the granules we observe are secondary lysosomes resulting from endocytic or autophagic activity of cells either during maturation in the bone marrow or following release into the circulation. Making this assumption, the explanation proposed for generation of giant granules in fibroblasts may explain the change in granule size in drug-treated CH PMNs. Thus, we suppose that abnormal membrane composition of CH mouse PMN endocytic vesicles may result in abnormal fusion reactions or delayed intralysosomal processing of ingested material. This would give rise to abnormal secondary lysosomes correctable by cholinergic drugs. This proposal requires rigorous experimental testing. However, the general concept that giant CH granules represent either secondary or autophagic vacuoles has been suggested

frequently because of the high incidence of abnormal inclusions visible in these lysosomes by electron microscopy.[37,38]

In addition to these morphologic changes following *in vivo* treatment with cholinergic agonists, the oral administration of bethanechol reduced spontaneous con A capping in CH PMNs. Whereas 56% of untreated CH mouse PMN formed caps with con A, only 22% of PMN from bethanechol-treated (10 weeks, oral administration) and 22% of untreated normal mouse PMNs formed caps.[16] This provides further evidence that microtubule function is correctable *in vivo* by cholinergic agents.

Conclusions

We consider that the impairment of microtubule assembly following binding events at the cell surface demonstrated indirectly in CH mouse cells (from con A capping studies) and directly in PMNs from a patient with CH syndrome (by electron microscopy) provides a possible explanation for the impaired inflammatory response characteristic of CH syndrome.

First, chemotactic movement of PMN is defective in CH mice and patients. Although the contractile force for cell movement appears to be provided by subcellular microfilaments,[51] directionality appears to be sensitive to colchicine and so is most likely determined by microtubules.[9] Since con A, C5a, and phagocytic particles all stimulate microtubule assembly in normal cells,[36,52,53] we suppose that chemotactic factors like C5a may fail to induce microtubule assembly in CH PMNs and hence fail to promote directional movement.

Second, CH PMNs show normal phagocytosis, but bacterial killing is impaired due to a reduced rate of fusion of lysosomes with phagocytic vesicles. Similarly, colchicine-treated normal PMNs show no impairment of phagocytosis,[10,13] but lysosomal degranulation is delayed.[10,30] This suggests that microtubule assembly induced by phagocytosis in normal cells is required for rapid lysosomal fusion with phagocytic vacuoles. By extension, failure of cytoplasmic microtubules to assemble following a phagocytic stimulus in CH PMNs would lead to a reduced rate of lysosomal degranulation.

Microtubule assembly following con A binding in PMNs from mice and the patient with CH syndrome can be normalized by concomitant exposure of the cells to cyclic GMP or cholinergic agonists. These data indicate that the defect in CH cells is not absence or structural changes in the tubulin *per se*, but rather absence of a signal to microtubule assembly that normally accompanies con A binding. This signal most likely involves cyclic GMP.

The concept that cyclic GMP may be involved in the regulation of microtubule assembly is not new. Thus, it has been established that increased cyclic GMP levels normally accompany con A binding in lymphocytes [54] and phagocytosis in PMNs.[32] Furthermore, the colchicine-sensitive process of chemotaxis [31] and lysosomal degranulation [30] in PMNs are both reported to be stimulated by exogenous cyclic GMP. These data raise the possibility that cyclic GMP and cholinergic agonists may normalize chemotaxis and lysosomal degranulation in CH PMNs.

Direct evidence for the latter response has recently been obtained. Boxer *et al.*[55] found that bethanechol and cyclic GMP correct both the impaired degranulation and impaired bactericidal activity towards *Staphylococcus aureus* of PMNs from an infant with CH syndrome. The dispersion of lysosomal granules observed here by electron microscopy in con A-treated normal cells and cyclic GMP and con A-treated CH cells may be prerequisite for degranulation.

The observation that CH mouse fibroblasts cultured in the continual presence of cholinergic agonists contain granules that are morphologically normal, at least at the level of the light microscope, indicates that the microtubule defect may also underlie the development of giant granules in CH cells. One possible explanation for these results is developed above.

The reduction in spontaneous con A capping and in the incidence of giant acridine orange–stained granules in CH mice treated *in vivo* with cholinergic agonists is of potential clinical importance. We suppose that sufficient drug can be maintained in the circulation to normalize microtubule integrity and hence to prevent development of abnormal lysosomes.

The nature of the defect in cyclic nucleotide metabolism in CH cells has not yet been elucidated. In addition, the precise role of cyclic GMP in microtubule assembly is not understood. Since cyclic GMP does not appear to directly stimulate microtubule assembly *in vitro*,[56] this compound is unlikely to be the final regulator of *in vivo* microtubule assembly. However, it may represent an intermediate step in permitting microtubule assembly to occur, perhaps by generating a second regulatory compound but more likely by stimulating events such as Ca^{2+} efflux [57] or turnover of GSH [24,25] that may be required for microtubule assembly *in vivo*.

Note Added in Proof

Further evidence for a microtubule defect in CH human PMNs and fibroblasts has been obtained since preparation of this manuscript. First, among PMNs from 3 additional children with CHS, spontaneous con A capping, indicating impaired microtubule assembly, was observed on 50%, 53%, and 38% of cells. Both cyclic GMP and ascorbate (see below) reduced the proportion of cells that capped with con A. However, PMNs from 2 adult male siblings with CHS failed to show a significant

elevation of con A capping above normal levels. Second, Hinds and Danes[58] reported that vinblastine induces microtubule paracrystal formation visible by phase contrast microscopy in only 20% of cells from four CH fibroblast lines, compared to over paracrystal formation in 80% of normal human fibroblasts. Third, we have observed reduced association of antitubulin antibody[59] with cytoplasmic filamentous structures presumed to be microtubules in confluent CH mouse fibroblasts as compared to normal mouse fibroblasts. Chediak-Higashi fibroblasts incubated overnight with carbachol (10^{-5} M) showed enhancement of filamentous staining with antitubulin.

Most importantly, Boxer *et al.*[60] have elucidated the cyclic nucleotide defect in CH PMNs: cells from 2 patients showed a tenfold elevation in cyclic AMP as compared to control. These authors also found that 10 mM ascorbic acid, which raises cyclic GMP in normal leukocytes,[61] reduced cyclic AMP to near normal and corrected the impaired chemotaxis and degranulation of the CH PMN *in vitro*. Furthermore, a patient treated with 200 mg day ascorbate has remained free of infection and her PMNs show essentially normal cyclic nucleotide levels, normal chemotaxis, and normal bactericidal activity. All these responses are reversible by withdrawal of ascorbate. Thus, ascorbate may provide a simple clinical approach to symptomatic relief for patients with CH syndrome.

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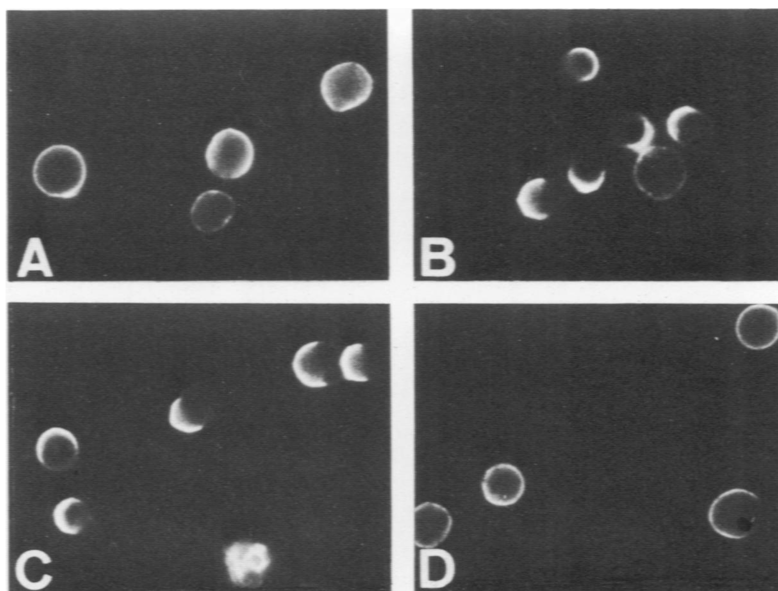


Figure 1—The distribution of con A on mouse PMNs. **A**—Normal PMN, uniform labeling predominates. **B**—CH PMN; most cells are capped. **C**—Colchicine-treated normal PMN; the cells are capped. **D**—Cyclic GMP-treated CH PMN; labeling is uniform. See Oliver *et al.*[15] for details.

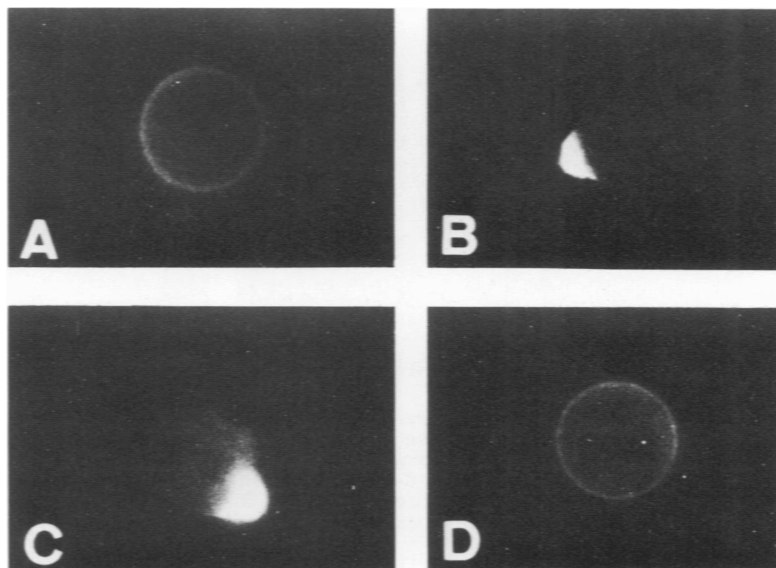


Figure 2—The distribution of con A on human PMNs. See legend to Figure 1. Experimental details are given in Oliver and Zurier.[16]

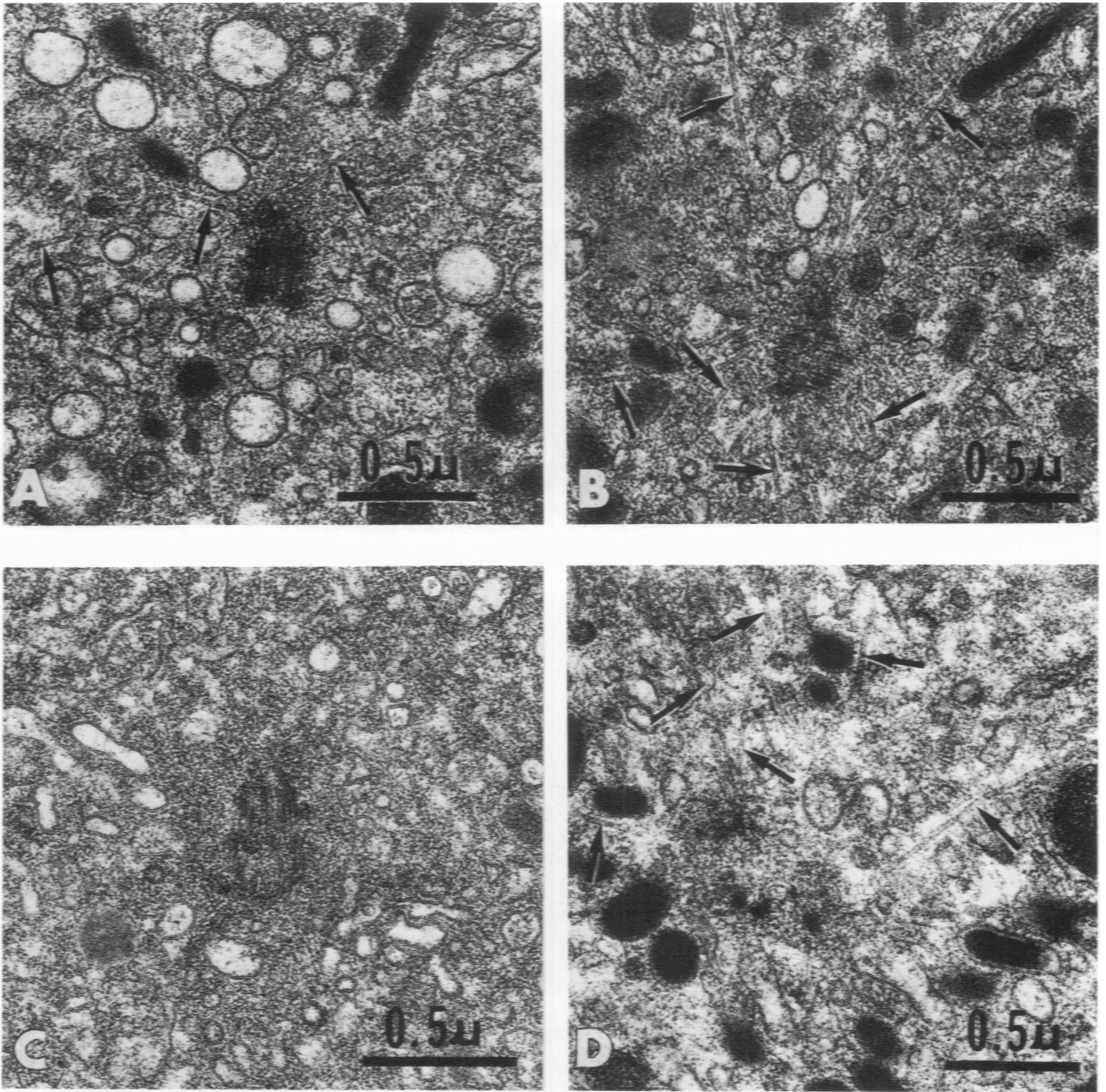
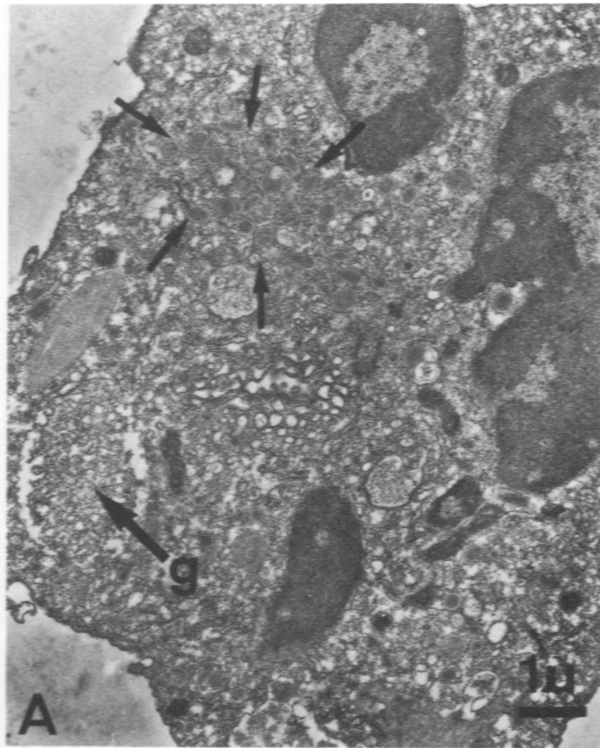


Figure 3—Electron micrographs of the centriolar region of normal and CH human PMN. Scale bar = 0.5 μ. **A**—Three microtubule profiles (*arrows*) are associated with the centriole in an untreated normal PMN (× 39,500). **B**—Con A-treated (100 μg/ml, 5 minutes) normal PMN displaying approximately 20 microtubules (*arrows* indicate 6 microtubules) radially emanating from the centriole (× 40,000). **C**—Centriole from a con A-treated CH PMN showing the absence of microtubules and halo of electron-dense ground substance (× 44,200). **D**—The centriolar region of CH PMN preincubated with carbachol (10^{-5} M, 30 minutes) and subsequently exposed to con A (100 μg/ml, 5 minutes). Six (of nine) microtubule profiles are indicated by the *arrows*. (× 40,000)



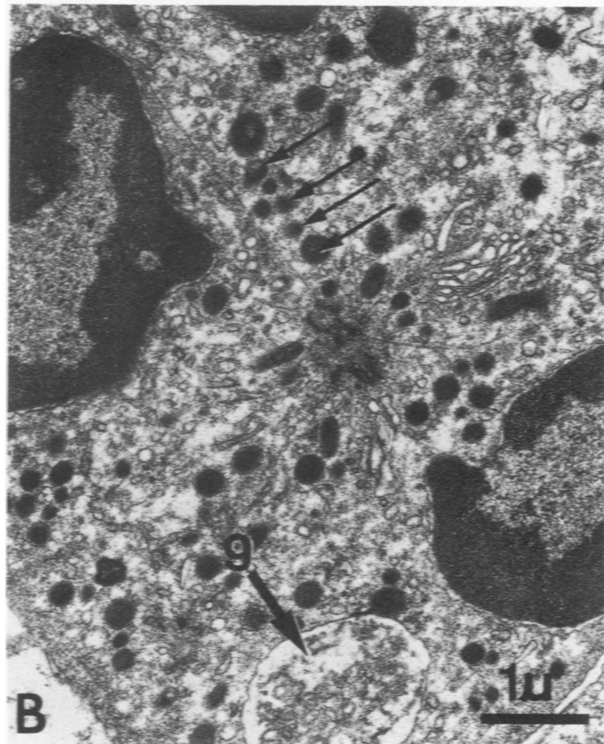


Figure 4—Low magnification electron micrographs of CH PMN. Scale bar = 1.0 μ. **A**—Con A-treated (100 μg/ml, 5 minutes) PMN showing large aggregate of cytoplasmic granules (*small arrows*) near the cell center. A prominent Golgi apparatus is adjacent to the centriole and a giant granule (*g*), pathognomic of the CH syndrome, is seen at the lower left. No microtubules are visible. (× 9300). **B**—Con A-treated CH PMN preincubated with cyclic GMP (10^3 M, 30 minutes). Note the long microtubule profiles radiating from the centrioles and the linear arrangement of cytoplasmic granules along a microtubular profile (*small arrows*). A giant granule (*g*) is depicted at the bottom of the micrograph. (× 13,100)

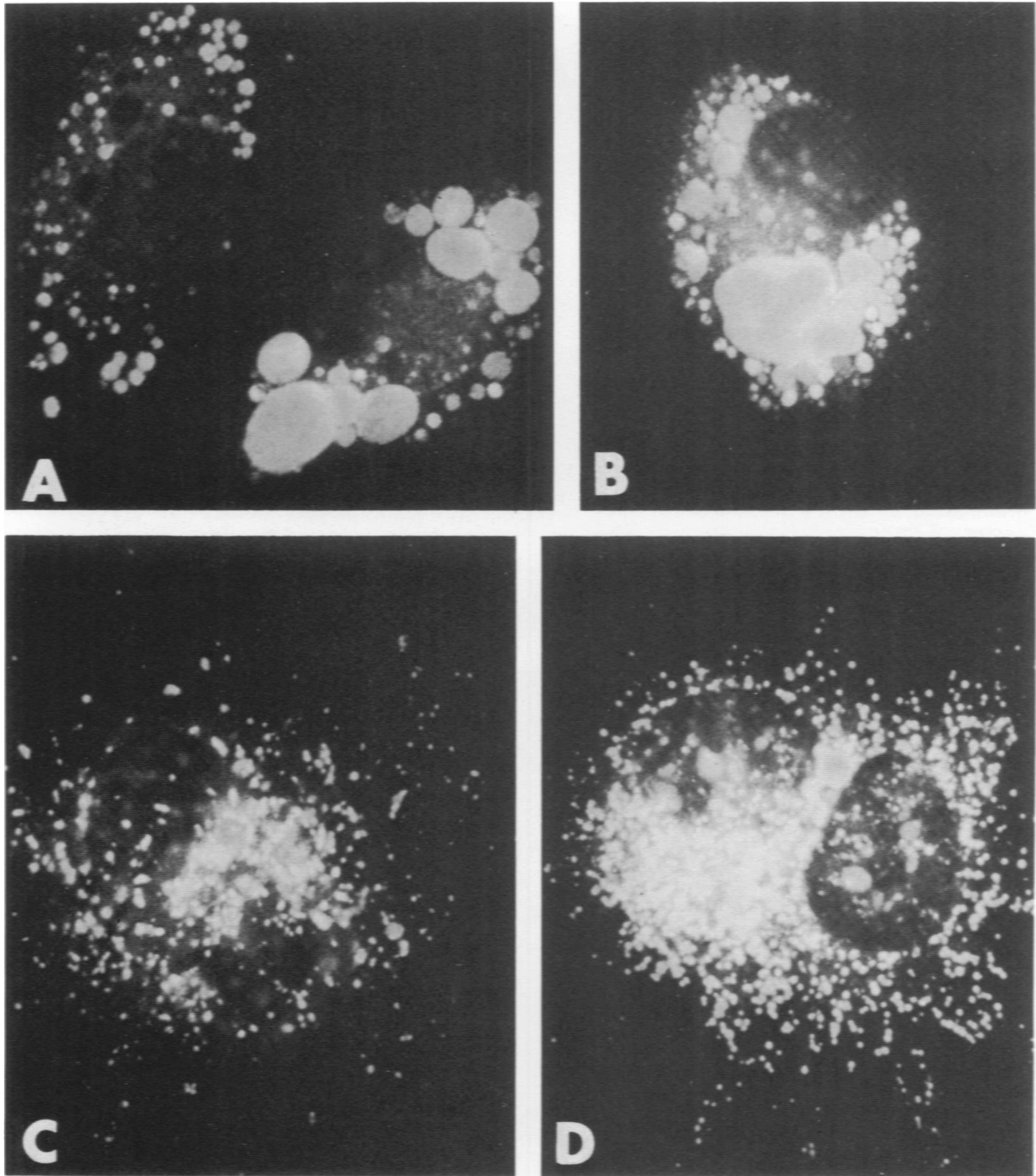


Figure 5—Distribution of lysosomes in normal and CH mouse fibroblasts. Primary fibroblasts were grown to confluency (2 weeks) in the presence and absence of carbachol or PMA. Lysosomes were stained with acridine orange (1 : 10^{6} in PBS, 5 minutes at 37 C). **A and B**—Confluent CH fibroblasts without drug. Giant lysosomes are clustered in the perinuclear area. **C**—CH fibroblasts grown with 10^{-5} M carbachol. Lysosomes are small and dispersed. A similar distribution of granules is seen in cells grown with 10^{-6} M and 10^{-8} M carbachol as well as with 10 ng/ml PMA. **D**—Normal fibroblasts containing small dispersed granules. Carbachol and PMA have no effect on granule morphology in these cells. Note that in normal and carbachol-treated fibroblasts lysosomes are dispersed well into peripheral processes. In CH fibroblasts virtually no peripheral lysosomes are observed. Experimental details are given in Oliver *et al.*[38]

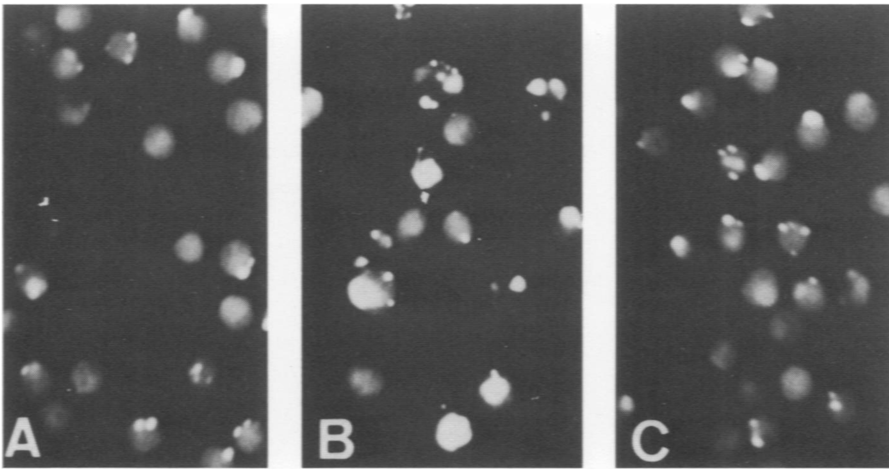


Figure 6—Granule morphology in leukocytes from normal and CH mice. Lysosomes were stained with acridine orange (1:10^{6}, 5 minutes at 37 C) and observed by fluorescence microscopy. **A**—Normal mouse leukocytes showing small granules. **B**—CH mouse leukocytes showing a mixture of giant granules and relatively normal granules. **C**—Leukocytes from a CH mouse given bethanechol (400 μg/ml *ad libitum* in the drinking water) for approximately 10 weeks. Most granules are small. Further details are given in Oliver and Zurier.[16]

[*End of Article*]