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Comparison of Arginase Activity in Red Blood Cells of Lower Mammals, Primates, and Man: Evolution to High Activity in Primates

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SUMMARY

Arginase activity in red blood cells (RBC) of various mammalian species including man was determined. In nonprimate species, the activity generally fell below the level of detectability of the assay: < 1.0 μmol urea/g hemoglobin per hr. Activities in higher nonhuman primates were equal to or of the same order of magnitude as those in man (approximately 950 μmol/g hemoglobin per hr). RBC arginase deficiency with normal liver arginase activity has been shown to segregate as an autosomal codominant trait in *Macaca fascicularis* established and bred in captivity. This study confirms the presence of this polymorphism in wild populations trapped in several geographic areas and demonstrates the absence of immunologically cross-reactive material in the RBC of RBC arginase-deficient animals. These data when taken together suggest that the expression of arginase in RBC is the result of a regulatory alteration, has evolved under positive selective pressure, and is not an example of the vestigial persistence of an arcane function. The expression of arginase in the RBC results in a marked drop in the arginine content of these cells.

INTRODUCTION

In 1972, Shih et al. discovered some male and female *Macaca fascicularis* at the New England Regional Primate Center with high blood arginine levels [1].

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Upon further investigation, the excess arginine was found to be confined to the red blood cells (RBC) and was due to arginase deficiency limited to the RBC, with normal enzyme activity seen in liver [1]. The deficiency was inherited in an autosomal codominant manner [1]. We extended these studies and showed that arginase activity in the liver of RBC-normal and RBC-deficient animals was identical by eight criteria: (1) pH optimum, (2) pH stability, (3) apparent K_m for arginine, (4) apparent K_i for ornithine and lysine, (5) requirement for and relative response to activation by different divalent cations, (6) heat stability at 68°C and 81°C without and with added manganese, respectively, (7) mobility in native polyacrylamide gel electrophoresis, and (8) immunoprecipitation with rabbit anti-human liver arginase antibodies [2].

Studies in normal humans and in those with hyperargininemia due to liver arginase deficiency demonstrate that normal man has uniformly high red cell arginase activity and that arginase activity is lost concurrently in both the liver and the RBC of affected individuals ([3, 4], and N. Whitman and S. D. Cederbaum, unpublished data, 1975). Immunologically cross-reactive material is present in the RBC of at least three arginase-deficient patients [5]. These data suggest that in man, and by implication in his close relative, *M. fascicularis,* both arginases are transcribed from the same gene locus. As a result, we concluded that red cell arginase deficiency in *M. fascicularis* was the result of a point mutation that resulted in the formation of an enzymatically and structurally unstable enzyme, the activity of which could be renewed in the liver but not in the RBC [2].

More recently, we examined 21 *M. fascicularis* animals largely trapped at different locations in the wild. The results of these studies reported below led us to study limited numbers of other mammalian species. On the basis of these studies, we now conclude that the expression of the liver arginase locus in the red cell evolved under positive selective pressure and that *M. fascicularis* is the transitional species in which this natural regulatory change began to become fixed in the population.

MATERIALS AND METHODS

Materials

All reagents were purchased from commercial sources and were the best grade available.

Animals

M. fascicularis (or, in some instances, their parents) were trapped in the wild and were resident at the Regional Primate Center at Davis, Calif., during these studies. All other primates were residents at the San Diego Zoo and were subjects for other studies for which blood drawing occurred. Other animals were residents in the UCLA vivarium and were the products of commercial sources or local breeding programs.

Red Blood Cells

Blood was usually obtained from anesthetized animals. A variety of intravenously administered agents were used depending upon the species. Although not studied sys-

tematically, work in man and the known properties of arginase did not cause us to believe that the enzyme activity would be adversely influenced by the anesthetics. Our previous experience suggested that it is unlikely that different reagents and protocols used to obtain blood samples in different venues and at different times influenced the results.

Whole heparinized blood (5–10 ml) was obtained from each animal and placed on ice. The blood was spun at 2,500 *g* for 30 min at 4°C and the RBC were then washed twice with 2 vol of sodium phosphate-buffered saline (PBS), pH 7.4. Double distilled deionized water (2–3 vol) was added to the final RBC preparation to rupture the cells, and the hemolysates were stored at −70°C until assay.

Arginase Assay

Arginase activity was determined by the conversion of ureido-labeled-[^{14}C]arginine (Research Products, Mount Prospect, Ill., 52–54 Ci/mol) to [^{14}C]urea and eventually to $^{14}CO_2$ as reported [6]. Hemoglobin concentration was measured using Drabkins reagent [7]. Arginase activity is expressed as μmol arginine hydrolyzed per *g* hemoglobin per hr.

Immunoprecipitation Studies

RBC extract from an animal with high arginase levels was diluted to an activity of 304 nmol/100 ml with 0.1 M Tris, pH 7.4. Each tube contained 20 μl of rabbit anti-human liver arginase, sufficient to precipitate 80%–85% of the arginase activity; 60 μl of undiluted control serum; 0.01 M Tris, pH 7.4, to bring tubes to equal volume; and 0–100 μl of RBC extract from a red cell arginase-deficient monkey, diluted to ½ that of the normal RBC hemoglobin concentration. Controls lacking active enzyme or antibody were also run. The tubes were incubated for 30 min at 37°C and then for 4 hrs at 4°C. The immune complexes were precipitated with *Staphylococcus* protein A (Enzyme Co., Boston, Mass.) by addition of a 10-fold binding excess for 10 min at 22°C. Arginase activity was determined in duplicate 50-μl aliquots of the supernatant. The preparation of rabbit anti-human liver arginase has been described [2].

RESULTS

Arginase Activity in RBC of M. fascicularis

The RBC arginase activity in 21 unrelated individual *M. fascicularis,* most trapped independently in the wild, was measured (table 1). In 11 animals, enzyme activity was higher than 1,500 μmol urea/g hemoglobin per hr, with a mean of 2,184 ± 526. They were classified as normal. Five had intermediate enzyme activities ranging from 88 to 1,150 and were classified as heterozygotes (698 ± 492). Five with levels of 12 or less were thought to be deficient. While misclassification of some individuals assigned to the heterozygote group is possible, it is less important at this time as we draw no inference as to the population genetics of the polymorphism. Definitive classification can be derived only from family studies or from gene studies, once the molecular biology of the polymorphism is understood.

Arginase Antigen Levels (CRM) in Red Cells of Arginase-deficient M. fascicularis

Increasing amounts of arginase-deficient red cell extract failed to displace active arginase molecules from antiarginase-antibody complexes into the supernatant (fig. 1). As the experiment was designed to add a 10-fold excess of

TABLE 1

ARGINASE ACTIVITY IN RBC HEMOLYSATES FROM DIFFERENT INDIVIDUAL *M. fascicularis*

	μmol UREA PRODUCED/g HEMOGLOBIN/HR		
	Identification no.	Activity	Origin
Normal activity			
(mean 2,181 ± 526) ...	1010	2,952	...
	17767	2,950	CPRC*
	17770	1,506	CPRC
	17771	1,700	Malasia
	17775	2,042	...
	17777	1,718	Malasia
	17785	2,034	Malasia
	17792	2,212	Malasia
	17798	2,000	Malasia
	17704	2,530	Malasia
	17824	1,954	Malasia
Intermediate activity			
(mean 698 ± 492)	964†	264	CPRC
	17766	1,174	CPRC
	17869	876	...
	17774†	88	Malasia
	17706	1,086	CPRC
Absent activity			
(mean 6.6 ± 4.0)	958	12.0	...
	1008	6.0	CPRC
	1098	6.0	CPRC
	17868	<1.0	Malasia
	17817	8.0	Malasia

NOTE: The experimental protocol is described in the text.
* Born at the California Regional Primate Center. Other animals trapped at different locations in the wild.
† The heterozygote status of these animals is probable, but not proven.

mutant red cell extract at the highest point, it is possible to conclude that CRM, if present, is at concentrations 5% or less than that in normals.

Arginase Activity in RBC Hemolysates of Different Mammals

Data from 13 mammalian species is presented in table 2. Except for man (in whom we have examined 200 individuals and in whom Naylor has examined 7,000) and rat and rabbit, we have examined only one or two subjects of each species and cannot be dogmatically certain that no polymorphism similar to that found in *M. fascicularis* exists. These data reveal that all lower mammals examined have no detectable red cell arginase activity whereas most primates had substantial arginase levels: > 20 μmol/g hemoglobin per hr. Two exceptions to this were for a baboon species, with no detectable activity in two individuals, and a single orangutan with low activity: < 10 μmol/g hemoglobin per hr. Only *M. fascicularis* and chimpanzees (see DISCUSSION) have the very high levels found in man.

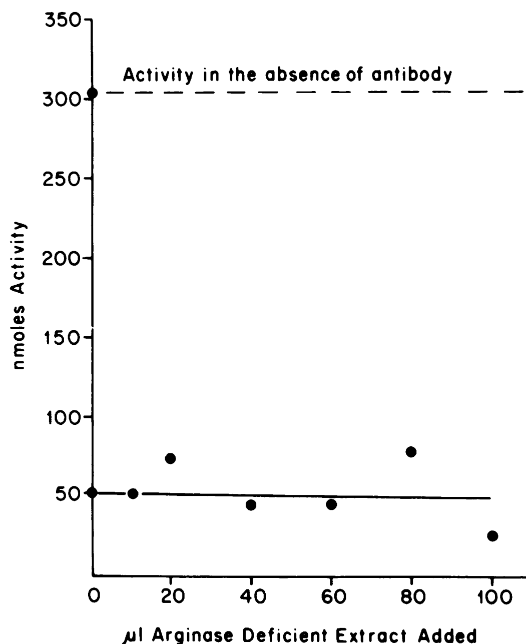

FIG. 1.—Immunoprecipitation inhibition by arginase-deficient *M. fascicularis* RBC extract. Increasing amounts of diluted extract (1:2 in 0.01 M Tris, pH 7.4) from an animal with RBC arginase deficiency was added to a fixed amount of extract from one with full arginase activity, diluted in 0.01 M Tris, pH 7.4, in the presence of rabbit anti-human liver arginase antibody. The activity in the supernatant was determined after staphylococcal protein A precipitation, as described in MATERIALS AND METHODS. At the largest addition of deficient extract, the concentration, on a gram hemoglobin basis, is 10 times that of the extract containing arginase. *The dotted horizontal line* is the residual activity in the absence of antibody, the activity that would be approached if there were significant amounts of material cross-reacting with antiarginase antibody, in the deficient RBC. The figure is composed of the average of triplicate values from the last two of six identical experiments. Although less clean, the other experiments point to the same conclusion. Enzyme activity is expressed as nmol arginine hydrolyzed per g hemoglobin per hr.

DISCUSSION

Autosomal codominant inheritance or arginase expression in the RBC of *M. fascicularis* was well demonstrated by the population and genetic studies of Shih et al. [1]. Our studies reported here demonstrate that this trait is widely distributed in natural *M. fascicularis* populations and that arginase expression does not confer any obvious advantage or disadvantage on the animal who carries it. Twenty-one subjects is too small a group from which to infer accurate population figures, but the crude gene frequency for the phenotypically expressing allele is 64% whereas that for the "silent" one is 36%.

Our previous work confirmed the data of Shih et al. that demonstrated normal arginase levels in the liver of red cell-deficient animals and showed that the properties of the enzyme were not demonstrably different from that in the livers of animals with high RBC activity [1, 2]. In human liver arginase-deficient patients with hyperargininemia, the red cell enzyme is deficient as well. Im-

TABLE 2

ARGINASE ACTIVITY IN RBC HEMOLYSATES FROM DIFFERENT MAMMALS

Species (no.)	Arginase activity (μmol urea/g hemoglobin/hr)
Mouse-Balb/c (2) *Mus musculus*	< 1
Rat (2) *Rattus norvegicus*	< 1
Rabbit (10) *Orcytolagus cuniculus*	< 1
Cat (2) *Felis catus*	< 1
Dog (1) *Canis familiares*	< 1
Douc Langur (1) *Pygathrix nemaeus*	200
Silver Leaf Monkey (1) *Presbytes cristatus*	420
Spider Monkey (1) *Ateles species*	387
Macaca fascicularis (21)	< 1–2,900
Orangutan (1) *Pongo pygmaeuos*	9.4
Baboon (2) *Papio species*	< 1
Gorilla (2) *Gorilla gorilla*	31.0, 20.6
Man (< 200) *Homo sapiens*	957 ± 206

munologic studies in three patients confirm a structural gene defect with normal amounts of CRM as the basis for this deficiency in the red cells and implicitly in the liver [5]. The data therefore explicitly indicate that both RBC and liver arginase are transcribed from the same structural gene. All precedent from comparisons between man and other higher primates suggest that it is highly probable that both liver and red cell activities are encoded in the same gene in *M. fascicularis* as well.

Deficient activity of an enzyme may be due to mutations involving a regulatory function or to mutations involving the sequence of the structural gene itself. Despite the evidence of normalcy of the gene product in liver and especially the absence of excess CRM in liver of RBC arginase-deficient animals, we postulated a structural gene mutation. We proposed that the unstable enzyme was rapidly destroyed but that the protein synthetic capacity of the liver permitted the maintenance of normal activity [2]. This explanation required a great many assumptions, but was made in an intellectual environment in which few mammalian regulatory genes were known and there was little appreciation of the diversity of regulatory potential that might reside in the exons themselves or in flanking or intercalary noncoding (introns) sequences. Our current hypotheses will be summarized below.

If either a regulatory alteration (or rearrangement) or an unprecedented gene duplication had occurred to account for arginase expression in some red cells, it would require, at least, an absence of CRM in arginase-negative cells. The data presented in figure 1 demonstrate that no CRM is demonstrable in arginase-negative red cell extracts. When the sensitivity of the experiments and the relative amounts of extract are considered, we can say confidently that the level of CRM must be less than 5% that found in normal cells.

The data so far discussed point to a polymorphism for a regulatory function that determines the expression of arginase in RBC in *M. fascicularis*. The phenomenon could, however, be either a biologic quirk limited to this species or part of a more general or highly directed evolutionary phenomenon. The universality of high RBC arginase in normal man pointed more to the latter. To answer this question, we sought to augment the more scattered data and occasionally unsupported statements in the literature. Arginase has been reported to be absent in red cells of goats, but not those of cows [8], and to be present in high amounts in chimpanzees [9]. The data in table 2 extend these data. Arginase is absent in the RBC of the lower mammals we examined whether totally herbivorous such as farm animals or rabbits or largely carnivorous such as cats and dogs. Except for the baboon, it is present in all higher primates; in some such as *M. fascicularis* and chimpanzees, it is present at levels comparable to those in man. Once again, the expression of red cell arginase bears no relation to dietary protein content, as *M. fascicularis* is completely herbivorous.

We now believe that the expression of arginase in RBC of higher primates (and in cows) has evolved under positive evolutionary pressure after having been extinguished in most lower mammals. The nature of this pressure is unclear, although marked lowering of red cell arginine levels is seen in *M. fascicularis* in whom the activity is found [1]. In this hypothesis, *M. fascicularis* is the transition species (or one of the transition species) in which this "natural" regulatory change is being fixed in the population, the fixation exemplified by man. Because this regulation appears to be expressed in a cis rather than a trans manner (each locus appears to be expressed independently), it is most probable that the heritable change resides in the gene itself or in its immediate vicinity. Such a change could range from that in a single base pair, permitting transcription in a tissue in which none has previously occurred, to the duplication of the gene with an entirely new set of regulatory signals. For reasons stated above, the latter appears unlikely. We have recently cloned cDNA probes for rat liver arginase and plan to use this probe to elucidate the mechanism of arginase expression in the red cell of *M. fascicularis* and man [10].

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