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Original paper

Clearance rates of biotinylated ferritins in canine: A possible physiological role of canine serum ferritin as an iron transporter

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Abstract

To elucidate the physiological role of canine serum ferritin, we measured clearance rates of biotinylated ferritins in beagle. Biotinylated canine tissue ferritins were cleared rapidly from circulation. The clearance time ($T_{1/2}$) of liver ferritin (H/L subunit ratio = 0.43) was 6.8 to 11.8 min, and that of heart ferritin (H/L = 3.69) was 9.3 to 25.0 min. $T_{1/2}$ of biotinylated canine liver ferritin was independent of iron content, whereas canine heart apoferritin ($T_{1/2}$ = 31.2 and 32.7 min) was more slowly removed from circulation than the holoferritin. On the other hand, biotinylated recombinant bovine H-chain ferritin homopolymer show a much slower rate of removal ($T_{1/2}$ = 153.8 and 155.0 min) compared with the L-chain ferritin homopolymer ($T_{1/2}$ = 26.4 and 31.3 min). The rapid clearance of canine tissue ferritin suggests that serum ferritin is an iron transporter in canines.

Introduction

Ferritin is a ubiquitous iron-binding protein with a molecular weight of 500,000, 24 subunits, and a relatively high iron content (2000-3000 Fe/molecule)(Worwood *et al.* 1976; Theil 1987; Harrison & Arosio 1996; Watanabe *et al.* 2000b). Intracellular ferritin is made up of variable proportions of L- and H-chains with approximate molecular weights of 20,000 and 21,000, respectively (Arosio *et al.* 1978; Theil 1987; Harrison & Arosio 1996). Human ferritin L- and H-chains have considerably different physiological and immunological properties (Luzzago *et al.* 1986; Levi *et al.* 1988, 1989) as the H-chain has ferroxidase, an essential enzyme for iron uptake, while the L-chain increases physicochemical stability and iron-core nucleation but has no ferroxidase activity (Levi *et al.* 1988, 1989, 1992; Lawson *et al.* 1991; Harrison & Arosio 1996). The subunit composition is tissue specific. The L-chain predominates in liver and spleen ferritins, while the H chain predominates in heart, red cells, and HeLa ferritins (Arosio *et al.* 1978; Harrison & Arosio 1996, Watanabe *et al.* 2000b).

Ferritin is found in serum at relatively low concentration (< 1 μg ml^{-1}) and ferritin concentrations of human, canine, bovine, and equine sera are positively correlated with body iron stores (Addison *et al.* 1972; Walters *et al.* 1973; Smith *et al.* 1984; Miyata & Furugouri 1987; Andrews *et al.* 1992). Serum ferritin composition and concentration vary among mammals. For example, human serum ferritin isolated from idiopathic hemochromatosis patients is very low in iron (0.02–0.07 μg Fe/μg of protein) (Worwood *et al.* 1976; Cragg *et al.* 1981). The serum ferritin consists of L- and G-chains, the latter of which is responsible for binding with concanavalin A (ConA) and is immunologically similar to the L-chain (Worwood *et al.* 1979; Cragg *et al.* 1981; Santambrogio *et al.* 1987). Fetal bovine serum ferritin has a high iron content (0.2 μg Fe/μg of protein) and is rich in L-chains but does not contain G-chains (Kakuta *et al.* 1997). Ca-

nine serum ferritin is rich in H-chain isoferritins (H/L subunit ratio = 3.46 ± 1.12 (SD)) and has a relatively high iron content (0.112 ± 0.017 μg Fe/μg of protein) despite having no G-chains (Watanabe *et al.* 2000a, b). Serum ferritin iron concentrations are high in bovine fetal serum (160 to 960 ng ml^{-1}) and canine serum (30 to 116 ng ml^{-1})(Kakuta *et al.* 1997; Watanabe *et al.* 2000b). Canine liver ferritin is rapidly removed from circulation ($T_{1/2} < 10$ min)(Pollock *et al.* 1978). In fetal bovine and canine sera, ferritin binds more than one thousand iron atoms and may play a role as an iron transporter while transferrin binds only two iron atoms.

Ferritin-binding proteins circulating in the serum and/or plasma of mammals have been found to consist of H-kininogen in human serum (Torti & Torti 1998), alpha-2-macroglobulin in rat and horse sera (Santambrogio & Massover 1989; Massover 1994), IgM and IgA in canine serum (Watanabe *et al.* 2000a), and fibrinogen in horse plasma (Orino *et al.* 1993). Human H-kininogen recognizes both the L- and H-chains of human ferritin (Torti & Torti 1998). A recent study shows that canine autoantibodies to ferritin preferentially recognize H-chain over L-chain (Orino *et al.* 2002). Therefore, clearance tests of ferritin need to consider ferritin subunit composition due to subunit specificity of the ferritin-binding proteins.

In this study, the physiological role of circulating serum ferritin in canines was examined using biotin-labeled canine liver and heart ferritins with H/L ratios of 0.43 and 3.69, respectively.

Materials and methods

Experimental animals

Four female and two male clinically healthy 3-year-old beagles with body weights of 9–12 kg were studied.

Tissues

Livers and hearts were obtained from the dogs under pentobarbital-induced anesthesia. The tissue samples were stored at -25 °C.

Preparation of ferritin and its apoferritin

Canine liver and heart ferritins were purified according to Watanabe *et al.* (2000a, b). Iron was removed from canine liver and heart ferritins by dialysis with 100 mM acetate buffer (pH 5.5) containing 100 mM thioglycolic acid as previously described (Levi *et al.* 1988).

Recombinant bovine L-chain and H-chain ferritin homopolymers

Bovine ferritin L- and H- chain cDNAs were expressed using a baculovirus expression system (Orino *et al.* 1997) with *Spodoptera frugiperda* (Sf-21) cells maintained in serum-free ESF921 medium (Expression Systems, CA, USA). The expressed ferritin subunits in culture were heated, salted-out, and purified by Sepharose CL-6B (Pharmacia, Sweden) gel chromatography (Orino *et al.* 2002). Iron concentrations of ferritin homopolymers were determined coulometrically as described (Kakuta *et al.* 1997).

Biotin-labeling

Ferritin was biotinylated according to the manufacturer's instruction with soluble sulfosuccinimidobiotin (PIERCE, Rockford, IL, USA). Molar ratios of sulfosuccinimidobiotin to ferritin of 50 : 1, 50 : 1, 200 : 1, and 400 : 1 for canine liver ferritin, canine heart ferritin, recombinant bovine L-chain ferritin homopolymer, and recombinant bovine H-chain homopolymer, respectively, were used.

Protein determination

Protein concentrations were determined (Lowry *et al.* 1951) with bovine serum albumin (BSA) (Boehringer Mannheim, Germany) as the standard.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Disc-PAGE

SDS-PAGE using a 4.5% polyacrylamide stacking gel and a 10% polyacrylamide running gel (Schägger & von Jagow 1987) was used to visualize ferritins used in this study. Disc-PAGE was performed using a 5% slab polyacrylamide gel and the buffer system of Davis (1964). Ferritin subunit bands stained with Coomassie Brilliant Blue R-250 were analyzed by densitometry at 565 nm using a Flying Spot Scanner (Shimadzu CS9000) (Shimadzu, Kyoto, Japan).

Clearance test of ferritin

Beagles were anesthetized with pentobarbital (20 mg kg^{-1}) and the biotinylated ferritin preparations (80 μg

each) were injected intravenously. Blood samples (1 ml each) were taken prior to injection and at frequent intervals after administration. The serum samples were stored at −25 °C until analysis.

Ferritin antibodies

Rabbit antibodies to bovine spleen ferritin and rat liver ferritin were produced according to immunization protocols of Kakuta *et al.* (1997) and Watanabe *et al.* (2000a), respectively.

Measurement of biotinylated ferritin by ELISA

Aliquots (100 μl) of rabbit antibodies (1000 ng ml^{-1} of rat liver ferritin antibody for biotinylated canine liver and heart ferritins, and 400 ng ml^{-1} of bovine spleen ferritin antibody for recombinant bovine L- and H-chain ferritin homopolymers) in phosphate-buffered saline (PBS: 20 mM sodium phosphate, 150 mM NaCl, pH 7.2) were added to wells of Immuno Plate Maxisorp F 96 microtiter plates (Nunc, Roskilde, Denmark) and incubated overnight at 4 °C. The antibody-coated plates were washed with PBS containing 0.05% Tween 20 (PBST) and masked with ELISA buffer (PBS containing 0.1% BSA and 0.1% Tween20) as described (Watanabe *et al.* 2000b). After 100 μl aliquots of canine serum diluted 11-fold with ELISA buffer were added to each well, the plates were incubated at 37 °C for 2 h. After washing with PBST, 100 μl of sheep anti-biotin antibody (Bethyl Laboratories, Montgomery, TX, USA) appropriately diluted with ELISA buffer was added, and the plates were incubated at 37 ∘C for 1.5 h. After washing with PBST, 100 μl of alkaline phosphatase-labeled rabbit anti-sheep IgG antibody (Chemicon International Inc., Temecula, CA, USA) appropriately diluted with ELISA buffer was added, and the plates were incubated at 37 °C for 1.5 h. After washing with PBST, the enzyme reaction was carried out using *p*-nitrophenyl phosphate as described (Orino *et al.* 1993).

Kinetics of clearance

The kinetic parameters of the clearance of biotinylated ferritin were determined by a pharmacokinetic analysis program (MULTI) using a non-linear square method (Yamaoka *et al.* 1981).

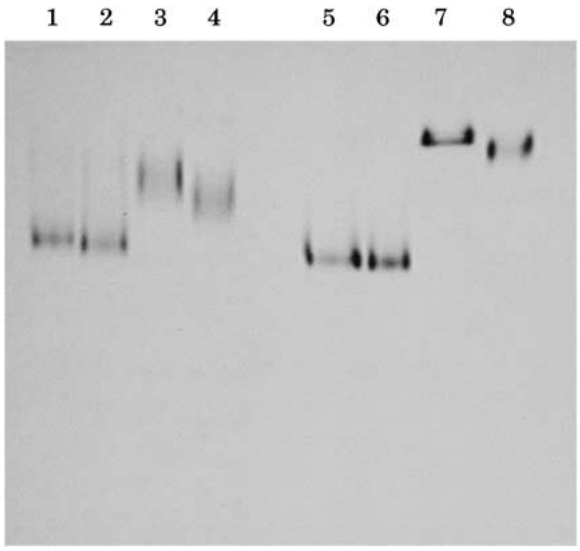

Fig. 1. Comparison of biotinylated and untreated ferritins and homopolymers by Disc-PAGE. The samples (2 μg each lane) are canine heart ferritin (lane 1), biotinylated canine heart ferritin (lane 2), canine liver ferritin (lane 3), biotinylated canine liver ferritin (lane 4), bovine H-chain ferritin homopolymer (lane 5), biotinylated bovine H-chain ferritin homopolymer (lane 6), bovine L-chain ferritin homopolymer (lane 7), and biotinylated bovine L-chain ferritin homopolymer (lane 8). Anode at bottom.

Statistical analysis

The value was expressed as means ± SD. The results of clearance experiments were analyzed by Student's *t*-test with the level of significance at $P < 0.05$.

Results

Biotinylated ferritin

Ferritin preparations were primarily monomeric and expressed bovine ferritin subunit cDNAs formed 24-mer molecules (Figure 1). Biotin-labeled ferritin samples migrated faster than unlabeled samples. The faster migration may be due to the reduction of a net positive charge between biotin moieties and amine of lysine of ferritin. However, the conformation of biotinylated ferritin did not differ from untreated ferritin except for change of a net charge by biotin-labeling. In addition, the removal of iron or biotin-labeling of ferritin did not affect the binding activity with anti-ferritin antibodies in ELISA or with anti-ferritin antibodies (ferritin-binding proteins) in canine sera (data not shown). The H/L subunit ratios of canine liver and heart ferritins are 0.43 and 3.69, respectively, according to previous data

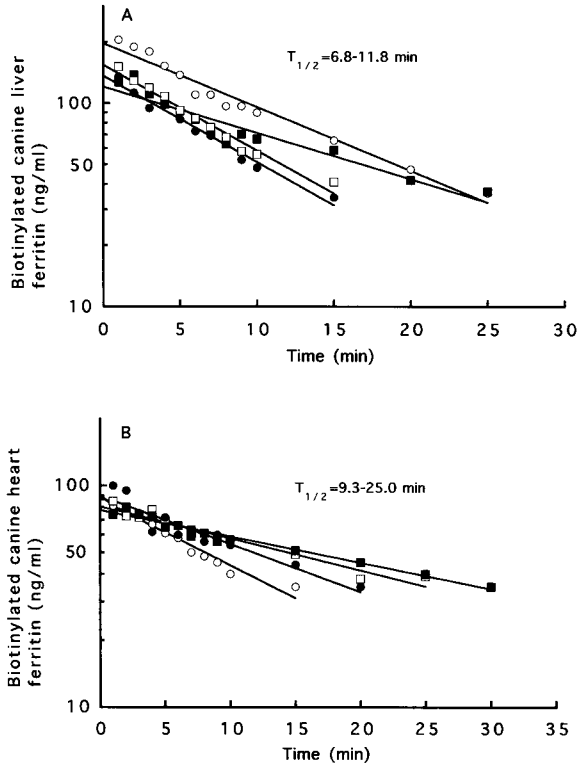

Fig. 2. Clearance of canine liver ferritin (A) and heart ferritin (B). Biotinylated canine tissue ferritins (80 μg each) were administrated intravenously in beagles. Symbols ○, ●, □, and ■ represent dogs 1, 2, 3, and 4, respectively, of Table 1 in change to same size A and Table 2 in B.

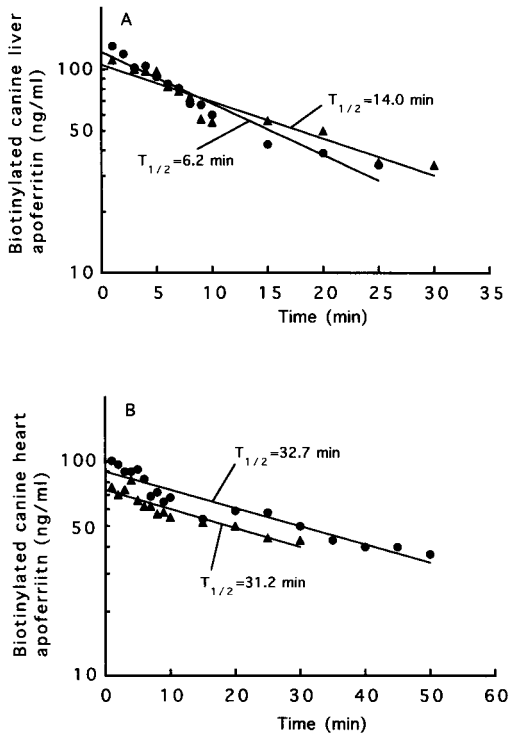

Fig. 3. Clearance of canine liver apoferritin (A) and heart apoferritin (B). Biotinylated canine apoferritins (80 μg each) were administrated intravenously in beagles.

(Watanabe *et al.* 2000b). Purified, expressed bovine ferritin homopolymers contained much less iron than before treatment (<less than 0.4 ng/μg of protein) (Orino *et al.* 2002).

Clearance analysis of ferritin

Assay detection limits of all sample types were 1 ng ml^{-1} and the recoveries of biotinylated ferritins from canine serum were almost 100%. ELISA standard curves of biotinylated ferritins were not affected even after addition of canine heart ferritin (final concentration: 100 ng ml^{-1}) although determinations of biotin samples were measured in the presence of H-chain predominant canine serum ferritin.

Clearance of biotinylated canine liver and heart ferritins injected into the four beagles fits a one-compartment model (Figure 2A & B). Following injection, canine liver and heart ferritins were both found only in the circulatory system as indicated by a volume of distribution (Vd) (l kg^{-1}) of less than 0.058 l kg^{-1} and 0.103 l kg^{-1} in liver and heart ferritins, respectively (Tables 1 & 2). Canine liver ferritin turnover time appeared to be unrelated to sex (Table 1). The half-life ($T_{1/2}$) of biotinylated canine liver ferritin ranged from 6.8 to 11.8 min (mean ± SD = 8.5 ± 2.4 min), and that of biotinylated canine heart ferritin ranged from 9.3 to 25.0 min (17.6 ± 6.6 min). Canine liver ferritin turnover was significantly rapid compared with canine heart ferritin ($p < 0.05$). Canine heart apoferritin turnover time was slower than holoferritin ($T_{1/2}$ = 31.2 and 32.7 min) although canine liver ferritin turnover was not affected by removal of iron (Figure 3A & B). Biotinylated bovine H-chain ferritin homopolymer was removed very slowly ($T_{1/2}$ = 153.8 and 155.0 min) from circulation compared to the L-chain ferritin homopolymer ($T_{1/2}$ = 26.4 and 31.3 min) (Figure 4A & B). The Vd values of canine tissue apoferritins and bovine ferritin homoplymers were between 0.057 and 0.135 l kg^{-1}, indicating that they were distributed only in the circulatory system after injection. There were no apparent relationships between ferritin clearance rates and serum ferritin concentrations or ferritin-binding activities (data not shown).

Table 1. Clearance analysis of biotinylated canine liver ferritin injected intravenously into beagles[a].

Dog No.	Body weight (kg)	Sex	Co (ng ml^{-1})	Kel (min^{-1})	$T_{1/2}$ (min)	AUC (μg min ml^{-1})	Vd (l kg^{-1})	CL (ml min^{-1} kg^{-1})
1	9.0	F	208	0.080	8.7	2.60	0.043	3.426
2	10.5	F	140	0.102	6.8	1.37	0.055	5.579
3	12.0	M	159	0.102	6.8	1.56	0.042	4.283
4	11.0	M	125	0.059	11.8	2.10	0.058	3.397

[a]Biotinylated canine liver ferritin was intravenously injected into four beagles, and the concentrations of the biotinylated canine liver ferritin of the sera obtained at time intervals were determined by ELISA.
Co, concentration at time 0 (zero); Kel, elimination of constant; $T_{1/2}$, biological half-life, AUC, area under the curve, Vd, volume of distribution, CL, clearance.

Table 2. Clearance analysis of biotinylated canine heart ferritin injected intravenously into beagles[a].

Dog No.	Body weight (kg)	Sex	Co (ng ml^{-1})	Kel (min^{-1})	$T_{1/2}$ (min)	AUC (μg min ml^{-1})	Vd (l kg^{-1})	CL (ml min^{-1} Kg^{-1})
1	10.0	F	90	0.074	9.3	1.21	0.089	6.612
2	9.0	F	87	0.043	16.0	1.99	0.103	4.460
3	10.0	F	82	0.035	20.0	2.35	0.098	3.400
4	10.0	F	78	0.028	25.0	2.80	0.103	2.854

[a]Biotinylated canine heart ferritin was intravenously injected into four beagles, and the concentrations of the biotinylated canine heart ferritin of the sera obtained at time intervals were determined by ELISA.
Co, concentration at time 0 (zero); Kel, elimination of constant; $T_{1/2}$, biological half-life, AUC, area under the curve, Vd, volume of distribution, CL, clearance.

Discussion

Cells take up circulating ferritin either by direct binding with ferritin receptors or through indirect binding mediated by specific receptors for ferritin-binding proteins (Santambrogio & Massover 1989; Massover 1994; Moss *et al.* 1992; Watanabe *et al.* 2000a). In humans, ferritin receptors are found on hepatic, lymphoid, and erythroid cells. Liver cells scavenge ferritin without differentiating L- and H-chains, whereas proliferating or differentiating cells such as lymphocytes and monocytes recognize isoferritins rich in H-chains with suppressive activities (Moss *et al.* 1992). In canines, liver ferritin is rich in L-chain isoferritin which is rapidly cleared from circulation ($T_{1/2}$ < 10 min)(Pollock *et al.* 1978), while serum ferritin is predominantly H-chains (Watanabe *et al.* 2000a). Autoantibodies to ferritin, ferritin-binding proteins, in canine serum preferentially recognize H-chain to L-chain (Orino *et al.* 2002), suggesting that the reticuloendothelial system is involved in the uptake of isoferritins rich in H chains. This study reports clearance analysis using canine tissue ferritins with different subunit compositions. Although Pollock *et al.* (1978) did not refer to sex difference in the clearance rates of canine liver ferritin, biological half-life of liver ferritin was not affected by dose of liver ferritin. This study with 2 males and 2 females in the analysis for liver ferritin was not affected by sex difference in clearance rates, although four females were used in heart ferritin experiment. Because heart ferritin (H-chain isoferritins) was removed more slowly from circulation than canine liver ferritin (L-chain isoferritins), canine serum ferritin may be rich in H-isoferritin. An alternative explanation lies in the difficulty for cells to take up immune complexes between H-isoferritins and anti-ferritin antibodies. Canine heart apoferritin showed slower clearance than the heart holoferritin although anti-ferritin antibodies in canine serum did not show iron dependent ferritin-binding activities (Orino *et al.* in press). The H-chain is essential to the incorporation of iron into ferritin. Whether specific receptors for iron-incorporated ferritin exist remains unknown.

Recrystallized canine liver ferritin was removed more rapidly from circulation than normal ferritin, however iron-free recrystallized canine liver ferritin showed slower clearance (Pollock *et al.* 1978). Canine heart ferritin showed faster mobility than liver ferritin on Disc-PAGE (Figure 1), indicating that H-chain of canine ferritin is more acidic than L-chain. Recrys-

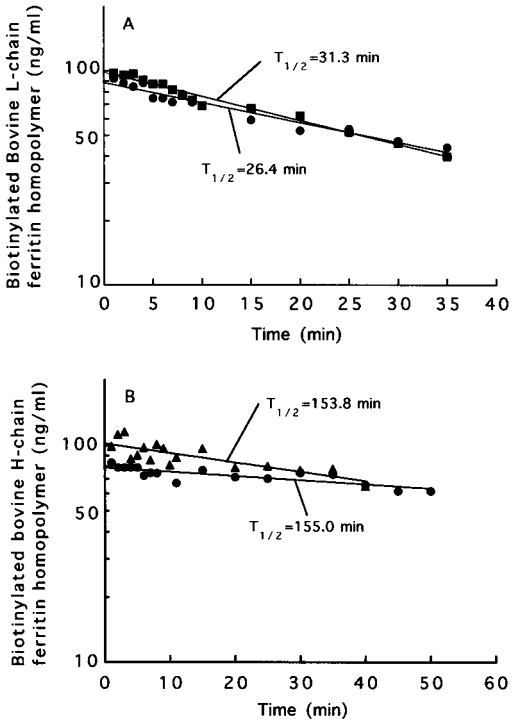

Fig. 4. Clearance of bovine L-chain ferritin homopolymer (A) and H-chain ferritin homopolymer (B). Biotinylated bovine ferritin homopolymers (each 80 μg) were administrated intravenously in beagles.

tallization selects out basic isoferritins (Pollock *et al.* 1978), increasing the proportion of the basic isoferritin in canine liver ferritin. The phenomenon of faster clearance after the recrystallization is similar to faster clearance of isoferritins rich in L-chains presented in this study. In contrast, this explanation is not supposed by the slower clearance of recrystallized ferritin after the removal of iron. Differences in affinity of anti-ferritin antibodies to ferritin subunits or apoferritins in ferritin determination may cause this inconsistency.

Faster clearance rates for L-chain isoferritins probably depend on ferritin uptake rates of ferritin receptors on the liver cells and not on the ferritin iron content. In this study, we did not identify which organ incorporates ferritin due to difficulties in injecting radioactive materials into dog. Biotin-labeled ferritin would allow us to analyze ferritin receptors using cultured cells and examine the bioavailability of ferritin iron.

Naturally-occurring ferritins are not exclusively composed of either L- and H-chain subunits, and amino acid sequence homology is highly conserved for the subunits across mammalian species (L > 79%, H > 91%)(Orino *et al.* 1997). Although the amino acid sequences of canine L- and H-chains have not been determined yet, canine autoantibodies to ferritin are not species specific (Orino *et al.* 2002). Unexpectedly, recombinant homopolymers were cleared from circulation slowly. Particularly, bovine H-chain homopolymer showed very slow clearance, perhaps because expressed bovine ferritin homopolymers are apoferritins. Further study is needed to clarify whether ferritin internalization is species-specific.

Biotin clearance is very slow (bovine: $T_{1/2}$ = approx. 8 h; pig: $T_{1/2}$ > 9 h) (Bryant *et al.* 1990; Frigg *et al.* 1993). We confirmed that repeated injections of biotinylated ferritin did not produce antibodies against biotin and biotin-moieties (data not shown), allowing us to inject different proteins into the same dogs. Ferritin-binding activity in serum of experimental animals after repeated administration of biotinylated ferritin did not differ from ferritin-binding activity prior to injection (data not shown). These results suggest that ferritin clearance rates are not influenced by biotin bioavailability and production of antibodies against biotinylated ferritin during clearance tests.

In humans, Con A-bound plasma ferritin shows much slower clearance ($T_{1/2}$ = approx. 50 h) than tissue ferritin (Worwood *et al.* 1982), suggesting that the circulating holoferritin released from damaged cells is selectively removed from circulation. In canines, ferritin iron labeled with ^{59}Fe is cleared at the same rate as ferritin protein without the release of iron from the ferritin (Pollock *et al.* 1978).

Transferrin iron turn over in canine plasma is 16.7 μg ml^{-1} day^{-1} (transferrin iron concentration: 1000 ng ml^{-1}; transferritn iron turnover ($T_{1/2}$): 60 min) (Smith 1989) because plasma iron turnover (μg ml^{-1} day^{-1}) is calculated by the formula {iron concentration (μg ml^{-1}) $\times$ 1000/$T_{1/2}$ (min)} (Smith 1989). Canine serum ferritin is predominantly composed of H-chains (Watanabe *et al.* 2000a). If ferritin iron turnover in canine circulation is estimated using a mean heart ferritin turnover rate ($T_{1/2}$) of 17.6 min and a mean serum ferritin iron concentration of 53 ng ml^{-1}, ferritin iron turnover is 3.0 μg ml^{-1} day^{-1} and ferritin/transferrin iron turnover ratio is 0.18. There is growing evidence that circulating ferritin plays a role as an iron-transporter for heme synthesis (Blight & Morgan 1983) and in fetal growth (Lamparelli *et al.* 1989; Kakuta *et al.* 1997). Rapid clearance of canine tissue ferritins observed in this study, therefore, leads to a conclusion

that canine serum ferritin plays a physiological role as an iron transporter.

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