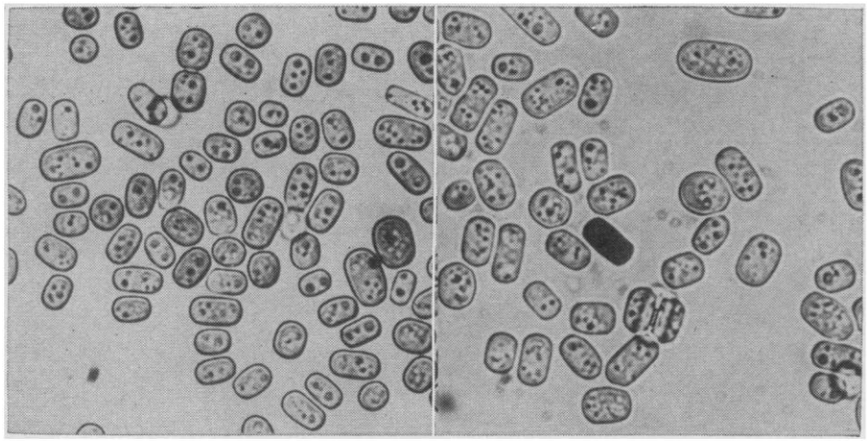

Fig. 1. *Geotrichum candidum* (×800). (Left) Typical arthrospores from a representative isolate from a worldwide collection. (Right) Arthrospores derived from a single ascospore of isolate PR-47. All cultures were grown simultaneously on potato-dextrose agar at 30°C for 24 hours and stained with crystal violet (200 μg/ml).

have no recent history of agricultural use.

On V-8 juice agar, malt extract, and most other natural media commonly used to culture fungi, 2-day-old cultures held at 25°C are typical of *Geotrichum candidum*, with aerial chains of arthrospores (Fig. 1), dichotomous hyphal branching at the periphery of the colony, and a fruity odor. Dominating the cultures in 3 to 5 days are hyaline oval to globose, punctate, median furrowed asci, each with a single smooth, thick-walled ascospore. The asci, formed only at septa, are preceded by the fusion of globose gametangia.

Although the wild-type cultures are self-fertile, compatible self-sterile clones were obtained as sectors and from conidia of the self-fertile wild type. Paired compatible self-sterile cultures always produced relatively long filamentous gametangia which fused in pairs at their apexes. Spore formation is analogous to that observed in species of *Rhizopus* in the Mucorales. Ascospores formed from the pairing of asexual Pr-47A_1 and Pr-47A_2 produced only the self-fertile Pr-47 type; Pr-11A_1 and Pr-11A_2 were respectively compatible with Pr-47A_2 and Pr-47A_1. Mating types were obtained by the isolation of single conidia from Pr-47 and Pr-11. Single ascospores from any source yielded only self-fertile clones. This behavior suggests that the wild-type self-fertile culture is diploid, although the site of meiosis is not yet known. Staining of vegetative and reproductive cells with Giemsa indicates that gametangia and mature ascospores each have a single nucleus, whereas arthrospores and vegetative hyphal cells have one to four nuclei. This condition suggests the possibility of somatic meiosis. The evolutionary tendency in the sexual strains appears to be toward self-fertility; nevertheless, self-sterile clones are most common in nature, and in this regard we have made several fertile crosses between field isolates of *G. candidum* from various parts of the world.

The perfect state of *G. candidum* is similar to *Endomyces reessii* van der Walt (*7*), whose asexual state may form part of the species complex of *G. candidum*. In this connection we have isolated self-sterile, cross-fertile mating types of *E. reessii*, and although morphologically similar to *G. candidum*, they did not form ascospores in any pairing with *G. candidum* (*8*).

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Diploid Azaguanine-Resistant Mutants of Cultured Human Fibroblasts

Abstract. *Two azaguanine-resistant clones of cultured, human fibroblasts were isolated from unrelated strains of karyotypically normal, male cells. The most resistant mutant has little hypoxanthine-guanine phosphoribosyltransferase activity, is virtually unable to incorporate hypoxanthine (a normal substrate of the enzyme), and resembles fibroblasts cultured from boys with the Lesch-Nyhan syndrome. The less resistant mutant has about one-third as much enzyme activity as its parent strain and is less able to utilize hypoxanthine. Both mutants are morphologically and karyotypically normal. These mutations may have occurred at the X-chromosomal, hypoxanthine-guanine phosphoribosyltransferase locus and may provide a realistic experimental model for studying mutation in human genetic material.*

We report here the isolation of azaguanine-resistant mutant cells from cultures of karyotypically normal, male human fibroblasts. Biochemically related mutants have previously been derived from strains of human and mouse cells (*1*, *2*, for example), which were already hyperdiploid and prone to additional karyotype variation. Azaguanine-resistant mutants of essentially diploid Chinese hamster cells have been described (*3*), but we believe that the mutants described below are the first diploid, biochemically defined mutants of human cells to be isolated in vitro.

Selection for resistance to 8-azaguanine (AG) is based on the activity of hypoxanthine-guanine phosphoribosyltransferase [HG-PRT; E.C. 2.4.2.8 (*4*)], which is specified in humans by a gene on the X chromosome (*5*). Normal substrates for the enzyme are hypoxanthine and guanine, which are converted to inosine 5′-monophosphate (IMP) and guanosine 5′-monophos-

phate, respectively. Cells having HG-PRT can also convert purine analogs, such as AG and 6-mercaptopurine, to their nucleotides, the incorporation of which results in inhibition or death. Conversely, cells with reduced amounts of the enzyme have impaired ability to incorporate the abnormal purines and are relatively resistant to them. This is illustrated by fibroblasts cultured from boys having the Lesch-Nyhan syndrome (*6*), which results from X-chromosomal mutations that cause a marked deficiency of HG-PRT activity (*7*). Figure 1 shows the growth attained by normal fibroblasts (strains 52 and 199) and by Lesch-Nyhan–derived fibroblasts (strain 252) in the presence of various concentrations of AG. Both normal strains are perceptibly inhibited at the lowest concentrations tested ($3.0 \times 10^{-7} M$) and are completely inhibited at about $2.7 \times 10^{-6} M$. Higher concentrations cause progressive detachment of cells from the cultured vessels. In contrast, inhibition of strain 252 is first detected at $2.2 \times 10^{-4} M$ and is still incomplete at $2.0 \times 10^{-3} M$, the highest concentration tested.

We are attempting to isolate mutants having deletions at the HG-PRT locus by selection with AG after x-irradiation of sensitive, male fibroblasts. In three small pilot experiments two mutants have been isolated, but we do not know whether the mutants were induced by the x-rays. Nevertheless, such mutants of diploid human cells have not been described, and we now report some of their properties.

Strain 52, the parental population used in experiment 1, was karyotypically normal XY, but its glucose 6-phosphate dehydrogenase was electrophoretically altered (*8*). Five milliliters of FCS-F10 (*9–12*) containing 2×10^6 cells was treated with 150 r of x-rays, which permitted survival of 70 percent of the cells as colony formers (*13*). The irradiated cell suspension was diluted, and 5-ml samples containing 8×10^4 cells were distributed into 24 dishes, 60 mm in diameter. On the third day after irradiation the FCS-F10 was replaced with CS-F10 (*9*) containing $2 \times 10^{-5} M$ AG. This medium was replaced every second day for 20 days. On day 21 the cells in the single colony that had formed were subcultured, propagated further in CS-F10 containing $5 \times 10^{-6} M$ AG, and named 52 AG^r-1.

In experiment 2, another XY strain (199) with a characteristic deficiency of glucose 6-phosphate dehydrogenase (*14*) was used. Cells (6×10^5) were irradiated with 220 r (40 percent survival). After 4 weeks of selection with CS-F10 containing $2 \times 10^{-5} M$ AG, a single colony was subcultured and propagated in CS-F10 with $2 \times 10^{-5} M$ AG. This strain is mutant 199 AG^r-1. Parental strains with characteristic glucose 6-phosphate dehydrogenase phenotypes were used in order to avoid uncertainties about presumed mutants being contaminants from Lesch-Nyhan cultures, which had normal dehydrogenase. Male cells were used, but we expect that female cells will have the same mutation rate at the HG-PRT locus; only one allele of this locus is expressed in cells with two X chromosomes, the unexpressed allele being on the inactive X chromosome (*15, 16*).

Both mutants were characterized in parallel with their respective parent strains and with strain 252. Mutant 199 AG^r-1 is very resistant to AG, resembling strain 252 in this respect (Fig. 1). Mutant 52 AG^r-1 has intermediate resistance. It becomes completely inhibited at $6.6 \times 10^{-5} M$ AG, a concentration which is approximately 20-fold greater than that needed to inhibit completely the sensitive parent strains but which has no inhibitory effect on strains 252 and 199 AG^r-1.

The various degrees of resistance to AG displayed by these five strains of cells are associated with different amounts of HG-PRT activity (Table 1). Cells grown to confluence in FCS-F10 medium were disrupted by sonic vibration. The enzyme activity was determined with a procedure modified in one important respect from that described by Littlefield (*1*) in that chromatography of the reaction mixtures was performed on Whatman 3MM paper (*17*) instead of ion-exchange paper. This permitted the determination of radioactivity in inosine as well as that in IMP, which is the immediate product of the enzyme reaction but which is rapidly degraded to inosine in whole fibroblast extracts. After 60 minutes of reaction, 80 to 90 percent of the radioactivity not in hypoxanthine is in inosine. However, this inosine is probably derived from IMP generated by HG-PRT for the following reasons. (i) Extracts of Lesch-Nyhan fibroblasts (strain 252) produce less than 2 percent as much inosine as control cells do. (ii) The inclusion of nonradioactive adenosine 5′-monophosphate ($2 \times 10^{-3} M$) in the reaction mixtures causes 80 to 85 percent of the nonhypoxanthine radioactivity to appear in IMP without altering the total number of counts in IMP and inosine, presumably because inclusion of the nonradioactive nucleotide lessens the opportunity for 5′-nucleotidase to attack the labeled IMP (*18*). (iii) Treatment of the crude cell extracts at 80°C inactivates nucleotidase more rapidly than HG-PRT, causing a larger proportion of the radioactivity not in substrate to reside in IMP in heated rather than in unheated extracts.

Table 1 shows that strain 52, which is most sensitive to AG, has the highest HG-PRT activity, that the least sensitive strains (252 and 199 AG^r-1) have the lowest activities, and that the mutant with an intermediate degree of sensitivity (52 AG^r-1) also has an intermediate specific activity, which is about 0.3 that of its parent strain.

Diminished HG-PRT activity impairs cellular incorporation of the normal purine metabolite hypoxanthine. The

Table 1. Hypoxanthine-guanine phosphoribosyltransferase activities of cultured AG-sensitive, parent fibroblasts (52), AG-resistant, mutant derivatives (52 AG^r-1 and 199 AG^r-1), and control, AG-resistant, Lesch-Nyhan mutant cells (252). Confluent monolayers were grown in dishes 60 mm in diameter in medium FCS-F10 (*9*). The monolayers were rinsed twice with and scraped into cold, 0.9 percent NaCl, collected by low-speed centrifugation, and resuspended in 1 to 3 ml of distilled water. The cells (on ice) were ruptured with ultrasonic treatment (30 to 40 seconds; Medtron unit). Reaction mixtures (50 μl) contained tris-HCl (*p*H 7.4), 180 nmole; $MgSO_4$, 270 nmole; 5′-phosphoribosyl pyrophosphate, 50 nmole; [^{14}C]hypoxanthine (4.15 μc/μmole), 8 nmole; bovine serum albumin, 100 μg; and amounts of total cell protein ranging up to 21 μg. Reactions were started by the addition of enzyme and were incubated at 37°C for 45 to 60 minutes. Reactions were terminated by the addition of 10 μl of 4*M* formic acid. Then nonradioactive hypoxanthine, inosine, and inosinic acid (0.085 μmole each) in a total volume of 50 μl were added to each reaction mixture, and 50 μl samples were chromatographed (ascending at room temperature) on Whatman 3 MM paper for 4.5 to 5 hours with 5 percent Na_2HPO_4 as solvent (*17*). The papers were dried in an oven. The spots containing inosine and inosinic acid were cut out and the radioactivity was counted in a scintillation counter in a mixture of PPO (2,5-diphenyloxazole, 22.74 g), POPOP [1,4-bis-2-(5-phenyloxazoly)benzene, 0.0379 g], and toluene (3.79 liters).

Cells	Radioactivity (count/min)	Protein (μg)	Specific activity
52	10,150	13.1	775
52 AG^r-1	1,195	4.7	254
199	14,500	21.0	691
199 AG^r-1	126	10.9	11.6
252	99	7.5	13.2

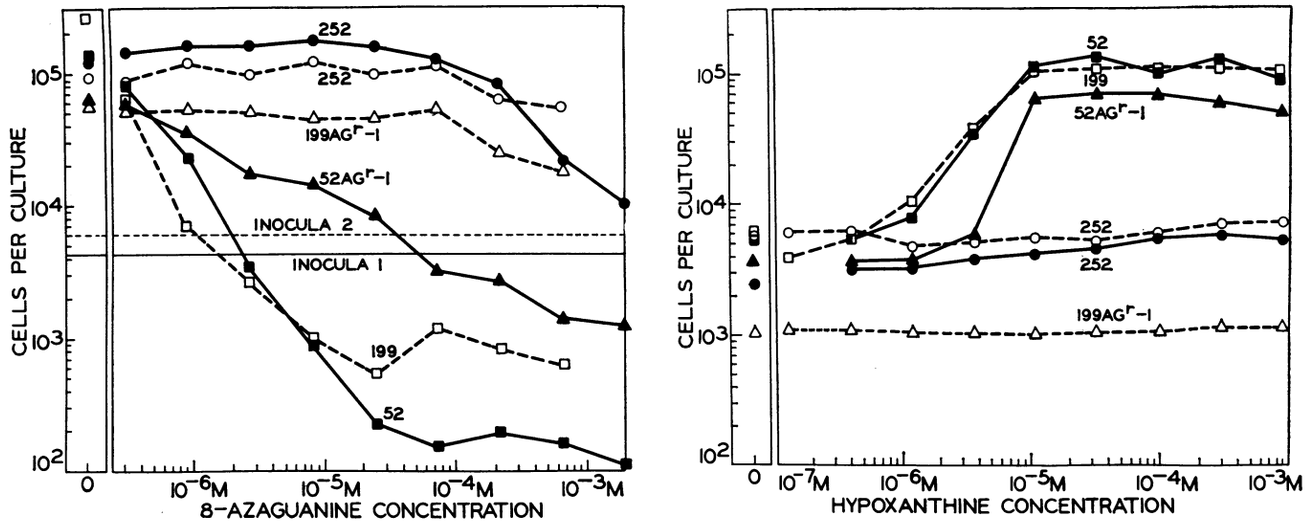

Fig. 1. Growth of human fibroblasts in the presence of 8-azaguanine. Strains 52 and 199 are sensitive parent strains; 52 AG^r-1 and 199 AG^r-1 are their mutant derivatives, and 252 was derived from a boy with the Lesch-Nyhan syndrome. Filled symbols represent data from experiment 1, and unfilled symbols connected with dashed lines represent data from experiment 2. "Inocula 1 and 2" represent the inoculum sizes of the three cell strains used in experiments 1 and 2. Cells in FCS-F10 (*9*) were inoculated into 13-mm diameter dishes (Bellco). After 15 to 24 hours each culture was presented with experimental medium [0.5 ml of CS-F10 (*9*) supplemented with test amounts of 8-azaguanine], which was renewed every second day. The cell populations in duplicate dishes on day 12 of experiment 1 and in triplicate dishes on day 9 of experiment 2 were determined with a Coulter counter (*11*). The averages of the counts are plotted here. Fig. 2. Growth (12-day interval, experiment 1; 9-day interval, experiment 2) of human fibroblasts forced to utilize exogenous hypoxanthine as their sole source of purines. The inocula and general procedures were those described in Fig. 1, with the exception that experimental mediums consisted of CS-F10 (*9*) supplemented with aminopterin ($10^{-7}M$), thymidine ($3 \times 10^{-5}M$), and various concentrations of hypoxanthine.

different levels of AG resistance and of enzyme activity displayed by the two new mutants had corollary expressions in different abilities to incorporate hypoxanthine from the culture medium. Growth after a requirement for exogenous purine was created by blocking purine biosynthesis with aminopterin in CS-F10 supplemented with various concentrations of hypoxanthine is shown in Fig. 2. The parental strains increased significantly when the concentration of hypoxanthine was $1.2 \times 10^{-6}M$ and achieved maximum growth for these conditions at $1.1 \times 10^{-5}M$. However, strain 252 did not increase significantly at any concentration tested. Mutant 199 AG^r-1 not only failed to use hypoxanthine for growth but suffered a large net loss of cells at all concentrations tested. Again, mutant 52 AG^r-1 displayed an intermediate phenotype by utilizing hypoxanthine for growth. However, the minimum concentration ($3.7 \times 10^{-6}M$) that evoked a growth response (50 percent increase of the mutant) was three times higher than that needed to promote growth of normal cells and elicited about a sevenfold increase of both parent strains.

Autoradiography of cells after brief growth in medium containing tritiated hypoxanthine shows strain 52 as heavily labeled (Fig. 3a), strain 252 as almost totally unlabeled (Fig. 3d), and 52 AG^r-1 as distinctly, but much less heavily labeled than its parent strain 52 (Fig. 3b). Mutant 199 AG^r-1, however, is almost unlabeled (Fig. 3c), resembling strain 252 in this respect.

Two important traits of the new mutants can be stated simply. (i) Neither mutant is a contaminant from another strain. Their glucose 6-phosphate dehydrogenase phenotypes correspond to their AG-sensitive parent strains. (ii) No characteristic karyotype abnormal-

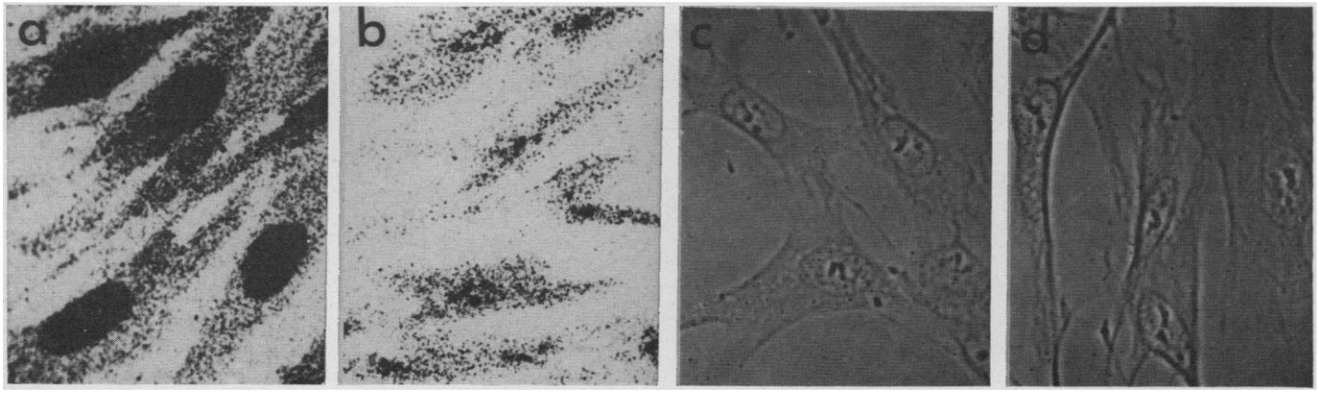

Fig. 3. Autoradiographic images of fibroblasts (×575) grown for 6 hours in FCS-F10 (*9*) containing [^{3}H]hypoxanthine (10 μc/ml; 3.14 c/mmole). The autoradiographic procedures have been described (*16*). (a) Parent strain 52; (b) mutant 52 AG^r-1; (c) mutant 199 AG^r-1; (d) Lesch-Nyhan strain 252; (a) and (b) are brightfield photographs. The low degree of labeling of (c) and (d) made it necessary to use phase-contrast photography in order to make the cells clearly evident.

ity has been detected in the parent or mutant strains.

The HG-PRT locus is ideal for two-way mutation studies with human cells. Selection for reversions of mutants toward wild type can be effected in a medium where growth depends on the ability to utilize hypoxanthine, while selection for a variety of changes from normal to reduced amounts of HG-PRT can be achieved with AG. The latter selection provides a reasonable, genetically tidy model system for determining mutation rates in human cells and utilizes readily available, normal male fibroblasts in standard culture mediums. HG-PRT deficiency is not, by itself, deleterious to cultured cells. However, enzyme deficiency resulting from a deletion or inactivation of a sizable portion of the X chromosome containing the HG-PRT locus is likely to be associated with lethality in cells having one X. Therefore, viable cells deficient in HG-PRT selected for with AG will almost always result from small mutational changes in euploid chromosome complements. Our results so far indicate an incidence of mutant cells that is about 10^{-6}, which may be an underestimate. By using artificial mixtures of mutant and normal cells, we found that the recovery of mutants (strain 252) as clones of viable cells after selection with $2 \times 10^{-5} M$ AG is related to the number of normal cells (strain 52) that is present. The number of mutant clones that form is not reduced by 10^4 normal cells per 60-mm dish, but 10^5 normal cells reduces the number to about 10 percent of maximum and the recovery of mutant clones is almost negligible in the presence of 10^6 normal cells per dish. This interaction between cells is not yet understood.

If the suggested incidence (10^{-6}) of AG-resistant mutants represents their spontaneous incidence in vivo, one may expect that resistant mutants already exist in the cell population whenever 6-mercaptopurine is used to treat leukemia. This is one reason why the leukemias regularly become permanently resistant to the purine analog (*19*), but other bases for resistance apparently exist (*19, 20*). However, spontaneous mutant cells are not frequent enough to interfere with a new method of diagnosing heterozygotes for the Lesch-Nyhan mutation, which is based on the ability of cells deficient in HG-PRT in a heterozygous fibroblast population to proliferate in the presence of AG (*21*). HG-PRT variation may play a role in other medical contexts, such as immunosuppression, where purine analog substrates of the enzyme are used.

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Fetal Kidney in Organ Culture: Abnormalities of Development Induced by Decreased Amounts of Potassium

Abstract. *Kidneys from fetal mice (10 to 18 days' gestation) were grown in organ culture. Concentrations of potassium less than 9 milliequivalents per liter (10- to 14-day kidneys), and less than 6 milliequivalents per liter (14- to 18-day kidneys), produced abnormal branching, failure of nephron induction, and occasional cystic dilatations of the ureteral bud. These studies emphasize the importance of an environment with a high concentration of potassium for development of the fetal kidney.*

Perey *et al.* (*1*) showed that newborn rabbits and rats injected with adrenocortical steroids on the day of birth developed cystic abnormalities of the kidney. Their data suggested that hypopotassemia, induced by the mineralocorticoids, was responsible for the morphologic changes observed. Microdissection of the kidneys from cystic animals (*2*) showed that the ureteral buds in the nephrogenic zone of the cortex failed to branch normally, did not regularly induce nephron formation, and terminated as blind-ending cystic structures. Although hypopotassemia was a likely cause of the renal defect observed, other possibilities such as a direct effect of the steroid upon the developing kidney were not excluded.

We have studied the effects of mediums with low and normal amounts of potassium on 1000 fetal kidneys grown in organ culture. Fetal mouse kidneys were chosen because their small size and ease of handling minimized the problems of central necrosis, apparently due to hypoxia, which occur during prolonged organ culture. Timed pregnancies of the mice were interrupted at 10 or 11 to 18 days of gestation; the fetuses were removed quickly and placed in Hanks balanced salt solution (*3*). The kidneys were removed with cataract knives under a dissecting microscope and placed on nucleopore membrane filters (*4*), supported by a stainless steel wire mesh in plastic organ-culture dishes (*5*). The cultures were incubated in a water-jacketed incubator at 37°C in 95 percent O_2 and 5 percent CO_2. Some of the cultures