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Characterization of autoantibodies to ferritin in canine serum

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Abstract

Ferritin-binding proteins circulating in mammalian blood are thought to be involved in the clearance of ferritin. The present study characterizes canine serum autoantibodies (IgM and IgA) that react with ferritin. Canine IgM and IgA bound to bovine spleen ferritin as well as canine liver ferritin. To examine the specificity of canine IgM and IgA to ferritin H and L subunits, we used canine heart ferritin and canine liver ferritin with H/L subunit ratios of 3.69 and 0.43, respectively. Canine IgM and IgA recognized both of the H- and L-subunit-rich isoferritins, showing that their binding activities to ferritin depend on the H-subunit content. Recombinant bovine H-chain ferritin homopolymer expressed in a baculovirus expression system bound more with IgM and IgA than the recombinant L-chain homopolymer expressed under the same conditions. These results suggest that canine IgM and IgA recognize H-subunit-rich isoferritins, and that H-subunit-rich isoferritins are cleared from the circulation more rapidly than L-subunit-rich isoferritins.

Introduction

Ferritin is an iron storage protein with a molecular weight of 500 000 composed of 24 subunits, that occurs ubiquitously in mammalian cells (Harrison & Arosio 1996). Tissue ferritin consists of a variable proportion of two subunits termed H and L, and its Fe/protein ratio is 0.2–0.3 (Worwood *et al.* 1976; Harrison & Arosio 1996; Watanabe *et al.* 2000b). The 21 000 Mr H chain is predominantly found in the heart, red cells, and among HeLa ferritins, whereas the 20 000 Mr L is predominantly found among liver and spleen ferritins (Harrison & Arosio 1996; Watanabe *et al.* 2000b). Human ferritin H- and L-subunits have considerably different physiological and immunological properties (Luzzago *et al.* 1986; Levi *et al.* 1988, 1989). The H subunit includes ferroxidase, which is important for iron uptake, whereas the L-subunit is involved in ferritin stability and iron-core nucleation but has no ferroxidase activity (Levi *et al.* 1988, 1989; Harrison & Arosio 1996).

Low concentrations of ferritin are found in serum of some mammals (<1 μg ml^{-1}) and the amounts are positively correlated with body iron storage (Addison *et al.* 1972; Walters *et al.* 1973; Smith *et al.* 1984; Andrews *et al.* 1992). Human ferritin purified from the serum of patients with idiopathic hemochromatosis is almost entirely composed of the L-subunit and the glycosylated (G) subunit (Mr = 23 000), the latter being responsible for concanavalin A (ConA)-binding and immunologically similar to the L (Worwood *et al.* 1979; Cragg *et al.* 1981; Santambrogio *et al.* 1987). The iron content of serum ferritin is very low (Fe/protein ratio = 0.02–0.07) (Worwood *et al.* 1976; Cragg *et al.* 1981). On the other hand, fetal bovine serum ferritin contains predominantly the L-chain (H/L = 0.03–0.27) but not the G-subunit, and high amounts of iron (Fe/protein ratio = 0.2) (Kakuta *et al.* 1977). Canine serum ferritin predominantly consists of the H-chain (H/L = 3.46 $\pm$ 1.12) and contains relatively high amounts of iron (Fe/protein ratio = 0.112 $\pm$ 0.017), but not the ConA-binding G

subunit (Watanabe *et al*. 2000a, b). Serum ferritins of mammals are biochemically different, and their physiological roles remain to be elucidated.

Ferritin-binding proteins circulating in the serum and/or plasma of mammals 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 H- and L-chains of human ferritin (Torti & Torti 1998). However, the specificity of the ferritin-binding proteins of other mammals to ferritin subunits has not been confirmed.

We found that canine IgM and IgA, but not IgG, have considerable ferritin-binding acitivities, and that serum ferritin exists as immune complexes. The present study characterizes autoanitibodies (IgM and IgA) to ferritin in canine serum.

Materials and methods

Blood and tissue

Blood samples were randomly collected from healthy dogs and canine hearts and livers were obtained under pentobarbital-induced anesthesia. Bovine spleens were collected from slaughtered cattle and cut into small pieces. All samples were stored at -20 °C.

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

SDS-PAGE proceeded according to the method of Schägger and von Jagow (1987) using a 4.5% polyacrylamide stacking gel and a 10% polyacrylamide running gel. 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).

Protein determination

The protein concentration was determined by the method of Lowry *et al*. (1951) with bovine serum albumin (BSA) (Boehringer Mannheim, Germany) as the standard.

Preparation of heart and liver ferritins

Canine heart and liver ferritin was purified according to the procedure described by Watanabe *et al*. (2000a,b). Bovine spleen ferritin was purified as described (Kakuta *et al*. 1997).

Recombinant bovine H- and L-chain homopolymers

Bovine ferritin H- and L-chain cDNAs were expressed using a baculovirus expression system as described (Orino *et al*. 1997). *Spodoptera frugiperda* (Sf-21) cells were maintained in serum-free ESF921 medium (Expression Systems, California, USA) and infected with recombinant baculovirus (AcRP23-*Lac*Z) carrying bovine H- or L-chains at a multiplicity of infection of 5–10 to produce recombinant ferritin subunits. Infected Sf-21 cells were incubated for 7–9 days at 28 °C, and the cells were centrifuged at $1000 \times g$ for 15 min. Triton X-100 (25% v/v) was added to the supernatant containing most of the expressed ferritin subunit proteins to a final concentration of 1.0%. The supernatant was placed at 4 °C overnight, and then stored at -20 °C until use. After thawing, the culture medium was heated and salted-out as described by Kakuta *et al*. (1997). The protein fraction containing expressed ferritin was dialyzed overnight against phosphate-buffered saline (PBS: 20 mM sodium phosphate, 150 mM NaCl, pH 7.2), and then passed through a column (2×100 cm) of Sepharose CL-6B (Pharmacia, Sweden) equilibrated with PBS at a flow rate of 10 ml h^{-1}. The peak fractions containing ferritin were collected.

Detection of ferritin-binding activities of canine antibodies

Ferritin binding activities of canine autoantibodies to ferritin were detected essentially as described (Watanabe *et al*. 2000a). One hundred microliters of 10 μg ml^{-1} ferritin samples in PBS were added to Immuno Plate Maxisorp F 96 microtiter plates (Nunc, Roskilde, Denmark), and placed overnight at 4 °C. Ferritin-coated plates were masked with the ELISA buffer (PBS containing 0.1% BSA and 0.1% Tween20) as described (Orino *et al*. 1993), and then 100 μl aliquot of canine serum diluted 20-fold with ELISA buffer was added to each well and the plates were incubated at 37 °C for 3 h. After washing, 100 μl of alkaline phosphatase-labeled antibody specific for canine IgM and IgA heavy chains (Bethyl Laboratories, Montgomery, Texas, USA) in ELISA buffer was added,

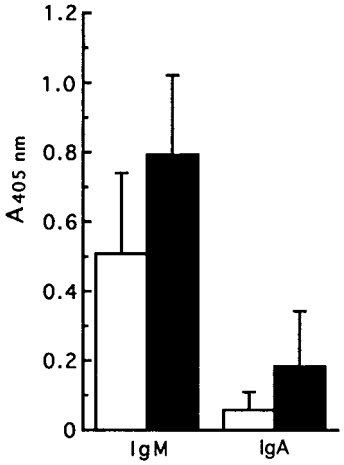

Fig. 1. Binding of canine IgM and IgA to canine liver and bovine spleen ferritins. Canine sera diluted 20-fold with ELISA buffer were added to each well of microtiter plates coated with canine liver (open bars) and bovine spleen ferritins (closed bars). Bound antibodies were detected using alkaline phosphatase-labeled antibodies specific for canine IgM and IgA heavy chains. Each value is the mean ±SD of 6 dogs.

and the plates were incubated at 37 °C for 3 h. After washing, the enzyme reaction proceeded as described (Orino *et al.* 1993).

Results

Ferritin samples

The H/L subunit ratios of ferritins from canine liver and heart, and bovine spleen as determined by densitometry were 0.43, 3.69, and 0.91, respectively. All bovine ferritin H- and L-subunits expressed spontaneously formed 24-mer aggregates as determined by Disc-PAGE (data not shown). The expressed ferritin contained little iron (0.4 ng/μg of protein) as determined coulometrically according to the method described by Kakuta *et al.* (1997)

Binding of canine IgM and IgA to canine liver and heart ferritins and bovine spleen ferritin

Canine IgM and IgA bound to bovine spleen ferritin as well as to canine liver ferritin (Figure 1), although differences among both ferritin-binding activities of 6 sera tested were evident, and in accordance with previous data (Watanabe *et al.* 2000a). Bovine spleen ferritin bound more tightly to IgA and IgM than canine liver ferritin, the H/L-subunit ratio of the former being 0.91 and that of the latter, 0.43. We determined

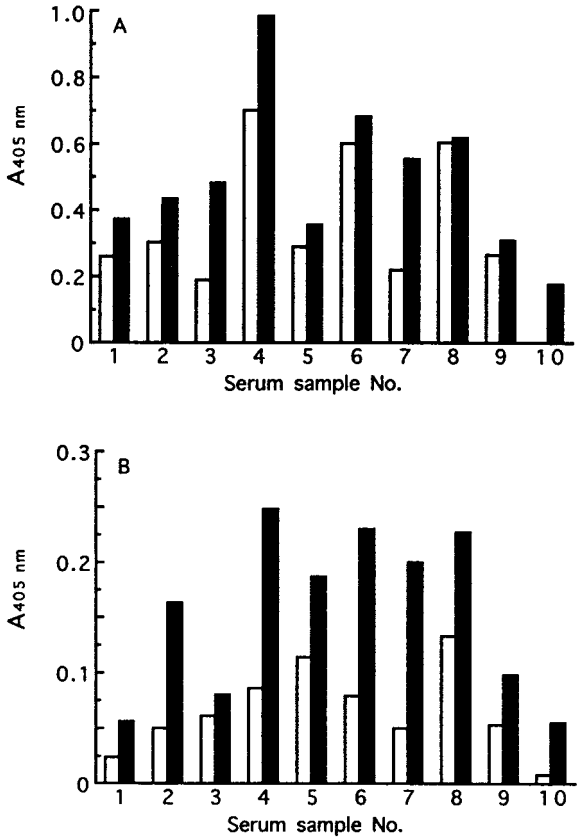

Fig. 2. Binding of canine IgM and IgA to canine liver and heart ferritins. Canine sera from 10 dogs were diluted 20-fold with ELISA buffer and added to wells of microtiter plates. Antibodies bound to canine liver ferritin (open bars) and canine heart ferritin (closed bars) were detected using alkaline phosphatase-labeled antibodies specific for canine IgM (A) and IgA (B) heavy chains.

the specificity of canine IgM and IgA for the ferritin H- and L-subunits using canine heart ferritin as H-subunit-rich isoferritin and canine liver ferritin as L-subunit-rich isoferritin (Figure 2). Canine IgM and IgA bound to both types of ferritin, and the binding appeared to be dependent on the content of H. IgM binding to both isoferritins in four canine serum samples (Nos. 5, 6, 8, and 9) showed essentially the same binding activities. Although canine heart and liver ferritins contained either L- or H-subunits exclusively, when they were coated on the plates, the distribution of subunits on the binding surfaces might not completely exclude minor subunits.

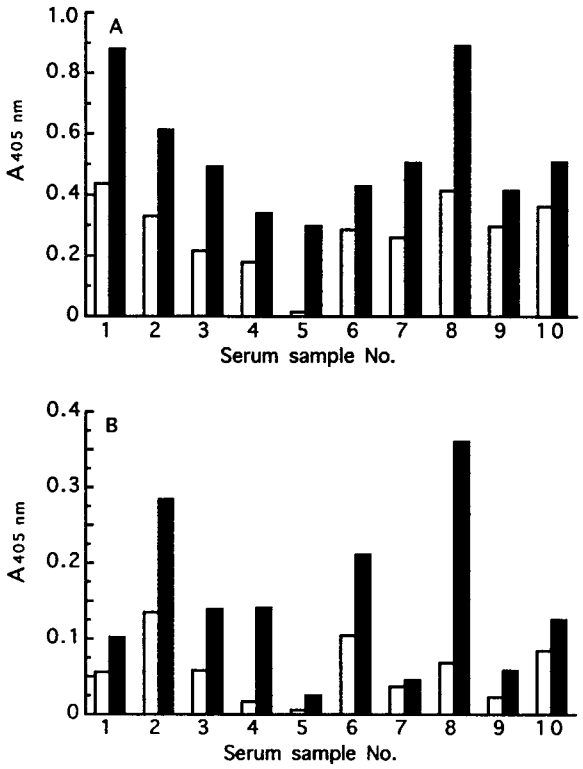

Fig. 3. Binding of canine IgM and IgA to recombinant bovine H- and L-chain ferritin monomers. Canine sera from 10 dogs were diluted 20-fold with ELISA buffer and added to wells of microtiter plates. Antibodies bound to bovine L-chain homopolymer (open bars) and H-chain homopolymer (closed bars) were detected using alkaline phosphatase-labeled antibodies specific for canine IgM (A) and IgA (B) heavy chains.

Specificity of canine IgM and IgA for bovine H- and L-chain homopolymers

To clarify the specificity of canine IgM and IgA to ferritin subunits, we used bovine H- and L-chain homopolymers expressed by a baculovirus expression system. Both IgM and IgA showed higher binding affinity for the H-chain homopolymer than for the L-chain homopolymer (Figure 3).

Discussion

Ferritin H- and L-subunits are immunologicaly different (Luzzago *et al.* 1986; Watanabe *et al.* 2000b). The present study examined whether canine IgM and IgA that bound to ferritin preferentially recognize the particular form. The ferritin-binding activities of IgM and IgA in canine sera did not show correlation with the serum ferritin concentrations (Watanabe *et al.* 2000a). Because canine IgM and IgA reacted with expressed ferritin H- and L-chain homopolymeres which had little iron, the ferritin-binding activities did not seem to depend on ferritin iron content. Canine IgM and IgA depended on the H-content for ferritin binding. Naturally-occurring ferritins do not completely exclude either the H- or the L-type subunit. Amino acid sequence homology between subunits across mammalian species is highly conserved (H > 91%, L > 79%) (Orino *et al.* 1997), although the amino acid sequences of the canine H- and L-chains have not been yet determined. We found that canine IgM and IgA had higher specificity for expressed bovine H-subunit than for expressed bovine L-subunit.

Some ferritin-binding proteins have been identified in the serum and/or plasma in mammals (Santambrogio & Massover 1989; Orino *et al.* 1993; Massover 1994; Torti & Torti 1998). In humans, ConA-binding ^{131}I-serum ferritin (a half-life $T_{1/2}$ is approximately 50 h) is removed more slowly than non-ConA-binding ^{131}I -spleen ferritin ($T_{1/2}$= 5 h) (Worwood *et al.* 1982). Human H-rich isoferritins probably bind to a serum component(s) to form complexes with ferritin, although ferritin itself does not seem to form immune complexes (Covell *et al.* 1984). Ferritin-binding protein(s) appear to be involved in the clearance of ferritin from the circulation, although their physiological roles remain to be elucidated. Clearance half times of liver ferritin (probably L-subunit-rich isoferritin) from the circulation of dogs was less than 10 min (Pollack *et al.* 1978). However, we found that canine serum ferritin was H-subunit-rich, and that the mean values of canine serum ferritin concentration (479 ± 286 ng ml^{-1}) and its iron/protein ratio (0.112 ± 0.017) were relatively high (Watanabe *et al.* 2000a, b). The H subunit containing ferroxidase is important for sequestering iron, while the L-subunit is not, suggesting that canine serum ferritin incorporates iron.

The present study suggests that H-subunit-rich isoferritin fractions in canine serum are cleared from the circulation more rapidly, and that serum ferritin plays a role as an iron-transporter in the circulation. Guinea-pig reticulocytes incorporate ^{125}I- or ^{59}Fe-labeled liver ferritin by receptor-mediated endocytosis as ferritin-iron, and utilize the iron for heme synthesis (Blight & Morgan 1983). Pregnant guinea-pigs injected ^{59}Fe- and ^{125}I-labeled tissue ferritin transport the iron to the fetus through the placenta (Lamparelli *et al.* 1989). Bovine fetal serum ferritin may contribute to iron transport in fetal bovine circulation because of a high iron content (about 20%) and a high percentage of

serum ferritin iron to total serum iron (8.8–28.5%) (Kakuta *et al*. 1997). These results indicate that ferritin plays a physiological role as an iron-transporter as well as transferrin.

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