CARBAMYLCHOLINE PREVENTS GIANT GRANULE FORMATION IN CULTURED FIBROBLASTS FROM BEIGE (CHEDIAK-HIGASHI) MICE

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The Chediak-Higashi (CH)[1] syndrome is an inherited disorder of humans (2) and certain mutant strains of mice (1, 7), mink (11), and cattle (16). It is characterized by partial oculocutaneous albinism, increased susceptibility to pyogenic infections, and several functional abnormalities of leukocytes including defective lysosomal degranulation and impaired chemotaxis (24). An accelerated phase of the disease, diagnosed as a lymphoma-like malignancy, commonly occurs in man (2). The presence of giant granules in virtually all granule-containing cells is pathognomonic of the disease.

We recently described an abnormality of concanavalin A (Con A) distribution on polymorphonuclear leukocytes (PMN) from CH mice that suggested a defect in microtubule (MT) assembly in these cells (15). In PMN from various sources as well as in lymphocytes, virus-transformed 3T3 fibroblasts (SV3T3), and other cells, the disassembly of MT, for example by cold or colchicine treatment, favors the aggregation of Con A into a surface cap (reviewed in reference 13). Consistent with this, PMN from normal black mice show a random surface distribution of Con A except after incubation with colchicine which permits cap formation. By contrast, Con A is capped spontaneously on PMN from the beige or CH mouse to the same degree as on colchicine-treated normal cells. Most importantly, we also showed that defective 3′5′ cyclic GMP (cyclic GMP) generation was the likely cause of the impaired MT function in CH cells. It was found that cyclic GMP and agents such as carbamylcholine (carbachol) and phorbol myristate acetate (PMA) that increase cyclic GMP levels (6) normalize the surface distribution of Con A on CH PMN and antagonize the colchicine effect on normal PMN.

We considered the unifying hypothesis that defective cyclic GMP generation could underly all characteristics of the disease, and selected the fibroblast for analysis of morphological features which might be expected to develop in long-term experiments.

In this report we show that agents that elevate cyclic GMP prevent the formation of giant granules in cultured embryonic fibroblasts from CH mice. We first demonstrate that cultured CH fibroblasts develop giant granules during incubation in vitro. We then show that CH cells incubated in medium supplemented with carbachol or PMA develop a new population of morphologically normal granules. In addition we report that CH cells are more susceptible than normal cells to shape changes induced by colchicine; this property is normalized by carbachol.

MATERIALS AND METHODS

Isolation and Culture of Embryonic Skin Fibroblasts

A CH (C57/6J, bg/bg) female mouse mated with a CH male and a black (C57/6J, +/+) female mated with a black male were sacrificed on the 14th day of gestation. The uteri were dissected out, the CH homozygotic or normal embryos were removed, and fibroblasts were isolated from the skin of the dorsal surface by the method of Moscona (12). The fibroblasts were maintained in

[1] *Abbreviations used in this paper:* carbachol, carbamylcholine; CH, Chediak-Higashi; Con A, concanavalin A; cyclic GMP, 3′5′ cyclic GMP; MT, microtubules; PMA, phorbol myristate acetate; PMN, polymorphonuclear leukocytes.

vitro in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin and streptomycin (Grand Island Biological Co., Grand Island, N.Y.) and containing 10% fetal calf serum (FCS). Cells were frozen in batches after 1 wk in culture and thawed at intervals for use. Vigorous growth was maintained for approximately 5 wk after thawing.

For the experiments reported here, cells (between 0.2 $\times$ 10^4 and 0.5 $\times$ 10^4) were plated in 3.5-cm petri dishes containing three 15-mm glass cover slips. The medium, with or without added drugs, was changed daily. The cultures reached confluency in 8–10 days. Preliminary experiments showed that the drug of major interest, carbachol, had no effect on the growth characteristics of the cells. Fibroblasts plated at approximately 10^4 cells per dish were maintained in medium with or without 10^{-5} M carbachol (the highest drug concentration employed in this study). Growth curves were obtained by daily counting of cells harvested by trypsinization from three dishes. As shown in Fig. 1, carbachol did not significantly affect the growth rate or the cell density at confluence of CH fibroblasts. Similar results were obtained with normal mouse fibroblasts (not shown).

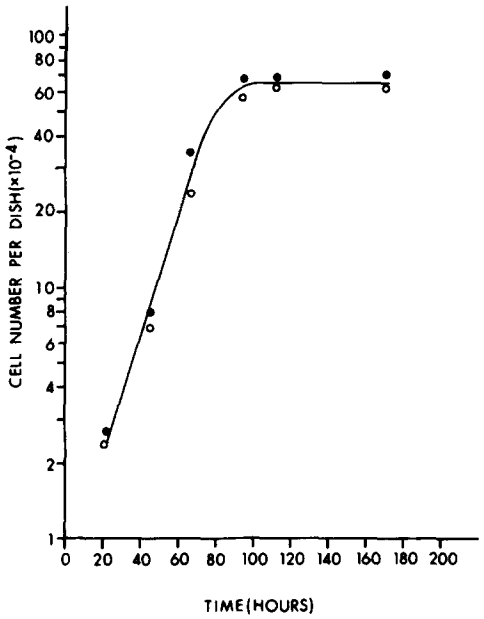

FIGURE 1 Growth curves of CH mouse fibroblasts. Cells (approximately 10^4 fibroblasts per culture dish) were grown to confluency in medium with or without added carbachol (10^{-5} M). At daily intervals, groups of three cultures were trypsinized, and the cells from each dish were counted in duplicate. Each point represents the average of six determinations. O, CH fibroblasts grown in medium alone. ●, CH fibroblasts grown in medium supplemented with carbachol.

Fluorescence Microscopy

Granule development was monitored by removing cover slips from the petri dishes at intervals and incubating the cells with acridine orange (1:10^6) in phosphate-buffered saline (PBS) for between 2 and 5 min at 37°C. The distribution of fluorescence on stained cells was examined by epi-illumination with a Zeiss Universal fluorescence microscope using an FITC filter and 500 dichroic mirror for excitation, a 40$\times$ planapochromatic objective, and 53 barrier filter. Acridine orange stains lysosomes an intense orange color against a background of green cytoplasm and nucleus (18, 25). 100 cells per cover slip were routinely scored for the presence or absence of giant granules. The distribution of granules was determined with confluent cultures since rapidly dividing CH cells contain a mixture in varying proportions of large and small granules, whereas confluent CH cells contain a majority of giant granules.

RESULTS

The morphology of lysosomal granules in CH fibroblasts is dramatically different from normal. Confluent CH fibroblasts (10–14 days in culture) show giant granules which cluster in the perinuclear area in about 60% of cells. Most granules in the remaining 40% of cells are also larger than normal. This is illustrated in Fig. 2 *a* and *b*. Two of the cells contain giant granules and the third shows a majority of granules which are considerably larger than those in normal cells (Fig. 2 *d*). In one cell a giant granule may be in the process of fusing with a smaller granule. Some granules which are not significantly larger than those in normal cells can also be seen.

In marked contrast with the untreated fibroblasts, CH cells grown in the presence of carbachol (10^{-5} M) contain only small granules in 91% of cells at confluence, and these granules are well dispersed throughout the cytoplasm (Fig. 2 *c*). One, or at most two large granules were observed in each of the remaining 9% of cells and may be partly due to dilution of giant granules present in the original cell population. The morphology of carbachol-treated CH fibroblasts is in fact identical to that of normal embryonic fibroblasts grown to confluence in either the presence or the absence of drug (Fig. 2 *d*).

The minimum effective dose of carbachol on fibroblast granule morphology was estimated by titrating granule size at confluence in cells grown in the presence of carbachol between 10^{-5} M and 10^{-10} M. Giant granule formation was prevented

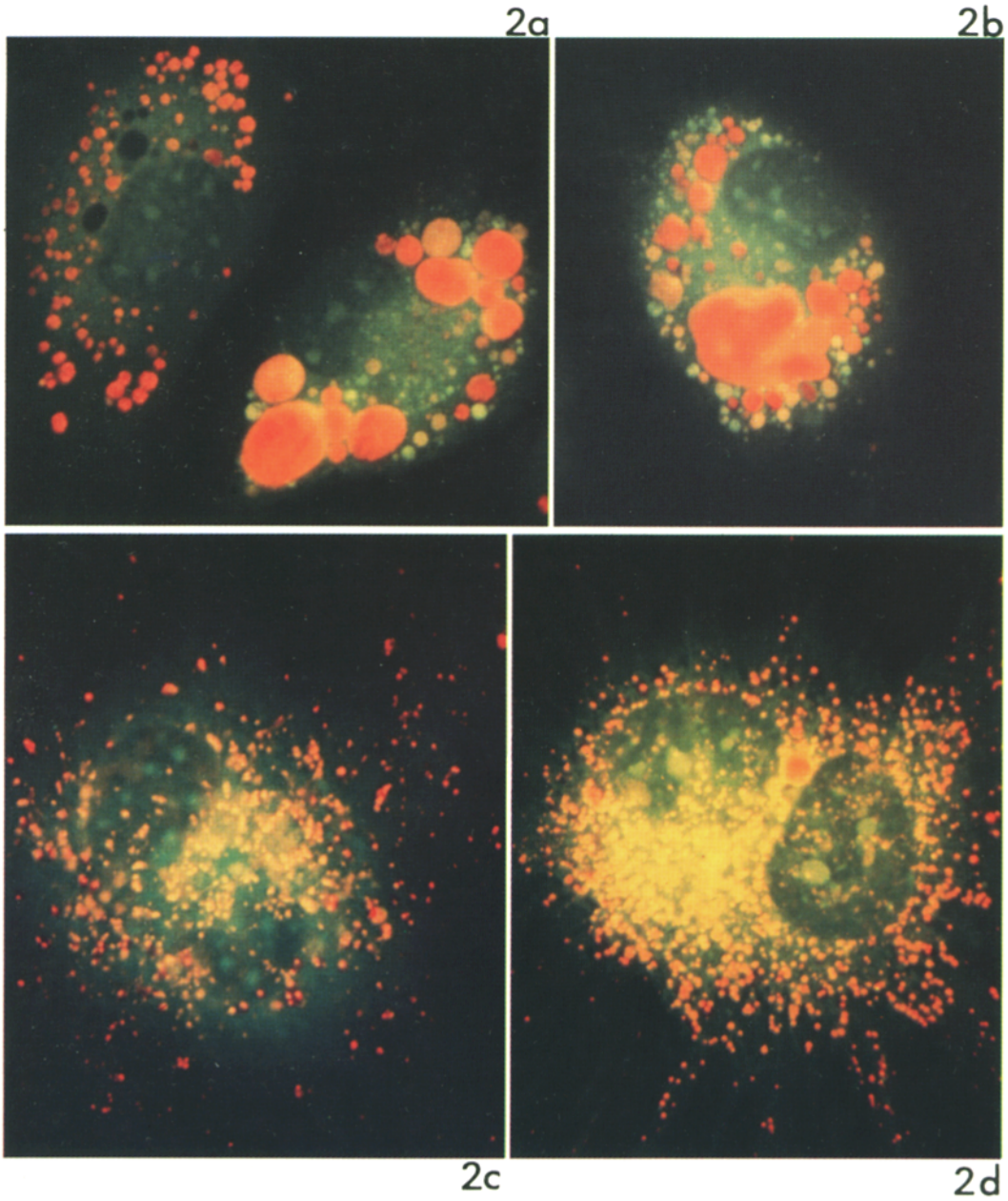

FIGURE 2 Distribution of lysosomes in normal and CH mouse fibroblasts. Primary fibroblasts were grown to confluency (2 wk) in the presence and absence of carbachol or PMA. Cells were stained with acridine orange and photographed with Kodak Ektachrome film. (*a*) and (*b*) CH fibroblasts without drug. 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. Initial magnification, 500.

at doses of carbachol between 10^{-5} M and 10^{-8} M and was depressed at 10^{-10} M. We also observed that cells treated with 10^{-5} M carbachol plus 10^{-5} M atropine, the classical antagonist of muscarinic drugs, developed giant granules in 40% of cells, compared with 9% of cells when only carbachol was present in the medium.

By contrast with cells exposed continuously to carbachol, cells grown to approximately 50% confluency (5 days) with carbachol and then maintained to full confluency (10 days) in medium lacking carbachol were indistinguishable from cells which were never exposed to the drug: either the granules regain the tendency to fuse once carbachol is removed or else new granules which fuse to form aberrant structures are generated during subsequent cell growth. Cells grown to confluence (10 days) without carbachol and then exposed to carbachol for a further 3–5 days show no apparent reversion of giant granules to small granules. Thus, it seems likely that carbachol prevents formation of abnormal granules. The drug probably does not modify the morphology of preformed granules.

Striking differences in colchicine sensitivity between CH fibroblasts and normal and carbachol-treated CH cells were also noted by light microscope examination of cell monolayers from near-confluent cultures. When colchicine (10^{-6} M) is included in the incubation medium during a 30-min incubation, CH cells become very small and rounded and are readily detached from the cover slip during gentle rinsing. By contrast, CH fibroblasts grown in the presence of carbachol do not show these shape changes in the presence of 10^{-6} M colchicine when incubated for 30 min. When incubation is continued beyond 1 h, the relatively colchicine-insensitive normal cells and carbachol-treated CH cells will eventually round up and detach from the substratum.

Inclusion of PMA (10^{-9} M) in the culture medium was as effective as carbachol in preventing giant granule formation. However, more detailed studies (dose-response and growth curves) were not performed with PMA since, in addition to its induction of increased cyclic GMP levels, it has other multiple sites of action (9, 26). Cyclic GMP itself was not used in these long-term experiments since it could not be expected to escape degradation when included in the tissue culture system.

DISCUSSION

In our previous study (15) we obtained evidence for a defect in MT function in PMN from CH mice that could be corrected by cyclic GMP or agents that promote cyclic GMP generation. This was based on a similar surface property (an extreme degree of Con A capping) in CH cells and colchicine-treated normal cells and reduction in cap formation to normal levels by elevation of cyclic GMP. These functional studies were consistent with the morphological evidence of Weissmann and co-workers (8, 22) for a role of cyclic GMP in promoting MT assembly in PMN. These authors have demonstrated that the number of MT observable in thin sections of normal human PMN is increased in the presence of zymosan particles or the C5a component of complement and is further increased by brief exposure of the cells to cyclic GMP, PMA, or cholinergic drugs. The mechanism by which cyclic GMP promotes MT assembly is not yet understood.

In the present study we report that CH fibroblasts are extremely sensitive to shape changes induced by colchicine. This again suggests defective MT function. Carbachol, a cholinergic drug that stimulates cyclic GMP generation in leukocytes, antagonizes the colchicine effect on CH fibroblast shape. This result is consistent with enhancement of MT stability after drug treatment.

In addition, we show that the aberrant giant lysosomal granules that are pathognomonic of the CH syndrome develop in cultured primary embryonic fibroblasts from CH mice. These granules are particularly prominent in confluent cultures, suggesting that they arise by fusion of preformed granules as previously suggested for the bone marrow cells of CH mink in vivo (5). We show here that inclusion in the growth medium of carbachol or PMA prevents the appearance of these abnormal structures.

Direct measurements of cyclic GMP levels of normal and CH cells before and after carbachol treatment are in progress. These data are required before we can conclude with certainty that improved MT function in PMN and fibroblasts and normal granule morphology in fibroblasts are directly related to increased cyclic GMP levels rather than to some other effect of the drug. The mechanism by which decreased cyclic GMP or impaired MT polymerization could lead to giant granule formation also remains to be established. One possibility is based on our previous observation that the composition of the membranes of phagocytic vesicles in normal rabbit PMN is altered by colchicine treatment, probably secondary to changes in surface protein mobility (14, 20).

Since endocytic vesicles may serve as the nuclei for lysosomal development in cells such as macrophages (4) and fibroblasts (10), it is possible that the membrane of the lysosome is abnormal in the MT-defective CH cell. For example, it may lack a surface-derived "recognition signal" which normally prevents fusion with other lysosomes. Alternatively, the aberrant granules could result from the inability of lysosomes of MT-defective cells to process materials normally. Thus, Patzelt and co-workers (17) have observed the reversible formation of large autophagic vacuoles in hepatic cells after inhibition of MT assembly with colchicine. Electron microscope studies of giant granules in CH cells have revealed abnormal inclusions (23) reminiscent of these colchicine-induced autophagic vacuoles.

Interestingly, CH fibroblasts are similar to SV40 virus-transformed 3T3 mouse embryonic fibroblasts in several respects. We consistently find the transformed cells to be more susceptible to shape change and detachment by colchicine than normal parental 3T3 fibroblasts. In addition, we previously reported that CH fibroblasts cap spontaneously with Con A and that SV3T3 fibroblasts also show Con A capping after brief exposure to colchicine (13, 21). Capping in both CH and colchicine-treated SV3T3 fibroblasts was prevented by cyclic GMP. By contrast, normal 3T3 and C57/6J +/+ fibroblasts show a random surface distribution of Con A before and after treatment with colchicine or cyclic GMP (13). This suggests that both CH and SV3T3 fibroblasts may show impaired MT assembly related to inadequate cyclic GMP generation, with the defect being more extreme in CH cells. In support of this, Rudland and co-workers (19) have reported that the ability of SV3T3 cells to modulate cyclic GMP levels, for example in response to serum growth factors, is impaired compared with nontransformed cells. Further, Brinkley and co-workers (3) have used a fluorescent antibody technique to show that intact MT are considerably more prominent in 3T3 than SV3T3 cells.

SUMMARY

Primary embryonic fibroblasts isolated from beige (Chediak-Higashi) mice develop pathognomonic giant granules in vitro. Inclusion in the culture medium of carbamylcholine (carbachol) or phorbol myristate acetate (PMA) results in the generation of morphologically normal granules.

Chediak-Higashi fibroblasts are highly susceptible to shape changes induced by colchicine. This abnormal property is also corrected by carbachol and PMA.

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