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Specificity and Prevalence of Natural Bovine Antimannan Antibodies

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Immune responses to the carbohydrate components of microorganisms, mediated both by antibodies and by lectins, are an important part of host defense. In the present experiments, the specificity and presence of natural bovine antibodies against mannan, a common fungal antigen, were examined by enzyme-linked immunosorbent assay (ELISA), using *Saccharomyces cerevisiae* mannan as an antigen. The results showed that all serum samples from animals of three age groups (newborn, calf, and adult) tested contained antimannan antibodies, and the titer of these antibodies increased significantly in adults. However, titers among individual adult cattle differed widely. Inhibition assays showed that yeast mannan was the strongest inhibitor. D-Mannose exhibited only a minor inhibitory effect at high concentrations. This suggests that most of these antibodies recognize an oligosaccharide-based epitope(s) different from those recognized by lectins. Cattle possess three serum C-type lectins (collectins) capable of recognizing mannan in a calcium-dependent manner. Addition of EDTA to the reaction did not reduce antibody binding, suggesting that the binding of these antibodies to mannan was not affected by the presence of collectin. The antibodies purified from either calf or adult serum by mannan-Sepharose affinity chromatography consisted of mainly immunoglobulin G (IgG) and a smaller amount of IgM. IgG1 was shown to be the dominant antimannan IgG isotype by isotype-specific ELISA. Together, these results demonstrate the production of natural antimannan antibodies in cattle in an age-dependent manner. These antibodies might be involved in defending the host against mannan-containing pathogens as a specific line of defense in conjunction with the innate response by lectins.

Immunity to carbohydrate antigens plays an important role in resistance to infectious agents (19, 24). Natural carbohydrate-specific antibodies are found in normal humans and animals. They are produced independently of immunization (3), and their presence is considered to be a result of the immune response to normal environmental antigens such as the bacterial flora in the gut. Examples include natural blood group alloagglutinins (33) and human anti-alpha-galactosyl antibodies (23). However, these natural anticarbohydrate antibodies might also be the result of subclinical or unrecognized infections. Studies with human anti-alpha-galactosyl antibodies have shown that these antibodies may play an important role in host defense (23).

In addition to anticarbohydrate antibodies, lectins present in serum and other body fluids also play an important role in host defense. A major group of these lectins consists of collectins (5, 7). Collectins are C-type lectins whose binding to ligands is calcium dependent. So far, five collectins have been identified: conglutinin, collectin 43 (CL-43), mannose-binding protein (MBP), and pulmonary surfactant proteins A (SP-A) and D (SP-D). Three of them (conglutinin, CL-43, and MBP) are found in serum, and the other two (SP-A and SP-D) are found in the lung (7). Conglutinin and CL-43 are found only in cattle (1, 5, 14). Thus, cattle are unique in that they possess three serum collectins (conglutinin, CL-43, and MBP) whereas other animals, including humans, are known to have only one (MBP). The sugar-binding preferences of these three bovine lectins are similar, as demonstrated by inhibition assays with monosaccharides; both MBP and conglutinin preferentially bind mannose and *N*-acetylglucosamine (GlcNAc), while CL-43 binds mannose and *N*-acetylmannosamine (7, 14). These collectins are an important part of innate immunity (5). They bind to their targets through their C-terminal carbohydrate recognition domains and activate complement or interact with phagocytic cells through their N-terminal domains, leading to the destruction of the targeted microorganisms. Collectins are especially important in newborns and the young, whose immune systems are not fully capable of mounting an efficient specific immune response. A congenital deficiency of MBP is responsible for numerous cases of immunodeficiency in children, who as a result suffer from recurrent infections (28–30). Adult humans with MBP deficiency, however, do not suffer from repeated infections as children do. This suggests that the development of specific humoral responses may have compensated for the loss of MBP.

Mannan or alpha-mannan is a common fungal antigen. It generally consists of an α(1-6)-linked mannose backbone to which short α(1-2)- and/or α(1-3)-linked mannose side chains are attached (11, 26). β(1-2)-linked mannose side chains may also be present (11, 25). The detailed structure may differ among species. The mannan antigens from the yeasts *Candida* spp. and *Saccharomyces cerevisiae* have been extensively studied (10, 11, 26). The *S. cerevisiae* mannan, which has structural and antigenic features similar to those of the *Candida* mannan, is commonly used as an affinity ligand for isolation or detection of collectins, especially MBP (7, 28). Mannans with similar structural features are also found in other fungi, including *Trichophyton mentagrophytes* (9) and *Aspergillus oryzae* (18), and in bacteria such as *Mycobacterium tuberculosis* and *Mycobacterium bovis* (8, 32). Some epitopes from yeast mannan cross-react with mannose-containing polysaccharides from other microorganisms, including *Serratia marcescens* (21) and *Salmonella thompson* (20).

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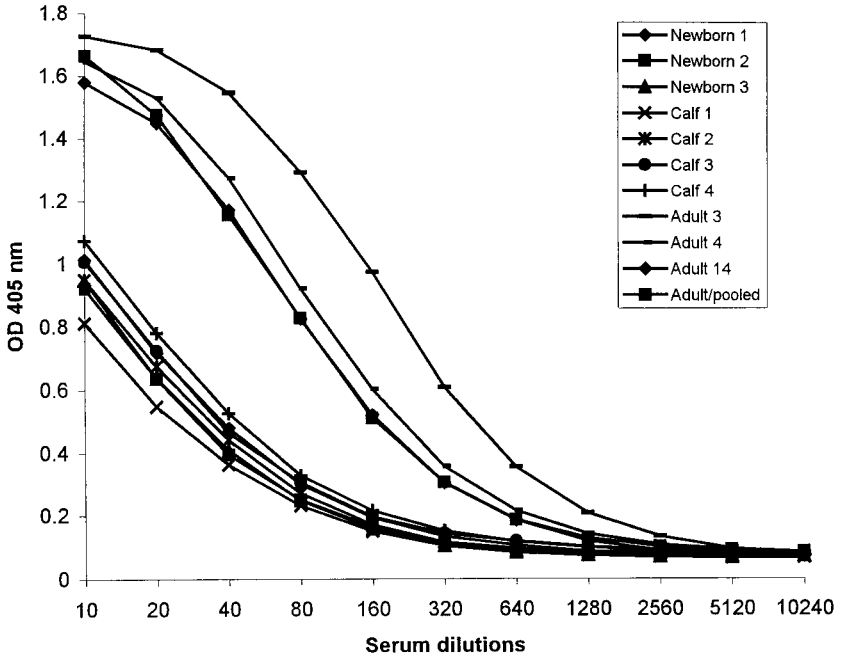

FIG. 1. Titration curves for 11 bovine serum samples (3 from newborns, 4 from calves, and 4 from adults).

Many of these fungi, including *Candida albicans*, are important pathogens. *C. albicans* is a normal inhabitant of the digestive tract, oral cavity, and vagina. Infections usually occur endogenously; i.e., they are caused by yeasts already present in the body. In cows, *C. albicans*, along with several other *Candida* species, can cause mastitis (13, 27). Vaginal infection by *Candida* in humans is particularly widespread (4). Antimannan antibodies have been found in normal and infected humans (15, 31), and they have been shown to be protective against yeast infections in experimental animals (4, 35). However, their protective role has not been directly demonstrated in humans. Recently, the anti-*S. cerevisiae* mannan antibodies found in normal humans have been suggested to be associated with inflammatory bowel disease (22).

Given the ubiquitous nature and the disease-causing potential of mannan-containing microorganisms, examination of antimannan antibodies could potentially yield important clues regarding immunity against these microorganisms. This is of particular interest with regard to cattle because they, unlike other mammals, possess three serum collectins, all of which are capable of binding to the mannan antigen. Studies of these antibodies in cattle may provide useful information on the role of antimannan antibodies and their relationship to lectin-mediated innate responses.

MATERIALS AND METHODS

Materials. Gamma-globulin-free bovine serum albumin, affinity-purified alkaline phosphatase-conjugated anti-bovine immunoglobulin G (IgG; heavy- and light-chain [H+L]) antibodies, and yeast (*S. cerevisiae*) mannan were obtained from Sigma Chemical Co. (St. Louis, Mo.). Monosaccharides, D-mannose, methyl D-mannose, GlcNAc, D-fucose, and D-glucose were also obtained from Sigma Chemical Co. Acemannan, a partially acetylated β(1-4)-linked mannan isolated from *Aloe vera*, was provided by Carrington Laboratories Inc. (Irving, Tex.). The alkaline phosphatase substrate *p*-nitrophenylphosphate was obtained from Pierce (Rockford, Ill.). Enzyme-linked immunosorbent assay (ELISA) plates were obtained from Nunc, Inc. (Naperville, Ill.).

Serum samples. One fetal, three newborn (1 to 10 days old), and five calf (~6 months old) bovine pooled serum samples were obtained from Gibco-BRL (Grand Island, N.Y.). Nineteen sera were obtained from adult cows at an age of 1 to >10 years at the Veterinary Medicine Park, Texas A&M University. All adult samples were collected in October 1996. An older sample, collected in May 1992 from one cow, was also used and designated no. 16-2. The new sample from this same cow was designated no. 16-1. All serum samples were kept at −20°C. A pooled adult sample was prepared by mixing equal volumes of individual adult samples, excluding sample no. 1, 6, 12, and 16-1 because of the limited amounts available.

ELISA. An indirect ELISA was used to measure the bovine antimannan antibodies. Plates were coated overnight at 4°C with yeast mannan (25 μg/ml) in 50 mM carbonate buffer (pH 9.6) (100 μl/well). The coated plates were washed three times with wash buffer (phosphate-buffered saline [PBS] with 0.02% NaN_3) and then blocked with the dilution buffer (3% bovine serum albumin, 0.05% Tween 20, and 0.02% NaN_3 in PBS) for 1 h. Serum samples diluted in the same buffer were added to the plates (100 μl/well), which were incubated at room temperature for 2 h. After the plates were washed as described above, the secondary antibody (alkaline phosphatase-conjugated anti-bovine IgG [H+L] antibodies), at a 1:1,000 dilution, was added (100 μl/well), and the plates were incubated at room temperature for 1 h. After being washed three times, each well received 100 μl of the substrate *p*-nitrophenylphosphate prepared according to the instructions of the manufacturer (Pierce). The reaction was stopped after a 10 min-incubation by addition of 50 μl of 2 M NaOH. The optical density (OD) at 405 nm was determined by using a microplate reader (Dynatech MR600).

For inhibition assays, sugars were first serially diluted twofold in the dilution buffer. Each dilution was then mixed with an equal volume of a diluted serum sample. The mixture was then kept at room temperature for 1 h before being added to the plates. The remaining steps were the same as described above. The inhibition efficiencies of various sugars were compared by determining the lowest concentrations that gave a 50% reduction in OD values.

Analysis of ELISA data. The titration curves of the newborn and calf serum samples were consistently parallel to each other but not to those of adults (Fig. 1). Thus, pooled adult serum was used as a reference for determining the titers of adult samples, and a pooled calf serum sample (no. 2) was used for the newborn and calf samples.

The titration end point was arbitrarily chosen as the highest serum dilution that gave an OD value twofold higher than the background (fetal bovine serum). The end point was assigned a value of 1 titer unit/100 μl. This gave calf sample no. 2 a unit range of 1 to 16 over five serial twofold dilutions (1:10 to 1:160) and the pooled adult sample a unit range of 1 to 64 over seven serial twofold dilution (1:10 to 1:640). The OD values were plotted against the logarithms of the titer units. A linear fit was made, and the log unit value of each sample was obtained by extrapolating against the regression line. The number of titer units per 0.1 ml of serum was calculated by an antilog transformation followed by multiplication by the sample dilution. All samples were tested at a 1:80 or 1:160 dilution so that the OD values would be within the standard curve range. The geometric means for different age groups were compared by the unpaired Student *t* test.

Measurement of antimannan IgA, IgG1, and IgG2. Antimannan IgG1, IgG2, and IgA were measured by an indirect ELISA using affinity-purified anti-bovine

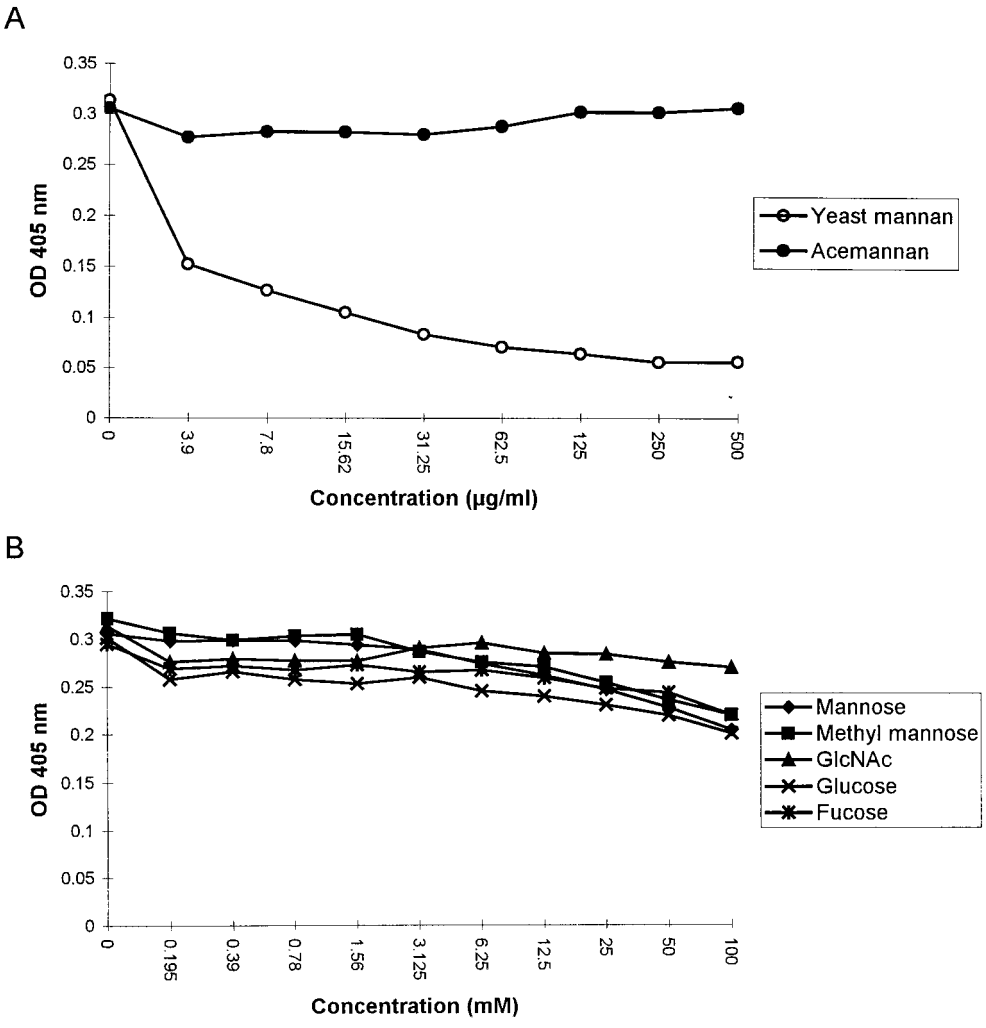

FIG. 2. The sugar inhibition assay. Calf serum sample no. 2 was used at a 1:80 dilution. (A) Monosaccharides; (B) polysaccharides.

IgA, IgG1, and IgG2 antibodies conjugated to horseradish peroxidase (Bethyl Laboratories, Montgomery, Tex.). The ELISA was performed as described above in a reagent excess manner. The conjugates and secondary antibodies were used at a 1:2,000 dilution. All serum samples were diluted 1:40. Tetramethylbenzidine (Pierce) was used as the substrate. The reaction was stopped after a 30-min incubation by addition of 100 μl of 2 M H_2SO_4. The OD at 450 nm was determined by using a microplate reader (Dynatech MR600).

Affinity chromatography. Five-milliliter columns of mannan-Sepharose beads (Sigma Chemical Co.) were used for isolation of bovine antimannan antibodies. The columns were first washed with 50 ml of loading buffer (20 mM Tris, 200 mM NaCl, 0.02% NaN_3, pH 7.4). Calf serum no. 2 or the pooled adult sample (10 ml) was mixed with an equal volume of the same buffer containing 5 mM $CaCl_2$ and loaded onto the column at a rate of 15 ml/h. The column was then washed with 100 ml of the buffer, bound proteins were eluted with 10 ml of elution buffer (0.5 M glycine, 0.15 M NaCl, pH 2.5), and 0.5-ml fractions were collected. Fractions containing detectable protein were pooled, dialyzed against PBS, and concentrated by using a 10-kDa-cutoff membrane concentrator (Amicon, Beverly, Mass.).

Gel electrophoresis and immunoblotting. Electrophoretic analysis of isolated proteins was carried out under denaturing conditions as previously described (12). Proteins were stained with Coomassie blue. For densitometric analysis of the protein bands, the stained gel images were acquired by using a GS-5000 digital imaging system (Alpha Infotech Co.) and the ODs of protein bands were measured by using the NIH Image program (version 1.6; National Institutes of Health).

For immunoblot analysis, proteins were blotted from the gel onto Immobilon polyvinylidene difluoride membranes. Blotted membranes were first blocked at room temperature for 2 h in TN buffer (25 mM Tris, 150 mM NaCl, pH 7.4) containing 3% Blotto and 0.05% Tween 20. They were then probed at room temperature for 1 h with the affinity-purified alkaline phosphatase-conjugated anti-bovine IgG (H+L) or anti-bovine IgM (μ) in TN buffer containing 1% Blotto and 0.05% Tween 20. After being washed with TN buffer containing 0.05% Tween 20, membranes were developed with the alkaline phosphatase substrate 5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium (BCIP-NBT; Sigma Chemical Co.).

RESULTS

Antimannan antibodies in serum were measured by ELISA, using yeast (*S. cerevisiae*) mannan as the antigen (see Materials and Methods). The polyclonal anti-bovine whole-molecule IgG (H+L) was used as the secondary antibody in the ELISA. It detects IgG but can also detect other classes of immunoglobulins, including IgM and IgA, since bovine light chain (lambda) is shared by all immunoglobulin classes (17). The titration curves for 11 serum samples (from three newborns, four calves, and four adults) are shown in Fig. 1. The curves for the newborn and calf sera were parallel to each other but not to those of adults. This suggests that the mannan-reacting antibodies of newborns and calves may differ in antibody isotype composition and/or affinity from those of adults. A pooled calf serum sample (no. 2) and a pooled adult serum sample were used as the references and were included in all assays.

Specificity of bovine antimannan antibodies. Inhibition assays with yeast mannan and other saccharides were used to

TABLE 1. Inhibition assays using calf and adult sera

Inhibitor	Concn	OD (405 nm) value minus background[a]		% Inhibition	
		Calf	Adult	Calf	Adult
None		0.36	0.382		
Yeast mannan	0.05 mg/ml	0.045	0.09	88	76
Acemannan	0.1 mg/ml	0.373	0.371	0	3
Methyl mannose	100 mM	0.266	0.34	26	11
Mannose	100 mM	0.305	0.354	15	7
GlcNAc	100 mM	0.365	0.362	0	5
Glucose	100 mM	0.345	0.333	4	15
Fucose	100 mM	0.369	0.392	0	0

[a] Calf serum (no. 2) was used at a 1:40 dilution, and the pooled adult serum was used at a 1:160 dilution.

determine the specificity of the bovine antimannan antibodies. The results using calf sample no. 2 are shown in Fig. 2. Only the yeast mannan exhibited strong inhibition (Fig. 2A). The β(1-4)-linked mannan (acemannan) and monosaccharides including mannose and methylmannose showed only minimal, if any, inhibition (Fig. 2). These results suggest that these antibodies are most likely directed against oligosaccharide sequences. Inhibition assays using the pooled adult sample were also performed, and a similar inhibition pattern was obtained (Table 1).

Bovine serum possesses three collectins, all of which are capable of binding to the yeast mannan in a calcium-dependent manner. To determine if these collectins affect the binding of the antimannan antibodies, EDTA was added to the assay during serum antibody incubation. There was no significant change in OD value in the presence of EDTA for either the calf or adult sample (Fig. 3). This suggests that collectins do not influence the binding of antimannan antibodies.

Presence of bovine antimannan antibodies in different age groups. Twenty-six bovine serum samples from cows of three age groups were tested for the presence of antimannan antibodies (Fig. 4). They included 3 pooled newborn (1 to 10 days) samples, 5 pooled calf (~6 months) samples, and 19 adult (>1 year) samples. All of them, including the newborn sera, contained antimannan antibodies (Fig. 4). There was no significant difference in the antibody titers between the newborn (mean titer, 194) and the calf (mean titer, 165) sera (Fig. 4). However, titers were significantly higher in adult samples ($P < 0.05$) than in samples from newborns or calves. Antibody titers of adult serum samples differed widely, with a mean of 836 and a standard deviation of 717 (Fig. 4).

Purification of bovine antimannan antibodies. The antimannan antibodies were purified from calf or adult serum by affinity chromatography using yeast mannan-Sepharose. $CaCl_2$ was added to the loading buffer to facilitate the isolation of bovine collectins. The gel electrophoresis analysis of proteins eluted from the column showed that these antibodies consisted of mostly IgG and a smaller amount of IgM (Fig. 5A). The antibody heavy and light chains were identified by immunoblot analysis (Fig. 5B and C). The protein migrating at about 200 kDa was the whole undenatured IgG molecule since it reacted with anti-IgG (H+L) antibody but not with anti-IgM heavy-chain (μ) antibody (Fig. 5).

The intensities of IgM and IgG heavy-chain bands were measured, and the IgM/IgG heavy-chain band intensity ratios for calves and adults were compared. There appeared to be relatively more IgM in adults (IgM/IgG heavy-chain ratio, 0.8) than in calves (IgM/IgG heavy-chain ratio, 0.71), although the difference was not statistically significant (chi-square test). No protein bands that corresponded to bovine collectins (conglutinin, MBP, or CL-43) were detected.

Antimannan IgA, IgG1, and IgG2. The antimannan IgA, IgG1, and IgG2 antibodies in two pooled newborn, two pooled calf, and one pooled adult sample, as well as nine individual

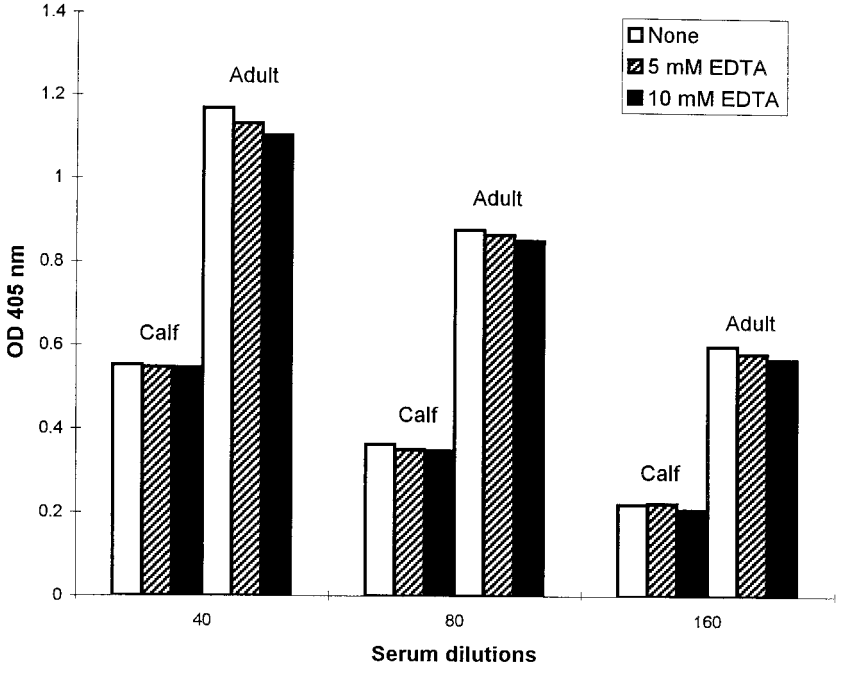

FIG. 3. Effect of EDTA on binding of bovine antimannan antibodies to mannan antigen. Calf serum no. 2 and the pooled adult serum were used.

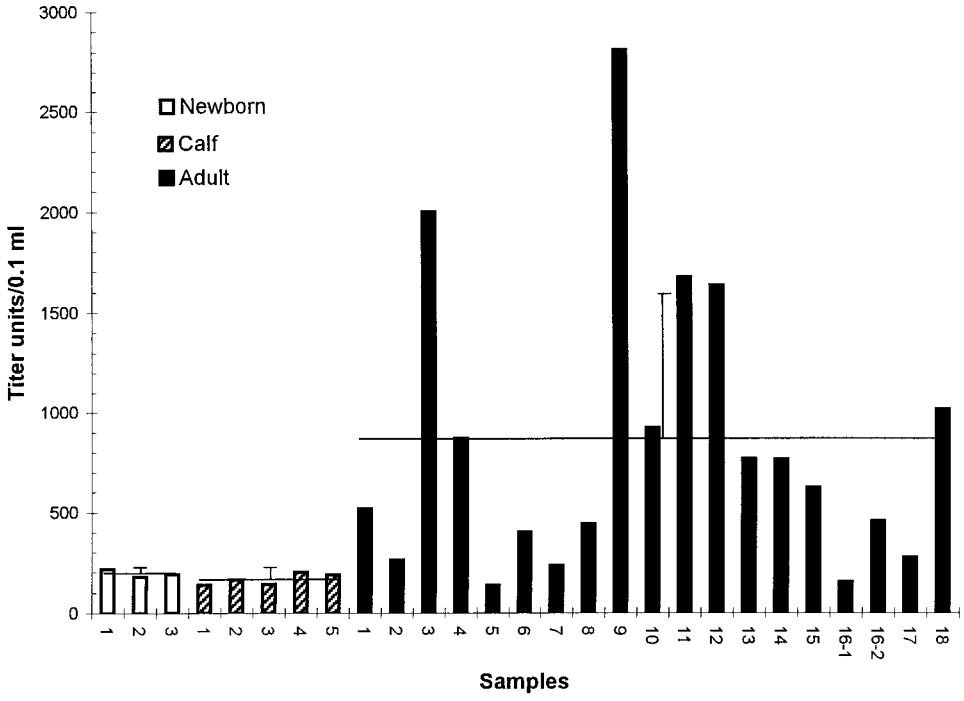

FIG. 4. Titers of antimannan antibodies in bovine serum samples. Samples were tested at a 1:80 or 1:160 dilution. The titers were obtained by extrapolating against the standard curve of calf sample no. 2 (for newborn and calf sera) or that of the pooled adult sample (for the adult sera) (see Materials and Methods). The horizontal bars indicate the average titers for each age group, and the standard deviation for each group is shown.

adult samples, were examined. The antimannan antibody was primarily of the IgG1 isotype in all samples except for one adult (no. 3), which had more IgG2 than IgG1 (Fig. 6A). The IgG1 level was significantly higher in adults than in newborns or calves ($P < 0.05$). IgA was present in all samples, and its level was significantly higher in newborns and calves than in adults ($P < 0.05$). IgA also accounted for a much higher percentage of the total immunoglobulin content (IgA, IgG1, and IgG2) in newborns and calves than in adults (Fig. 6B). The IgG2 levels of the nine individual adult samples differed widely, with a mean OD value of 0.116 and a standard deviation of 0.185. The IgG2 level in adults was higher than those in newborns and calves, but the difference was not statistically significant ($P = 0.09$).

DISCUSSION

The experiments described here demonstrated the presence of antimannan antibodies in normal cattle. The concentration of these antibodies was significantly higher in adults than in newborns or calves. However, the titers of the individual adults differed widely, suggesting that the adults may have been exposed to mannan-containing microorganisms to various degrees. These antibodies were also detected in the newborns (1 to 10 days old); in this case, they were probably derived from the mother's colostrum.

There are two IgG isotypes in cattle, IgG1 and IgG2 (2). IgG2 can be further divided into IgG2a and IgG2b. The IgG1 isotype was found to be the primary antimannan IgG in newborns, calves, and adults. IgG1 is the major antibody in bovine serum, which has an IgG1/IgG2 ratio of 1.2:1 (2). In cattle, there is no antibody transfer through the placenta, and the newborn obtains maternal antibodies through colostrum. IgG1 is also the major antibody in the colostrum (IgG1/IgG2 ratio, 16:1) and in milk (IgG1/IgG2 ratio, 116:1) (2). IgA is a very minor immunoglobulin compared to IgG in both serum and colostrum, although the IgA concentration in colostrum is significantly higher than that in serum (colostrum IgA/serum IgA ratio, 15:1) (2). This seems consistent with the findings that IgG1 was also the major antimannan antibody in newborns and

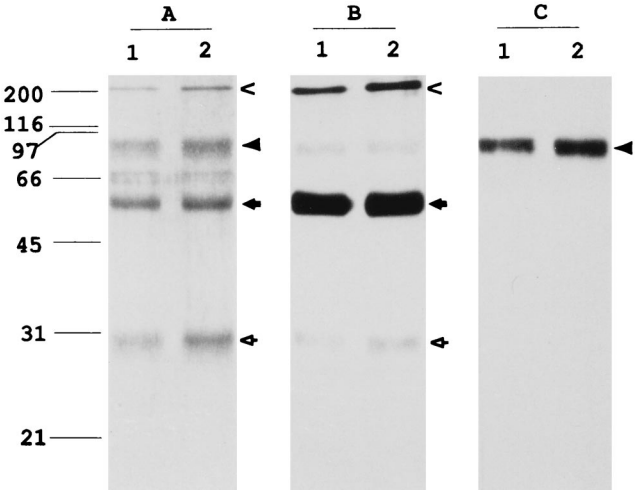

FIG. 5. Gel electrophoretic and immunoblot analyses of bovine serum proteins eluted from the mannan-Sepharose column. (A) Proteins on the blot stained with Coomassie blue; (B) proteins on the blot probed with alkaline phosphatase-conjugated anti-bovine whole-molecule IgG (H+L); (C) proteins on the blot probed with alkaline phosphatase-conjugated anti-bovine IgM heavy chain (μ). Lane 1, proteins from calf serum no. 2; lane 2, proteins from the pooled adult serum. The open arrowhead indicates the whole-molecule IgG, the closed arrowhead indicates the IgM heavy chain, the closed arrow indicates the IgG heavy chain, and the open arrow indicates the λ light chain. The positions of molecular mass standards (in kilodaltons) are indicated on the left side of the gel.

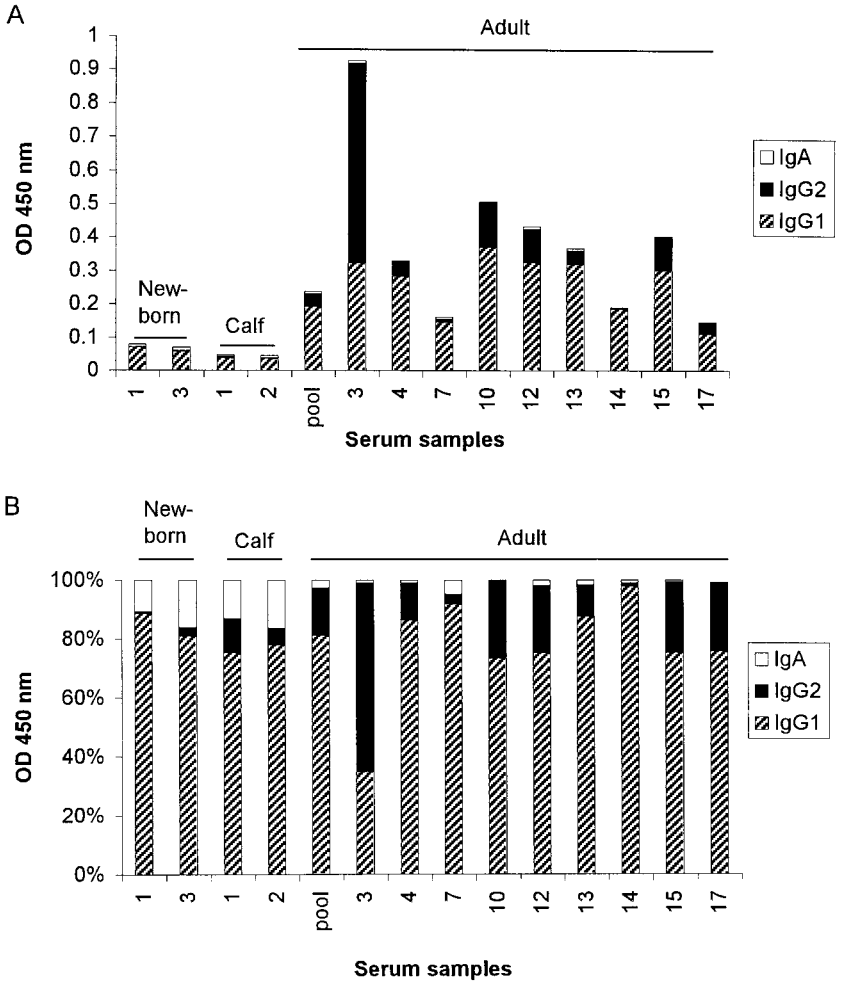

FIG. 6. Measurement and comparison of antimannan IgA, IgG1, and IgG2 responses. The antimannan IgA, IgG1, and IgG2 antibodies were measured by using class- or isotype-specific, horseradish peroxidase-conjugated antibodies as described in Materials and Methods. All sera were diluted 1:40. The serum sample numbers correspond to those in Fig. 4. (A) Antimannan IgA, IgG1, and IgG2 levels determined directly from OD values; (B) antimannan IgA, IgG1, and IgG2 levels by percentage.

calves and that the antimannan IgA levels in these groups were significantly higher than those in adults. IgG1 has been found in general to be the primary IgG isotype induced by infections or immunizations, whereas the IgG2 response differs widely. Both IgG1 and IgG2 are capable of fixing complement and have receptors on activated neutrophils, but only IgG2 has receptors on resting neutrophils (34). Under T-cell-independent conditions, gamma interferon increases the B-cell production of IgG2 but not that of IgG1 (6).

Mannan is a common antigen in yeast pathogens. In cows, *C. albicans* is an important cause of mastitis (13, 27). Thus, the presence of increased titers of antimannan antibodies in adult cows suggests that they may have been exposed to this or a related yeast pathogen(s). These antibodies could be beneficial in fighting current infections and in prevention of future infections by such mannan-containing microorganisms (4, 35).

All three bovine collectins (conglutinin, CL-43, and MBP) can react with the mannose residue with high affinity (7). The binding of these lectins to their ligands requires calcium. In purified antimannan antibody preparations from either calf or adult serum, no proteins that corresponded to bovine collectins were detected, although calcium was added to the buffer during the purification process (see Materials and Methods). Thus, it seems likely that the antimannan antibodies are present in the serum in a much larger quantity. Consistent with this suggestion is the fact that binding of the antibodies to yeast mannan was not influenced by addition of EDTA.

Most of the antimannan antibodies were found to react with an oligosaccharide-based epitope(s) on the mannan antigen; i.e., the reaction of these antibodies with the mannan antigen was only minimally inhibited by the monosaccharide D-mannose or methyl D-mannose. This is in contrast to the reaction of the lectins with their ligands, which is effectively inhibited by relevant monosaccharides (7). Most lectins, including collectins, recognize individual terminal sugar residues on a polysaccharide, although they may favor those sugar residues in certain linkages. Studies with antibodies against antigenic factors of *Candida* spp. mannan have also shown that antimannan antibodies recognize complex structures on mannan (10, 25, 26).

Lectins are a part of the innate immune system, reacting with any microorganisms possessing the target sugar residues, i.e., common structural patterns of microorganisms (16). On the other hand, antibodies are produced only after animals are exposed to foreign antigens and their reaction is extended beyond those common microbial structures (recognized by lec-

tins) to those unique to the particular invading microorganism. Thus, the antibody response may act as a secondary, but more specific, line of defense, strengthening overall immunity and compensating for any deficiency in lectin function. The presence of three different plasma lectins in cattle may enhance the development of such an antibody response, since it has been shown that the development of specific immunity is directly related to the initial response of innate immunity (16).

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