Species Comparison of Methemoglobin Reductase

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Methemoglobin (MHb) formation is effective in treating cyanide (CN) poisoning. Endogenous activity of the enzyme MHb reductase (MR) reflects the capacity to reduce MHb and thus represents a key factor for evaluating anti-CN efficacy of MHb formers. MR activity was measured in whole blood of nine animal species and was compared with human MR activity. The animals in this comparative study included seven nonhuman primate (NHP) species, the beagle dog, and the ferret. Although exhibiting higher MR activity than in humans, the rhesus and aotus NHPs' average MR activity was the closest to humans', with raw data from each NHP showing overlap with human raw data. The beagle dog, used extensively to study anti-CN characteristics of MHb formers, was the sole species that displayed MR activity lower than in humans, with no data overlap. Based on MR activity, the rhesus and aotus NHPs may each represent a more accurate model for predicting human responses to MHb formers. The data from this study provides a unique interspecies enzyme comparison, which should facilitate future rational development of anti-CN MHb formers. Exp Biol Med 228:79–83, 2003

Key words: methemoglobin reductase; species comparison; nonhuman primates; cyanide; animal models

Cyanide (CN) is a primary nitrile present in all living animals and it also occurs in abundance in many plants such as sorghum, cherry, almond, bamboo, and cassava (1). CN is inexpensive and easy to manufacture from readily available components, and is accessible for a wide array of commercial activities. For example, CN is used in various industrial applications, such as electroplating, mining, and in the production of many synthetic fiber materials such as nylon (1). However, because it shows rapid, profound toxicity at adequate concentrations, CN has also been employed as a chemical threat agent. Although CN may not be an efficient large-scale offensive persistent chemical weapon, it has been used in military and terrorist operations (2), and it remains a recognized chemical warfare threat (3, 4). Historical use of CN as a military or terrorist weapon has been reviewed elsewhere (1, 5).

Due to CN's toxic nature and rapid onset of action, treatment must be administered as soon as possible after poisoning. Clinically, a prophylactic could be developed and administered prior to exposure (1). Many compounds that form methemoglobin (MHb) effectively counter CN toxicity (6–14). Mechanistically, CN has a stronger affinity for MHb than for cytochrome oxidase (15), the putative molecular receptor for CN toxicity (1), to form cyanomethemoglobin. Because of CN's toxicity profile, a long-acting MHb former has been recommended, potentially for use as a pretreatment (6, 16, 17). However, MHb itself cannot transport oxygen, and careful monitoring of MHb levels minimizes harmful side effects that can occur when MHb levels rise above about 20% of the total hemoglobin. These side effects include dyspnea, exercise intolerance, headache, fatigue (~20%–50% MHb), tachypnea, seizures, central nervous system depression and coma (~50%–70% MHb), and ultimately death (>70% MHb).

An MHb former for use as an anti-CN pretreatment is under consideration. In the 1940s, some cautioned against using an MHb former as a CN pretreatment in a military setting because of the potential reduction in the overall physical performance efficiency of military personnel pretreated with an MHb former (18). Nevertheless, newer MHb formers are now available, and research evaluating this approach continues. As part of this effort, the most appropriate and practical available nonhuman model must be identified. Model selection criteria for studies evaluating MHb formers include physiological and genetic enzyme profile similarities with humans (19).

Endogenous activity of the enzyme MHb reductase (MR) reflects an organism's capacity to reduce MHb and is therefore an important factor in evaluating the anti-CN efficacy of MHb formers. Under normal conditions, this NADH-dependent enzyme (also referred to as NADH MR,

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ferricyanide reductase, NADH diaphorase, or cytochrome b_5 reductase) is the only system within the erythrocyte that maintains hemoglobin in its oxygen-carrying reduced state. Toxicologically, MR is the rate-limiting enzyme controlling the toxicokinetics of the reduction of MHb, thus directly affecting the anti-CN efficacy of MHb formers. Species with lower MR activities convert MHb back to hemoglobin slower than do species with higher activities (20). It is noteworthy that a second form of this enzyme, which is NADPH dependent, is less active in mammals in converting MHb back to hemoglobin than is the NADH-dependent form (20). Deficiencies in the reductase systems and resulting clinical manifestations have been described elsewhere (21).

MR has been measured in humans, as well as in a variety of nonhuman species, including cattle, cat, dog, fruit bat, goat, guinea pig, horse, mouse, Northern brown bandicoot, platypus, rabbit, rat, red kangaroo, sheep, short-beaked echidna, wallaby, and wombat (22–24). Although studies evaluating MHb formers have been conducted in nonhuman primates (NHPs) (25–27), MR activity in NHPs has not been systematically evaluated (28, 29). Lacking specific data, Marrs *et al.* (30) speculated that MR activity in NHPs would likely resemble that observed in man, rather than the higher activity in species such as rodents.

This study evaluated normal MR activity in various NHP subspecies, and compared their MR activity with normal human MR activity. For additional comparison, MR was also evaluated in two other species, the ferret, and the beagle dog, the latter of which is often used in MHb studies (30). Presumably, the species with MR activity that most closely resembles that of man would provide the closest model to man for development of an effective, safe MHb class of CN pretreatments or improved antidotes and their active metabolites (31, 32).

Material and Methods

Whole blood samples from humans (Mayo Medical Laboratories, Rochester, MN), NHPs (United States Army Medical Research Institute of Chemical Defense [USAMRICD], the Southwest Regional Primate Research Center, and the New England Regional Primate Research Center), ferrets (Armed Forces Radiobiological Research Institute [AFRRI]), and beagle dogs (the Walter Reed Army Institute of Research [WRAIR] and AFRRI) were collected in acid citrate-dextrose (ACD) tubes (Table I). All samples were shipped cold (not frozen) overnight to the Mayo Medical Laboratories where normal, endogenous MR activities were determined using a standard enzyme-catalyzed reaction as depicted in Figure 1. Under these conditions, the samples are stable for 2 days at ambient temperature and for more than 20 days at 4°C. The standard Mayo Medical Laboratory MR assay was performed at 30°C for 12 min in the presence of Tris-HCl buffer, 2 m*M* NADH with or without 2 m*M* $K_3Fe(CN)_6$, and a 1:20 hemolysate mix as described previously (33–35). NADH oxidation was analyzed spectrophotometrically at 340 nm.

Preliminary *t* tests were performed as necessary to assess whether gender (i.e., beagle dogs, chimpanzees, and African Green NHPs only), anesthesia condition (AFRRI beagle dogs only), and/or laboratory (i.e., beagle dogs from WRAIR or AFRRI) were a significant source of variability. An analysis of variance (ANOVA) was subsequently performed on all species and a Dunnett's test was used to compare the human MR data against that of the other spe-

Table I. Test Groups for Measurement of Endogenous MR Activity

	n	M/F	Anesthestic(s)
Human data set			
Human *(Homo sapiens)*	30	NA	NA
Nonhuman primate data set			
African Green *(Chlorocebus aethiops)*	19	15/4	Telazol (3.0 mg/kg)
Aotus *(Aotus sp.)*	12	11/1	Ketamine (25 mg)
Baboon *(Papio anubis)*	10	10/0	Ketamine (10.0 mg/kg)
Chimpanzee *(Pan troglodytes)*	6	3/3	Telazol (10.0 mg/kg)
Cynomolgus *(Macaca fasicularis)*	6	6/0	Acepromazine (0.7 mg/kg) Ketamine (7.0 mg/kg)
Marmoset *(Callithrix jacchus jacchus)*	6	6/0	Ketamine (0.2 ml)
Rhesus *(Macaca mulatta)*	15	15/0	Telazol (3.0 mg/kg)
Other nonhuman species data set			
Ferret *(Mustela putorius furo)*	5	5/0	Ketamine (0.25 mg/kg) Xylazine (2.0 mg/kg)
Beagle *(Canis familaris, beagle)* (WRAIR)	15	12/3	No anesthesia
Beagle *(Canis familaris, beagle)* (AFRRI)[a]	7	0/7	No anesthesia
Beagle *(Canis familaris, beagle)* (AFRRI)[a]	7	0/7	Acepromazine (0.2 mg/kg) Diazepam (0.025 mg/kg) Ketamine (5.0 mg/kg) Isoflurane (1.5–2.0%)

[a] This single group of beagle dogs was sampled both under anesthesia as well as with no anesthesia in order to ascertain whether anesthesia affected MR activity. M, male; F, female; NA, not available.

$$2\,K_3Fe(CN)_6 + NADH \xrightarrow[\text{reductase}]{\text{ferricyanide}} 2\,K_2Fe(CN)_6 + NAD^+ + H^+$$

Figure 1. Principle of the MR assay conducted at Mayo Medical Laboratories (Rochester, MN). The activity at 30°C was followed by spectrophotometric analysis by measuring oxidation of NADH at 340 nm.

cies. For all analyses, statistical significance was maintained at $P < 0.05$.

Results

MR activity was quantified as International Units per gram of hemoglobin (IU/g Hb) following Mayo Medical Laboratories' standard procedures, and as used by others (36). See Beutler (33) for a more detailed description of the use of this unit of measurement. Analyses (*t* tests) indicated no significant effect of gender on MR activity. Therefore, MR activity data sets for the WRAIR beagles, chimpanzees, and African green NHPs were collapsed across gender for subsequent analyses. In addition, paired *t* tests conducted on the MR activity in beagle dogs sampled with or without anesthesia revealed no significant differences. Therefore, for subsequent analyses on these data, anesthesia condition was not included as a variable, and values for groups were averaged. A *t* test indicated that the MR activity in the beagle dogs from WRAIR was significantly higher than that observed in the beagle dogs from AFRRI. Therefore, these two groups of beagle dogs were subsequently evaluated as unique data sets.

The species differed statistically from each other (one-way ANOVA). Dunnett's tests to compare each species with humans indicated that all species were significantly different from humans. However, MR data as presented in a Box-and-Whisker plot (Fig. 2) (Box-and-Whisker plots are summary data plots based on the median, quartiles, and extreme values of a data set. The box represents the interquartile range [25th–75th percentiles] that contains 50% of the values. The whiskers are lines that extend from the box to the largest and smallest observed values that are less than 1.5 box lengths from either end of the box. The line across the box indicated the median of the data set. Outliers are 1.5–3 box lengths from the end of the box. Extremes are more than 3 box lengths from the end of the box.) illustrate that rhesus and aotus NHPs were quite similar to humans, with a great deal of overlap. Using the 25th and 75th percentiles, the beagle dog, the chimpanzee, the baboon, and the ferret all displayed higher MR activity, and failed to overlap with human MR activity (Fig. 2). However, MR data illustrate that rhesus and aotus NHPs were quite similar to humans, with a great deal of overlap. From raw data, the beagle dog was the only species tested that did not overlap with humans.

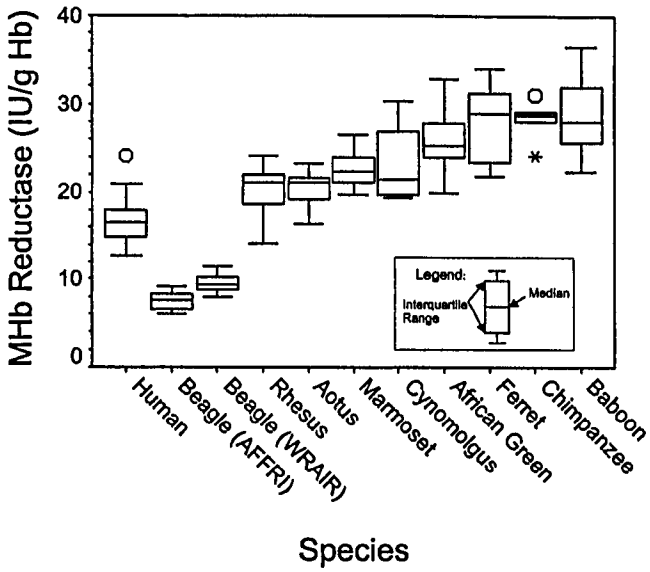

Figure 2. Box-and-Whisker plot of MR data. O and an asterisk represent outlier and extreme scores, respectively.

Discussion

In the present cross-species evaluation of endogenous MR, humans, seven NHP subspecies and two additional comparative nonhuman species were studied. When combined with previous reports (20, 22, 23), these results expand the database of known MR activity among nonhumans, and provide information vital for the selection and successful application of a nonhuman model for anti-CN MHb former research. Among the NHPs, comparison of MR activity revealed differences across subspecies. The rhesus and aotus NHPs exhibited MR activity closest to humans. These data support the continued use of the rhesus NHP in MHb former research (12). The aotus NHP may also be an alternative nonhuman model. The lower MR activity in the beagle dog relative to MR activity in humans, however, suggests caution when using this species as a nonhuman model in this particular area of research.

It is noteworthy that Srivastava and colleagues measured erythrocyte MR activity in a variety of nonhuman species, including the rhesus NHP and the beagle dog (29, 37). In one study (29), beagle dog MR activity was higher than the other species evaluated (rhesus NHP, rat, and mastomys). However, in a more recent study (37), beagle dog erythrocyte MR activity was reported to be significantly lower than MR activity in the rat, mastomys, mouse, and hamster. Furthermore, the beagle dog was described as being relatively MR deficient (37). Although the nature of differing MR results between these two studies (i.e., Refs. 29, 37) remain unclear, our data, showing low MR activity in the beagle dog, would generally support the results of the second study by Srivastava *et al.* (37).

The presence or absence of anesthetic agents was not addressed in previous MR species comparison studies (22, 23). It was determined in the present study that anesthesia did not significantly affect MR activity in the beagle dog. However, this limited evaluation should not preclude a more thorough study of this variable.

The authors recognize that the MR assay used in this study, although widely employed and accepted, uses an artificial substrate and does not measure MR directly. MR activity using this convenient artificial substrate is known to differ in magnitude from that determined using direct measurement; however, relative changes across experimental conditions within each methodology are similar (34).

In conclusion, a careful consideration of MR activity would be most prudent when selecting a nonhuman model for MHb studies. Data support the comparability of both the rhesus and aotus NHPs to humans, as MR activities were most similar. Relatedly, Huser (38) suggested that for hematologic studies, the rhesus NHP is the most preferable NHP model for man. Finally, the rhesus NHP data indicate that from a comparative point of view, this species more accurately resembles the human with respect to metabolic disposition (31).

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