Conglutinin, Mannan-Binding Protein, and Serum Amyloid P Component Concentrations in Sera from Cows: Changes Associated with Mastitis

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ABSTRACT. To study the roles of conglutinin (Kg), mannan-binding protein (MBP), and serum amyloid P component (SAP) in the protection of cattle against infections, the concentrations of these proteins in the sera from cows with mastitis were determined by sandwich enzyme-linked immunosorbent assays. The Kg and MBP concentrations in the sera from cows with mastitis were much lower than those from uninfected (or clinically healthy) ones, and increased after recovery. No significant difference in the SAP concentrations was found between healthy and infected cows. With the sera from cows experimentally infected with bovine leukemia virus, the Kg concentrations were also lower than those from uninfected ones, whereas the MBP concentrations were not. From these findings, the Kg concentration is suggested to be serologically a possible indicator for clinical diagnosis of treatment for mastitis although both Kg and MBP concentrations were found to decrease in cows with mastitis and to increase after recovery.—**KEY WORDS:** bovine, conglutinin, mannan-binding protein, mastitis, serum amyloid P component.

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Bovine serum contains conglutinin (Kg), mannan-binding protein (MBP), IgM, and IgG which are Ca^{2+} -dependently reactive with invertase and/or mannan [13, 14, 25, 26] in addition to C-reactive protein (CRP) and serum amyloid P component (SAP) [17, 18]. The serum CRP and SAP concentrations increase rapidly in human and mouse with acute inflammation caused by infection or tissue injury [15, 20, 21], whereas they did not significantly increase in cows with acute inflammation [17, 19]. In contrast to these two serum proteins, the serum Kg concentrations of cattle have been influenced by the season, nutritional state, parturition, and infections [5, 9–12, 22]. Moreover, Kg is well-known to bind to complement [14] and to show antibacterial activity [7, 9]. To study the roles of Kg, MBP, and SAP in the protection of cattle against infections, therefore, attempts were made to compare the Kg, MBP, and SAP concentrations in sera from uninfected (or clinically healthy) and infected cows by sandwich enzyme-linked immunosorbent assays (ELISA) which have been recently developed in this laboratory [1].

MATERIALS AND METHODS

Bovine sera and purification of Kg, MBP, and SAP: Bovine sera for sandwich ELISA were obtained from the blood samples collected from healthy and infected cattle (female Holsteins) in the areas of Ashoro Branch, Hokkaido Tokachi Agricultural Mutual-Aid Association, Ashoro, Hokkaido and of National Institute of Animal Health, Tsukuba, Ibaraki, Japan. Clinical diagnosis of mastitis was done by agglutination of leukocytes in the milk using the reagent for the PL test (Nihon Zenyaku Kogyo. Co., Fukushima, Japan) which is a slightly modified method of California Mastitis Test (CMT) [23]. The PL test is used to determine pH (P) and the number of leukocytes (L) in the suspected milk. When leukocytes in the milk from the suspected cows were found to be agglutinated in the PL test, the cow was diagnosed as mastitis. The sera from cows with mastitis were obtained from blood samples collected between 1 and 2 days after the mastitis was recognized and clinical treatment with kanamycin was initiated. After no agglutination of leukocytes in the milk from the treated cows was confirmed in the PL test 21 days after the final clinical treatment was done, the sera were obtained from the blood samples from the cows.

Bovine serum samples were also obtained from 2 cows aged 2.5 and 3.5 years with apparently

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persistent lymphocytosis induced by subcutaneous injection of bovine leukemia virus (BLV)-infected peripheral blood lymphocytes (1×10^7 cells) 2 times for 6 months. These two cows were found to be clinically healthy after they produced antibodies against the specific antigens and core proteins of BLV, which were detected in agar gel immunodiffusion tests.

Bovine serum for isolation of Kg, MBP, and SAP was obtained from a local slaughter house. Kg, MBP, and SAP were isolated by affinity chromatography on mannan-Sepharose 4B or Seakem HE agarose gels followed by gel filtration as described previously [13, 18, 26].

Identification of microorganisms: Identification of microorganisms in the milk from the suspected cows was basically carried out by the methods described previously [2, 3].

Preparation of antisera and biotinylation: Antisera were prepared by immunizing rabbits with purified Kg, MBP, and SAP by the immunization schedule as described previously [24]. Antibodies were purified by affinity chromatography on purified Kg-, MBP-, or SAP-coupled Sepharose 4B [4]. Biotinylation was performed according to the methods described previously [8]. One hundred μg of NHS-LC-Biotin (Pierce, IL, U.S.A.) was added to one ml of purified antibody (1 mg/ml) equilibrated with 0.1 M NaHCO_3 . The mixture was allowed to stand at room temperature for 4 hr. After then, the mixture was subjected to a PD-10 column (Pharmacia, Uppsala, Sweden) equilibrated with 0.15 M phosphate buffered saline, pH 7.4 (PBS).

Enzyme-linked immunosorbent assays (ELISA): Sandwich ELISA was carried out in quadruplicate with microtiter ELISA plate (Corning Inc. N.Y., U.S.A.). Each well of ELISA plates was coated at 4°C overnight with 100 μl of purified antibody (4–5 $\mu\text{g}/\text{ml}$). After washing the plates 3 times with PBS containing 0.05% Tween 20, 200 μl of 0.25% gelatin in PBS was added to each well for blocking at 37°C for 1 hr. After the plates were similarly washed, 100 μl of diluted bovine sera or purified serum protein was added to each well. The plates were incubated at 37°C for 1 hr. After washing, 100 μl of biotinylated antibody (diluted to 1:1,000–1:5,000) was added to each well and the mixture was incubated at 37°C for 1 hr. After washing the plates, 100 μl of ExtrAvidin-Peroxidase (Sigma Chemical Co., St. Louis, U.S.A., diluted to 1:10,000) was added to each well. After incubation at 37°C for 30 min, the

plates were similarly washed. One hundred μl of the substrate solution containing 0.03% H_2O_2 and 1 mg/ml *o*-phenylenediamine in 0.1 M citrate-phosphate buffer, pH 5.0 was finally added to each well and allowed to develop color in the dark at room temperature for 30 min. To stop the reaction, 25 μl of 10 N H_2SO_4 was added to each well. The enzyme-substrate reaction was determined spectrophotometrically with a microplate photometer (Corona Electric Co., Tokyo, Japan) at 492 nm. A buffer blank or negative controls were also assayed for any nonspecific color reaction. Absorbances obtained with four determinations were averaged, from which the average blank value was subtracted. The net absorbance were plotted against Kg, MBP, and SAP concentrations (ng/ml).

RESULTS

Determination of Kg, MBP, and SAP concentrations in sera from healthy cows: With coating antibodies at 4–5 $\mu\text{g}/\text{ml}$, dose-response curves were obtained over range of 5 to 250 ng/ml of purified Kg, MBP, and SAP. The ELISA system was found to be reliable for determination of these serum proteins at concentrations as low as 5 ng/ml as reported previously [1]. Table 1 summarizes the concentrations of Kg, MBP, and SAP in sera from 10 healthy cows aged 2–7.5 years. The values are reported as $\mu\text{g}/\text{ml}$ and represent the mean $\pm$ SD.

Determination of Kg, MBP, and SAP concentrations in sera from infected cows: Table 1 also summarizes the concentrations of Kg, MBP, and SAP in sera from 5 cows aged 4–7 years with mastitis and in those from the same cows after recovery. The results are presented as $\mu\text{g}/\text{ml}$ and represent the mean $\pm$ SD. Figure 1 shows the Kg, MBP, and SAP concentrations in each serum obtained from infected, and clinically treated and recovered cows as

Table 1. The Kg, MBP, and SAP concentrations in the sera from healthy, infected, and recovered cows

cow	concentration $\mu\text{g}/\text{ml}$ (mean $\pm$ SD)		
	Kg	MBP	SAP
Healthy ^{a)}	56.5 $\pm$ 14.4	2.37 $\pm$ 0.87	11.4 $\pm$ 3.9
Infected ^{b)}	1.4 $\pm$ 1.1	0.77 $\pm$ 0.41	12.5 $\pm$ 3.3
Recovered ^{c)}	28.9 $\pm$ 13.6	2.48 $\pm$ 0.82	16.0 $\pm$ 4.3

a) Sera from 10 healthy cows aged 2–7.5 years, b) Sera from 5 cows with mastitis aged 4–7 years as listed in Table 2, c) Sera from the same 5 cows after recovery by clinical treatment as listed in Table 2.

Fig. 1. Serum concentrations ($\mu\text{g/ml}$) of conglutinin (Kg), mannan-binding protein (MBP), and serum amyloid P component (SAP) in the sera obtained from healthy and infected cows determined by sandwich ELISA. Healthy, healthy cows; Mastitis, cows suffered from mastitis; Recovered, clinically treated and recovered cows; BLV, cows experimentally infected with bovine leukemia virus.

compared with those from healthy cows. As shown in Fig. 1, much lower Kg concentrations were found in the sera from cows suffered from mastitis as listed in Table 2. Following treatment, the Kg concentrations in the sera from the same recovered cows were found to trend to rise to the normal concentrations

Table 2. Different sera obtained from infected adult cows

Cow No.	Age(yr)	Clinical diagnosis	PL test	Infected with
2	6	Mastitis	(+)	<i>S. uberis</i>
		Recovered	(-)	
		Mastitis	(+)	NT
3	6	Mastitis	(+)	<i>K. pneumonia</i>
		Recovered	(-)	
4	7	Mastitis	(+)	<i>P. aeruginosa</i>
		Recovered	(-)	
5	4	Mastitis	(+)	<i>E. coli</i>
		Recovered	(-)	
6	4	Mastitis	(+)	NT
		Recovered	(-)	
7	2.5		NT	BLV
		Lymphocytosis		
8	3.5		NT	BLV
		Lymphocytosis		

PL test, simplified agglutination test with leukocytes in milk and PL test reagent; (+), positive agglutination; (-), negative agglutination; NT, not tested; BLV, bovine leukemia virus.

in healthy cows (Fig. 1).

Low Kg concentrations were also found in the sera from cows aged 2.5–3.5 years experimentally infected with BLV (Fig. 1 and Table 2).

On the other hand, the Kg concentrations (64 and 41 $\mu\text{g/ml}$) in the sera from two calves aged 8 months which were experimentally splenectomized were found to be much higher than those (3.45 $\mu\text{g/ml}$) from healthy calves.

With respect to MBP, The MBP concentrations in the sera from cows with mastitis decreased and then increased after recovery (Table 1 and Fig. 1). However, the MBP concentrations (2.0 and 2.2 $\mu\text{g/ml}$) in the sera from two cows experimentally infected with BLV were quite similar to those (2.37 $\pm$ 0.87 $\mu\text{g/ml}$) from healthy cows (Table 1 and Fig. 1). The MBP concentrations in the sera from two experimentally splenectomized calves were similar to those from healthy calves.

In contrast to Kg and MBP, the SAP concentrations was found to be similar in the sera from healthy and infected cows (Table 1 and Fig. 1). No difference in the SAP concentrations was found between experimentally splenectomized and unoperated calves.

DISCUSSION

According to the previous reports [5, 9, 11, 22], the lowest or lower Kg concentrations were found in

cattle with acute infections, whereas the Kg rose to adult normal concentrations in about 4 weeks after recovery. Similar fluctuation in the Kg concentrations was found in the sera from cows with mastitis. The Kg concentrations were found to decrease in the sera from cows with mastitis. After treatment and recovery, the Kg concentrations increased in the sera from the recovered cows. Low Kg concentrations were also found in the sera from the cows aged 2.5–3.5 years experimentally infected with BLV (Fig. 1). These findings suggest that the Kg concentrations in the sera may be associated with the physiological condition of the cows with mastitis or BLV. This is supported by the present finding that the cow (cow no. 2 as shown in Fig. 1) possessing the low Kg concentrations again suffered from mastitis although it showed negative PL test after clinical treatment with kanamycin (Table 2). Thus, the Kg concentrations may be serologically a more useful indicator for clinical treatment of mastitis than a simplified agglutination test using the PL reagent.

On the other hand, the physiological condition of cattle seems to be a very important factor when the Kg concentrations are determined since fluctuations in the Kg concentrations have been reported to be reflected by the physiological condition of cattle [5, 9–12, 22]. This is supported by the findings on the Kg concentrations in sera from two calves aged 8 months which were experimentally splenectomized. The Kg concentrations (64 and 41 $\mu\text{g/ml}$) in the sera from the two experimentally splenectomized calves were found to be much higher than those (3.45 $\mu\text{g/ml}$) from healthy calves.

The MBP concentrations in sera from healthy cows were not so high as compared with the Kg concentrations (Table 1 and Fig. 1). However, the MBP concentrations in the sera from cows with mastitis were found to be lower and they increased to the normal levels after recovery as found for the case with Kg. It has been reported that human serum MBP showing low concentrations of MBP were found in children whose sera are unable to opsonize baker's yeast, who are known to suffer from severe recurrent infection [27]. In contrast to the low MBP concentrations in the sera from cows with mastitis, however, the normal MBP concentrations were found in the sera from two cows experimentally infected with BLV. In addition, the cow (cow no. 2) again suffered from mastitis although it showed healthy MBP concentration during and after clinical treatment. These findings

suggest that the MBP concentrations in bovine sera may not be influenced by the physiological condition of cows with all of bovine infectious diseases. Thus, bovine serum MBP may not be an acute phase reactant as found for human MBP [6] since the low MBP concentrations in the sera from cows with mastitis are contrary to the rapid increase in the haptoglobulin concentrations in the sera from cows with the same disease [16].

The SAP concentrations were found to be similar in the sera from healthy and infected cows (Table 1) although the Kg and MBP concentrations decreased in cows with infectious diseases and increased after recovery. Thus, bovine SAP may not be an acute phase reactant although it is one of lactation-associated proteins as reported previously [19].

From the present findings, the change in the Kg concentrations is suggested to be serologically an useful indicator to clinical diagnosis of treatment for mastitis.

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