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Characterization of ferritin and ferritin-binding proteins in canine serum

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Abstract

Ferritin and ferritin-binding proteins in canine serum were characterized. A certain percentage of ferritin in canine serum, but no tissue ferritin, was precipitated by centrifugation at 16, 000 $\times$ g for 30 min. The precipitated ferritin was found to contain two subunits corresponding to the H and L subunits of canine liver ferritin by immunoblotting, the H subunit being predominant. More ferritin was precipitated from canine sera which had been incubated with anti-rat liver ferritin antibody than from untreated sera, and the H chain also predominated. To evaluate the possibility that the autoantibody was responsible for the precipitation of canine serum ferritin, the ferritin-binding activities of canine antibodies were examined using liver ferritin-coated microtiter plates and alkaline phosphatase-labeled antibodies specific for canine IgM, IgA, and IgG heavy chains. The results showed that IgM and IgA, but not IgG, had considerable ferritin-binding activities. Given these results, we suggest that there is H-chain-rich isoferritin in canine serum, and that ferritin exists as an immune complex.

Introduction

The iron storage protein ferritin, which consists of a protein shell of 24 subunits enclosing a core of ferric iron, ubiquitously occurs in organs and cells of mammals (Theil 1987; Worwood 1990). Tissue ferritins are composed of a variable proportion of two types of subunits, H and L. The H chain predominates in heart, red blood cells, and HeLa cells ferritins, and has a molecular weight of 21 kDa, whereas the L chain predominates in liver and spleen ferritins, and has a molecular weight of 19 kDa (Arosio *et al*. 1978; Worwood 1990).

In a large variety of mammals including humans, horses, cattle, dogs, and cats, previous research indicates that a relatively low concentration of ferritin ($<$ 1 μg ml^{-1}) is present in normal serum, and that the serum ferritin level is positively correlated with body iron storage (Addison *et al*. 1972; Walters *et al*. 1973; Smith *et al*. 1984; Miyata *et al*. 1987; Andrews *et al*. 1992, 1994). Human ferritin purified from the serum of patients with idiopathic hemochromatosis was found to be mainly composed of the L subunit and the glycosylated (G) subunit, the latter with a molecular weight of 23 kDa, which bound to concanavalin A and was immunologically similar to the L (Worwood *et al*. 1979; Cragg *et al*. 1981; Santambrogio *et al*. 1987). Recently the L chain was found to be predominant in fetal bovine serum ferritin by immunoblotting, but the G chain appeared to be absent (Kakuta *et al*. 1997).

In addition to ferritin, there are ferritin-binding proteins in serum and/or plasma: H-kininogen in human serum (Torti & Torti 1998), alpha-2-macroglobulin in rabbit and horse sera (Santambrogio & Massover 1989; Massover 1994), and fibrinogen in horse plasma (Orino *et al*. 1993b). However, the physiological role of the ferritin-binding proteins remains to be clearly established.

Numerous clinical studies on canine serum ferritin have been undertaken (Weeks *et al*. 1990; Newlands *et al*. 1994; Gookin *et al*. 1998), however, its biochem-

ical properties remain unclear. In the present study, it was found that canine serum contained H-chain-rich isoferritin, and that anti-ferritin autoantibodies were ferritin-binding proteins in canine sera.

Materials and methods

Blood and liver

Blood samples were collected from randomly-selected dogs, and a whole liver was obtained from a dog euthanized using succinylcholine chloride. Blood and liver were obtained from female Wistar albino rats aged 9 weeks (Clea Japan, Tokyo, Japan) under anesthesia with pentobarbital. The serum samples and livers were stored at −25 °C until use.

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

SDS-PAGE was performed according to the method of Schägger and von Jagow (1987), using 4.5% polyacrylamide stacking gel and 10% polyacrylamide running gel. Densitometry of ferritin subunit bands stained with Coomassie Brilliant Blue R250 was scanned at 565 nm using a Flying Spot Scanner (Shimazu CS 9000)(Shimazu, Kyoto, Japan).

Immunoblotting of canine serum ferritin was carried out using a procedure described previously (Watanabe *et al*. 1995) and antiserum to rat liver ferritin was used as a probe. Densitometry of ferritin subunit bands immunostained on the blotting membrane was scanned at 535 nm.

Protein determination

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

Purification of ferritin from canine and rat livers

Ferritin was purified from canine and rat livers using the procedure described for purification of bovine spleen ferritin (Kakuta *et al*. 1997), except that Pefabloc SC (Merck, Darmstadt, Germany) was used in place of phenylmethylsulfonyl fluoride as a serine proteinase inhibitor.

Figure 1 shows the SDS-PAGE pattern of purified proteins. The molecular weights of heavy and light chains of canine ferritin were 21.1 kDa and 18.7 kDa, respectively, and those of heavy and light chains of rat ferritin were 21.1 kDa and 19.3 kDa, respectively. The H/L subunit ratios of canine and rat ferritins were 0.28 and 0.54, respectively.

Figure 1. SDS-PAGE of purified canine and rat liver ferritins. Samples are canine ferritin (2 μg, lane 1), rat ferritin (2 μg, lane 2), and marker proteins (2 μg each, lane 3): ovalbumin (45.0 kDa), lactate dehydrogenase (36.0 kDa), adenylate kinase (21.7 kDa), myoglobin (17.2 kDa), cytochrome *c* (12.4 kDa). Anode at bottom.

Preparation of antibodies

Antisera to rat liver ferritin were produced in female Japanese white rabbits (Clea Japan) using an immunization protocol described previously (Orino *et al*. 1993a). Specific antibodies were purified from the antisera by affinity chromatography on ferritin-bound Sepharose 4B, which was prepared by coupling 10 mg of purified ferritin to 10 ml of CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden).

Immunodiffusion

Double immunodiffusion was performed essentially according to the method of Ouchterlony (1953).

Sandwich enzyme-linked immunosorbent assay (ELISA)

Ferritin in rat sera was determined by sandwich ELISA using the procedure described for measuring

horse serum ferritin (Orino *et al*. 1993a). ELISA buffer containing gelatin instead of bovine serum albumin (see below) was used. The concentration of the affinity-purified antibodies to rat liver ferritin for coating was 400 ng ml^{-1} and that of alkaline phosphatase-labeled anti-rat liver ferritin antibodies prepared using glutaraldehyde (Avrameas 1969) was 200 ng ml^{-1}. Ferritin in canine sera was determined by the same ELISA system except that canine liver ferritin instead of rat liver ferritin was used as a standard.

Detection of ferritin-binding activities of canine antibodies

One hundred microliters of 10 μg ml^{-1} canine liver ferritin, rat liver ferritin, or bovine spleen ferritin in phosphate-buffered saline (PBS: 20 mM sodium phosphate, 150 mM NaCl, pH 7.2) were added to each well of the Immuno Plate Maxisorp F96 microtiter plates (Nunc, Roskilde, Denmark), and the plates were kept overnight at 4 °C. The ferritin-coated plates were then washed and masked as described for sandwich ELISA (Orino *et al*. 1993a), and a 100 μl aliquot of canine serum diluted 20- or 100-fold with ELISA buffer (PBS containing 0.1% gelatin and 0.1% Tween 20) was added to each well and the plates were incubated at 37 °C for 2 h. After washing, a 100 μl aliquot of alkaline phosphatase-labeled antibody specific for canine IgM, IgA, or IgG heavy chains (Bethyl Laboratories, Montgomery, TX, USA), which had been appropriately diluted with ELISA buffer, was added and the plates incubated at 37 °C for 2 h. After washing, the enzyme reaction was performed as in the previously described sandwich ELISA.

Statistics

Statistical significance was determined using Student's *t*-test.

Results

Immunological cross-reaction between canine and rat liver ferritins

A single precipitin line was seen between purified canine or rat liver ferritin and antiserum to rat liver ferritin, and the lines for both ferritins merged with a spur (Figure 2), indicating partial identity but suggesting that some epitopes present in rat ferritin were not present in canine ferritin. Figure 3 shows that a sandwich ELISA system developed for rat ferritin was also applicable to the determination of canine ferritin. Recoveries of 22.7 ng ml^{-1} and 45.5 ng ml^{-1} canine liver ferritin added to the canine sera diluted 11-fold with ELISA buffer were 99.0 $\pm$ 8.1% (mean $\pm$ SD, $n = 4$) and 97.5 $\pm$ 5.4% ($n = 4$), respectively. Heat treatment of diluted canine sera at 75 °C for 15 min had no effect on serum ferritin values. Intra-assay coefficients of variation from six measures of ferritin in two canine sera were 4.1% (98 ± 4 ng ml^{-1}) and 4.4% (182 ± 8 ng ml^{-1}). Inter-assay coefficients of variation from five measures of ferritin in two canine sera were 15.2% (92 ± 14 ng ml^{-1}) and 7.7% (196 ± 15 ng ml^{-1}).

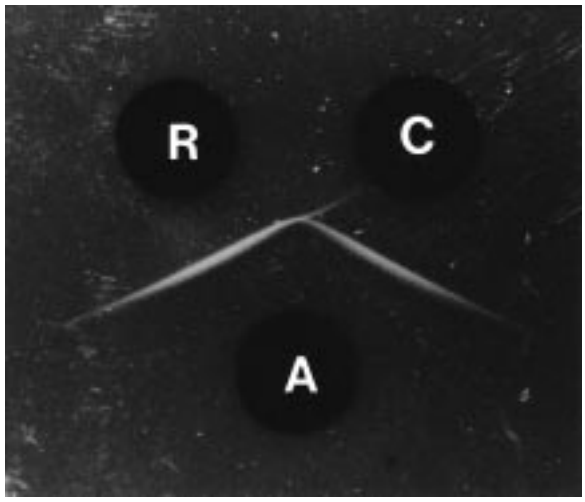

Figure 2. Immunological cross-reaction between rat and canine liver ferritins. A: antiserum to rat liver ferritin, R: rat liver ferritin, C: canine liver ferritin.

Precipitable ferritin in canine serum

When canine sera were centrifuged at $16,000 \times g$ for 30 min at 4 °C, the ferritin levels in the sera were found to be significantly ($P < 0.001$) decreased when compared with those in uncentrifuged sera (Figure 4). In contrast, centrifugation had no effects on the ferritin levels in rat sera. Purified canine liver ferritin (200 ng ml^{-1} in ELISA buffer) did not precipitate at all using this method. As appreciable amounts of pellet were obtained after centrifugation of the canine sera, the pellets were subjected to immunoblotting using anti-rat liver ferritin antibody as a probe. As shown in Figure 5, although many bands were seen on the blotting membrane, the bands specifically reacting with the antibody could be identified when compared with the control membrane. Two bands corresponding to canine liver ferritin H and L subunits were

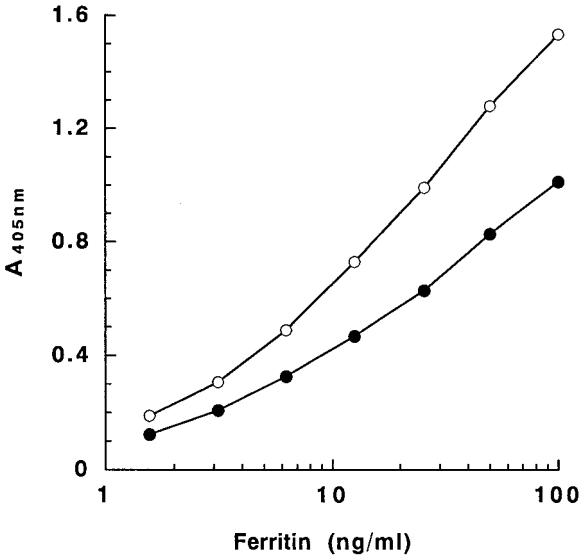

Figure 3. ELISA standard curves for rat and canine liver ferritins assayed with anti-rat ferritin antibodies. ○: rat liver ferritin, ●: canine liver ferritin.

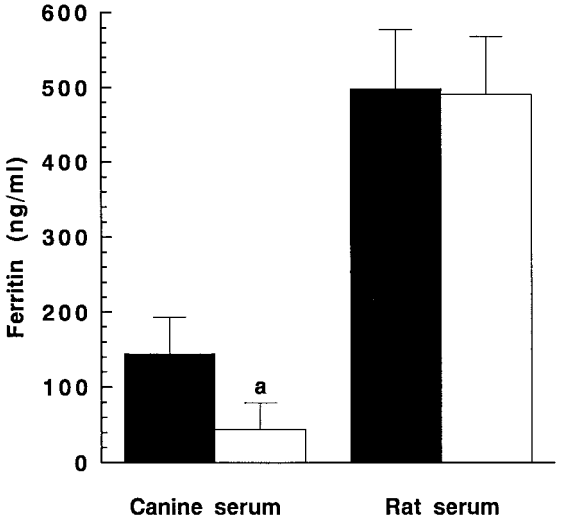

Figure 4. Effects of centrifugation on ferritin concentration in canine and rat sera. Canine and rat sera were centrifuged at 16, 000 × *g* for 30 min at 4 °C, and ferritin in obtained supernatants (open bars) or in uncentrifuged sera (closed bars) was determined by ELISA. Each value is the mean ± SD of 12 dogs and 9 rats. a: Significantly different from the uncentrifuged canine sera ($P < 0.001$).

specifically detected, and the H chain was predominant (Figure 5, lanes 2, 4 and 6). As not all the ferritin in the canine serum was thought to have precipitated (Figure 4), purified antibody to rat liver ferritin was added to the canine serum and incubated overnight. The mixture was centrifuged under the same condition as described above. Ferritin levels were determined by ELISA and ferritin subunits by immunoblotting using the resulting supernatant and precipitate. Little ferritin remained in the supernatant, indicating that almost all ferritin was precipitated from the serum with the added antibody. Immunoblotting results showed that more ferritin was precipitated from the antibody-added serum than from the unadded serum, and that the H chain was also predominant (Figure 5, lanes 3, 5 and 7). The mean ferritin H/L subunit ratio obtained from six serum samples was 3.46±1.12 (SD). The specific band which had a larger molecular weight than the H chain and seemed to correspond to the G chain was not detected.

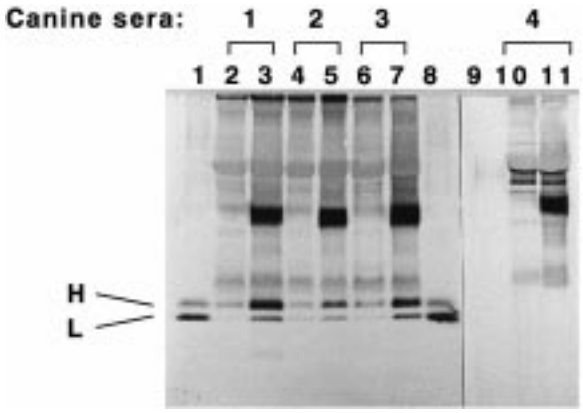

Figure 5. Immunoblotting of canine serum ferritin. 1 ml each of four different canine sera was incubated overnight at 4 °C with (lanes 3, 5, 7, and 11) and without (lanes 2, 4, 6, and 10) 6 μg of purified anti-rat ferritin antibody, and then centrifuged at 16, 000 × *g* for 30 min at 4 °C. Resulting pellets were subjected to immunoblotting using anti-rat ferritin antiserum (lanes 1–8) or preimmune serum (lanes 9–11). Lanes 1, 8, and 9 were purified canine liver ferritin (500 ng). H and L are ferritin subunits. Anode at bottom.

Binding of canine IgM and IgA to liver ferritin

Given the above result that canine serum ferritin was precipitated by low-speed centrifugation, but no tissue ferritin was precipitated, we considered that an autoantibody might be responsible for the serum ferritin precipitation. To evaluate this hypothesis, we examined the ferritin-binding activities of canine antibodies using alkaline phosphatase-labeled antibodies specific for canine IgM, IgA, and IgG heavy chains. IgM and IgA, but not IgG, were found to have considerable ferritin-binding activities (Figure 6). No relationship between the ferritin-binding activity of IgM or IgA and serum ferritin concentration was found to exist (data not shown). IgM and IgA in canine serum

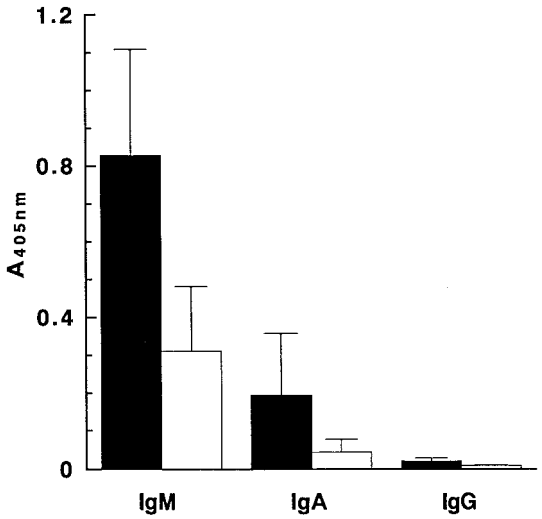

Figure 6. Binding of canine IgM and IgA to canine liver ferritin. Canine sera diluted 20- (closed bars) or 100-fold (open bars) with ELISA buffer were added to canine liver ferritin-coated wells of microtiter plates. Antibodies bound to the wells were detected by alkaline phosphatase-labeled antibodies specific to canine IgM, IgA, and IgG heavy chains. Each value is the mean ± SD of 10 dogs.

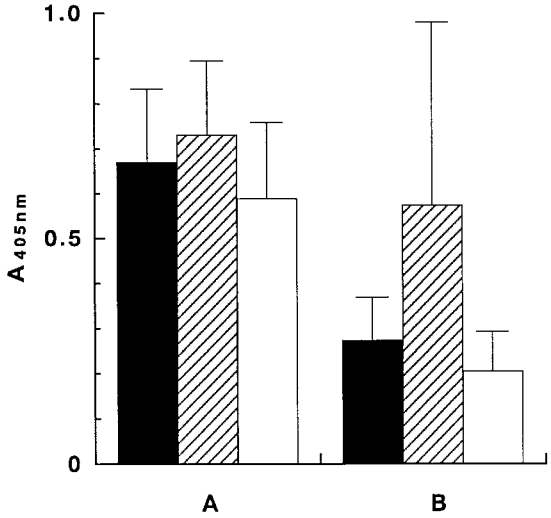

Figure 7. Binding of canine IgM and IgA to rat liver and bovine spleen ferritins. Canine sera diluted 20-fold with ELISA buffer were added to wells of microtiter plates coated with canine liver ferritin (closed bars), rat liver ferritin (hatched bars) and bovine spleen ferritin (open bars). Antibodies bound to the wells were detected by alkaline phosphatase-labeled antibodies specific to canine IgM (A) and IgA (B) heavy chains. Each value is the mean ± SD of 6 dogs.

also bound to rat liver and bovine spleen ferritins (Figure 7).

Discussion

The present study revealed that the H subunit was predominant in canine serum ferritin. This contrasts with the findings that human serum ferritin has only a trace of H chain (Cragg *et al.* 1981; Santambrogio *et al.* 1987), and that the L chain is predominant in fetal bovine serum ferritin (H/L ratio: 0.03–0.27) (Kakuta *et al.* 1997).

Anti-human liver ferritin antiserum and anti-L-subunit monoclonal antibodies have been shown to have a lower affinity for the G subunit of human serum ferritin than for the L subunit (Santambrogio *et al.* 1987). Therefore, it is too early to suggest that the G subunit is not present in canine serum ferritin despite the non-detection of the specific band which seemed to correspond to the G chain in the protein by immunoblotting using the antiserum to rat liver ferritin as a probe (Figure 5). We believe that analysis of the subunit composition and carbohydrate of the purified canine serum ferritin is necessary to provide conclusive evidence of this.

Canine serum ferritin, in which the H-chain predominates, has been measured by sandwich ELISA using the L-chain-rich canine liver ferritin as a standard (Weeks *et al.* 1990; Andrews *et al.* 1992). As the H and L chains of ferritin have a large immunological difference (Luzzago *et al.* 1986), canine serum ferritin levels previously reported might be underestimated. We recommend that the H-chain-rich ferritin purified from canine heart is used as a standard in ELISA for canine serum ferritin.

Human serum ferritin has been reported to have a relatively low iron content (0.02–0.07 μg Fe/μg protein) (Cragg *et al.* 1981; Pootrakul *et al.* 1988), whereas ferritin in fetal bovine serum has a relatively high iron content (about 0.2 μg Fe/μg protein) (Kakuta *et al.* 1997). The iron content of canine serum ferritin remains to be elucidated.

Although tissue ferritin is precipitated by ultracentrifugation at more than $100,000 \times g$ because of its high iron content, it is not precipitated at all by low-speed centrifugation at $16,000 \times g$. In the present study, a part of, but not all, canine serum ferritin was precipitated by low-speed centrifugation, and autoantibodies IgM and IgA with ferritin-binding activities were detected. These results suggest that canine serum ferritin exists as an immune complex. Antigen-antibody complexes are rapidly eliminated from the blood by the reticulo-endothelial system *in vivo* (Benacerraf *et al.* 1959). Antibodies to ferritin in canine

serum may be responsible for rapid sequestration of the ferritin from the circulation of the dog (Pollock *et al*. 1978).

To our knowledge, this is the first identification of an anti-ferritin antibody as a ferritin-binding protein. The presence or absence of the autoantibody which recognizes ferritin in animals other than dogs remains to be examined. Human serum ferritin does not seem to act as the immune complex (Celada *et al*. 1982; Covell *et al*. 1984).

The fact that alpha-2-macroglobulin has been identified as a ferritin-binder in rabbit and horse sera (Santambrogio & Massover 1989; Massover 1994) suggests that a binding of ferritin is a general functional capability of the protein in mammals. Although fibrinogen (Orino *et al*. 1993b) and H-kininogen (Torti & Torti 1998) have been shown to have ferritin-binding activities in horses and humans, respectively, it has yet to be proven whether these proteins are the general ferritin-binders in mammals.

Studies have detected an inhibitory effect of serum on the immunoassay of ferritin in humans and horses, which seems to be due to the masking of ferritin epitopes by ferritin-binding protein(s), because heat or detergent treatment of the serum that dissociates ferritin from ferritin-associated proteins increases the ferritin levels (Niitsu *et al*. 1988; Orino *et al*. 1993a). In the present study, although the autoantibody to ferritin was present in canine serum, ELISA of ferritin in the serum did not reveal the 'serum inhibition' effect. This could be attributed to the lower ferritin-binding avidity of the autoantibody than the rabbit anti-ferritin antibody used in the assay system or to the differences in epitopes recognized by each antibody.

References

Addison GM, Beamish MR, Hales CN, Hodgkins M, Jacobs A, Llewellin P. 1972 An immunoradiometric assay for ferritin in the serum of normal subjects and patients with iron deficiency and iron overload. *J Clin Pathol* **25,** 326–329.

Andrews GA, Chavey PS, Smith JE. 1994 Enzyme-linked immunosorbent assay to measure serum ferritin and the relationship between serum ferritin and nonheme iron stores in cats. *Vet Pathol* **31,** 674–678.

Andrews GA, Smith JE, Gray M, Chavey PS, Weeks BR. 1992 An improved ferritin assay for canine sera. *Vet Clin Pathol* **21,** 57–60.

Arosio P, Adelman TG, Drysdale JW. 1978 On ferritin heterogeneity. Further evidence for heteropolymers. *J Biol Chem* **253,** 4451–4458.

Avrameas S. 1969 Coupling of enzymes to proteins with glutaraldehyde. Use of conjugates for the detection of antigens and antibodies. *Immunochemistry* **6,** 43–52.

Benacerraf B, Sebestyen M, Cooper NS. 1959 The clearance of antigen antibody complexes from the blood by the reticuloendothelial system. *J Immunol* **82,** 131–137.

Celada A, Barnet M, Aguado MT, Cruchaud A, Lambert PH. 1982 Ferritin levels and circulating immune complexes in patients with solid tumors. *Bull Cancer* **69,** 22–27.

Covell AM, Jacobs A, Worwood M. 1984 Interaction of ferritin with serum: implications for ferritin turnover. *Clin Chim Acta* **139,** 75–84.

Cragg SJ, Wagstaff M, Worwood M. 1981 Detection of a glycosylated subunit in human serum ferritin. *Biochem J* **199,** 565–571.

Gookin JL, Bunch SE, Rush LJ, Grindem CB. 1998 Evaluation of microcytosis in 18 Shibas. *J Am Vet Med Assoc* **212,** 1258–1259.

Kakuta K, Orino K, Yamamoto S, Watanabe K. 1997 High levels of ferritin and its iron in fetal bovine serum. *Comp Biochem Physiol* **118A,** 165–169.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951 Protein measurement with the Folin phenol reagent. *J Biol Chem* **193,** 265–275.

Luzzago A, Arosio P, Iacobello C, *et al*. 1986 Immunochemical characterization of human liver and heart ferritins with monoclonal antibodies. *Biochim Biophys Acta* **872,** 61–71.

Massover WH. 1994 α_2-Macroglobulin: a ferritin-binding protein. *Ann N Y Acad Sci* **737,** 468–471.

Miyata Y, Furugouri K. 1987 The relationship between serum ferritin concentration and tissue non-heme iron or tissue ferritin in dairy cattle. *Jpn J Vet Sci* **49,** 1157–1159.

Newlands CE, Houston DM, Vasconcelos DY. 1994 Hyperferritinemia associated with malignant histiocytosis in a dog. *J Am Vet Med Assoc* **205,** 849–851.

Niitsu Y, Goto Y, Arisato N, Sasaki K, Kohgo Y. 1988 Ferritin-binding proteins in serum. *Br J Haematol* **68,** 496–497.

Orino K, Saji M, Ozaki Y, Ohya T, Yamamoto S, Watanabe K. 1993a Inhibitory effects of horse serum on immunoassay of horse ferritin. *J Vet Med Sci* **55,** 45–49.

Orino K, Yamamoto S, Watanabe K. 1993b Fibrinogen as a ferritin-binding protein in horse plasma. *J Vet Med Sci* **55,** 785–787.

Ouchterlony Ö. 1953 Antigen-antibody reactions in gels. IV. Types of reactions in coordinated systems of diffusion. *Acta Pathol Microbiol Scand* **32,** 231–240.

Pollock AS, Lipschitz DA, Cook JD. 1978 The kinetics of serum ferritin. *Proc Soc Exp Biol Med* **157,** 481–485.

Pootrakul P, Josephson B, Huebers HA, Finch CA. 1988 Quantitation of ferritin iron in plasma, an explanation for non-transferrin iron. *Blood* **71,** 1120–1123.

Santambrogio P, Cozzi A, Levi S, Arosio P. 1987 Human serum ferritin G-peptide is recognized by anti-L ferritin subunit antibodies and concanavalin-A. *Br J Haematol* **65,** 235–237.

Santambrogio P, Massover WH. 1989 Rabbit serum alpha-2-macroglobulin binds to liver ferritin: association causes a heterogeneity of ferritin molecules. *Br J Haematol* **71,** 281–290.

Schägger H, von Jagow G. 1987 Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal Biochem* **166,** 368–379.

Smith JE, Moore K, Cipriano JE, Morris PG. 1984 Serum ferritin as a measure of stored iron in horses. *J Nutr* **114,** 677–681.

Theil EC. 1987 Ferritin: structure, gene regulation, and cellular function in animals, plants, and microorganisms. *Annu Rev Biochem* **56,** 289–315.

Torti SV, Torti FM. 1998 Human H-kininogen is a ferritin-binding protein. *J Biol Chem* **273,** 13630–13635.

Walters GO, Miller FM, Worwood M. 1973 Serum ferritin concentration and iron stores in normal subjects. *J Clin Path* **26,** 770–772.

Watanabe K, Yahikozawa S, Orino K, Yamamoto S. 1995 Immunological cross- reaction between lactoferrin and transferrin. *J Vet Med Sci* **57,** 519–521.

Weeks BR, Smith JE, Stadler CK. 1990 Effect of dietary iron content on hematologic and other measures of iron adequacy in dogs. *J Am Vet Med Assoc* **196,** 749–753.

Worwood M. 1990 Ferritin. *Blood Rev* **4,** 259–269.

Worwood M, Cragg SJ, Wagstaff M, Jacobs A. 1979 Binding of human serum ferritin to concanavalin A. *Clin Sci* **56,** 83–87.