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Tissue Distribution and Multiplicity of Enzymes That Generate and Scavenge Reactive Oxygen Species in Japanese Monkey

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ABSTRACT—Five enzymes involved in the generation and scavenging of reactive oxygen species, *i.e.*, NADH/NADPH oxidase, xanthine oxidase (XOD), superoxide dismutase (SOD), ascorbate peroxidase, and catalase were assayed in various tissues of the Japanese monkey. Their activities were largely different between tissues. Generally, small intestine, kidney, and cerebellum contained larger amounts of these enzymes than other tissues. Multiplicities of these enzymes were analyzed by staining of their enzymatic activities after electrophoresis. The number of isozymes was 2 in the case of NADPH oxidase and catalase, and 3 in the case of XOD, SOD, and ascorbate peroxidase. The expression of these isozymes differed between tissues, suggesting the occurrence of tissue-specific systems to generate and scavenge reactive oxygen species in the Japanese monkey.

INTRODUCTION

Reactive oxygen species (ROS) are produced as a consequence of aerobic respiration and substrate oxidation. Major ROS are known to be superoxide anion (O_2^-) and hydroxyl radical (·OH). Several enzymes are known to be involved in generating ROS (Halliwell and Gutteridge, 1984). These enzymes include NADH/NADPH oxidase and xanthine oxidase (XOD). Since ROS are highly reactive (Iyer *et al*., 1961; Tauber and Babior, 1977; Babior 1982), the occurrence of high levels of ROS may cause metabolic malfunctions and damage to biological macromolecules, resulting in the generating of various diseases including cancer (Matés *et al*., 1999.), although low levels of ROS are indispensable in many biochemical processes such as the defense system against micro-organisms (Michell, 1984). The accumulation of ROS in the cell would certainly be prevented. Superoxide dismutase (SOD), catalase, and ascorbate peroxidase are known to be typical enzymes that scavenge ROS. Major processes of the generation and scavenging of ROS and enzymes involved in respective reactions are summarized in Fig. 1. Cellular oxygen radical homeostasis might be regulated by these enzymes.

The levels of enzymes generating and scavenging ROS are known to be different in various tissues (Hashimoto, 1974; Marklund, 1984). In humans, a variety of tissue-specific diseases resulting from the imbalance of antioxidant enzymes have been reported (Matés *et al*., 1999). Since the occurrence of multiple forms has been reported in some of these enzymes (Nishimura *et al*. 1964; Beckman *et al*. 1973), the different expression of multiple forms may be implicated in pathogenesis of diseases. The levels of enzymes to generate and scavenge ROS in tissues might be species-specific, since, for example, SOD activity has been shown to be positively correlated to the metabolic activity of mammals and may have a role in determining their life-span (Tolmasoff *et al*., 1980).

The enzymes generating and scavenging ROS have been scarcely studied in non-human primates such as monkeys and apes. Since monkeys are evolutionarily related to humans, some diseases caused by ROS might be common to both. In this respect, monkeys are thought to be suitable models to study ROS-induced human diseases. These enzymes might also be useful markers to estimate environmental stress on monkeys in different habitats, since monkeys in the wild and/or under captivity are exposed to environmental oxidative stress such as ozone and oxides of nitrogen (Halliwell and Cross, 1994).

In this paper, as a first step to clarify the ROS-regulating system in monkeys, we analyzed the levels and multiplicities of enzymes to generate and scavenge ROS in various tissues of the Japanese monkey. The level of each enzyme was quite different between tissues, and multiple isozymes were found in each enzyme. From these results, ROS-generating and scavenging systems in monkeys are discussed.

MATERIALS AND METHODS

Chemicals

Cytochrome c, nitroblue tetrazolium, NADH, and NADPH, SOD, and XOD were purchased from Sigma Chem. Co., St Louis, MO, 3,3'-diaminobenzidine tetrahydrochloride, ferric chloride, flavin-adenine

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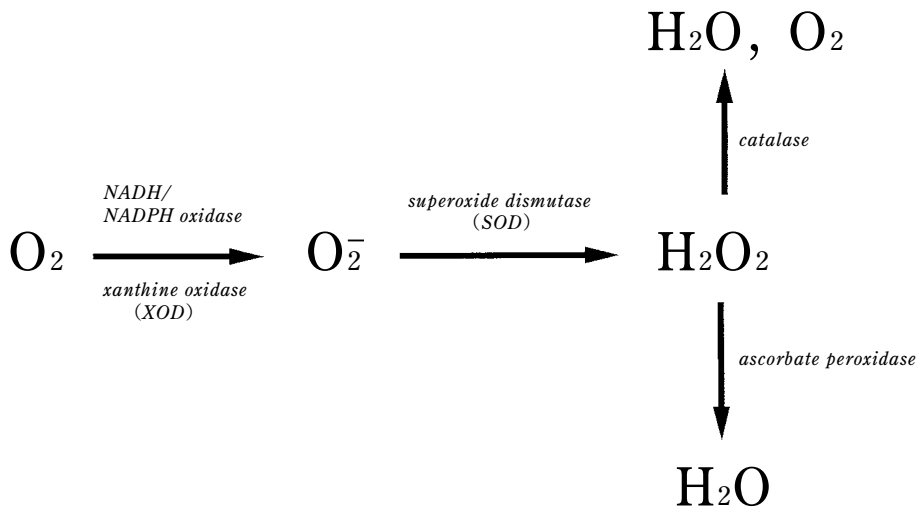

Fig. 1. Major pathways of the generation and scavenging of reactive oxygen species. NADH/NADPH oxidase and xanthine oxidase (XOD) (EC 1.1.3.22) catalyze reactions to produce superoxide anions (O_2^-). Superoxide dismutase (SOD) (EC 1.15.1.1) catalyze the dismutation of O_2^- to H_2O_2. The decomposition of H_2O_2 is catalyzed by catalase (EC 1.11.1.6) and its reduction by ascorbate peroxidase (EC 1.11.1.11).

dinucleotide, nitroblue tetrazolium, potassium ferricyanide, and riboflavine from Wako Pure Ind., Osaka, and ascorbate, tetramethylethylenediamine (TEMED), and xanthine from Katayama Chem., Osaka. All other chemicals were of reagent grade.

Preparation of monkey tissue homogenate

Three young male Japanese monkeys (*Macaca fuscata*) were used. Several ml of blood was collected from the basilic vein by syringe under anesthesia with ketamine hydrochloride. Serum was obtained by centrifugation of the blood. Cerebrum, cerebellum, heart, lung, stomach, small intestine, liver, pancreas, spleen, kidney, and muscle were removed from monkeys immediately after death by exsanguination via bilateral carotid arteries under deep anesthesia with ketamin hydrochloride and sodium pentobarbital, in accordance with guidelines of the Primate Research Institute, Kyoto University. These tissues were stored frozen at –80°C until use. After thawed, tissues were homogenized in 5 volumes of 0.1 M MOPS buffer, pH 7.5, containing 0.1 mM EDTA and 1 mM dithiothreitol with a mechanical homogenizer. Each homogenate was centrifuged at 20,000×g for 5 min and the supernatant was used for assaying enzymatic activities and for detecting enzymatic activities after non-denaturing polyacrylamide gel electrophoresis. Although some of these enzymes such as NADPH and NADH oxidases have been shown to be membrane-associated, most of their activities were recovered in the centrifugal supernatant of the crude homogenate prepared after freezing-thawing (data not shown). For long-term storage, each supernatant was mixed with an equal volume of glycerol and kept at –20°C.

Assay of enzymatic activities

Each assay procedure is described briefly as follows. Incubation temperature was 25°C. Spectrophotometric measurement was carried out with a spectrophotometer (model U-3210; Hitachi, Ltd., Tokyo).

NADH/NADPH oxidase : According to the method of Azzi *et al.* (1975). Reaction mixture contained 20 mM TES-KOH buffer, pH 7.0, 80 μM NADH/NADPH, 40 μM partially acetylated cytochrome c, and an appropriate amount of tissue homogenate supernatant. The increase of A_{550} was measured.

XOD : According to the method of Hashimoto (1974). Reaction mixture contained 10 mM potassium phosphate buffer, pH 7.5, 0.13 mM xanthine, 0.2 mM oxonate, and an approximate amount of tissue homogenate supernatant. The decrease of A_{292} was measured.

SOD : According to the method of Asada *et al.* (1973). Reaction mixture contained 50 mM potassium phosphate buffer, pH 7.8, 0.1 mM xanthine, 0.1 mM EDTA, 10 μM cytochrome c, 0.04% XOD, and an appropriate amount of tissue homogenate supernatant. The increase of A_{550} was measured.

Catalase : According to the method of Beers and Sizer (1952). Reaction mixture contained 90 mM potassium phosphate buffer, pH 7.0, 0.08% H_2O_2, and an appropriate amount of tissue homogenate supernatant. The decrease of A_{240} was measured.

Ascorbate peroxidase : According to the method of Tezuka *et al.* (1997). Reactin mixture contained 50 mM potassium phosphate buffer, pH 7.0, 0.4 mM ascorbate, 0.2 mM H_2O_2, and an appropriate amount of tissue homogenate supernatant. The decrease of A_{290} was measured.

Activity staining of enzymes

Electrophoretic separation of ROS-generating and scavenging enzymes in the tissue homogenate supernatant was carried out using slab gels of polyacrylamide. The composition of the gel was the same as that described by Ornstein (1964) and Davis (1964). Staining procedure of each enzyme activity was described briefly as follows.

NADH/NADPH oxidase : According to the method of Tamoto *et al.* (1983). Staining solution contained 50 mM potassium phosphate buffer, pH 7.4, 10 μM flavin adenine dinucleotide, 2.45 mM nitroblue tetrazolium, and 0.5 mM NADH/NADPH.

XOD : According to the method of Özer *et al.* (1998). The gel was immersed successively in the following 3 solutions for 30, 20, and 10 min, respectively. Solutions 1, 2, and 3 contained 50 mM sodium phosphate buffer, pH 7.0, and 2 mM sodium ascorbate; 50 mM sodium phosphate buffer, pH 7.0, 4 mM sodium ascorbate, and 2 mM H_2O_2; 50 mM Tris-HCl buffer, pH 7.6, 0.5 mM xanthine, 30 mM TEMED, and 2.45 mM nitroblue tetrazolium, respectively.

SOD : According to the method of Beauchamp and Fridovich (1971). The gel was immersed successively in the following 2 solutions for 20 and 15 min, respectively. Solutions 1 and 2 contained 0.245 mM nitroblue tetrazolium; 36 mM potassium phosphate buffer, pH 7.8, 28 μM riboflavine, and 28 mM TEMED. The gel was then illuminated for 10 min.

Catalase : According to the method of Wayne and Diaz (1986). The gel was immersed successively in the following 4 solutions for 30, 20, 10, and 10 min, respectively. Solutions 1, 2, 3, and 4 con-

tained phosphate buffered saline, pH 7.0, and 0.25 mM thimerosal; phosphate buffered saline, pH 7.0, 1.4 mM 3,3'-diaminobenzidine tetrahydrochloride, and 2 mM H_2O_2; 2 mM H_2O_2; 0.16 mM ferric chloride and 0.06 mM potassium ferricyanide, respectively.

Ascorbate peroxidase : According to the method of Mittler and Zilinskas (1993). The gel was immersed successively in the following 3 solutions for 30, 20, and 10 min, respectively. Solutions 1, 2, and 3 contained 50 mM sodium phosphate buffer, pH 7.0, and 2 mM sodium ascorbate; 50 mM sodium phosphate buffer, pH 7.0, 4 mM sodium ascorbate, and 2 mM H_2O_2; 50 mM sodium phosphate buffer, pH 7.0, 28 mM TEMED, and 2.45 mM nitroblue tetrazolium, respectively.

After activity staining, the gel was scanned by using a scanner (model GT-9500; Seiko Epson Corp., Suwa, Japan). All scanned data were treated with NIH-image (ver. 1.62) software.

Protein determination

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

RESULTS

The levels of ROS-generating and scavenging enzymes in monkey tissues

Six enzymes involved in the generation and scavenging of ROS, i.e., NADH/NADPH oxidase, XOD, SOD, ascorbate peroxidase, and catalase, were assayed in various tissues of the Japanese monkey (Fig. 2). Two enzymes generating superoxide anions, i.e., NADH and NADPH oxidases, were contained at high levels in the small intestine and kidney but were at low levels in the liver and spleen. They showed similar tissue distributions except that the level of NADH oxidase was significantly higher than that of NADPH oxidase in the stomach, muscle, and serum. The other superoxide anions-generating enzyme, XOD, was contained in various tissues at high levels in the kidney, pancreas, lung, and cerebellum. The level in the serum was found to be comparable to those in tissues. SOD, which catalyzes the dismutation of superoxide anions to O_2 and less reactive species H_2O_2, was contained at high levels in the kidney and cerebellum, followed by the small intestine. The low level in the lung as well as the serum was marked. Ascorbate peroxidase and catalase are enzymes positioning at the final step of scavenging ROS. Catalase were distributed at high levels in various tissues except for the cerebrum and muscle. Ascorbate peroxidase was contained at high level in the small intestine.

The results of tissue distribution of these six enzymes showed that the levels of most of ROS-generating and scav-

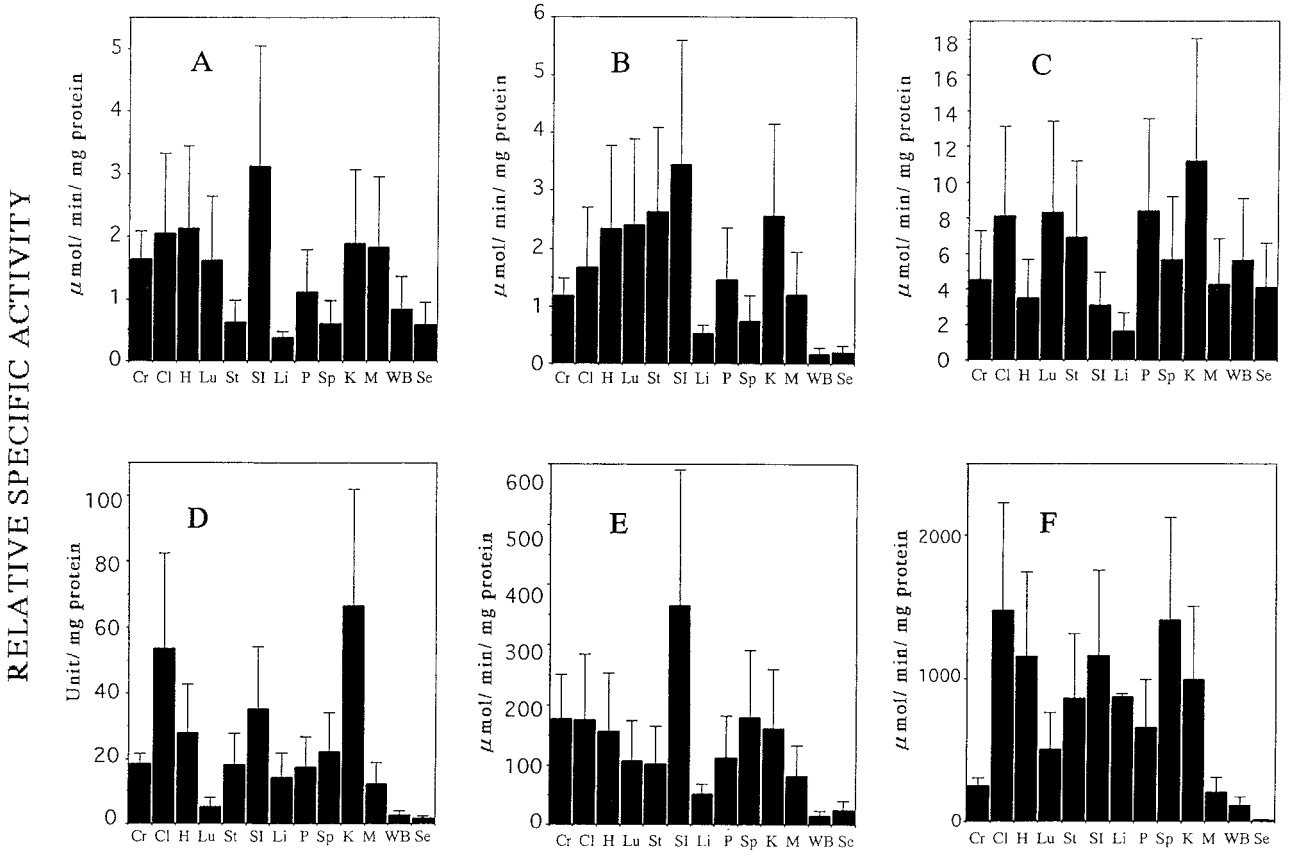

Fig. 2. Relative specific activities of NADH oxidase (A), NADPH oxidase (B), xanthine oxidase (XOD) (C), superoxide dismutase (SOD) (D), ascorbate peroxidase (E), and catalase (F) in various tissues of the Japanese monkey. Activity of each enzyme is expressed as μmol/min under the present assay conditions given in Materials and Methods. Exceptionally, the activity of SOD is shown by units defined by Asada et al. (1973). Relative specific activity was calculated as the total activity/ the amount of the total soluble protein in each tissue. Data are indicated as mean ± SEM. Cr, cerebrum; Cl, cerebellum; H, heart; Lu, lung; St, stomach; SI, small intestine; Li, liver; P, pancreas; Sp, spleen; K, kidney; M, muscle; WB, whole blood; Se, serum

enging enzymes were higher in the small intestine, kidney, and cerebellum than in other tissues. Lung and muscle were specified as that the levels of ROS-generating enzymes were relatively high between tissues and those of ROS-scavenging enzymes were relatively low. In the liver, the levels of both ROS-generating and scavenging enzymes were low. The results also showed that the levels of ROS-scavenging enzymes seem to be higher than those of ROS-generating enzymes in each tissue, although, since the assay condition of each enzyme differed, it was difficult to compare the activity values between enzymes.

Multiplicity of ROS-generating and scavenging enzymes

Some isozymes were found in each enzyme by activity staining. The typical isozyme bands of each enzyme are shown in Fig. 3 and the entire results are summarized in Table 1. The results of five enzymes except for NADH oxidase are given, since the activity staining of NADH oxidase was unsuccessful.

NADPH oxidase : Two isozyme bands were detected in all tissues but not in whole blood and serum. The relative intensity of these two bands differed between tissues, and the tissues where the band-1 was stained more intensely than band-2 were the cerebellum, small intestine, spleen, and kidney. Only band-2 was detected in whole blood. Since no appreciable band was found in the serum, the band in whole blood may be derived from blood cells.

XOD : Two bands were detected in most tissues except for the muscle, where an additional slowest-moving band (band-3) was detected. The fast-moving band-1 isozyme was more intense than the slow-moving band-2 isozyme in the lung, stomach, pancreas, and serum. The reverse relationship was found in the spleen and kidney. The slowest-moving band was shown to be stained more intensely than the other two bands in the muscle.

SOD : Three activity bands were detected in all tissues except for the serum where no band was detected. The slowest-moving band (band-3) was stained most intensely among 3 bands in several tissues including the cerebrum and cerebellum. Exceptionally, in the heart, the fast-moving band (band-1) was stained most intensely. The intensity of band-2 was weaker than those of the other two bands in most tissues.

Ascorbate peroxidase : Two bands were detected in all tissues except for the serum. In the lung, stomach, small intestine, spleen and whole blood, slow-moving band (band-2) was stained more intensely than fast-moving band (band-1). In the cerebrum and kidney, the reverse results were obtained. In the serum, only band-1 was detected.

Catalase : Two bands were detected in all tissues but not in whole blood and serum, although the intensity of band-2 was very weak in most tissues. The intensity of band-2 was

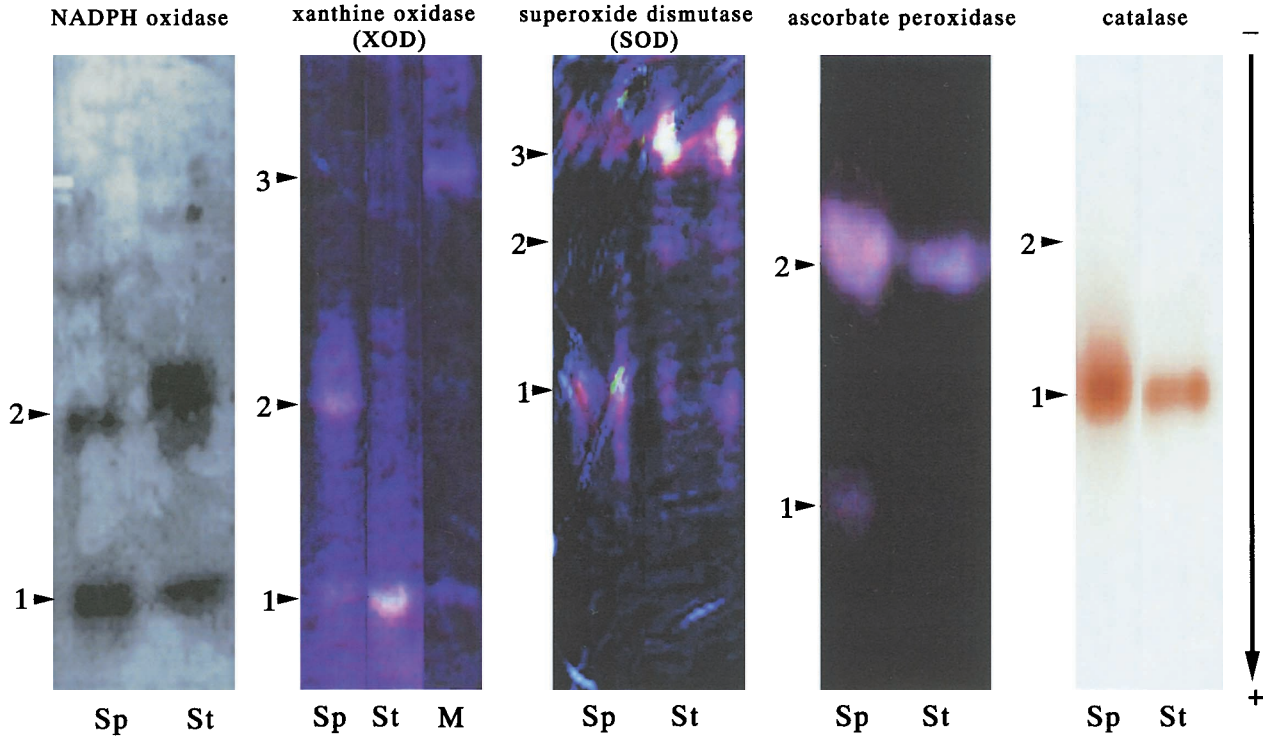

Fig. 3. Typical results of activity staining of ROS-generating and scavenging enzymes. The results of spleen (Sp) and stomach (St) are given, with the result of muscle (M) in the case of xanthine oxidase. A portion of the crude homogenate supernatant was subjected to electrophoresis on a 7% polyacrylamide gel in Tris-glycine buffer, pH 8.3. The staining procedure of each enzyme is given in Materials and Methods. The upper half of each gel is shown since no activity band was detected in the lower half. Since the band-2 of catalase was scarcely observed in the spleen and stomach, only the position of this band is given.

Table 1. Relative levels of multiple forms of NADPH oxidase, xanthine oxidase (XOD), superoxide dismutase (SOD), ascorbate peroxidase, and catalase in various tissues of the Japanese monkey estimated from activity staining of respective enzymes after polyacrylamide gel electrophoresis. Typical samples of stained gels are given in Fig. 3. Cr, cerebrum; Cl, cerebellum; H, heart; Lu, lung; St, stomach; SI, small intestine; Li, liver; P, pancreas; Sp, spleen; K, kidney; M, muscle; WB, whole blood; Se, serum

	Cr	Cl	H	Lu	St	SI	Li	P	Sp	K	M	WB	Se
NADPH oxidase													
band-1	+	++	+	++	+	+++	+	+	++	++	+	−	−
band-2	+	+	++	+	++	+	+	+	+	+	+	++	−
XOD													
band-1	+	+	+	++	+++	+	+	++	+	+	+	++	++
band-2	+	+	+	+	±	+	+	+	++	++	±	++	+
band-3	−	−	−	−	−	−	−	−	−	−	++	−	−
SOD													
band-1	+	+	+++	+	+	+	+	+	++	+	+	+	−
band-2	±	±	±	±	±	+	±	±	±	+	±	+	−
band-3	++	+++	++	+	++	++	++	++	++	+++	+	+	−
ascorbate peroxidase													
band-1	++	+	+	±	±	+	+	+	+	++	+	+	+
band-2	+	+	+	++	++	++	+	+	+++	+	+	+++	−
catalase													
band-1	++	++	+	++	++	+	+	+	+++	+	++	+++	−
band-2	±	±	±	±	±	±	+	±	±	+	±	−	−

comparable to that of band-1 only in the liver and kidney. No band was detected in the serum.

DISCUSSION

First we discuss the distributions and multiplicities of 6 typical enzymes to generate or scavenge ROS. NADH and NADPH oxidases are known to be membrane associated enzymes. Although they have been shown to be different enzymes, a complex formation has been reported in some tissues/cells such as endothelial cells (Bayraktutan *et al*., 1998). Their similar distribution in monkey tissues might show that they act as a complex enzyme system. NADH/NADPH oxidase has been studied extensively in blood cells such as macrophages and neutrophils, since they have an important role of defense against micro-organisms in blood by generating ROS (Michell, 1984). High levels of these enzymes in the small intestine and lung may agree well in that these tissues have similar defense systems against infection of environmental micro-organisms. The occurrence of a single electrophoretic band of NADPH oxidase has been reported in guinea-pig polymorphonuclear leukocytes (Tamoto *et al*., 1983), being consistent with the results obtained in Japanese monkey blood. In most monkey tissues, however, the other slow-moving band was found. The multiplicity of NADPH oxidase might correlate with the complicating structure of this enzyme.

XOD, as well as NADH/NADPH oxidase, is known to be an important enzyme to generate ROS. This enzyme has been shown to be localized in hepatocytes, gastrointestinal mucosal cells and endothelial lining cells of various tissues in humans (Moriwaki *et al*., 1993). Similar results have been obtained in some other mammals (Huh *et al*., 1976). High levels in the monkey lung, kidney, and stomach are consistent with these results, although relatively low levels in the monkey small intestine and liver gave inconsistent results. Electrophoretic analysis of XOD from various tissues of the horse (Seeley *et al*., 1984) and humans (Duley *et al*., 1985) has shown that this enzyme exists as a single form. It was found in the present study, however, that three forms of this enzyme exist in Japanese monkey. The high resolution of XOD isozymes might be due to the application of the sensitive analytical method of Nazmi *et al*. (1998). These different forms might be products of different genes and might cause the occurrence of tissue-specific ROS-generating systems in monkeys.

SOD is an important enzyme to scavenge superoxide anions to less reactive H_2O_2. Three different isozymes, Cu/Zn-SOD, Mn-SOD, and extracellular SOD are known. Since the level of extracellular SOD is known to be much lower than those of the other two forms, the levels of SOD in monkey tissues in the present study may be summed values of levels of Cu/Zn-SOD and Mn-SOD. Deducing from the results of human SODs, these two SODs were visualized separately by activity staining after electrophoresis. The fast-moving band-1 and slow-moving band-3 were thought to be Mn-SOD and Cu/Zn-SOD, respectively (Yasuyama *et al*., 1988). The intermediate band-2 might be an isoform of Cu/Zn-SOD, and is unlikely to be extracellular SOD since its intensity was comparable to those of bands 1 and 2 in some tissues such as the small intestine. On these assumptions, it is possible to say that Cu/Zn-SOD is contained at higher levels than Mn-SOD in several tissues including the cerebrum, stomach and kidney. Mn-SOD is contained at higher levels than Cu/Zn SOD in the heart, being consistent with the results from other mammals (Marklund, 1984). Relative levels of Cu/Zn-SOD have been shown to be predominant in rodents and Mn-SOD predominant in carnivores and unglates (Marklund, 1984). The Japanese monkey resembles rodents in this respect. The species-specificity has also been reported in the case of the total SOD levels, showing that SOD activity is correlated with

the metabolic activity of the tissue in large mammals and contained high SOD levels (Tolmasoff *et al*., 1980).

Ascorbate peroxidase and catalase were contained at high levels in various monkey tissues except for blood. Since ascorbate peroxidase is known to have important roles in plant tissues to scavenge ROS (Asada and Takahashi, 1987), this enzyme has been studied more extensively in plant tissues than in mammalian tissues. However, its high level in various monkey tissues suggests that this enzyme is also important in higher animals as has been found in the bovine eye (Wada *et al*., 1998). To date there are few reports for isozymes of ascorbate peroxidase in mammalian tissues. In higher plants, there have been found three isozymes, and two isozymes localized in chloroplast and one in cytosol (Mittler and Zilinskas 1993). It is difficult to deduce the localization of two isozymes found in monkey tissues at present.

Catalase is distributed widely in monkey tissues as reported for other mammalian tissues. Although two isozymes were found in Japanese monkey tissues excluding blood cells, which contained only one isozyme, this number was lower than those of isozymes in other mammals. Three to five isozymes have been reported in rat and mouse liver (Nishimura *et al*., 1964; Holms and Masters, 1970; Masters, 1982; Mainferme and Wattiaux, 1982), and 3 isozymes in human liver (Nishimura *et al*., 1964).

We should mention the ROS-generating and scavenging systems in monkey tissues. Low levels of ROS are indispensable in many biological processes inducing defense against micro-organisms (Halliwell and Cross, 1994). In the present study, we could find significant levels of ROS-generating NADH/NADPH oxidase in the small intestine and lung, suggesting that these enzymes generate ROS and contribute to killing infectious micro-organisms in these tissues. On the other hand, high levels of ROS might result in oxidative stress, which can cause severe metabolic malfunctions such as cancer and other diseases (Halliwell and Cross, 1994). The balance of enzymes to generate and scavenge ROS is thought to be important in each tissue to maintain its normal activity. It is appropriate to postulate the occurrence of tissue-specific ROS-generating and scavenging systems in monkey, considering from the different tissue distributions of ROS-generating and scavenging enzymes and the presence of multiple forms in each enzyme which might have different specificities. Since the investigation of these enzymes have scarcely been carried out in monkey, the present results are thought to be fundamental for further studies such as gene expression of these enzymes. Additionally, high levels of ROS-generating and scavenging enzymes in the monkey nervous system, especially in the cerebellum, is noteworthy, since the monkey is a suitable animal model to study the highly-developed nervous system of humans.

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