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Genetic Variation in the Carbonic Anhydrase Isozymes of Macaque Monkeys. II. Inheritance of Red Cell Carbonic Anhydrase Levels in Different Carbonic Anhydrase I Genotypes of the Pig-Tailed Macaque, *Macaca nemestrina*[1]

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The inheritance of red blood cell levels of carbonic anhydrase isozymes (CA I and CA II) has been studied in different carbonic anhydrase I genotypes of the pig-tailed macaque, Macaca nemestrina. *Quantitation of CA I isozymes in a series of animals indicates that the total CA I concentration is the sum of the average effects of each CA I structural allele and that the average effects are independent of the various allelic combinations. The relative average effects were 0.32:0.95:1.0 for the* CA I^a, CA I^b, *and* CA I^c *structural genes, respectively. It is also demonstrated that the level of CA II is related to the CA I genotypes. Multiple regression analysis demonstrated that each dose of CA I–deficiency gene present decreased the CA II concentration by approximately 30%, with this decrease in CA II level being solely related to the dose of CA I–deficiency gene and not to the level of CA I. The CA I–deficient animals produce CA I products that are similar to the common CA Ia, CA Ib, CA Ic electrophoretic types. Limited mating data indicate that the CA I components in CA I–deficient animals are inherited codominantly.*

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INTRODUCTION

Extensive electrophoretic and quantitative variation for the two isozymes of erythrocyte carbonic anhydrase has been reported for *Macaca nemestrina* (Headings and Tashian, 1971; Tashian *et al.*, 1971).

In the preceding paper (Magid *et al.*, 1973), it was demonstrated that a quantitative relationship exists between the electrophoretic types of CA I and the levels of that isozyme in the erythrocytes and also that there is an association between the concentrations of CA I and CA II.

In this paper, the carbonic anhydrase system in *M. nemestrina* is further characterized, with particular reference to (1) the correlation between CA I concentration and CA I genotypes, (2) the correlation between CA I and CA II concentrations, and (3) the inheritance of CA I electrophoretic types in CA I–deficient homozygotes.

Genetic Nomenclature

The structural alleles at the *CA I* locus will be designated I^a, I^b, I^c, and I^d, and the structural alleles at the *CA II* locus will be designated II^{a_2} and II^b. The CA I–deficiency gene and the normal allele at this locus will be designated X^- and X^+, respectively. For example, an animal that is phenotypically CA Iab will have the genotype X^+I^a/X^+I^b. It will be demonstrated later in this paper that CA I–deficient animals can have CA I electrophoretic types that are apparently identical to the common CA I electrophoretic types. Therefore, CA I–deficient animals whose CA I electrophoretic types are known will have the following genotypes:

$$X^-I^a/X^-I^a,\ X^-I^a/X^-I^b, \text{ etc.}$$

A suppressed CA I structural gene whose CA I electrophoretic type is unknown will be designated X^-I^x.

EXPERIMENTAL PROCEDURES

Materials

Whole blood samples were obtained from a breeding colony of *M. nemestrina* at the Washington Regional Primate Center, Medical Lake, Washington.

Quantitation of Carbonic Anhydrase Isozymes

The concentrations of each carbonic anhydrase form (CA I and CA II) were determined in crude hemolysates with a radioimmunosorbent assay (Magid *et al.*, 1973). CA I phenotypes were determined using vertical starch gel electrophoresis as previously described (Tashian *et al.*, 1971).

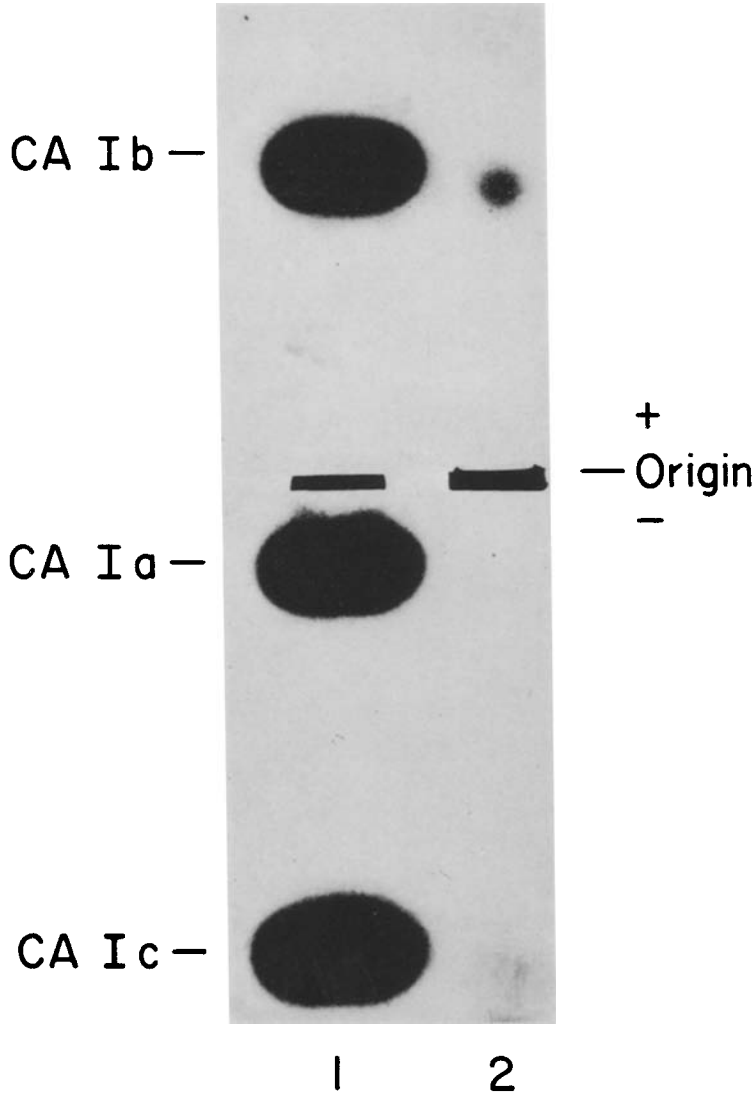

Fig. 1. Starch gel electrophoresis of (1) a marker sample made up of equal concentrations of purified CA Ia, CA Ib, and CA Ic and (2) a concentrated chloroform–ethanol extract of a CA I–deficient hemolysate. The CA I–deficient pattern exhibits esterase staining in the CA Ib and CA Ic regions of the starch gel. The material in these regions cross-reacts with anti–CA I serum.

Multiple Regression Analysis

Multiple regression analysis was performed with the aid of a University of Michigan program (BMDO3R) written for the IBM 360/67 computer. The program was written by the Health Sciences Computing Facility, University of California, Los Angeles.

Detection of CA I Electrophoretic Types in CA I–Deficient Animals

The present technique couples electrophoresis in starch gel with product analysis by the radioimmunosorbent assay (Magid *et al.*, 1973). Two-milliliter samples of hemolysate from CA I–deficient animals were extracted in chloroform and ethanol (Tashian *et al.*, 1966), and the precipitated hemoglobin was removed by centrifugation ($10{,}000 \times g$ for 30 min at 4 C). The supernatant was then concentrated by lyophilization, and this powder was redissolved in 0.2 ml of distilled water. By this procedure, a five- to seven-fold concentration of carbonic anhydrase was realized. The concentrated enzyme solutions were then subjected to vertical starch gel electrophoresis (Tashian, 1969), and the regions corresponding to CA Ia, Ib, and Ic were cut from the gel with the aid of a marker sample containing all three electrophoretic types (see Fig. 1). Each region of starch gel was subsequently homogenized, and th eCA I was eluted into 2 ml of assay buffer (Wide, 1969) and analyzed for CA I cross-reactivity by the radioimmunosorbent assay. This method uses the radioassay qualitatively; any region that inhibits the binding of I^{125}-labeled antigen is considered to contain CA I cross-reacting material. Approximately 10% of a purified CA Ia preparation added to the starch gel at a concentration of 3 μg/ml was recovered in the eluate from the CA Ia region of the starch gel.

RESULTS AND DISCUSSION

Quantitation of Carbonic Anhydrase Isozymes

CA I and CA II isozyme concentrations were recently determined in animals with different CA I phenotypes (Magid *et al.*, 1973). Results of these quantitations indicated a relationship between the CA I phenotype of an animal and its CA I concentration, and also indicated an association between CA I and CA II concentrations. The concentrations of the CA I and CA II isozymes have been determined for an additional series of animals. The results of both studies were combined and are presented in Table I.

An attempt has been made in Table I to group animals with respect to their assumed CA I genotypes, rather than to their CA I phenotypes. This has been done to preserve as much genetic information as possible. Because

Table I. CA I and CA II Concentrations Associated with Each CA I Genotype

Proposed CA I genotype	CA I concentration (μg/mg Hb) $\bar{x} \pm$ SD	CA II concentration (μg/mg Hb) $\bar{x} \pm$ SD
X^-I^x/X^-I^x (16)[a]	0.002 ± 0.002	1.07 ± 0.16
X^-I^x/X^+I^a (19)	2.71 ± 0.68	1.92 ± 0.31
X^+I^a/X^+I^a (3)	6.57	2.25
X^-I^x/X^+I^b (21)	8.09 ± 1.28	1.89 ± 0.32
X^+I^a/X^+I^b (17)	11.02 ± 1.95	2.64 ± 0.35
X^+I^a/X^+I^c (5)	11.54 ± 0.88	2.47 ± 0.22
X^+I^b/X^+I^c (2)	18.50	2.25

[a] Number of animals tested.

of the small family size and long generation time of primates, it is very difficult to identify the genotypes of all animals with certainty. Animals that are heterozygous for different electrophoretic types (i.e., X^+I^a/X^+I^b, X^+I^a/X^+I^c, X^+I^b/X^+I^c) or those homozygous for the deficiency gene X^-I^x/X^-I^x, can be classified from starch gel patterns. Those animals whose phenotype is CA Ia or CA Ib (only one animal with the CA Ic phenotype was found in the mating population) may or may not be heterozygous for the deficiency gene. In most instances, heterozygotes in these classes can be determined using the available mating data. For example, in the case where a CA Ia male is mated to a CA Iab female to produce a CA Ib offspring, it is obvious that the genotype of the male parent is X^-I^x/X^+I^a and that of the offspring is X^-I^x/X^+I^b. The only critical matings that can be used to determine homozygosity for CA Ia or CA Ib animals would be those matings of CA Iab parents to produce CA Ia or CA Ib offspring. The genotypes of these offspring would therefore be X^+I^a/X^+I^a or X^+I^b/X^+I^b, respectively. Unfortunately, matings of this type are rare. However, if all animals of phenotype Ia or Ib are classified as genotypically X^-I^x/X^+I^a or X^-I^x/X^+I^b, respectively, the error of misclassification would be small, since only 15% of all phenotypically Ia or Ib animals (on the basis of calculations from population gene frequencies) are expected to be X^+I^a/X^+I^a or X^+I^b/X^+I^b.

It is obvious from Table I that CA I concentration is correlated with the CA I genotype ($r = 0.94$). Closer inspection reveals the total CA I concentration to be the sum of the average effects of each allele and the average effects to be independent of the various allelic combinations. Determinations of the concentration of CA I electrophoretic types in CA Iab heterozygotes have also been made using quantitative cellulose acetate electrophoresis (Ruis-Reys and Ramirez-Zorilla, 1968) or column chromatography on

DEAE A50 (Tanis *et al.*, 1970). The concentrations of CA Ia and Ib in these heterozygotes are the same as the predicted average effects from Table I.

Similar findings have been reported for human red cell acid phosphatases (Spencer *et al.*, 1964). Three acid phosphatase alleles (P^a, P^b, and P^c) were studied in a randomly selected group of English adults. The relative contribution of each allele to the total acid phosphatase activity was found to be 2:3:4 for P^a, P^b, and P^c, respectively. Whether the activity differences were related to protein concentration differences or to specific activity differences was not determined at that time. Acid phosphatases were later partially purified from the red cells of homozygous P^a/P^a and P^b/P^b individuals (Scott 1966). Kinetic properties of these two enzymes differ slightly, but the differences do not appear sufficient to explain the marked difference in total activity observed in human red cells.

The cause of the concentration difference between CA Ia and CA Ib has not been determined. Evidence to be presented elsewhere (DeSimone *et al.*, 1973*b*) indicates that this difference is not related to different rates of synthesis.

Association Between CA I Genotypes and the Concentration of CA I and CA II

CA II concentration appears to be associated with either the CA I genotype or the CA I concentration, or with both of these factors (Table I). To partition the association, a multiple linear regression analysis was performed with CA II concentration as the dependent variable. The CA I genotype, the CA I concentration, and the interaction term for CA I genotype and CA I concentration were the independent variables. Partial regression coefficients, adjusted for CA I concentration, were computed for two scales of CA I genotype. One scale ranks CA I genotype with respect to the dose of nondeficiency genes present and therefore focuses on the effect of the deficiency gene. The other classification takes advantage of information available from each of the seven CA I genotypes. Values from 1 through 7 were assigned for a direct ranking of the average concentration of CA I for each CA I genotype.

Results of the analysis using the dose of nondeficiency genes for ranking CA I genotype are presented in Table II. The only significant partial regression coefficient is that due to the dose of nondeficiency genes ($P<0.01$). Although the CA I concentration is correlated with the dose of nondeficiency gene (simple correlation coefficient 0.83), the CA I concentration, adjusted for CA I genotype differences, does not appear to be a factor in determining the concentration of CA II. When the analysis was performed using all seven genotype classes, no changes in the regression were observed.

Table III lists the CA II concentrations associated with each dose of the

Table II. Multiple Regression Analysis: Dependent Variable, [CA II]; Independent Variables, [CA I], CA I Genotype Value, and [CA I] × CA I Genotype Value[a]

Variable	Partial regression coefficient	F value	Significance
[CA I]	0.038	1.73	N.S.
CA I genotype value	0.734	61.63	$P<0.01$
[CA I] × CA I genotype value	0.013	1.50	N.S.

[a] Multiple regression equation: [CA II] $= k+b_1$. [CA I] $+b_{.2}\times$(CA I genotype value) $+b_{12}$ [CA I] × (CA I genotype value).

Table III. CA II Concentrations Associated with Dosage of CA I–Deficiency Gene

CA I genotype	CA II concentration (μg/mg Hb) $\bar{x}\pm$SD	Conditional correlation coefficient
X^-I^x/X^-I^x (16)[a]	1.07 ± 0.16	-0.02
X^-I^x/X^+I^{yb} (40)	1.90 ± 0.31	0.040
X^+I^y/X^+I^y (27)	2.53 ± 0.33	0.080

[a] Number of animals tested.
[b] y is a general designation for a nonsuppressed CA I structural allele.

normal (X^+) gene. Also included are the conditional correlation coefficients of CA I concentration on CA II concentration, given the dose of the X^+ gene. Each dose of deficiency gene (X^-) reduces the CA II concentration by approximately 30%, with the effect of dose being additive. The fact that the conditional correlation coefficients are close to zero also indicates that the CA I concentration is probably not a determining factor in the regulation of CA II levels.

Inheritance of Electrophoretically Distinguishable CA I Products in CA I–Deficient Homozygotes

By concentrating large volumes of blood (Headings and Tashian, 1971), it was possible to demonstrate that a small amount of CA I is synthesized in homozygous CA I–deficient animals. A component was distinguishable on starch gel electrophoresis that (1) migrates in the position of CA Ia, (2) has a similar enzymatic activity for β-naphthyl acetate, (3) is inhibited by Diamox, a specific inhibitor of carbonic anhydrase, and (4) is immunologically identical to purified CA I on immunodiffusion. In all bloods

Table IV. Inheritance of CA I Electrophoretic Types in CA I–Deficient Homozygotes

Proposed genotype of male parent[a]		Proposed genotypes of female parent		Proposed genotypes of offspring
X^-I^a/X^-I^c	×	X^-I^b/X^-I^c	→	X^-I^a/X^-I^b
	×	X^-I^b/X^-I^c	→	X^-I^a/X^-I^b
	×	X^-I^b/X^-I^c	→	X^-I^b/X^-I^c

[a] The same male was mated to the three females listed in the second column.

examined, both from pooled samples and single individuals, only a component which could be classified as CA Ia was found.

To determine whether the CA I product in CA I–deficient animals is associated only with the CA Ia electrophoretic type, an additional series of animals was typed by the concentration procedure of Headings and Tashian (1971). A sufficient quantity of blood was available from each of five additional CA I–deficient animals to permit typing. In this small sample of animals, the CA Ia, CA Ib, and CA Ic electrophoretic types were present. In addition, a hemolysate was identified with esterase activity in both the CA Ib and CA Ic regions of the gel (Fig. 1). This indicates that some CA I–deficient animals may also be heterozygous for CA I electrophoretic types. It seems likely, therefore, that the mutation responsible for the CA I deficiency has occurred at a site which is distinct from those responsible for the electrophoretic variants of CA I.

Because it is difficult to obtain large volumes of blood from single animals, a sensitive immunological procedure, described above, was used for additional typing of CA I–deficient animals. The results of the typing of a small series of animals by this procedure demonstrated the presence, in hemolysates, of CA Ia, CA Ib, and CA Ic electrophoretic types.

During the process of screening the breeding colony at the Washington Regional Primate Center for CA I and CA II variants, it was found that three matings were available that might possibly give some information regarding the inheritance of the CA I electrophoretic types in CA I–deficient animals. The mating data presented in Table IV, although based on only three crosses, are nevertheless compatible with a codominant mode of inheritance.

Possible Genetic Control Mechanisms

The results of experiments relating to the quantitative control of the carbonic anhydrases in *M. nemestrina* red blood cells have revealed a complex system

suggesting many possible control mechanisms. It has previously been suggested (Tashian *et al.*, 1971) that the CA I–deficiency mutation acts only in the *cis* position and has the effect of either suppressing the synthesis of CA I or interfering in some manner with the formation or stability of CA I. Since no incorporation of C^{14}-L-serine into CA I from reticulocytes of CA I–deficient animals could be demonstrated (DeSimone *et al.*, 1973*b*), it is possible that (1) CA I may be preferentially lost as newly forming or newly formed molecules, or (2) CA I is synthesized at a 5000-fold reduced rate. The CA I–deficiency mutation affects the concentration of at least three of the CA I electrophoretic types but does not measurably change their catalytic, electrophoretic, or immunological properties. It seems unlikely, therefore, that the CA I–deficiency mutation could cause a gross structural change which renders the CA I molecule to be almost completely unstable and at the same time does not change any of its other chemical properties.

The reduction of CA II concentration associated with the CA I–deficiency phenotype has been shown to result from a decreased rate of synthesis rather than an increased rate of degradation (DeSimone *et al.*, 1973*b*). This observation, together with the finding of a dosage effect of the CA–I deficiency gene on CA II concentration, may have some bearing on the nature of the CA I–deficiency mutation. Since the decreased rate of synthesis of CA II is not related to any feedback mechanism by CA I protein, it may be assumed that the CA I–deficiency mutation exerts its effect through either its mRNA or its DNA. The possibility that reduced synthesis of CA II is related to a control element shared by both CA I and CA II is unlikely, because no *trans* effect of the CA I–deficiency mutation is observed on CA I synthesis by the homologous chromosome.

Carbonic anhydrase isozymes are probably the products of gene duplication (*cf.* Tashian *et al.*, 1972), and their structural genes appear to be closely linked (DeSimone *et al.*, 1973*a*). As such, the CA I–deficiency mutation could exert a polar effect on CA II synthesis.

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