Mannan-binding protein (MBP) is a C-type serum lectin that is an important constituent of the innate immune defense because it activates the complement system via the lectin pathway. While the pig has been proposed to be an attractive source of xenotransplantable tissues and organs, little is known about porcine MBP. In our previous studies, phosphomannan, but not mannan, was found to be an effective inhibitor of the C1q-independent bactericidal activity of newborn piglet serum against some rough strains of Gram-negative bacteria. In contrast, the inhibitory activities of phosphomannan and mannan were very similar in the case of MBP-dependent bactericidal activity against rough strains of Escherichia coli K-12 and S-16. Based on these findings, we inferred that an MBP-like lectin with slightly or completely different carbohydrate binding specificity might exist in newborn piglet serum and be responsible for the C1q-independent bactericidal activity. Herein we report that a novel phosphomannan-binding lectin (PMBL) of 33 kDa under reducing conditions was isolated from both newborn and adult porcine serum and characterized. Porcine PMBL functionally activated the complement system via the lectin pathway triggered by binding with both phosphomannan (P-mannan) and mannan, which, unlike MBP, was effectively inhibited by mannose 6-phosphate- or galactose-containing oligosaccharides. Our observations suggest that porcine PMBL plays a critical role in the innate immune defense from the newborn stage to adulthood, and the establishment of a newborn piglet experimental model for the innate immune defense from the newborn stage to adulthood is a valuable step toward elucidation of the physiological function and molecular mechanism of lectin pathway.

Innate immunity was formerly thought to be a nonspecific immune response characterized by phagocytosis. However, innate immunity exhibits considerable specificity and is capable of discriminating between pathogens and self, as proposed in the concept of pattern recognition molecules, including C1q and mannann-binding protein (MBP), for host defense, which has the ability to specifically recognize pathogens and facilitate their removal. MBP, also referred to as mannan-binding lectin (MBL), is a Ca²⁺-dependent (C-type) serum lectin exhibiting primary specificity for mannose, fucose, and N-acetylgalactosamine (1), and belongs to a family of proteins called the collectins (2), in which carbohydrate recognition domains (CRD) are found in association with collagen-like domains (CLD) (3). MBP has been shown to have complement-dependent bactericidal activity, to serve as a direct opsonin, and to mediate the binding and uptake of bacteria that express a mannose-rich O-polysaccharide by monocytes and neutrophils (4–6). Furthermore, MBP can facilitate the uptake of apoptotic cells by macrophages and immature dendritic cells (7, 8). MBP functions as a β-inhibitor of the influenza virus (9) and protects cells from HIV infection by binding to gp120, a high mannose-type oligosaccharide-containing envelope glycoprotein on HIV (10). MBP may also play an important role in other common serious diseases like atherosclerosis (11) and chronic pulmonary disease (12), and MBP deficiency could impair normal innate immune function and increase susceptibility to infection (13). Recently, MBP was shown to exhibit novel cytotoxic activity against colorectal carcinoma cells in in vivo experiments, which we propose to term MBP-dependent cell-mediated cytotoxicity (MDCC) (14, 15).

The complement system, which consists of three activation pathways, is involved in both innate and acquired immunity (16–18). The classical pathway is activated by antigen-antibody complexes and is a major effector of antibody-mediated immunity. The other two, the lectin and alternative, pathways function in innate immune defense. The lectin pathway involves

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3 The abbreviations used are: MBP, mannan-binding protein; PMBL, phosphomannan-binding lectin; CRD, carbohydrate recognition domain; CLD, collagen-like domain; RACE, rapid amplification of cDNA ends; MDCC, MBP-dependent cell-mediated cytotoxicity; P-mannan, phosphomannan; P-disaccharide, phosphodisaccharide; P-trisaccharide, phosphotrisaccharide; Man, mannose; GlcNAc, N-acetylgalactosamine; Man-6-P, mannose 6-phosphate; Fuc, fucose; Gal, galactose; SPR, surface plasmon resonance; HRP, horseradish peroxidase; ELISA, enzyme-linked immunosorbent assay; CBB, Coomassie Brilliant Blue; MASP, MBP-associated serine proteases.
Complement Activation and Carbohydrate Specificity of PMBL

carbohydrate recognition by MBP and the subsequent activation of associated unique enzymes, MBP-associated serine proteases (MASPs) (19, 20). The alternative pathway does not involve specific recognition molecules. Experiments have indicated that the lectin pathway is also amplified by the alternative pathway (21). Thus, both the classical and lectin pathways, supported by the alternative pathway, lead to the final, nonenzymatic assembly of the bactericidal membrane attack complex.

MBP has been described in many species (22). MBP occurs in two distinct forms in rodents and other animals including the rhesus monkey, MBP-A and MBP-C, which differ in their carbohydrate binding specificity (23). However, only one form has been found in the chicken, rabbit, and man, due to conversion of a gene into a pseudogene between monkeys and apes (22).

Xenotransplantation of porcine tissues has been proposed to be a means of alleviating the shortage of organs and tissues needed for the treatment of human organ failure and cellular diseases (24), yet little is known about porcine MBP. It is a well-known fact that newborn piglet serum is extremely deficient in all classes of immunoglobulins, and the level of complement in newborn piglet serum is very low, usually ranging from 2 to 5 CH50 in the case of the classical complement pathway. We previously reported that the C1q-independent bactericidal activity of newborn piglet serum was completely inhibited by P-mannan, but not by mannan (25), and MBP was shown to exhibit complement-dependent bactericidal activity, inhibiting with both P-mannan and mannan, against rough strains of E. coli K-12 and S-16 (5). Therefore, we inferred that there are some MBP-like lectins with slightly or completely different carbohydrate binding specificities in newborn piglet serum, which are responsible for the antibody-independent bactericidal activity.

As an extension of these studies, here we report that a novel P-mannan-binding lectin (PMBL) of 33 kDa under reducing conditions, followed by transfer to a nitrocellulose membrane. The membrane was probed with primary guinea pig anti-rabbit MBP antibodies and the subsequent activity via the lectin pathway triggered by binding with both P-mannan and mannan, which, unlike MBP, was effectively inhibited by both Man-6-P- and Gal-containing oligosaccharides.

EXPERIMENTAL PROCEDURES

Materials

Newborn and adult porcine sera were supplied by Japan SLC, Inc. (Shizuoka, Japan) and Invitrogen/Invitrogen (Copenhagen, Denmark), respectively. Purified rabbit and human MBPs and guinea pig anti-rabbit MBP polyclonal antibodies were prepared in our laboratory. A standard calibration kit for gel filtration, Epoxy-activated Sepharose 6B resins, and Hitrap Q Sepharose HP and Superose 4B columns were purchased from Amersham Biosciences. P-mannan, (Man-6-P)-Man (phosphotrisaccharide), and Man-(Man-6-P)-Man (phosphotrisaccharide) were purified from Hansenula capsulata (NRRL Y-1842) culture medium in our laboratory as described previously (25).

Purified mannan from Saccharomyces cerevisiae and human complement C4 were obtained from Sigma. BIAcore sensor chips, SA and CM5, were purchased from BIACORE (Uppsala, Sweden). Monosaccharides for inhibition assaying were purchased from Wako (Osaka, Japan) and Sigma. All chemicals for gel electrophoresis and Western blotting were supplied by ATTO (Tokyo, Japan), Bio-Rad, and Pierce.

Preparation of Biotin-labeled P-Mannan and P-Mannan-Sepharose 4B Affinity Resins

For the preparation of biotin-labeled P-mannan and P-mannan-Sepharose 4B affinity resins, P-mannan from H. capsulata (NRRL Y-1842) was coupled to Biotin-LC-Hydrazide (Pierce) and epoxy-activated Sepharose 4B, respectively, according to the manufacturer’s instructions.

Purification of Newborn and Adult Porcine Serum Lectins by P-Mannan-Sepharose 4B Affinity Chromatography

Newborn and adult porcine lectins were purified from newborn and adult porcine sera, respectively, by affinity chromatography on a P-mannan-Sepharose 4B column as described previously (1).

Separation of Lectins from Adult Porcine Serum by Hitrap Q Anion Exchange Chromatography

The adult porcine serum lectin eluate from the P-mannan-Sepharose 4B column was dialyzed against buffer C (20 mM Tris-HCl, 5 mM EDTA, pH 6.7), and then the dialysate was applied to a Hitrap Q column, which had been equilibrated with the same buffer. Elution of porcine serum lectins was carried out with buffer C containing a linear gradient of NaCl, from 0 to 500 mM.

Determination of Molecular Weights of Porcine Serum Lectins by Superose 6 Gel Filtration

Gel filtration of the purified adult porcine serum lectins, 20 μg each, was performed with a FPLC system (Amersham Biosciences) on a Superose 6 column (Amersham Biosciences), which had been equilibrated with buffer D (TBS containing 10 mM EDTA, pH 8.0). Calibration was carried out with a standard calibration kit (Amersham Biosciences).

SDS-PAGE and Western Blot Analyses

The purified newborn and adult porcine serum lectins were resolved on a 5–20% Tris-HCl gradient gel (ATTO) under reducing conditions, respectively, and then stained with Coomassie Brilliant Blue (CBB). The purified adult porcine serum lectins were also determined by Western blot analysis. The samples were separated on a 5–20% Tris-HCl gradient gel under reducing conditions, followed by transfer to a nitrocellulose membrane. The membrane was probed with primary guinea pig anti-rabbit MBP polyclonal antibodies followed by secondary anti-guinea pig HRP-conjugated IgG (Zymed Laboratories Inc.), and then developed by the enhanced chemiluminescent method (SuperSignal West Pico Chemiluminescent...
Protein Sequencing of Purified Newborn and Adult Porcine Serum Lectins

The purified newborn porcine PMBL was digested with lysylendopeptidase, and then separated with a reverse phase HPLC LC-10AD system (SHIMADZU, Kyoto, Japan). The sequences of the separated peptides of newborn porcine PMBL and the N-terminal amino acid sequences of adult porcine serum lectins were determined, respectively, with an LC/MS/MS Applied Biosystems 477A (Foster, CA).

PCR-based Cloning of a Porcine PMBL cDNA

PCR with Degenerated Oligonucleotides—Degenerated oligonucleotides designed on the basis of the amino acid sequences of the lysylendopeptidase-digested peptides shown in Fig. 1, were purchased from Cluachem, Co., Ltd. (Kyoto, Japan). PCR was performed using adult pig liver cDNA as a template, 2.5 units of Taq polymerase (Nippon Gene), and (1) an E2N+18 s1 primer (5′-GTIATHACNTGYGGNATHCC) and an E2D-33 a2 primer (5′-AANGCNACRTCNNGNGCAT), or (2) an E2D-33 s1 primer (5′-CARGAYATGGCNCCNGAYGT) and an E2D-29 a1 primer (5′-TCNGCNACNCRTGRTCTT) (100 pmol each) in a reaction mixture (25 µl). I: inosine, H: A or C or T, Y: C or T, R: A or G, N: A or C or G or T. Amplification was carried out with 30 cycles of 94 °C for 30 s, 50 °C for 1 min and 72 °C for 1 min, using a Zymoreactor II thermal cycler (ATTO Corp., Tokyo, Japan). The primary PCR product from primer combination 1 was used as a template for a secondary PCR with a nested E2N+18 s2 primer (5′-GGIATHCCNGTNACNAAYGG) and an E2D-33 a1 primer (5′-ACRTCNGGNCCCATRTCTYT) (100 pmol each). The primary PCR product from primer combination 2 was used as a template for a secondary PCR with a nested E2D-33 s2 primer (5′-ATGCGNCNGAYGTNGCNTT) and an E2D-29 a2 primer (5′-ACNCCRTGRTCTTNGGYTC) (100 pmol each). Amplification was carried out with 30 cycles of 94 °C for 30 s, 50 °C for 1 min and 72 °C for 1 min. The PCR products were subcloned into the pcRII vector (Invitrogen, Carlsbad, CA) and characterized by DNA sequencing with a DNA sequencer (Model 373A, Perking Elmer, Corp., Applied Biosystem, Division, CA).
central portion containing E2D-33 originating primers was amplified by RT-PCR using a sense primer (5'-GGGGAGATGGACGCAAAGG) and an antisense primer (5'-CTC-TTCCAGTGTCTGAGGT). The amplified PCR products were subcloned in the pCRII vector and characterized by DNA sequencing.

5'-Rapid Amplification of cDNA Ends (5'-RACE)—The first strand cDNA was synthesized with reverse transcriptase using a 5'-RACE a1 primer (5'-CCCTGGTTCTCCCTTTCTC). After polyadenylation to the 5' termini, amplification was performed by PCR using an oligo(dT) primer and a 5'-RACE a2 primer (5'-CCCTTTCTCCTCTTGGGTCC). The primary PCR products were used in a secondary PCR with an oligo(dT) primer and a nested 5'-RACE a3 primer (5'-CCTTTGCGTCCATCTCGCCC). The amplified PCR products were subcloned into the pCRII vector and characterized by DNA sequencing.

3'-Rapid Amplification of cDNA Ends (3'-RACE)—The first strand cDNA was synthesized with reverse transcriptase using a 3'-RACE s1 primer (5'-GGGAGGAAGGATGACCTACA). Amplification was performed by PCR using a 3'-RACE s2 primer (5'-GATGACCTACAGCAACTGGA) and an oligo(dT) primer. The primary PCR products were used in a secondary PCR with a nested 3'-RACE s3 primer (5'-CTACAGCAAACGAGGAGTA) and an oligo(dT) primer. The amplified PCR products were subcloned into the pCRII vector and characterized by DNA sequencing.

Carbohydrate Binding and Inhibition Specificity of Porcine Serum Lectins

Immunoassay 96-well plates were coated with 100 μl of 100 μg/ml PV-mannose in sodium/bicarbonate buffer, pH 9.6, and then incubated overnight at 4 °C as described. The plates were washed three times with PBS buffer containing 0.05% Tween-20, followed by once wash with TBS buffer also containing 0.05% Tween-20 (TBS-T). Porcine
PMBL, porcine MBP or rabbit MBP 200 ng was added to each well in TBS-T buffer containing 5 mM CaCl$_2$ in the presence of monosaccharide, followed by incubation at 25 °C for 2 h. The plates were then washed three times with TBS-T buffer containing 5 mM CaCl$_2$, and then primary guinea pig anti-rabbit MBP polyclonal antibodies were added, followed by secondary anti-guinea pig HRP-conjugated IgG. The plates were then washed three times, 50 μl of o-phenylenediamine was added to each well, and then the plates were read at 490 nm with an ARVO SXc 1420 Multilabel Counter (PerkinElmer, Wallac Oy, Finland). All ELISA experiments were performed in triplicate.

**Solid Phase Binding Assay Based on Surface Plasmon Resonance (SPR)**

The affinity between porcine PMBL or rabbit MBP and P-mannan or mannan was measured using a BIAcore X instrument (BIACORE). Biotin-labeled P-mannan and mannan were covalently immobilized on the sensor chips, SA and CM5, by the Biotin-Streptavidin and amine-coupling methods, respectively, according to the manufacturer’s instructions. All measurements were carried out at 25 °C and at the flow rate of 20 μl/min for both the association and dissociation phases in the HBS-P buffer (10 mM Hepes, 150 mM NaCl, 0.005% polysorbate 20, pH 7.0) supplied by the manufacturer. The interaction was monitored as the change in the SPR response. Association and dissociation rate constants were calculated by nonlinear fitting of the primary sensorgram data using BIAevaluation 3.0 software.

**Complement C4 Cleavage Assay**

A complement C4 cleavage assay was performed as described previously (26). Briefly, microtiter wells were coated overnight at room temperature with 1 mg of P-mannan or mannna in 100 μl of coating buffer. Residual protein binding sites were blocked with 0.1% (w/v) human serum albumin in TBS for 1 h. After washing with TBS plus 0.005% Tween 20 and 5 mM CaCl$_2$ (TBS/Ca$^{2+}$), each well was filled with a MBP dilution, 0–1000 ng, or various saccharides for inhibition assay- ing in 100 μl of MBP binding buffer at room temperature for 1.5 h. After washing thoroughly, wells then received MASPs from diluted MBP-deficient human serum, followed by incubation overnight at 4 °C. At the end of the incubation, 100 μl of 2 mg/ml human C4 diluted in C4 dilution buffer was added to each well, followed by incubation at 37 °C for 1.5 h. After washing with TBS/Tw/Ca$^{2+}$, the wells were washed with TBS/Tw/Ca$^{2+}$, and then deposited C4b was detected by adding HRP-AvidinD in TBS/Tw/Ca$^{2+}$ at room temperature for 1 h. HRP activity was determined by adding 100 ml of Sure Blue TMB to each well, and then the plate was read at 450 nm. The optical density induced by porcine PMBL was determined in triplicate and compared with human and rabbit MBPs assigned arbitrary activity values. All experiments were performed in triplicate and were repeated a minimum of three times.

**Statistical Analysis**

The results are expressed as the mean ± S.D. of data obtained from three experiments performed in triplicate. The statistical significance was determined by the Student’s t-test. p < 0.05 was considered significant.

**RESULTS**

**Isolation and Cloning of PMBL from Newborn Porcine Serum**—As an extension of our previous studies, a novel PMBL was isolated from newborn piglet serum using a P-mannan-Sepharose 4B affinity column in the presence of monosaccharide, and then primary guinea pig anti-rabbit MBP polyclonal antibodies were added, followed by secondary anti-guinea pig HRP-conjugated IgG. The plates were then washed three times, 50 μl of o-phenylenediamine was added to each well, and then the plates were read at 490 nm with an ARVO SXc 1420 Multilabel Counter (PerkinElmer, Wallac Oy, Finland). All ELISA experiments were performed in triplicate and were repeated a minimum of three times.

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Complement Activation and Carbohydrate Specificity of PMBL

A  (kDa)  CBB staining  (kDa)  Western blot

B  

C  

D  

(a) porcine P-mannan-binding lectin (PMBL)
porcine 33 kDa lectin

(b) porcine mannan-binding lectin (MBP-C)
porcine 31 kDa lectin
cleotide primers for PCR. First, fragments of about 450 bp and 150 bp were amplified with an E2N+18 s2 primer and an E2D-33 a1 primer, and an E2D-33 s2 primer and an E2D-29 a2 primer, respectively. The PCR with an E2N+18 s1 primer and an E2D-29 a1 primer, followed by the nesting PCR with an E2N+18 s2 primer and an E2D-29 a2 primer, did not amplify any specific fragments. The amplified fragments were subcloned into the pCRII vector and then sequenced. Next, based on the determined sequences, completely matched oligonucleotide primers were synthesized to amplify the middle part of the cDNA, which overlapped with these two fragments, and to determine the 5’- and 3’-sequences of the cDNA by means of the 5’-RACE and 3’-RACE strategies. The fragments obtained on RT-PCR of the middle portion, and 5’-RACE and 3’-RACE were subcloned into the pCRII vector and then sequenced. The whole cDNA of the lectin was covered by joining these three fragments.

However, the 5’- and 3’-ends remained completely unclear. To confirm the predicted exons, and to determine the 5’- and 3’-exons of porcine PMBL mRNA, 5’- and 3’-RACE experiments were performed using porcine liver RNA as a template, followed by sequencing.

The merged sequence of the cDNA fragment and deduced amino acid sequence are shown in Fig. 2A. The 1010-bp cDNA contains a 747-bp open reading frame, and 175-bp 5’-untranslated regions. The three lysylendopeptidase-digested peptides identified on mass spectrometry can be seen in the open reading frame of the deduced amino acid sequence, though it did not entirely coincide with the digested peptide sequences.

Judging from the deduced amino acid sequence, the novel lectin is composed of 249 amino acid residues, and includes a cysteine-rich region, a CLD domain and a CRD domain. It has 19 Gly-X-Y repeats and a Gly-X-Gly interruption at the eighth repeat, which are found in other mammalian MBPs (27). The latter 17 of 18 residues conserved in the CRDs of animal C-type lectins are present in the C terminus of this lectin. Four cysteine residues among the 18 consensus amino acids, which may be important for proper folding, are conserved in this lectin. Hydropathy analysis (Fig. 2B) showed that the first 20 amino acids are highly hydrophilic, indicating a typical signal peptide, and also indicated that porcine PMBL contains a hydrophilic collagen-like domain and a typical carbohydrate-recognition domain similar to that of many other serum C-type lectins (2, 3). The mature protein is composed of 229 amino acid residues with a predicted molecular mass of 26,514 Da. The porcine PMBL has an identical sequence to that previously registered in the NCBI GenBank™ (E37364), however, nothing has been reported yet about its characterization and function.

Sequence Homology with Other MBPs—To compare the amino acid sequence of the porcine PMBL with those of other MBPs, their amino acid sequences were aligned (Fig. 2C). Sequence analysis showed that the lectin exhibited 61.4% similarity with human MBP (28), 69.9% with mouse MBP-A, and 52.0% with MBP-C (29), 66.9% with rat MBP-A and 53.6% with MBP-C (30), 80.0% with rhesus MBP-A, and 59.0% with MBP-C (30), and 54.6% with rat MBP (31), respectively. The porcine PMBL exhibited the highest homology with rhesus MBP-A and high homology with serum-type MBPs. In contrast, the sequence identity to porcine L-MBP (32, NCBI GenBank™: AF164576) with PMBL was only 56.7%.

Isolation and Identification of Lectins from Adult Porcine Serum—To determine whether PMBL could also be isolated from adult porcine serum, similar to in the case of newborn piglet serum, the pretreated adult porcine serum was applied to a P-mannan-Sepharose 4B affinity column in the presence of 20 mM CaCl₂, and the bound proteins were eluted with 50 mM mannose. Surprisingly, the purified lectins gave two distinct bands corresponding to 33 and 31 kDa on 5–20% gradient SDS-PAGE analysis with staining with CBB under reducing conditions (left panel in Fig. 3A). Western blot analysis confirmed that both the 33- and 31-kDa bands were recognized by guinea pig anti-PMBL.

FIGURE 4. Phylogenic trees of animal MBPs. Phylogenic relationships of the amino acid sequences of porcine PMBL and several animal MBPs were determined by the neighbor-joining (N-J) method. A indicates serum-type and C indicates liver-type, respectively.

FIGURE 3. Purification and characterization of adult porcine PMBL. A, SDS-PAGE (left) and Western blotting (right) analyses of purified adult PMBL. The experiments were described under “Experimental Procedures.” 1 μg of protein sample purified at different step was resolved on SDS-PAGE under reducing conditions and then stained with CBB (left panel), or transferred to a nitrocellulose membrane followed by Western blotting analysis with anti-rabbit MBP pAb (right panel). Lane 1, mannose fraction eluted from an affinity column. Lanes 2 and 3, fractions A and B eluted from an ion-exchange column, respectively, as described in E. B, Hitrap Q anion exchange chromatography for separation of adult porcine PMBLs. The adult porcine PMBL eluted from the P-mannan-Sepharose 6B column was applied to a Hitrap Q column, and elution was carried out with buffer C containing a linear gradient of NaCl, from 0 molar to 500 molar, as shown on the right. C, the elution profile of the purified adult porcine PMBLs on a Superose 6 gel filtration column. The adult porcine PMBLs separated on the ion-exchange column were applied to a Superose 6 gel filtration column to determine their native molecular masses. The arrows indicate the peak positions of the eluted standards: T, thyroglobulin (MW 669,000); F, ferritin (MW 440,000); C, catalase (MW 232,000). D, the N-terminal amino acid sequences of the two purified adult porcine lectins, 33 kDa (a) and 31 kDa (b), determined with a protein sequencer. The N-terminal amino acid sequences deduced and determined from the nucleotide sequence and those of the purified adult porcine lectins were compared, respectively. The residues in boxes are identical with the N-terminal amino acid sequences of the mature proteins, and the residues at the left of the boxes are the signal peptide sequences. The arrows indicate the signal peptide cleavage sites.
pig anti-rabbit MBP polyclonal antibodies, as shown in the right panel in Fig. 3A. To separate these two lectins, the eluate from the P-mannan-Sepharose 4B column was subjected to Hitrap Q anion exchange chromatography with a linear gradient of NaCl, from 0 to 500 mM. As shown in Fig. 3B, the two lectins were eluted as peak 1 comprising fractions 7–19 and peak 2 comprising fractions 25–30 at NaCl concentrations of around 225 and 500 mM, respectively. With SDS-PAGE and Western blot analyses, peaks 1 and 2 each gave a single band corresponding to 33 and 31 kDa under reducing conditions, respectively, both of which were recognized by anti-rabbit MBP antibodies (Fig. 3A).

To determine the molecular masses of the two lectins under natural conditions, the peak 1 and 2 eluates were applied to a Superose 6 gel filtration column. The results showed that molecules presenting the 33- and 31-kDa lectins were mainly eluted with materials with masses of about 700 and 650 kDa, respectively (Fig. 3C).

To identify the 33- and 31-kDa lectins purified from adult porcine serum, the sequences of the 16 N-terminal amino acid residues of the 33- and 31-kDa lectins were determined with a protein sequencer, as shown in Fig. 3D. The 33- and 31-kDa lectins were both cleaved at a site between the last amino acid of the signal peptide deduced from the cDNA sequence and the first amino acid of the mature protein identified with a sequencer. The results showed that the 33-kDa lectin from adult porcine serum is the same lectin as that from newborn piglet serum, and was designated as PMBL in this study, and the 31-kDa lectin is the liver-type and was designated as porcine MBP-C in this study.

**Phylogenetic Tree of Porcine Serum Lectins and Animal MBPs—** The phylogenetic relationship between the porcine serum lectins and several animal MBPs was analyzed using the neighbor-joining method. A phylogenetic tree was constructed based on multiple sequence alignments of the CRDs of all identified MBPs. As shown in Fig. 4, the phylogenetic tree clearly shows that mammal MBPs form a tight cluster, gene duplication, therefore, leading to two different MBP forms; one is serum-type MBP (S-MBP, or MBP-A), and the other is liver-type MBP (L-MBP or MBP-C). The porcine 33-kDa PMBL is the serum-type, suggesting that it may have almost the same function as S-MBP, and the porcine 31-kDa lectin is the liver-type and was designated as porcine MBP-C in this study.

**Carbohydrate Binding Specificities of Porcine PMBL and L-MBP—** The carbohydrate binding specificities of porcine PMBL and L-MBP were compared with that of rabbit MBP by means of the monosaccharide inhibition assay. As summarized in Fig. 5, the results of the inhibition experiments demonstrated unambiguously that Man, GlcNAc, and Fuc at concentrations of 50–100 mM behaved similarly as to the binding inhibition of porcine PMBL, MBP-C, and rabbit MBP; however, if the concentrations of these monosaccharides were below 50 mM, they showed higher binding inhibition of porcine MBP-C and PMBL than that of rabbit MBP (Fig. 5, A–C). Only Man-6-P specifically inhibited the binding of porcine PMBL, i.e. not that of MBP-C and rabbit MBP (Fig. 5D). These data demonstrate that porcine PMBL exhibits Ca$^{2+}$-dependent binding to

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**FIGURE 5. Mannosaccharide inhibition of porcine lectin- or rabbit MBP-binding with PV-mannose.** 96-well immunoassay plates were coated with PV-mannose, and then incubated with porcine PMBL or rabbit MBP in the presence of mannose (A), GlcNAc (B), L-fucose (C), or mannose 6-phosphate (D). After washing well, o-phenylenediamin, as a substrate, was added to each well, and then the plates were read at 490 nm using a Multilabel Counter. All ELISA experiments were performed in triplicate.
Man, GlcNAc, and Fuc, similar to porcine MBP-C and rabbit MBP, and shows especially significant binding to Man-6-P, different from porcine MBP-C and rabbit MBP.

**Determination and Comparison of the Kinetic Parameters for the Porcine PMBL Interaction with P-Mannan and Mannan by SPR Analysis**—To quantitatively characterize the porcine PMBL/P-mannan or/mannan interaction using a more sensitive system, P-mannan and mannan were immobilized on a BIAcore Sensor Chip, respectively. As shown in Fig. 6, the kinetic parameters were calculated from the SPR sensorgrams of porcine PMBL and rabbit MBP. The two tables in Fig. 6 show the association rate ($k_a$), dissociation rate ($k_d$), and dissociation ($K_D$) constants for the binding of porcine PMBL and rabbit MBP.

**FIGURE 6.** The interaction between porcine PMBL or rabbit MBP and P-mannan or mannan on SPR. A, SPR measurement of the interaction between porcine PMBL (left panel) or rabbit MBP (right panel) and P-mannan. Biotin-labeled P-mannan was covalently immobilized on a sensor chip SA, and then the affinity between porcine PMBL or rabbit MBP and phosphomannan was measured using BIAcore X as described under "Experimental Procedures." The interaction parameters of the kinetics and affinities were calculated from the association and dissociation phases of the sensorgrams as shown in the bottom panel. B, SPR measurement of the interaction between porcine PMBL (left panel) or rabbit MBP (right panel) and mannan. Purified mannan was covalently immobilized on a sensor chip CM5, and then the affinity between porcine PMBL or rabbit MBP and mannan was measured using BIAcore X as described under "Experimental Procedures." The interaction parameters of the kinetics and affinities were calculated from the association and dissociation phases of the sensorgrams, as shown in the bottom panel. Kinetic parameters: The $k_a$ ($s^{-1} M^{-1}$) and $k_d$ ($s^{-1}$) values were determined by SPR analysis. The $K_D$ value was $k_a/k_d$.

Complement Activation and Carbohydrate Specificity of PMBL

Complement Activation of Porcine PMBL via the Lectin Pathway—To further determine whether porcine PMBL is involved in complement activation via the lectin pathway, a C4 cleavage assay was performed. Porcine PMBL was assayed in a P-mannan- or mannan-dependent C4 cleavage test by trapping MBP-MASP complexes on P-mannan- or mannan-coated plates and then measuring MASP-2 mediated C4 cleavage by detecting C4b deposition. This test specifically measures lectin pathway activation via MBP without interference from the classical pathway. As shown in Fig. 7, porcine PMBL-dependent C4 cleavage capacity, triggered by binding with either P-mannan or mannan, was significantly more effective and a little less effective, respectively, than for both human and rabbit MBP. Meanwhile, PMBL-dependent C4 cleavage capacity was distinctly observed in a dose-dependent manner compared with the capacity of human and rabbit MBPs. The C4 cleavage capacity of porcine PMBL triggered by binding with P-mannan was observed with ~6.25 ng/well, it being equivalent to that of both human and rabbit MBP with more than 50 ng/well, respectively, in the presence of MBP-deficient human serum (Fig. 7). On the other hand, Agah et al. (32) reported that the porcine 31-kDa lectin (designated as porcine MBL in their
study) was less effective than human MBP in restoring C3 deposition assay.

Inhibition of the Complement Activation of Porcine PMBL via the Lectin Pathway by Saccharides—The inhibition of the complement activation of porcine PMBL by various monosaccharides and di/trisaccharides via the lectin pathway was examined in more detail using the complement C4 cleavage assay. As shown in Fig. 8, the addition of various monosaccharides effectively abolished the porcine PMBL-dependent C4 cleavage capacity, demonstrating that porcine PMBL can be specifically inhibited by Man, Fuc, and GlcNAc at the concentration of 100 mM, similar to human and rabbit MBPs, in this assay, with triggering by binding with both P-mannan and mann. Interestingly, 100 mM Man-6-P was able to completely abolish the porcine PMBL-dependent C4 cleavage capacity triggered by binding with mann, and ~70% of the inhibition with P-mannan, compared with the human and rabbit MBPs. In addition, 100 mM Gal could also completely abolish the porcine PMBL-dependent C4 cleavage capacity triggered by binding with mann, but not with P-mannan, compared with the human and rabbit MBPs (Fig. 8). One significant finding is that porcine PMBL binds P-mannan with about 300-fold higher affinity compared with rabbit MBP, as judged on BIAcore SPR analysis described above. Taken together, the data suggest that porcine PMBL functionally activates the complement system via the lectin pathway triggered by binding with different carbohydrate ligands expressed on infectious microorganisms in newborn and adult porcine sera compared with human and rabbit MBPs, which is extremely important for the innate immune defense system of newborn piglets.

Next, the C4 cleavage assay, which monitors complement activation via the lectin pathway, was also used to determine porcine PMBL responses to pure phospho-di/trisaccharide preparations derived from H. capsulata (NRRL Y-1842)-culture medium. The inhibition of complement activation via the lectin pathway of porcine PMBL occurred with both (Man-6-P)-Man and Man-(Man-6-P)-Man were corresponded with their concentrations, and compared with that of human and rabbit MBPs. As summarized in Fig. 9, the results of the inhibition experiments significantly demonstrated that C4 activation on mannan-coated wells could be completely inhibited by co-incubating the three kinds of MBPs (25 ng/well) with both (Man-6-P)-Man and Man-(Man-6-P)-Man at 5 μM (Fig. 9, B and D), and also could be inhibited to roughly equal extents by co-incubating human and rabbit MBPs, but not porcine PMBL, with both (Man-6-P)-Man and Man-(Man-6-P)-Man at 5–20 μM on P-mannan-coated wells (Fig. 9, A and C). Meanwhile, the inhibition of the C4 cleavage capacity of porcine PMBL triggered by binding with P-mannan was similar in the presence of (Man-6-P)-Man and Man-(Man-6-P)-Man at concentrations of more than 25 μM, respectively (small panels in Fig. 9, A and C), suggesting that porcine PMBL exhibited much higher affinity than human and rabbit MBPs to P-mannan-dependent binding. Similar MBP binding inhibition occurred with both
Man-(Man-6-P)-Man and Man-(Man-6-P)-Man when the ELISA wells were coated with P-mannan and mannan, respectively (data not shown). Moreover, as shown in Fig. 9, the effect of the phosphotrisaccharide, Man-(Man-6-P)-Man, was correspondingly higher than that of the phosphodisaccharide, (Man-6-P)-Man, on both P-mannan- and mannan-coated wells.

**DISCUSSION**

It is a well known fact that the serum of newborn piglets is extremely deficient in all classes of immunoglobulins, which results from the special type of placentation in this animal species that prevents the transfer of antibodies from the sow to the fetus. The level of complement in newborn piglet serum is very low, usually ranging from 2 to 5 CH50 in the case of the classical pathway for complement. Nevertheless, the bactericidal activity against rough strains of Gram-negative bacteria can as a rule be detected not only in newborns, but also in fetal pig serum starting from the 35th day of gestation, a fact which indicates that antibodies are not involved in the bacterial killing. This is in agreement with the view that rough strains of Gram-negative bacteria may be killed through antibody-independent activation of the complement cascade, which is completely inhibited not only by EGTA and Mg$^{2+}$, but also by the extracellular P-mannan from *H. capsulata*, but not by the mannan from *S. cerevisiae*. However, the mechanism of complement activation has not been elucidated yet (25). Meanwhile, our previous
The complement system and the natural antibody repertoire provide a critical first-line defense against infection in animals. Both defense systems cooperate within the innate immune response. The complement system is an integral part of innate antimicrobial immune defense, and mediates humoral and cellular interactions within the immune response. Complement may be activated via three different routes: the classical pathway, the alternative pathway, and the recently described lectin pathway. The binding of natural antibodies to microbial surfaces opsonizes invading microorganisms and activates complement via the classical pathway. Lectins, carbohydrate-binding proteins, serve as weapons against pathogens by aggregating and opsonizing them. In the lectin pathway, MBP acts as the recognition molecule and activates complement in association with MASPs, C1r/C1s-like serine proteases capable of cleaving complements C4, C2, and C3. Activation of the complement system promotes the three main biological activities: opsonization of pathogens, chemotaxis and activation of leukocytes, and direct killing of pathogens. Recently, accumulating evidence shows that the complement system acts as an adjuvant, enhancing and directing the adaptive immune response, and also functions to dispose of apoptotic cells (16, 17). The pattern recognition features of porcine PMBL binding appear to have evolved in order to permit the widest range of potential microbial structures to be recognized with the most efficient use of genetic information.

Xenotransplantation, the transplantation of organs, tissues, and cells from animals, preferably pig-to-human, might be considered to be the transplantation method of the future. Organs from domestic animals are available in unlimited numbers for xenogeneic transplantation and could swiftly solve the problem of organ shortages. However, the immunological mechanisms, and the antigenicity of cells and their surface structures will remain obstinately unaltered. In this regard, hyperacute rejection of discordant xenografts, such as in the case of pig-to-human or pig-to-rhesus combinations is caused by activation of complement after deposition of xenoreactive natural antibodies to cell-surface antigens on the grafts (33). Under certain circumstances, when antidonor antibodies and complement-mediated immune responses are inhibited for a few days, grafts can survive indefinitely, despite the return of antidonor antibodies and complement, a phenomenon referred to as “accommodation.” However, little is known about porcine MBP and its complement activation in porcine serum. Here, we have chosen newborn piglets as a future unique experimental model for xenotransplantation and infection studies, because newborn piglets lack maternal antibodies and only traces of immunoglobulins produced prenataIy are present in their sera. Our data indicate that porcine PMBL exhibits 61.4% identity with human MBP, 69.9% with mouse MBP-A, 66.9% with rat MBP-A, 54.6% with rabbit MBP, and 80.0% with rhesus MBP-A at the amino acid sequence level, respectively, suggesting that porcine PMBL exhibits the highest homology with rhesus MBP-A and high homology with S-MBPs, and may have almost the same functions as S-MBP on complement activation via the lectin pathway in newborn and adult porcine sera. Moreover, the inhibition experiments on their carbohydrate binding specificities indicated that Man, GlcNAC, and Fuc behaved similarly as to the binding inhibition of porcine PMBL, MBP-C, and rabbit MBP; however, porcine PMBL shows specifically significant binding to Man-6-P and Gal, different from porcine MBP-C and human and rabbit MBP. BIAcore SPR analysis of the porcine PMBL interaction with P-mannan and mannan demonstrated that porcine PMBL exhibits about 300-fold higher affinity as to binding with P-mannan and 5-fold higher affinity as to binding with mannan compared with rabbit MBP, while it also indicated that porcine PMBL binds with P-mannan a little more strongly (about 11-fold) than binding with mannan. Furthermore, based on the results of lectin pathway activation experiments involving the C4 cleavage assay, the data suggest that porcine PMBL functionally activates the complement via the lectin pathway triggered by binding with both P-mannan and mannan, and it is significantly more effective and a little less effective, respectively, than both human and rabbit MBP. The mechanism underlying the different capacities of complement activation triggered by binding with different carbohydrate
Complement Activation and Carbohydrate Specificity of PMBL

The results of this study suggest that an intact complement system is crucial for innate immune defense. The porcine PMBL-mediated lectin pathway for complement activation triggered by binding with different carbohydrate ligands, which may be expressed on infectious microorganisms in newborn and adult porcine sera compared with human and rabbit MBPs, may be expressed on infectious microorganisms in newborn and adult porcine sera compared with human and rabbit MBPs. Some infectious diseases involving MBP.

Other components of innate immunity such as scavenger receptors (34), Fc receptors (35), cytokines (36), and antimicrobial peptides (37) may provide residual protection against low infectious doses. Based on our observations, the establishment of the newborn piglet as a unique experimental model for innate immune system studies is a valuable step toward elucidation of the physiological function and molecular mechanism of the PMBL-involved lectin pathway and also provides a knowledge-based approach for xenotransplantation of newborn piglet tissues and organs, and there will be future therapeutic applications of the latter in some infectious diseases involving MBP.

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