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cystine
cystinosis
fibroblast
genetic disease
leukocytes

Biochemical, Morphologic, and Cytogenetic Studies of Leukocytes Growing in Continuous Culture from Normal Individuals and from Patients with Cystinosis

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Extract

Continuous leukocyte cultures were established from the blood of three siblings with classical nephropathic cystinosis and from their mother. The cells from patients with cystinosis (CC) contained at least 15- to 20-fold more cystine than cultured leukocytes from normal individuals (NC). The transport of cystine-^{35}S into CC was similar to uptake into NC when tested at pH 5.0 and pH 7.4.

The cultured CC could not be distinguished by ultrastructure from NC. Crystalline inclusions which could easily have been mistaken for cystine were observed in the lysosome of a normal cell. The modal number of chromosomes in CC and NC lines was 46. There was a marked degree of aneuploidy. Tetraploidy was present in 3.8–9.6% of metaphases. Structural rearrangements and breaks were seen in all cell lines.

Speculation

Continuous leukocyte cultures may prove a useful experimental tool for further studies of the basic metabolic derangement responsible for abnormal intracellular accumulations of cystine in cystinotic cells (CC). The cultures are a convenient source of relatively large amounts of pure cellular material for investigative use. Because they grow in suspension, cultured leukocytes are well suited for investigation of transport phenomena in normal human cells (NC) as well as those with genetic diseases. The absence of a defect in the uptake of cystine at pH 5.0 in CC fails to support the hypothesis of a lysosomal transport abnormality for cystine in these cells, yet cannot be regarded as firm refutation of that hypothesis. Elucidation of the basic metabolic defect in cystinosis may require studies of the enzymology and transport systems of isolated lysosomes; such transport studies may have formidable technical problems.

The demonstration of intralysosomal crystals which could easily have been mistaken for cystine in one line of cultured NC emphasizes the need for great caution in assigning pathophysiologic significance to any rare crystalline bodies seen only in occasional lysosomes or other organelles of CC.

The chromosome abnormalities, and perhaps the capacity to proliferate, of continuously cultured leukocytes may be related to infection with herpes-like virus, as has been previously suggested.

Introduction

Cystinosis is a recessively inherited autosomal disease which in its characteristic nephropathic form results in the Fanconi syndrome and glomerular insufficiency during childhood with death in uremia before puberty [5, 44]. Clinical variants of lesser severity have been recognized [9, 19, 22, 31]. Cystinotic tissues contain large excesses of cystine, which are often visible in appropriately prepared tissues as intracellular crystalline deposits. Cystinotic white cells [35] and cultured fibroblasts [37, 38] contain increased intracellular cystine levels, and the metabolic defect responsible for cystinosis, which has so far eluded delineation, may be investigated in these cells. Considerable evidence suggests that cystinosis is a lysosomal storage disease [21, 30, 39–41, 43, 50].

Continuous suspension cultures of leukocytes may be derived from the peripheral blood of patients with lymphomas and leukemias and also from normal individuals [4, 15, 18, 26, 32]. The present report describes several of the properties of such cell lines cultured from normal individuals and those with cystinosis and illustrates the feasibility of utilizing these cells for investigations of the pathogenesis of cystinosis.

Culture Technique

Cell lines were developed by a modification of the method of Gerber and Monroe [18]. Peripheral blood, 50–100 ml, was drawn into 7.5–15 ml commercially available acid-citrate-dextrose (ACD) anticoagulant solution (Fenwal) in a syringe prewarmed to 37°. The blood was transferred to 50-ml plastic tubes [53] and allowed to sediment for 3–6 hr. The supernatant, including the interface region but with as few erythrocytes as possible, was removed and centrifuged at 400 $\times$ *g* for 10 min. The cell pellet was washed once with prewarmed calcium-free phosphate-buffered saline, pH 7.4, and resuspended in prewarmed medium [54] containing 20% fetal calf serum and 50 μg/ml neomycin in 8-ounce milk dilution bottles. Cell density was adjusted to 3–5 $\times$ 10^6/ml medium. The flasks were gassed with 95% air-5% CO_2, capped, placed on their sides, and incubated at 37°. During all manipulations caution was taken to avoid cooling the cultures to significantly below 37°.

Viable cell counts were obtained at intervals of several days using 0.1% solutions of trypan blue in 0.9% saline. The cultures were maintained at population densities of at least 5 $\times$ 10^5/ml. To decrease the volume of medium as the cell number declined, the bottles were briefly centrifuged, and the supernatant medium was aspirated. When the medium in a culture became acid (as judged by the phenol red indicator) one-half of the spent medium was replaced with prewarmed fresh medium. If the cells in a culture became too few for maintenance in the 8-ounce bottles, the cells were transferred to smaller cylindrical vessels and the "feeder layer" scraped from the side of the milk dilution bottle was included in the transfer; the milk dilution bottle with residual feeder layer was then refed with fresh medium and incubated without further disturbance as a reserve.

After 2–3 months, the small free floating cells in most cultures were replaced by a pleomorphic group of leukocytes in which large blastic cells were apparent. Usually, this was followed by an increase in cell number, increasing acidity of the medium, and appearance of grossly visible clumps in the culture. These occurrences signified the establishment of a proliferating cell line. In several cases, the successful culture was established in the reserve milk dilution bottle instead of the smaller bottle which had been tended more assiduously.

Once proliferating, the lines were grown easily by periodically feeding an equal volume of fresh medium added to the spent acid medium one to two times per week. The population density in the cultures was approximately 0.3–1.2 $\times$ 10^6 cells/ml. Generation times were not determined precisely, but were about 2–5 days. Cultures were not continuously stirred or shaken. Larger batches were grown in cylindrical glass bottles, which were manually agitated every 1–2 days.

The cell lines were frozen in a viable state by modification of methods used for freezing cultured skin fibroblasts. The vehicle for freezing was RPMI 1640 containing 30% fetal calf serum and 10% dimethyl sulfoxide, and cell density was 10 $\times$ 10^6/ml. The frozen vials were stored in liquid nitrogen tanks. For recovery, the vials were rapidly thawed, the medium containing the dimethyl sulfoxide (DMSO) was removed, and the cells were suspended in fresh medium [51].

We successfully established cell lines using the methods described above in five of six attempts. The three cystinotic lines (CC) were derived from the blood of three siblings with classical nephropathic cystinosis; the carrier line was from their mother; a normal line was from an unrelated normal adult (*CS*, Table II). Three additional normal cell (NC) lines also were used in this study [52]. All patients who were sources of successful cultures had a positive test for Epstein-Barr (herpes-like) virus antibody in their serum, as determined by Gerber and Monroe [18].

All cell lines were maintained in culture for over 6

months, with no grossly detectable change in their properties.

Intracellular Cystine Content

Method

A technique modified from that of Schneider, Bradley and Seegmiller [35] was used. Cells were centrifuged at 400 × g for 2 min and medium was aspirated. The cells were then alternately suspended and recentrifuged in three changes of phosphate-buffered saline at 4°. The final cell pellet was taken up in 1–2 ml 1.5% sulfosalicylic acid and frozen until analysis. Cystine was determined on the protein-free supernatant with an amino acid analyzer [55] specially modified for high sensitivity, using the gradient elution system [10].

Results

The levels of cystine in three CC, one carrier, and three NC lines are shown in Table I. Cystinotic values fluctuated somewhat during the 6 months in culture, and the values in the table indicate the range after the cells were proliferating for at least 2 months. There was no trend toward a persistent alteration in the cystine levels during the culture period. The CC contained at least 15- to 20-fold more cystine than did the NC. The intracellular cystine level of the cell line cultured from the one obligate heterozygote for cystinosis was close to the control value.

Transport Studies

Method

The uptake of cystine-^{35}S by CC and cultured NC was measured at pH 5.0 and pH 7.4 by modification of the technique of Clausen, Rodbell, and Dunand [8].

The cells in 200 ml growth medium (population density 0.3–1.2 × 10^6 cells/ml) were washed twice at 4°

Table I. Range of intracellular cystine content of continuous white cell lines

Subjects	Half-cystine, μmoles × 10^1/g cell protein	Total protein/ sample, mg
Cystinosis type 1		
IM	3.7–31.0	4.40–13.0
TM	4.0–5.4	5.00–5.07
LM	3.1–13.6	2.57–8.43
Heterozygote		
RM	0.2–0.3	11.
Normal		
XC	0.2–0.3	8.76
XG	≤0.2–0.3	5.00
CS	≤0.2–0.3	2.90

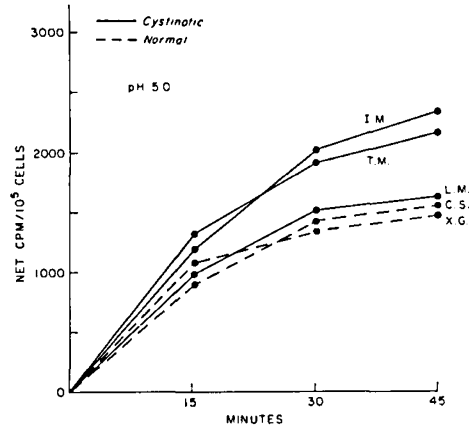

Fig. 1. Uptake of cystine-^{35}S by CC and cultured NC at pH 5.0. See text for details.

in 10 ml 0.9% saline with sedimentation at 100 × g for 2 min. Then 5–10 × 10^6 cells were incubated at 37° in 3 ml medium containing $MgCl_2$ (2.5 mM), $CaCl_2$ (0.1 mM), KCl (10 mM), NaCl (130 mM), bovine serum albumin (fatty acid-poor; 10 mg/ml) [56], cystine-^{35}S (31.5 mCi/mmole, 0.28 mM) [57], and Na phosphate (10 mM); final pH was adjusted to 5.0 or 7.4 with HCl or NaOH.

At 0, 15, 30, and 45 min triplicate 0.2-ml aliquots were removed from each incubation tube and layered over 0.1 ml ice-cold incubation mixture without cystine and albumin (wash fluid) in polyethylene microfuge tubes (Beckman). Cell pellets were prepared and counted as described [8] except for one additional wash. Viable cell counts were performed with 0.1% trypan blue at the beginning and end of the incubation. The cells remained viable during the 45-min incubation period at either pH, 5.0 (mean survival 103%) or 7.4 (mean survival 96%).

Results

Figures 1 and 2 show the uptake of radioactivity by CC and cultured NC incubated in medium containing cystine-^{35}S at pH 5.0 and pH 7.4; uptake is expressed as counts per minute above the zero time values per mean number of viable cells. Each point represents the average of triplicate determinations for each cell line.

At neither pH did CC show a diminished uptake when compared with control NC.

Electron Microscopy

Methods

Cell pellets were formed by centrifugation at 800–1,000 × g for 5–10 min. Pellets were fixed in

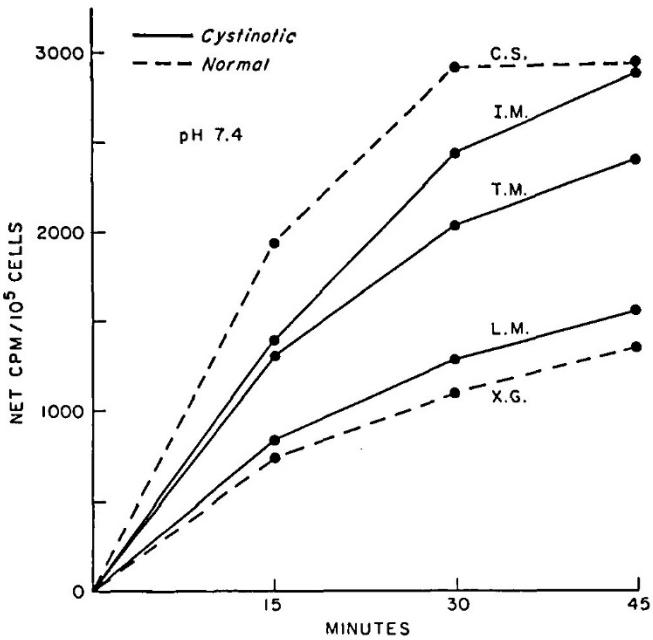

Fig. 2. Uptake of cystine-^{35}S by CC and cultured NC at pH 7.4.

Fig. 3. Cultured lymphoid cell from a patient with cystinosis (*IM*). Electron-dense tubular structures form an elaborate network within dilated channels of the endoplasmic reticulum. × 32,000.

2.5–3% glutaraldehyde and prepared for electron microscopy according to techniques previously reported [2, 3]. Ultrathin epoxy sections were doubly stained with 5% uranyl acetate in methanol and with saturated lead citrate [49], before examination in an electron microscope [58].

Results

All white cell lines showed similar ultrastructural features and could not be distinguished qualitatively either from each other or from cells of previously described lymphoid cell lines [2, 13, 26, 28]. A spectrum of lymphoreticular cells was observed in each culture sample, although the predominant cell would be described as "blast-like" [13] with sparse granular endoplasmic reticulum, prominent Golgi zones, and moderate numbers of mitochondria. In the lines presently examined, plasmocytic type cells and large histiocytic cells [2] were not common. One of the control cultures but none of the cystinotic samples contained cells with herpes type intranuclear virus particles (EB virus). Dense microtubular structures (Fig. 3) within the endoplasmic reticulum, previously described in lymphoid cell lines of neoplastic and nonneoplastic origin [3, 6, 12, 26, 28], were abundant in cells of two patients with cystinosis and in one of the controls. Enlarged mitochondria with paracrystalline inclusions (Fig. 4) were seen in occasional cells of the cystinotic cultures and resembled mitochondrial inclusions previously described in lymphoma cells [3]. An active Golgi apparatus was common in all of the cells examined, and

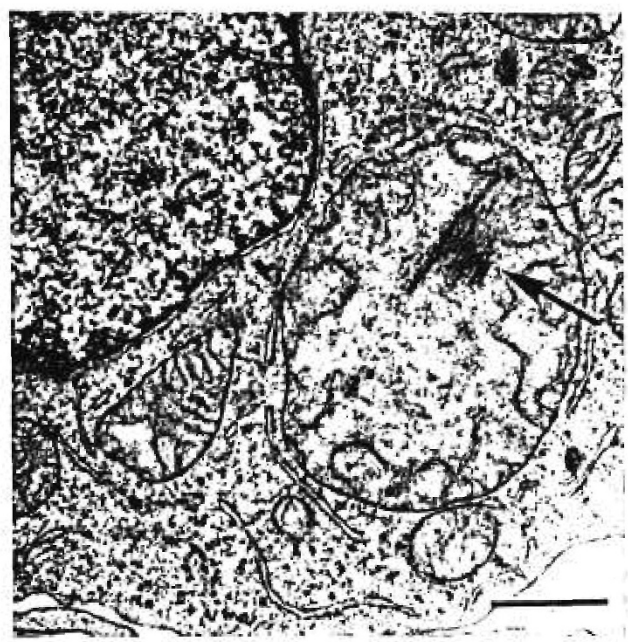

Fig. 4. Cultured lymphoid cell from patient with cystinosis (*IM*). Enlarged mitochondrion contains a paracrystalline inclusion (arrow). × 32,000.

many cells contained heterogeneous dense structures which would be classified as lysosomes. Many of these lysosomes contained fragments of membrane or other debris which could have been cellular in origin (Fig. 5). Numbers of lysosomal bodies varied within each sample, but appeared as numerous as in some previously studied lymphoid cell lines [2, 12, 13]. Curiously, hollow lancellate structures which might easily have been interpreted as cystine crystals were they present in a CC line were identified in one lysosome of a control culture (Fig. 6).

Cytogenetics

Method

Two CC, one carrier, and four continuous NC lines, all approximately 6 months old, were examined cytogenetically [1].

Results

The modal number of chromosomes in all lines was 46 (Fig. 7). There was a marked degree of aneuploidy and an increased frequency of tetraploidy in all lines examined (Table II). Tetraploidy was present in from 3.8–9.6% of the metaphases (Fig. 8). In all lines the frequency of hypodiploidy was greater than hyperdiploidy. In the hypodiploid lines, chromosome loss was random and no clearly defined clones were recognized. The majority of those missing, however, were from G, F, or E groups. Structural rearrangements and breaks were seen in both CC and NC lines (Fig. 9). No clear markers were apparent, although one normal subject (*C*) had a ring chromosome from the C group present in 6% of his cells.

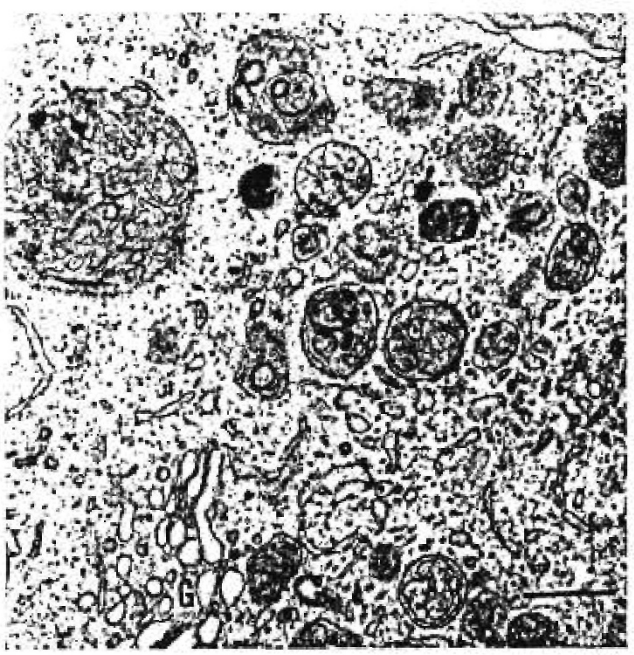

Fig. 5. Culture lymphoid cell from cystinosis carrier (*RM*). Cluster of lysosomal bodies with membranous inclusions located in the region of the Golgi apparatus (G). × 26,000.

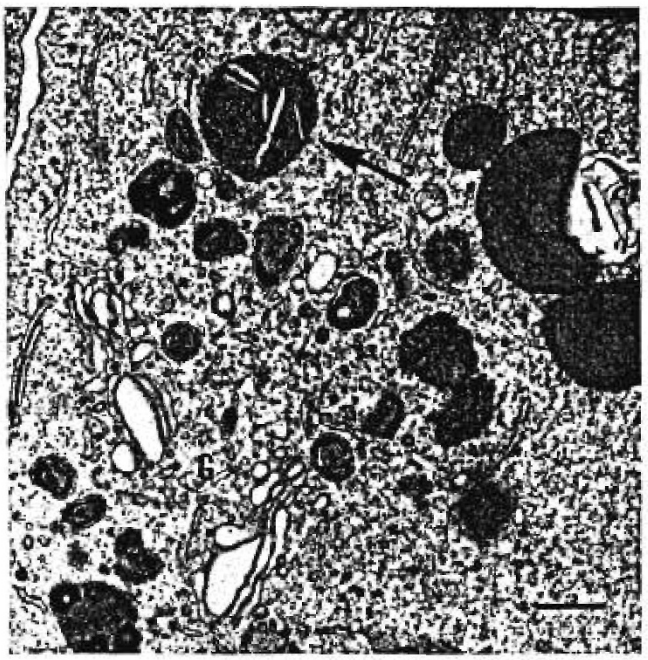

Fig. 6. Cultured lymphoid cell from a healthy control (*CS*). Dense lysosomal bodies are typically clustered in the Golgi region (G). One body (*arrow*) contains hollow inclusions resembling crystals. Bar equals 0.5 μ.

Discussion

The present study indicates that leukocyte lines from individuals with cystinosis, maintained for some months in culture, expressed and maintained expression of the increased intracellular cystine content which was the biochemical characteristic of cystinotic cells. This has been demonstrated previously for cystinotic fibroblasts in culture [37, 38]. The cystine levels in the cultured CC were somewhat lower than the levels found in the fibroblasts [37] or in peripheral CC (uncultured) [35]. Differences in the intensity of crystal deposition in cystinotic organs have been appreciated for many years, and different classes of cystinotic peripheral white cells have different cystine levels [42]. Leukocyte cell lines are more difficult to establish than fibroblast cultures, but once proliferation begins they are much easier to maintain and more easily grown for study, and, unlike fibroblasts, these cells may probably

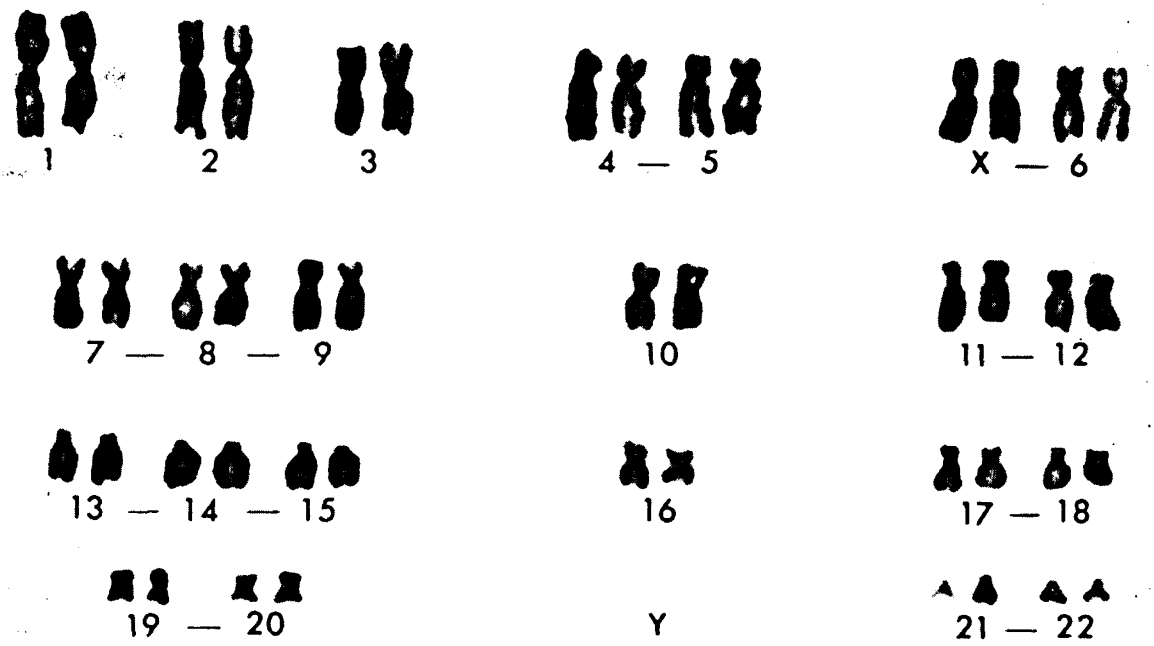

Fig. 7. Karyotype of normal continuous lymphocyte line derived from *subject CS*. Chromosomes were arranged according to Al-Aish [1.]

Table II. Cytogenetic findings in long-term leukocyte cultures from cystinosis, carrier, and normal subjects

Subject	Sex	Condition	No. of chromosomes						Total cells counted	Structural alterations						Comment
			<45	45	46	46–91	92	Endo-mitosis		Gap	Breaks	Frag-ment	Dicen-tric	Ring	Trira-dius	
TM	F	Cystinosis	3	6	56	3	5		73	3	2		1	1		
IM	M	Cystinosis	6	4	36		3	1	50		1	1	1			
R	F	Carrier	7	6	33	1	5		52		2					
CS	F	Normal	65	44	350	7	37		503	8	13	46	7		1	
G	M	Normal	10	7	31	3	2		53		2	1	1			
C	M	Normal	9	5	23	1	3		51		1	2		3		All rings in C group
E	M	Normal	9	8	31		1	1	50	1	1	2				

be indefinitely maintained in culture. The cultured white cells have been used for enzymologic studies of cystinosis which will be reported elsewhere [48]. The present study highlights certain other properties of these cells including the facility with which they may be used to investigate transport phenomena.

Uncultured cystinotic white cells are not deficient in activity of the mechanism for cysteine transport across the outer cell membrane [36]. There is good evidence that cysteine and cystine have different transport systems in peripheral white cells [36] (where cystine uptake is relatively poor [33]), rat kidney cortex [45], and lower organisms [46]. Cultured leukocytes have adapted to an extracellular source of cystine which is entirely in disulfide form in the culture medium. The normal uptake of cystine by CC at physiologic pH (7.4) was not surprising and was consonant with the normal growth rate of CC in culture, where cystine is an essential amino acid [14, 17].

The uptake studies at pH 5.0 represented an attempt to examine indirectly a possible transport system for cystine which might be operative at the level of the lysosomal membrane. Cystinosis appears to be a lysosomal cystine storage disease [21, 30, 39–41, 43, 50], and an abnormality in a membrane permease facilitating the efflux of cystine from the lysosomes could account for such storage. The interior pH of the lysosome is regarded as considerably more acid than the rest of the cell cytoplasm or extracellular fluid [23]. Furthermore, the membranes of at least certain kinds of lysosomes are in part derived from the cell membrane through invagination of the surface membrane. The inside of the membrane of vacuoles so formed is homologous with the outside of the cell membrane

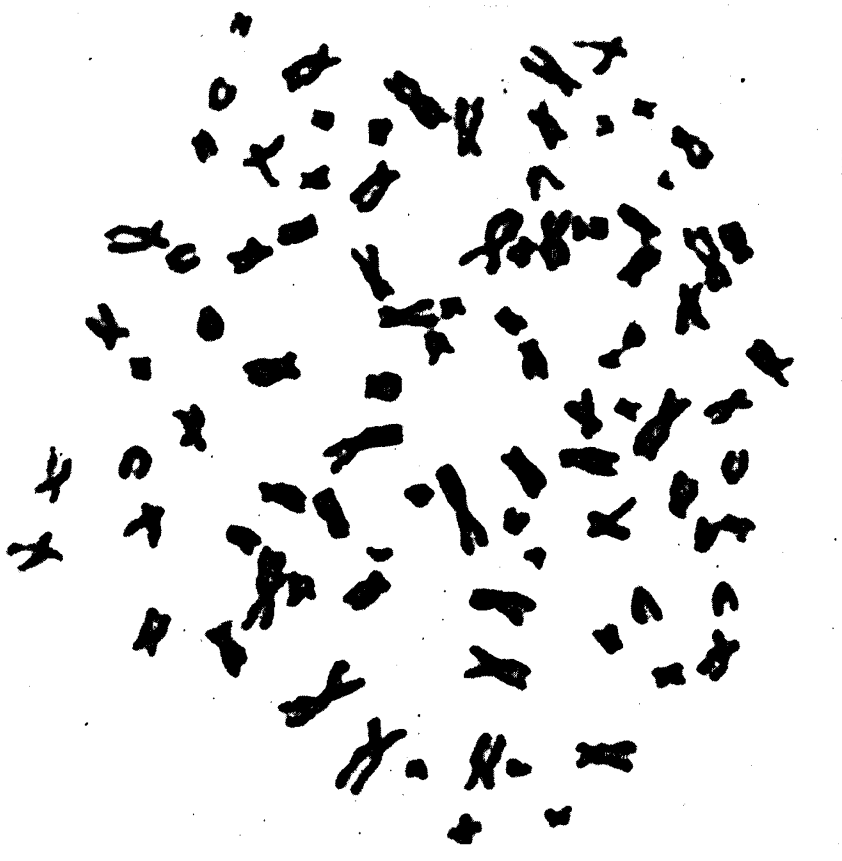

Fig. 8. A tetraploid metaphase from *subject CS* showing 92 distinct chromosomes.

[23]; the compositions of both membranes are similar [47]. We reasoned, therefore, that measurement of cystine movement across the outer cell membrane from outside to inside at acid pH might indicate the activity of a system which facilitates the efflux of cystine from lysosomes.

The uptake of cystine at acid pH into CC did not appear deficient in this study. No further attempt was made to define whether uptake proceeded by active transport or other mechanism. Although the results failed to support the hypothesis of a lysosomal membrane abnormality in cystinosis, because of the assumptions involved they did not rule out this possibility.

The cultured white cells from patients with cystinosis could not be distinguished by ultrastructure from cells derived from normal individuals, despite biochemical evidence of consistently higher intracellular quantities of cystine. Similar findings have been observed in cystinotic fibroblasts [20], although other observers have described what they regard as characteristic alterations but no crystal formation in cystinotic fibroblast lysosomes [43]. Presumably, in the cultured leukocytes, the excess cystine was sequestered within lysosomes, but crystalline structures, which might have provided a morphologic marker, were not observed by us. The limited sampling power of the electron microscope does not permit us to exclude that rare cystine crystal might condense in an occasional CC; however, this was certainly not the pattern observed in the several hundred cell sections which were examined. The presence of crystalline inclusions, which could easily have been mistaken for cystine crystals in a lysosome of a normal cell, indicates the need for extreme caution in drawing conclusions about the pathophysiologic significance of rare crystals that might be found in those CC where crystal deposition is not prominent. Intralysosomal membrane fragments of the same appearance as those experimentally induced by chloroquine [16] were found in CC and non-CC, and appeared to represent evidence of autophagy [11] by the lysosomes.

The mitochondrial matrix fibrils observed in the cultured leukocytes might conceivably be misinterpreted as cystine. This is not a moot point, in view of one report that crystals have been observed within ap-

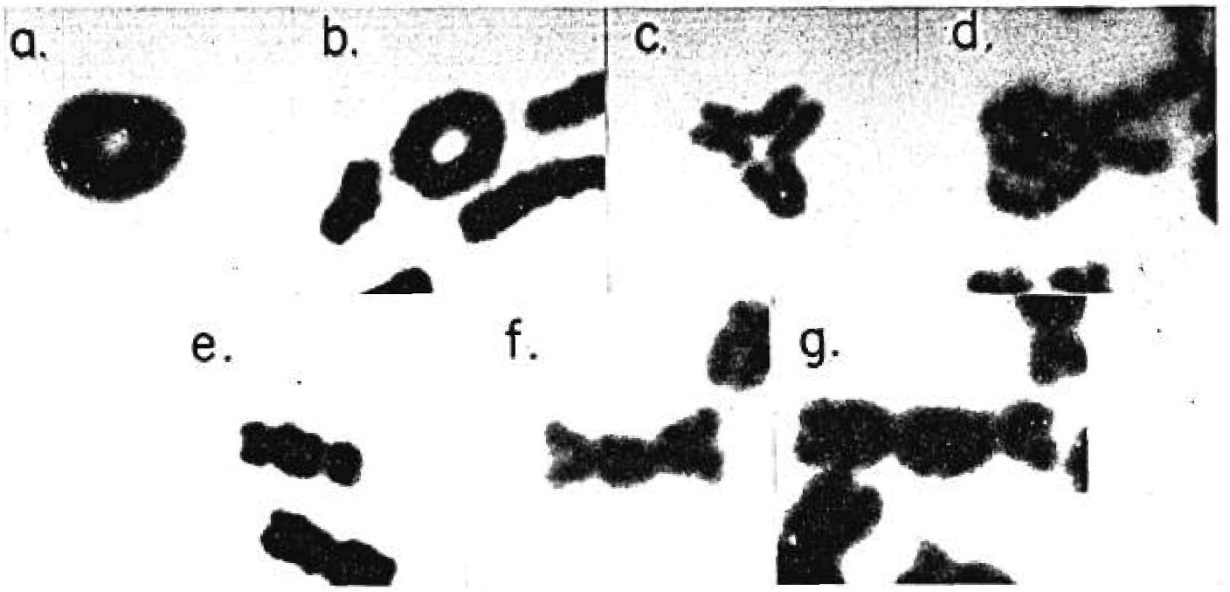

Fig. 9. Structural rearrangements and alterations. *a* and *b*: Ring C. *c*: Triradius. *d*: Unusual secondary association ("pregnant chromosome") *e*, *f*, and *g*: Dicentric chromosomes.

parent mitochondria in an intestinal biopsy from a case of cystinosis [27]. The exact nature of the crystals that we observed in the mitochondria is unknown, but may represent mitochondrial deoxyribonucleic acid (DNA) [3] or crystallized mitochondrial enzymes [34].

Patients with Fanconi's syndrome of renal tubular dysfunction, with or without cystinosis, have not been reported to have chromosome breakage in short-term lymphocyte cultures. In continuous cultures, CC showed no significant cytogenetic differences from those of NC. All lines have diploid modal counts. Within the lines, hypodiploidy and tetraploidy were the most frequently observed variants from the modal numbers, a pattern previously noted in long-term leukocyte lines [7, 25].

Breaks and structural rearrangements were prevalent in all lines. Viruses are recognized to cause chromosomal breaks *in vivo* and *in vitro* [24, 29]. The presence of antibodies to herpes-like virus was noted in donors of our established long-term leukocyte lines, and virus particles similar to those observed in four of six lines by Gerber and Monroe [18] were seen in one of our lines. Thus, it is possible that the breaks and structural rearrangements observed here might be related to the presence of virus.

Summary

Leukocytes growing in continuous culture have been derived from normal individuals and subjects homozygous and heterozygous for nephropathic cystinosis. The cultured leukocytes from patients with cystinosis have abnormally elevated levels of nonprotein cystine. Distinctive morphologic abnormalities were not found in the cystinotic cells by electron microscopy. All cell lines had a primarily diploid chromosome complement, although hypodiploidy, tetraploidy, and other chromosome abnormalities were observed. The cystinotic cells showed no defect in the uptake of exogenously supplied cystine-^{35}S either at neutral or at acid pH.

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