

thelium has been implicated in the rapid uptake and degradation of a variety of metabolites, and angiotensin-converting enzyme has been found in vesicles lining the plasma surface of the endothelium (17). It is possible that carbonic anhydrase is also associated with the plasma surface of the endothelial cells. The presence of additional enzyme within the cells or interstitium is suggested by the finding that  $^{14}\text{CO}_2$  tends to be lost following enzyme inhibition. Presumably, once  $^{14}\text{CO}_2$  has entered the tissue, enzyme inhibition hinders equilibration with tissue  $\text{HCO}_3^-$  and the gas escapes into the alveoli.

The relatively small differences found between the  $^{14}\text{CO}_2$  and  $\text{H}^{14}\text{CO}_3^-$  curves under control conditions appear to be due in part to failure of some of the perfusion fluid to be adequately exposed to tissue carbonic anhydrase. Evidence for this was obtained in three additional studies in which bovine erythrocyte carbonic anhydrase (5 mg/100 ml, 4000 Wilbur-Anderson units per milligram; Sigma) was added to the perfusion fluid after the control runs. This served to reduce  $r$  value differences by 22 to 73 percent. Residual differences after addition of enzyme may be related to incomplete mixing of the injection solutions. A simple model calculation suggests that no less than 86 percent of the plasma flow through the pulmonary vasculature has access to carbonic anhydrase (18).

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10. If exposure of the  $^{14}\text{C}$ -labeled compound to carbonic anhydrase only occurred before it arrived at the capillaries, 95 percent would be in the form of  $\text{HCO}_3^-$  at the exchange site and the control curves would resemble those of  $\text{HCO}_3^-$  after the administration of acetazolamide. Exposure

to enzyme beyond the exchange surface (in the pulmonary veins) would have no effect on tissue distribution.

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14. Both  $^{22}\text{Na}^+$  and  $^{36}\text{Cl}^-$  have access to a small portion of the extravascular volume accessible to  $^3\text{H}_2\text{O}$  during a single transit through the lung:  $r_{22\text{Na}^+} 0.10 \pm .02$  (standard error of the mean)

and  $r_{36\text{Cl}^-} 0.11 \pm .02$  in a series of five runs in five lungs in which both isotopes were incorporated.

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18. It was assumed that a fraction of the capillaries have no carbonic anhydrase and therefore have  $r$  values for  $^{14}\text{CO}_2$  and  $\text{H}^{14}\text{CO}_3^-$  corresponding to these  $r$  values after the administration of acetazolamide. The remainder of capillaries have  $r$  values equal to the average of control values for  $^{14}\text{CO}_2$  and  $\text{H}^{14}\text{CO}_3^-$ .
19. The anaerobic collector was funded by the California Lung Association. There was additional support from NIH grants HL-18606 and 5K04 HL-00132-04.

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## Inherited Medullary Thyroid Carcinoma: A Final Monoclonal Mutation in One of Multiple Clones of Susceptible Cells

**Abstract.** *Inherited medullary thyroid carcinomas contain one form of glucose-6-phosphate dehydrogenase (G6PD) in black female patients who are mosaic in normal tissues for G6PD types A and B. The same individual may have several tumors each containing either G6PD A or G6PD B. The data suggest that the inherited defect is an initial mutation producing multiple clones of defective cells; each tumor then arises as a final mutation in one clone of these cells.*

Recently we reported that medullary thyroid carcinoma and pheochromocytoma tissue obtained from a black female patient heterozygous for glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49; G6PD) forms A and B contained only the B form of the enzyme (1). These data gave biochemical evidence that the final mutation in formation of these inherited tumors was a clonal event. In Knudson's theory of genetic tumor formation (2) at least two mutational events must occur; the first mutation is the inherited defect which renders the involved cells susceptible to neoplastic change. The second event is a somatic mutational change which completes tumor formation. Our above data for G6PD types define only the nature of the final mutational event for inherited medullary thyroid carcinoma. In the present report, we give biochemical evidence that the initial inherited mutation in this disease produces multiple clones of cells susceptible to neoplastic change; each tumor formed is then the result of one or more mutational changes in one clone of the susceptible cells.

To continue our studies of the A and B forms of G6PD in black female patients with inherited medullary thyroid carcinoma we have used electrophoresis as in (1) according to the method of Ellis and Alperin (3) with the following modifications. The normal and tumor tissues were homogenized in 0.1M tris-HCl buffer (pH .8) containing 2 mM NADP (4)

and centrifuged at 4°C, 2000 rev/min; the supernatant was centrifuged again. The final clear supernatant gave discrete bands of G6PD types A and B with a minimum of trailing during electrophoresis. Tissues obtained at surgery were either used immediately or stored at -70°C for no longer than 1 month.

Four patients have now been studied for the clonal origin of inherited medullary thyroid carcinoma. The first patient was described previously (1) and the three others are from another black kindred. In patients 1, 2, and 3 (Fig. 1), the normal tissues studied (red blood cells in all patients and thyroid in two) contained equal amounts of both forms of G6PD while all the tumor tissues sampled contained either the A or B G6PD. In two of these three patients, only one thyroid tumor was available for study while patient 3 had small lesions in both lobes of the thyroid gland. In each case, the entire available tumor tissue was used for the analysis of G6PD forms. The data confirm our earlier suggestion that the final mutation in the formation of inherited medullary thyroid carcinoma occurs as a clonal event (1).

In patient 4 (Fig. 2), larger amounts of tumor tissue from both sides of the thyroid gland were available for study. This patient was also important because studies of her red cells (Fig. 2) indicated an unequal heterozygosity for the A and B forms of G6PD, with a predominance of the B form. This type of the hetero-

zygote state for G6PD has been described (5). The lesion in the right thyroid lobe was a small single nodule, while the tumor from the left was much larger and appeared to contain several nodules which had begun to coalesce. In study 1 (Fig. 2) a section was taken from one area of the left tumor and one-half of the right nodule was used. In duplicate runs tissue from the tumor on the left contained only G6PD A, and tissue from the tumor on the right contained only G6PD B. A small remnant of left normal thyroid tissue gave the same AB pattern as the patient's red cells. In study 2 (Fig. 2) we used the remainder of the right nodule and several further sections from the left. The right tumor again contained only G6PD B, while one sample from the left gave a strong AB pattern and the rest of the samples showed B only. The fact that this patient's red cells contained a marked predominance of B over A made it unlikely that the strong A band in study 1 (Fig. 2) and the equal intensity A and B bands in study 2 came from normal tissue contaminating the tumor lesion. In order to further rule out such contamination, we compared the biochemistry of the AB homogenate to one of the sections which contained G6PD B only. The activity of histaminase (E.C. 1.4.3.6), an enzyme found in medullary thyroid carcinoma (6), was measured and compared to the protein content of each homogenate. The specific activity of the G6PD AB homogenate was 49 unit/mg, and of the G6PD B homogenate, 44.3 unit/mg; multiple studies of normal thyroid in the past have never yielded values higher than 0.03 to 1.5 unit/mg (6). Both sections therefore appeared to contain predominantly tumor tissue in equal amounts. The AB pattern apparently resulted from our obtaining a section of tissue which contained a combination of two tumor nodules. It is possible that the mixed pattern could represent the existence of a multiclonal tumor in this patient. This seems unlikely given our abundance of data indicating clonal origin of the medullary carcinoma in the other patients and of the tumor on the right side in this patient. Also, in study 3 (Fig. 2) in this patient, the remainder of the large left tumor was sectioned and each of four homogenates contained only G6PD B.

The data from patient 4 (Fig. 2) now place the final mutation into perspective with the initial inherited defect in the formation of inherited medullary thyroid carcinoma. The finding of tumors that contain either A or B G6PD in this individual indicates that the inherited muta-

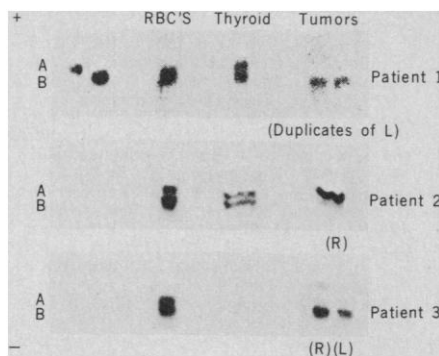


Fig. 1. The electrophoretic patterns of G6PD in normal and tumor tissues from the first three patients studied (L, tumor lesion removed from the left side of the thyroid; R, tumor lesion from the right side of the thyroid). In all studies, thyroid and tumor tissues were always run together at the same time. The red cells were usually run separately to screen the patient for G6PD heterozygosity.

tion results in the production of multiple clones of cells susceptible to neoplastic change; these are the developing cells which subsequently form the parafollicular cells of the thyroid. The parafollicular cells are thought to arise embryologically from a portion of the neural crest (7). We previously speculated (1) that if all inherited medullary thyroid carcinoma and pheochromocytoma tissue, from black individuals heterozygote for G6PD A and B, always contained the same G6PD form in a given patient, this would be evidence that the initial inherited defect occurred as an early monoclonal mutation in the developing neural crest. A small clone of defective stem cells from which tumors can eventually

arise would then populate the thyroid and adrenal regions. The data described here suggest, however, that our alternative proposal is the case (1). Multiple cells of the developing neural crest containing both the A and B forms of G6PD inherit the initial mutation which render them susceptible to neoplastic change; these cells then are at increased risk for tumor formation. Each tumor subsequently arising in the thyroid area must then originate from a single or a very small clone of the genetically susceptible cells.

Our data provide biochemical evidence for an inherited tumor in man arising through a combination of events that render multiple clones of cells susceptible to a final monoclonal mutation. The final lesions of inherited trichoepitheliomas (8) and neurofibromas (9) appear to be of multiclonal origin by analysis of G6PD isoenzymes. This suggests that the final form of these tumors originates directly from multiple clones of mutated cells. These tumors then may not follow the mutational development pattern proposed by Knudson for other inherited tumors (2), and the final tumor lesions may be the direct result of the initial inherited multiclonal mutation. Alternatively, the mutational events subsequent to the initial inherited event might occur simultaneously in multiple clones of the susceptible cells. Trichoepitheliomas and neurofibromas are benign lesions, whereas medullary thyroid carcinoma is a malignancy. This fact alone, however, cannot account for the different G6PD findings since we have found inherited pheochromocytoma, a benign lesion, to have a clonal origin (1).

Further studies of G6PD in black female patients with inherited medullary thyroid carcinoma, pheochromocytoma, and parathyroid hyperplasia [multiple endocrine neoplasia type II (MEN-II)] and with other forms of multiple endocrine neoplasia may help to elucidate the events leading to tumor formation. In MEN-II syndrome, final tumor formation of medullary thyroid carcinoma and pheochromocytoma may be preceded by a stage of hyperplasia (10). It will be of interest to know whether the hyperplasia develops directly from the multiple clones of susceptible cells arising in the initial inherited event or whether the subsequent monoclonal mutations have already occurred in the hyperplastic areas. Also, the nature of the parathyroid lesions in MEN-II remains to be defined. There is evidence (11) that the parathyroid hyperplasia or adenoma formation is a direct manifestation of the in-

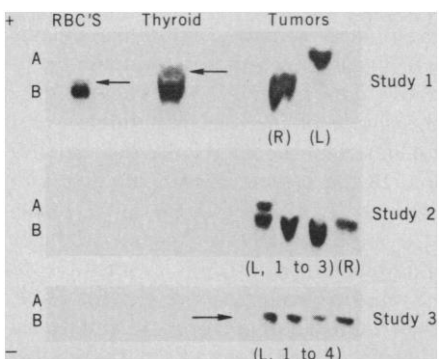


Fig. 2. The electrophoretic patterns of G6PD in normal and tumor tissues from patient 4. Freshly prepared tissue homogenates were used for each study; as in Fig. 1, the red cells were studied separately. The symbols L and R are used as in Fig. 1, and the numbers next to the symbols in studies 2 and 3 refer to separate cuts of tumor tissue from that side of the thyroid. The arrows in study 1 point to the A band in an electrophoretic study of the patient's red cells; the arrow in study 3 points to the B position for G6PD.

herited defect rather than a secondary event. However, it is not known whether the development of the parathyroid glands is from the neural crest or the "APUD" (amine, precursor uptake, L-DOPA decarboxylase) series of cells (12) which is thought to give rise to the other lesions in the MEN-II syndrome. Investigation of the G6PD content of the parathyroid tumors in black heterozygotes might help settle the question of whether these tumors follow the same pattern of mutational events as does the medullary thyroid carcinoma in these patients. We had no clinical reason to remove parathyroid tissue from three of our patients, and a hyperplastic parathyroid from the fourth was not available for study (1).

Patients with multiple endocrine neoplasia type I inherit defects which result in simultaneous parathyroid, pituitary, and pancreatic tumors (13). Studies of G6PD isoenzymes might indicate whether the series of mutational events for these neoplasms are similar to those for inherited medullary thyroid carcinoma.

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## Ammonia Intoxication in the Near-Adult Cat as a Result of a Dietary Deficiency of Arginine

**Abstract.** Near-adult cats, fasted overnight, and given a single meal of a complete amino acid diet without arginine, developed hyperammonemia and showed clinical symptoms of ammonia toxicity within 2 hours. One cat (2.7 kilograms) died 4.5 hours after ingesting only 8 grams of the diet. Since ornithine also prevented hyperammonemia, it appears that the domestic cat cannot synthesize ornithine.

Despite the popularity of the cat as a household pet in the United States (1) and other Western countries, virtually nothing is known regarding its dietary requirement for amino acids (2). The domestic cat and presumably other felids have a much higher requirement for protein in their diet for maintenance than most other adult mammals; for example, about 20 percent of the dietary calories as protein is required by the adult cat (3) compared to 4 to 8 percent for other mammals, such as the rat, dog, sheep, and man (2, 4). This high requirement for protein appears to be due to the inability of the cat to regulate the activity of the nitrogen catabolic enzymes (such as hepatic transaminases and urea cycle enzymes) (5). Other peculiarities of the cat's nutrition include its inability to un-

dertake the conversions of  $\beta$ -carotene to vitamin A and of tryptophan to niacin, and an inability to synthesize sufficient taurine to prevent central retinal degeneration (6).

In a series of experiments designed to define the indispensable amino acids for the growing kitten, we have been giving kittens the diet shown in Table 1. Individual amino acids have been sequentially deleted from the amino acid mixture, and the effect on food intake, growth, and free amino acid concentration in the plasma was measured. In the course of these studies, arginine was deleted from the amino acid mixture. When kittens were switched (without fasting) from an arginine-containing diet to one without arginine, they rapidly lost weight (about 100 g/day) compared to a gradual weight loss of about 15 g/day when any other indispensable amino acid was deleted from the diet (7). Vomitus was found in the cages, there was hemoconcentration and virtual complete refusal to eat. Similar observations were also made on two further groups of six kittens given an arginine-free diet.

To study further the effect of an arginine-free diet on the cat, 16 individually caged cats (6 to 8 months old) of a mean ( $\pm$  S.E.) body weight of  $2590 \pm 90$  g and from four different litters were used. They had previously received a complete, purified amino acid diet (Table 1) and were divided into two groups on the basis of litter, sex, and body weight. Food was withheld from both groups from 5 p.m. the evening before the experiment. On the morning of the experi-

Table 1. Composition of the semipurified amino acid diet.

Dietary components	Percent of diet
Amino acid mixture*	34.7
Turkey fat	25.0
Starch	19.3
Sucrose	15.7
Salt mixture †	4.0
Vitamin premix (19)	1.0
Choline chloride	0.33
Total	100.0

\*Contained the following amino acids as a percentage of the diet: L-histidine  $\cdot$  HCl  $\cdot$  H<sub>2</sub>O, 1.2; L-isoleucine, 1.8; L-leucine, 2.4; L-lysine  $\cdot$  HCl, 2.8; L-methionine, 1.1; L-cystine, 0.8; L-phenylalanine, 1.5; L-tyrosine, 1.0; L-threonine, 1.4; L-tryptophan, 0.4; L-valine, 1.8; L-arginine  $\cdot$  HCl, 2.0; L-asparagine, 2.0; L-serine, 1.0; L-proline, 2.0; glycine, 2.0; L-glutamic acid, 6.0; L-alanine, 1.0; and sodium acetate, 2.5, to balance the hydrochlorides. †Hegsted's Salt Mix, Nutritional Biochemicals Corp.