Mutant Enzymatic and Cytological Phenotypes in Cultured Human Fibroblasts

Abstract. *Fibroblasts were cultured from the cells of two children who shared some characteristics of Hurler syndrome, but they did not show corneal clouding and excessive excretion of mucopolysaccharides. The fibroblasts differ from those of controls and of patients with typical Hurler syndrome or other mucopolysaccharidoses in that they have abundant cytoplasmic inclusions, striking diminutions in beta-glucuronidase, and elevations in acid phosphatase.*

We have been screening inherited abnormalities for biochemical phenotypes which could be useful for genetic experiments with somatic cells. Studies have been made of fibroblasts cultured from six patients diagnosed as having Hurler syndrome (*1*), a disease in which there is a derangement in the metabolism of acid mucopolysaccharides (AMPS). In two of our patients, corneal clouding was absent, and urinary excretion of AMPS was barely, if at all, above normal. Cultures derived from these two patients proved so different from the others that we conclude that different biochemical abnormalities are present in the two groups of patients, despite their many similarities. The clinical picture (*2*) also rules out the diagnosis by any of the known mucopolysaccharidoses, as well as that of familial neurovisceral lipidosis, sometimes called pseudo-Hurler syndrome (*3*).

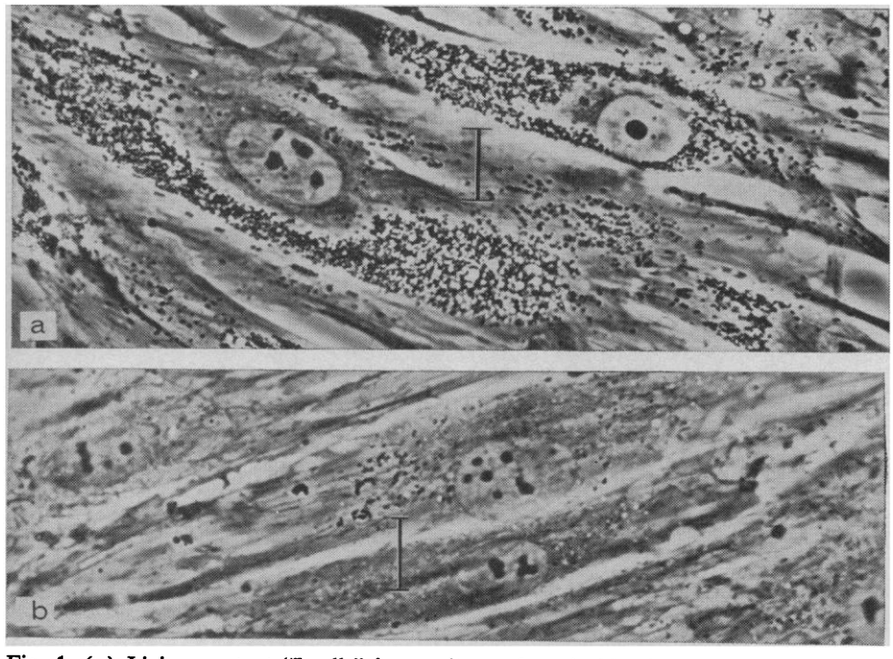

Fig. 1. (a) Living, mutant "I cells" in a culture of strain No. 216 photographed with a Zeiss phase-contrast microscope and Kodak High Contrast film. There is relative absence of inclusions from areas adjacent to the nuclei; these may be Golgi areas. (b) Living cells of control strain No. 47 cultured and photographed as the "I cells" were (markers indicate 15 μ).

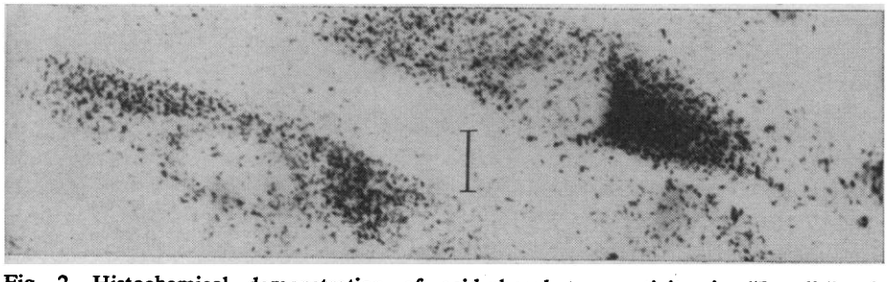

Fig. 2. Histochemical demonstration of acid-phosphatase activity in "I cells" of strain No. 216 (*7*). The cytoplasms and nuclei are rather clearly defined by the deposits of azo dye. Photographed with bright field and a green filter on Kodak High Contrast film (marker indicates 15 μ).

That fibroblasts cultured from skin biopsies of patients with typical Hurler syndrome synthesize and accumulate AMPS at supranormal rates (*4*) explains their cytoplasmic metachromasia upon staining with toluidine blue (*5*). About 10 percent of such fibroblasts are either highly vacuolated or contain a large number of refractile granules when examined with the phase-contrast microscope. Some cells have both traits. The most granular among these fibroblasts show the most intense metachromasia (*6*).

The two unusual cell strains included in this study have other, quite different, properties. They are derived from the skin biopsies of two apparently unrelated patients, a 5-year-old girl (No. 163 below) and a 4-year-old boy (No. 216 below). We have named these cultured fibroblasts "I cells" because all of them contain an abundance of dark, cytoplasmic inclusions which surround the nucleus and a juxtanuclear zone [(*7*) and Fig. 1a]. The morphology of the inclusions in strain No. 163 suggested lysosomal pathology when studied with the electron microscope; this concept was supported by the demonstration of intense acid phosphatase activity with naphthol-ASMX-phosphate as histochemical substrate [(*7*) and Fig. 2]. Only a few fibroblasts cultured from patients with the known mucopolysaccharidoses studied show this histochemical trait, and, then to a decidedly lesser degree. This trait is virtually absent from control strains.

The hereditary nature of the "I-cell" trait is demonstrated by small but significant numbers of the mutant cells, apparently occurring as clones, in cultures derived from the father and paternal uncle of patient No. 163 (*7*).

To investigate the possible role of lysosomes in the pathogenesis of these two cell strains, the study of enzymes characteristic of lysosomes (*8*) was undertaken. The general experimental format was that described by DeMars (*9*). Duplicate determinations of enzyme activities and total cell protein (*10*) in fibroblast monolayers were made at intervals during the growth of replicate cultures of each cell strain. Acid-phosphatase was determined by two methods, and β-glucuronidase was also determined (*11*). The cell strains studied in four experiments are listed in Table 1. Since repeat experiments with the same strains gave very similar results, the graphs present typical data sampled from several experiments, which are summarized. The cultures

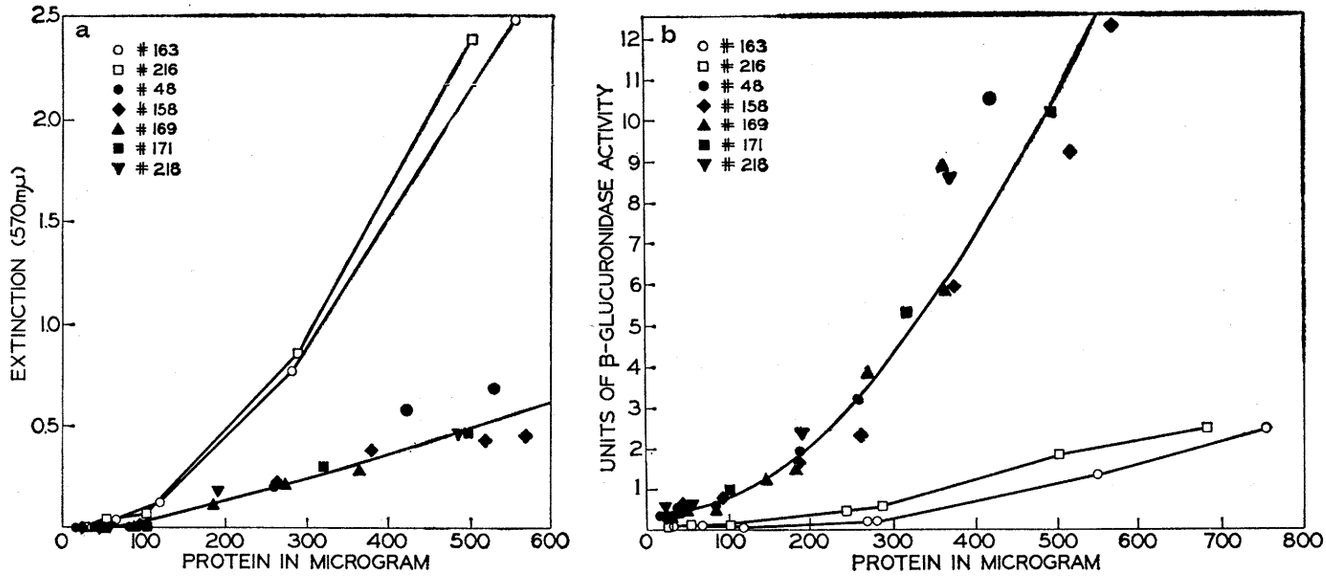

Fig. 3. (a) Acid phosphatase activities during the growth of "I cells" and various comparison strains. Naphthol-ASMX-phosphate was used as substrate (*11*). See Table 1 for definitions of the cell strains. (b) Beta-glucuronidase activities (*11*). The ordinates represent the total units of enzyme activity per culture at various growth levels, indicated on the abcissa.

comprise two groups with regard to acid-phosphatase activity with naphthol-ASMX-phosphate as substrate (Fig. 3a). The first "low-activity" group consists of all strains that are not "I cells." The second "high-activity" group contains the two "I-cell" strains, Nos. 163 and 216. Their specific activities are similar and are approximately five times the average of the "low-activity" group at the time the cells are confluent. This quantitatively confirms the more qualitative histochemical distinctions.

When the increase of β-glucuronidase relative to total culture protein during growth was studied [compare Gorman (*11*)], the cultures also fell into the same two groups of strains defined, but now with reversed relationship between their enzyme activities (Fig. 3b). The two "I-cell" strains comprised a "low-activity" group having about one-fifth the specific activity of β-glucuronidase in the "high-activity" group containing all other strains. No significant differences between strains in *p*-nitrophenyl phosphatase activity were found.

The correlation between the abundant inclusions in "I cells" and their altered hydrolytic enzyme activities is obvious. A tentative identification of the inclusions with lysosomes has proved a fruitful working hypothesis. However, we still lack explicit information on the chemical nature of the inclusions. Possibly, they have no physiological counterpart in normal cells and are merely accumulations of unidentified substances that interfere with normal activity of the hydrolases studied.

Two of the enzyme activities are anomalous in opposite directions in the "I-cell" strains. These may be explained as mutations affecting the structure of the enzyme molecules or mutations that, instead, influence the activities of structurally normal polypeptides. Separate structural mutations for each enzyme would require coincidence of two individually improbable events. A single mutation affecting the structure of a polypeptide subunit common to both enzymes could account for the observations.

In contrast, a single mutation affecting the makeup of a subcellular structure could alter the activities of enzymes associated with the structure, elevating some and diminishing others.

Experimental approaches to sorting out these hypotheses with the cultured cells are obvious. The promising start made in the biochemical characterization of the "I cells" should enhance the utility of the living cell phenotypes for genetic studies in vitro.

Table 1. Cell strains studied. For descriptions of the syndromes, see (*1*).

Type of donor	Cell strain (No.) and donor
"I-cell" donors	163 (female)
	216 (male)
Hurler syndrome	134, 143, 144, 158 (males)
Hunter syndrome	171, 174, 175, 187, 219 (males)
Sanfilippo syndrome	180 (female)
Scheie syndrome	185 (female)
Patients' relatives	169 (father of No. 163)
	168 (mother of No. 163)
	217 (father of No. 216)
	218 (mother of No. 216)
	193 (uncle of No. 163)
	172 (mother of No. 171)
Unrelated controls	47 (female)
	48, 69, 159 (males)

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11. All enzyme assays were performed in duplicate directly on monolayers of cells grown in glass petri dishes (30 mm in diameter). Medium F10 (Grand Island Biol. Corp.) with 15-percent fetal bovine serum was renewed every 2nd day. The cell monolayers were rinsed twice with 0.9 percent NaCl and fixed with acetone for 20 seconds at room temperature. Beta-glucuronidase was assayed with J. A. Gorman's method (Ph.D. thesis, Univ. of Wisconsin, 1963) with a 6-hour incubation time. *P*-nitrophenyl phosphatase determinations (*9*) were performed with 0.2*M* acetate buffer at *p*H 5.0 and an incubation time of 1 hour. Activity against naphthol-ASMX-phosphate was quantitated with an unpublished method devised by Dr. F. A. Walker and is briefly described here with his permission. Reaction mixture 1 ml per assay: acetate buffer, 0.2*M*, *p*H 5.3; $MnCl_2$, 0.0025*M*; naphthol-ASMX-phos-

phate (Sigma), 0.15 mg per ml; fast blue RR salt (Sigma), 0.50 mg per ml; incubation time, 2 hours at 37°C. The monolayers were rinsed twice with 95-percent ethanol and were extracted with 2 ml of a 1/100 dilution of freshly prepared solution of absolute ethanol saturated with KOH in absolute ethanol for 30 minutes at 60°C. The extracts were transferred to conical centrifuge tubes to which were added 2 ml of the following mixture (in volumes): conentrated HCl, 3; absolute ethanol, 47; $CHCl_3$, 50. The precipitates that formed were removed by centrifugation, and the optical densities at 570 mμ of the supernatants were determined.

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New Method for the Detection of Light Deflection by Solar Gravity

Abstract. *The prediction of Einstein's theory of general relativity that light will be deflected by the sun may be tested by sending radio waves from the earth to Venus or Mercury when either passes behind the sun and detecting the echoes with a radar interferometer.*

One of the three classical tests of general relativity originally proposed by Einstein concerns the prediction that the path of starlight will be deflected by the sun (*1*). Although attempts to detect this frequency-independent deflection have been made during almost every total solar eclipse since 1919, the results have been quite discordant and have verified the prediction only to within about ± 20 percent (*2*). The purpose of this report is to point out and discuss a new method with the potential for measuring the gravitational bending of light to substantially higher accuracy. The essential element of this method is the use of a radar interferometer. Since the maximum predicted bending is 1.75 arc sec for light just grazing the limb of the sun, conventional radar systems would be unable to detect such a deflection: the finest angular resolution at present obtainable with an interplanetary radar system is about 4 arc min—over 100 times greater than the largest predicted general relativistic effect. By employing two radar receivers in an interferometer arrangement, however, the error in determining the angle of arrival of a signal can be decreased approximately in inverse proportion to the receiver separation. Such interferometric techniques cannot be used effectively at optical wavelengths for this purpose because the phase ambiguities introduced by the atmosphere are practically impossible to unravel (*3*). By contrast, at centimeter wavelengths, the atmosphere introduces relatively few rotations of the phase vector which can probably be determined to a small fraction of a rotation.

A specific realization of this method could involve illuminating Venus or Mercury with a radiation of 8×10^3 Mhz (X-band) when either passes behind the sun and receiving the radio waves over a base line of approximately 10 km (*4*). The deflection predicted by general relativity would then be measurable to within 10 to 15 percent. Longer base lines could yield proportionately higher accuracies.

The theoretical expression for the bending can be obtained, for example, by calculating in isotropic coordinates (*5*) the difference η in angle of arrival between a ray propagating rectilinearly and according to general relativity. To first order in r_0, we find

$$\eta = \frac{2r_0}{r_e} \tan\left(\frac{\theta}{2}\right) \qquad (1)$$

where $r_0 \approx 1.5$ km denotes the so-called gravitational radius of the sun (*6*), r_e the heliocentric coordinate distance of the earth observer at the time of detection, and θ the planet-sun-observer angle with the planet's position being at the time of reflection. This apparently new result is remarkable in showing that the gravitational deflection undergone by an electromagnetic ray is independent of the position on a given radial line from which the ray originates. The farther out the source is on the radial line, the farther the ray's closest approach is from the sun, but the longer its path to the receiver; these two opposing influences on the total deflection just cancel as indicated by Eq. 1.

Near superior conjunction when the radar signal to a planet passes closest to the sun, Eq. 1 may be written in the following way

$$\eta \approx \frac{4r_0}{d}\left(\frac{r_p}{r}\right); \quad d \ll r_e, r_p \qquad (2)$$

where r_p is the heliocentric distance of the planet at reflection, r the rectilinear distance between the earth observer and planet, and $d = (r_e\ r_p/r) \sin\theta$ the approximate distance of closest approach of the signal to the sun. Because of the finite planet-observer separation, the maximum deflection of $4r_0/d (\approx 1.75$ arc sec) is not attained. Thus, with Venus the target and with the signal grazing the limb of the sun, $\eta \approx 0.73$ arc sec.

For a two-element interferometer operating at X-band with a 10-km base line, a change of one fringe would correspond to a difference in angle-of-arrival of at least 0.7 arc sec. But, depending on the signal-to-noise ratio and other factors, the phase within a fringe may be measurable to within about 10°, implying an error in the determination of the angle of arrival of only about 0.02 arc sec for this situation. Larger separations would lead to proportionately smaller errors.

To determine the feasibility of such an experiment, we must consider the possible limitations on d, the refractive effects of the earth's atmosphere and the solar corona, the angular size of the target planet, the uncertainty in the orbital positions of the target and antennas, the possible ambiguities in the comparison of the echo phases received at the two sites, the absolute phase stability required between receiver elements, and the necessary radar-system sensitivity. We discuss each in turn.

The minimum usable value of d is set mainly by three constraints: the noncoplanarity of the orbits of earth and target planet, the radio noise emitted by the sun, and the turbulence of the inner corona. The resultant limitation, although depending on the radar system and varying with the solar cycle and from conjunction to conjunction, lies at about $d = 3r_s$ (r_s is the solar radius) and implies that the largest detectable general relativistic deflection will be about 0.25 arc sec for planetary echoes.

Variations in the earth's atmosphere set limits on the accuracy with which ground receivers can be used to infer the exo-atmospheric direction of propagation of radio signals. At the very low elevation angle of 7°, the 2-day root-mean-square fluctuations σ in the estimate of azimuth angle for X-band signals are about 2 arc sec for a 1-km base line (*7, 8*). These fluctuations decrease with the cosecant of the elevation angle [thus, σ (45°) ≈ 0.3 arc