Generation and Characterization of Anti-phenyl Sulfate Monoclonal Antibodies and a Potential Use for Phenyl Sulfate Analysis in Human Blood

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Patients with chronic kidney disease (CKD) have increased blood levels of phenyl sulfate (PS), a circulating uremic toxin. In this study, we produced anti-PS monoclonal antibodies (mAbs) and characterized their cross-reactivity to structural PS analogs. To induce PS-specific mAbs, we synthesized 4-mercaptophenyl sulfate with a sulfhydryl group at the para-position of PS and conjugated it to carrier proteins via bifunctional linkers. Using these PS conjugates as immunogens and as antigens for enzyme-linked immunosorbent assay (ELISA) screening, we produced by a hybridoma method two novel mAbs (YK33.1 and YKS19.2) that react with PS conjugates independent of carrier and linker structures. Although all of the PS analogs tested, with the exception of indoxyl sulfate, were cross-reactive to both mAbs in phosphate buffered saline (PBS), PS specificity for YKS19.2 was enhanced in human plasma and serum. YKS19.2 mAb was cross-reactive only with o-cresyl sulfate, which is absent in human blood. PS sensitivity for YKS19.2 mAb increased to an IC₅₀ of 10.4 µg/mL when 0.1% Tween 20 was added in a primary competitive reaction. To explore potential clinical applications, we determined concentrations of PS in serum samples from 19 CKD patients by inhibition ELISA using YKS19.2 mAb and compared them to those found using an LC-MS/MS method. A good correlation was observed between each value (R²=0.825). Therefore, the unique antigen specificity of YKS19.2 mAb could be useful for prescreening of patients with accumulated PS or for comprehensive analysis of uremic toxins that have a PS-like structure.

Key words phenyl sulfate; monoclonal antibody; enzyme-linked immunosorbent assay; LC-MS; chronic kidney disease

Chronic kidney disease (CKD) is a primary cause of end-stage renal disease and is becoming a serious health problem, particularly in developed countries. Advanced CKD patients require dialysis or renal transplantation. Severely reduced renal function causes complications such as cardiovascular and metabolic bone diseases, which further decrease the patient’s quality of life. No treatment can restore damaged renal tissues. However, the progression of CKD can be delayed or possibly prevented if the disease is medicated appropriately and managed through lifestyle modification at an early stage. Preventive and palliative therapy can also appropriately and be delayed or possibly prevented if the disease is medicated managed through lifestyle modification at an early stage. Preventive and palliative therapy can also appropriately and be delayed or possibly prevented if the disease is medicated managed through lifestyle modification at an early stage. Preventive and palliative therapy can also appropriately and be delayed or possibly prevented if the disease is medicated.
investigated comprehensively. A recent study in rats suggested that PS might be predictive of early-stage CKD because its plasma levels were associated with the progression of renal disease.\textsuperscript{19} These findings allow us to speculate that PS plays a pathological role in the progression of CKD and to explore the potential for PS to act as a novel diagnostic marker for CKD. A clinically applicable, rapid and convenient analytical method for PS provides a way to address these issues and test the possibility that increased blood PS levels may detect early renal damage in CKD patients.

Analysis of PS in biological samples is typically performed using chromatography and mass spectrometry, such as LC-UV, GC-MS, and LC-MS.\textsuperscript{19–24} Although these instrumental analyses are accurate and sensitive, they are very expensive and time-consuming, which makes it difficult to use them in routine clinical analysis for many samples. A rapid, inexpensive, and high-throughput analysis using enzyme-linked immunosorbent assay (ELISA) or immune-strip assay is preferable for regular use, such as medical examinations in clinics and hospitals. However, such immunomessays require a well-characterized antibody (Ab) for the specificity and cross-reactivity of the cognate antigen (Ag). To the best of our knowledge, a monoclonal antibody (mAb) specific to PS has not yet been reported.

In this study, we produced the first novel monoclonal antibodies (mAbs) against PS and characterized their cross-reactivity to structural PS analogs, including InS and pCS. To investigate a potential use in screening patients with accumulated PS, we assayed serum levels in CKD patients by inhibition ELISA using an anti-PS mAb and compared them to PS levels determined by LC-MS/MS analysis.

**MATERIALS AND METHODS**

**Animals and Cells** BALB/c mice were obtained from CLEA Japan (Tokyo, Japan) and maintained under specific pathogen-free conditions at the animal facility at Tohoku University. The protocol was approved by the Institutional Animal Care and Use Committee at Tohoku University. Mouse myeloma SP2/O cells (CRL-1581) were purchased from American Type Culture Collection (Rockville, MD, U.S.A.) and maintained in RPMI-1640 supplemented with 10% fetal calf serum and 50 \( \mu \)M \( \beta \)-mercaptoethanol.

**Reagents** Bovine serum albumin (BSA), ovalbumin (OVA), and keyhole limpet hemocyanin (KLH) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Sigma (St. Louis, MO, U.S.A.), and MP-Biomedicals (Tokyo, Japan), respectively. Horse-radish peroxidase (HRP)- conjugated goat anti-mouse immunoglobulin G (IgG) Ab and HRP-conjugated streptavidin (stv) were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, U.S.A.) and BioLegend (San Diego, CA, U.S.A.), respectively. Horseradish peroxidase (HRP)- conjugated goat anti-rabbit immunoglobulin G (IgG) Ab and 3,3',5,5'-Tetramethylbenzidine (TMB) solution (1X) was obtained from BioRad Laboratories (Hercules, CA, U.S.A.) to remove unreacted linker. Collected fractions (1.5 mL) were mixed with PS-SH (15 \( \mu \)mol) immediately after synthesis.

**Preparation of 4-Mercaptophenyl Sulfate Potassium Salt (PS-SH)** PS-SH was synthesized from bis(4-hydroxyphenyl)disulfide via disulfanediyllbis(4,1-phenylene)bis(sulfate) potassium salt (Fig. 1). Sulfation of bis(4-hydroxyphenyl) disulfide with pyridine-sulfur trioxide complex yielded disulfanediyllbis(4,1-phenylene)bis(sulfate) potassium salt as previously reported.\textsuperscript{25} Subsequent reduction with polymer-bound triphenylphosphine yielded PS-SH. Synthesized structures were confirmed by \( ^1 \)H-NMR and high-resolution mass spectrometry.

**Preparation of PS Conjugates** Carrier proteins such as BSA (3 mg), OVA (10 mg), and KLH (10 mg) dissolved in PBS (1 mL) were mixed at a 1:147, 1:40, or 1:400x ratio with a heterobifunctional linker KMUS, respectively, according to the manufacturer’s instructions. Following a 30-min incubation with gentle rotation, we applied maleimide-activated carrier proteins to the PD-10 column (BioRad, Hercules, CA, U.S.A.) to remove unreacted linker. Collected fractions (1.5 mL) were mixed with PS-SH (15 \( \mu \)mol) immediately after synthesis. Following overnight incubation at room temperature, we dialyzed the resulting conjugates (PS-KMUS-BSA, -OVA, -KLH) in PBS three times to remove unreacted and oxidized PS-SH and followed this with filter sterilization. The protein concentration was determined by bicinchoninic acid protein assay. BSA (10 mg) was activated with GMBS and SMCC at a 1:100x ratio and then reacted with PS-SH to generate PS conjugates with different linkage structures (PS-GMBS-BSA and PS-SMCC-BSA) as described above.

**Generation of mAbs** Female 8- to 10-week-old BALB/c mice were immunized intraperitoneally (i.p.) with 100 \( \mu \)g PS-KMUS-KLH conjugate emulsified in 200 \( \mu \)L of complete Freund’s adjuvant (Difco, Lawrence, KS, U.S.A.) and, 4 weeks later, boosted with the same dose of the conjugate emulsified in an incomplete adjuvant. Nine days (clone YK33.1) or 16 d (clone YKS19.2) after the secondary immunization, mice received i.p. PS-KMUS-KLH (100 \( \mu \)g; dissolved in 200 \( \mu \)L PBS), and 3d thereafter the fusion experiments were performed. We fused spleen cells from two (first fusion experiment) or one (second fusion experiment) immunized mice/mouse with SP2/O myeloma cells using polyethylene glycol 1500 (Roche Applied Science, Indianapolis, IN, U.S.A.) according to the standard procedure. Following hypoxanthine-aminopterin-thymidine (HAT) medium selection (Invitrogen, Carlsbad, CA, U.S.A.), supernatants from hybridoma clones were screened by indirect ELISA against PS conjugates and unconjugated carrier proteins (see below). Single clones were isolated by repeated limiting dilution cloning. Established hybridoma
were cultivated in hybridoma SFM medium (Invitrogen), after which mAbs were purified from the conditioned medium by affinity chromatography on HiTrap Protein G HP Columns (GE Healthcare, Buckinghamshire, U.K.), dialyzed in PBS, and sterilized through a 0.22 µm sterile Millex-GV filter (Millipore, Billerica, MA, U.S.A.). Subclasses of mAbs were determined using IsoQuick™ strips for mouse monoclonal isotyping (Sigma). Biotinylated (Bio-) Abs were prepared using EZ-Link NHS-LC-Biotin according to the manufacturer’s instructions.

**Preparation of PS Analog**  
- o-Cresyl sulfate (oCS), p-nitrophenyl sulfate (pNPS), p-chlorophenyl sulfate (pCPS), quinolinol sulfate (QS), 1-naphthol sulfate (1NS), 2-naphthol sulfate (2NS), and 4-methylumbelliferyl sulfate (4MUS) were synthesized from their corresponding phenol derivatives. Sulfation of each phenol derivative with a pyridine-sulfur trioxide complex yielded potassium sulfate salt, as reported previously.25)

**ELISA**  
Wells of 96-well microplates (Greiner Bio-one, Kremsmunster, Austria) or Nunc Maxisorp 96-well microplates (Thermo Fisher Scientific) were coated with PS conjugates in 50 µL PBS overnight, washed four times with 150 µL PBS, and blocked with 200 µL 1% BSA in PBS. Unless otherwise noted, incubation in microplates was carried out at 4°C. Following 2 h incubation, blocking buffer was washed off with PBS once, and 50 µL Bio-mAbs (YK33.1, 1 µg/mL; YKS19.2, 0.5 µg/mL) or cell culture supernatant was incubated for 1 h in the indicated matrix, followed by incubation with an HRP-conjugated goat anti-mouse IgG (0.4 µg/mL) or stv (0.5 µg/mL) in blocking buffer. For competitive inhibition assays, 25 µL Bio-mAbs (YK33.1, 1 µg/mL; YKS19.2, 0.5 µg/mL) or cell culture supernatant was incubated for 1 h, followed by incubation with an HRP-conjugated goat anti-mouse IgG (0.4 µg/mL) or stv (0.5 µg/mL) in blocking buffer. For competitive inhibition assays, 25 µL Bio-mAbs (YK33.1, 1 µg/mL; YKS19.2, 0.5 µg/mL) or cell culture supernatant was incubated for 1 h, followed by incubation with an HRP-conjugated goat anti-mouse IgG (0.4 µg/mL) or stv (0.5 µg/mL) in blocking buffer. For competitive inhibition assays, 25 µL Bio-mAbs (YK33.1, 1 µg/mL; YKS19.2, 0.5 µg/mL) or cell culture supernatant was incubated for 1 h, followed by incubation with an HRP-conjugated goat anti-mouse IgG (0.4 µg/mL) or stv (0.5 µg/mL) in blocking buffer. For competitive inhibition assays, 25 µL Bio-mAbs (YK33.1, 1 µg/mL; YKS19.2, 0.5 µg/mL) or cell culture supernatant was incubated for 1 h, followed by incubation with an HRP-conjugated goat anti-mouse IgG (0.4 µg/mL) or stv (0.5 µg/mL) in blocking buffer.

**Deproteinization**  
Human pooled plasma or PS-spiked plasma samples (25 µL) were mixed with 75 µL 0.1% formic acid in acetonitrile and deproteinated via sonication for 10 min in an ultrasonic bath (AS ONE, Osaka, Japan) in a 1.5 mL centrifugation tube. Following centrifugation at 16400×g for 15 min at 4°C, 90 µL supernatant was transferred to a new tube and dehydrated under reduced pressure with a SpeedVac centrifugal concentrator (TOMY Digital Biotechnology, Tokyo, Japan) for 45 min at 60°C. The resulting residue was reconstituted with 25 µL PBS.

**Analysis of CKD Patient Serum**  
PS serum levels from 19 stage 2–5 CKD patients who visited the nephrology outpatient clinic at Shinmatsudo Central General Hospital (Matsudo, Japan) were determined by developed ELISA as described below. No patient received dialysis. PS-SMCC-BSA (0.1 µg/mL) was coated and blocked on Nunc Maxisorp 96-well microplate as described above. Bio-YKS19.2 (25 µL) dissolved in PBS at a concentration of 0.5 µg/mL containing 0.2% Tween 20 was incubated with serum samples (25 µL) for 1 h, followed by incubation with HRP-conjugated stv (0.5 µg/mL) in blocking buffer. For competitive inhibition assays, 25 µL Bio-mAbs (YK33.1, 1 µg/mL; YKS19.2, 0.5 µg/mL) was incubated with 25 µL sample or competitor. In some experiments, Tween 20 was added to the primary mAb reaction at various concentrations. The plate was washed four or five times with PBS containing 0.05% Tween 20 (PBS-T) after incubation with primary Ab and secondary reagents. Bound mAbs on plates were measured by the addition of 1× TMB solution (50 µL). After a sufficient period of time, the reaction was stopped with the addition of 50 µL 1 M phosphoric acid, and the resulting color intensity was determined by spectrophotometry at 450 nm using a Multiskan™ FC Microplate Photometer (Thermo Fisher Scientific).

This study was performed according to the Declaration of
Helsinki and approved by the Ethics Committee of the Tohoku University School of Medicine. Informed consent was obtained from all patients.

Statistical Analyses All statistical analyses were performed using JMP ver. 13.0.0 (SAS Institute, Cary, NC, U.S.A.). Correlations were evaluated according to Pearson’s correlation analysis.

RESULTS AND DISCUSSION

Preparation of PS Conjugates PS is not a strong enough immunogen to induce an Ab response in mice. To confer immunogenicity, PS must be conjugated with carrier proteins as previously demonstrated in other hapten-specific mAbs generated by a hybridoma method. In designing PS conjugates, we speculated that a sulfate group in PS could be the only unique Ag determinant to generate mAbs reactive to PS and its derivatives. It is well accepted in immunology that conjugation of a carrier protein to the farthest position from the targeted structure in the hapten greatly contributes to the elicitation of an anti-hapten Ab with preferential specificity. Therefore, we chemically synthesized PS-SH, which has a sulfhydryl group at the para-position of PS, and conjugated it to carrier proteins using a KMUS heterobifunctional linker with an NHS-ester and a maleimide reactive group at opposite ends of a long alkane spacer arm (Fig. 1). KLH, BSA, and OVA were reacted with primary amine-reactive NHS-ester of the KMUS linker, and then KMUS-activated carriers were covalently conjugated to PS-SH via a sulfhydryl-reactive maleimide group. Resultant PS-KMUS conjugates were used as Ags for immunization and ELISA for mAb screening and characterization.

Generation of Anti-PS mAbs Of the three PS-KMUS conjugates prepared, PS-KMUS-KLH was selected as an immunogen because of the greater immunogenicity of KLH in mice compared to BSA and OVA. T helper 2 (Th2)-prone BALB/c mice were chosen as an immunizing host. After double-immunization with complete and incomplete Freund’s adjuvants, BALB/c mice were boosted i.p. with PS-KMUS-KLH. Consequent increases in the serum titer of KLH as well as both PS-KMUS-BSA and -OVA conjugates with different carrier proteins were confirmed by indirect ELISA (data not shown). By contrast, immunization with complete Freund’s adjuvant alone was not sufficient to increase anti-PS-KMUS-BSA and -OVA titers, although the anti-KLH titer was elevated. This could be because of the very low immunogenicity of the PS portion of KLH conjugates. Repeated immunization was required to induce PS-specific mAbs in BALB/c mice.

Because elevated serum titers were observed, spleen cells were prepared from mice that had been immunized three times (twice with adjuvant, once without) and subjected to fusion experiments using myeloma. After HAT selection, we screened positive wells by indirect ELISA by comparing the reactivity of supernatants to PS conjugates and carrier proteins. We used PS-KMUS-BSA and -OVA as coated antigens in ELISA screening to efficiently exclude the hybridoma-producing anti-KLH mAbs. We obtained nearly 721 positive wells by repeating two independent fusion experiments. Of these, the upper positive 96 wells and 24 wells of the highest order reactivity to PS-KMUS-BSA in the first and second fusion experiments, respectively, were further tested for reactiv-

Fig. 2. Reactivity of Hybridoma Culture Supernatant (Clone YK33.1 and Clone YKS19.2) with PS Conjugates with Various Carrier Proteins and Linkers

Hybridoma culture supernatants (50 µL; A, C, clone YK33.1; B, D, clone YKS19.2) were incubated with various PS conjugates (A, B, 10 µg/mL, n=1; C, D, 5 µg/mL, n=3) coated on 96-well microplates in 50 µL volume followed by an HRP-conjugated polyclonal antibody. Bound mAbs were visualized using TMB substrates and measured on a plate reader. Bars indicate means±S.E. (C, D).
Reactivity to BSA, OVA, and PS-KMUS-OVA. Twelve wells showed reactivity to PS-KMUS-BSA and -OVA but not BSA and OVA. Therefore, we obtained the hybridoma clones from these independent wells by limiting dilution cloning and subjected supernatants to an inhibition ELISA against PS. We finally narrowed down the selection to two hybridoma clones producing YK33.1 and YKS19.2 mAbs with preferential specificity to the PS portion of the conjugates. The subclasses of these mAbs were both mouse IgG1/κ.

**Characterization of Anti-PS mAbs** To precisely characterize the Ag specificity and sensitivity of the two anti-PS mAbs YK33.1 and YKS19.2, we cultivated these hybridoma clones in serum-free medium and purified mAbs by protein G affinity chromatography. We first confirmed the results obtained during the course of hybridoma screening regarding recognition of the PS portion of the conjugates by these mAbs.
YK33.1 and YKS19.2 mAbs were incubated with PS-KMUS-KLH, -BSA, and -OVA conjugates coated on 96-well microplates, and their binding was analyzed by indirect ELISA. Although these mAbs bound to any PS-KMUS conjugates, they were unreactive to both native and KMUS-activated carrier proteins (Figs. 2A, B). In addition, we changed the linker bridging between the PS and carrier proteins to an SMCC or a GMBS with a short cycloalkane and alkyl spacer arm, respectively, to exclude the possibility that the linker structure may have been contributing to Ag recognition by the mAbs. Both YK33.1 and YKS19.2 mAbs were reactive with PS-linker conjugates independent of the carrier protein and spacer arm structure (Figs. 2C, D). These results indicate that the PS portion of the conjugates is an essential antigenic determinant for YK33.1 and YKS19.2 mAbs.

Next we addressed whether PS competes for mAb reaction to PS conjugates by inhibition ELISA. When Bio-mAbs were incubated with coated PS-SMCC-BSA conjugates in the presence of free PS at different concentrations in PBS, an inhibition curve showed almost linearity on a plot at the PS concentration between 10 and 1000 µg/mL (Figs. 3A, B). IC_{50} values against the binding of YK33.1 and YKS19.2 mAbs were 100 and 250 µg/mL, respectively. Following this, we further investigated the specificity of these mAbs by determining their cross-reactivity against various structural PS analogs and InS derivatives (Supplementary Fig. 1). By adding different concentrations of competitors (from 10 to 1000 µg/mL) to the reaction, we obtained inhibition curves against coated PS conjugates by inhibition ELISA (Figs. 3A, B). pNPS, pCS, oCS, Phenyl sulfate, 1-Naphthol sulfate, 2-Naphthol sulfate, 4-Methylumbelliferyl sulfate, Indoxyl sulfate, and p-Cresyl sulfate were unreactive to both native and KMUS-activated carrier proteins (Supplementary Fig. 2). In contrast to YKS19.2 mAb, PS-KMUS-OVA and -KLH conjugates were used as coated antigens for YKS19.2 and YK33.1 mAbs in PBS, whereas the InS structure is distinguishable from PS by these mAbs.

Increased Specificity of an Anti-PS mAb in Human Plasma and Serum Various substances in biological samples can interfere with the Ag–Ab interaction in immunoassays. Considering future clinical applications, we tested cross-reactivity when human serum and plasma were used as a matrix in the assay. Inhibition curves by free PS and its analogous competitors were obtained as performed in PBS. Although YK33.1 mAb showed cross-reactivity to almost all PS analogs in PBS, the specificity of this mAb was drastically improved in human plasma (Fig. 3D) and serum (Fig. 3E). oCS, which has not been reported to be present in human blood, was the only cross-reactive analog with an IC_{50} comparable to PS (Table 1). In addition, this increased specificity of YKS19.2 mAb was also observed in serum when PS-KMUS-OVA and -KLH conjugates were used as coated antigens (Supplementary Fig. 2). In contrast to YKS19.2 mAb, the specificity of YK33.1 mAb was not greatly changed in human plasma (Fig. 3C). Following this, we tested monkey and mouse plasma to investigate whether a similar matrix effect was observed. The increased specificity was inherent to human serum and plasma, and neither monkey nor mouse plasma affected the specificity of YKS19.2 mAb (Fig. 4). This species-specific effect allows us to speculate that blood protein(s) may play an important role in the increased specificity of YKS19.2 mAb. To clarify this issue, we deproteinated plasma by acetonitrile-based precipitation and used it as a matrix in the competitive inhibition assay. As shown in Fig. 5, the specificity of YKS19.2 mAb was lower in protein-depleted plasma than it was in plasma. This finding suggests that the interaction of PS and its analogs with blood protein(s) could be a mechanism for increased specificity of YKS19.2 mAb. Therefore, we further tested whether human albumin, which is one of the abundant proteins in serum and plasma, could contribute to the increased specificity of YKS19.2. However, reconstitution in 5% human serum albumin (HSA)-containing PBS did not change the YKS19.2 specificity, suggesting that HSA is not a primary interactive protein for YKS19.2.

In previous reports, the Ag–Ab reaction was improved in the presence of nonionic detergents such as Tween 20, presumably because of epitope renaturation. Therefore, the effects of the addition of Tween 20 to the primary Ab reaction on the specificity of YKS19.2 to PS were investigated. Tween 20 at concentrations of 0.05% and 0.1% markedly decreased the IC_{50} to 14.3 and 15.3 µg/mL, respectively (Table 2). Considering maximum absorbance (B_{max}) values, we decided to use 0.1% Tween 20 as an addition agent to the primary reaction to increase the specificity to PS. A similar effect was also observed.

| Compound                      | PBS (µg/mL) | Plasma (µg/mL) |
|-------------------------------|-------------|----------------|
| o-Cresyl sulfate              | 54.3        | 13.0           |
| p-Nitrophenyl sulfate         | 7.5         | >100           |
| p-Chlorophenyl sulfate        | 48.3        | >100           |
| Quinolinol sulfate            | 434.7       | >100           |
| Phenyl sulfate                | 275.9       | 29.7           |
| 1-Naphthol sulfate            | 179.4       | >100           |
| 2-Naphthol sulfate            | >500        | >100           |
| 4-Methylumbelliferyl sulfate  | 139.3       | >100           |
| Indoxyl sulfate               | >500        | >100           |
| p-Cresyl sulfate              | 278.1       | >100           |

The IC_{50} of PS and its analogs was determined by inhibition ELISA (Fig. 3) using Bio-YKS19.2 mAb in PBS and plasma matrix (n=3) in the range of 1, 10, 50, 100, 250, and 500 µg/mL or 1, 5, 10, 25, 50, and 100 µg/mL, respectively.
in plasma without a negative impact on cross-reactivity (Table 3). The detection limit of PS was 2.5 µg/mL (Student’s t-test, n=3, p<0.05 vs. B0 value). Competitive inhibition curves of PS in each matrix are shown in Supplementary Fig. 3.

**Analysis of CKD Patient Serum** To explore the potential applicability of a YKS19.2 mAb, we determined concentrations of PS in serum samples from 19 stage 2–5 CKD patients using both an inhibition ELISA and a validated LC-MS/MS method. A good correlation between ELISA and LC-MS/MS was obtained (R²=0.825; Fig. 6). However, ELISA showed a higher PS concentration than the LC-MS/MS method. This result suggests that YKS19.2 might be cross-reactive to undetermined serum molecule(s) with similar pathological kinetics to PS. Otherwise, serum matrix might affect the measurements in a YKS19.2 inhibition ELISA because nutritional status possibly varies among the tested CKD patients. To address this point, we measured serum total proteins and albumin as indexes of nutritional status and analyzed their correlations with PS levels (Supplementary Fig. 4). Total proteins and albumin levels were not correlated with the PS concentration. Presumably, nutritional status may not influence the measurement by inhibition ELISA, at least this time. The reason for this overestimation is currently unknown, but it may have to do with cross-reactivity to as yet unidentified compounds with sulfate groups in serum. The finding that YKS19.2 specificity is increased in human blood is useful, but further study is required to clarify this point prior to clinical application of YKS19.2 mAb. For example, immunoprecipitation by YKS19.2 and compound analysis by a LC-MS/MS may provide a clue to identify the potential cross-reactive substance(s) for Ab and/or substance(s) interfering with the PS-Ab reaction.

**CONCLUSION**

In this study, we generated two anti-PS mAbs with preferential specificity for PS derivatives rather than InS derivatives. YKS19.2 mAb in particular showed elevated specificity for PS in human serum and plasma. Sensitivity was increased when Tween 20 was added to the primary competitive Ab reaction at a concentration of 0.1%. The unique Ag specificity of YKS19.2 mAb could prove to be an advantage in prescreening for patients with high concentrations of PS. Otherwise, these mAbs could be useful as anti-PS derivative mAbs for comprehensive analyses of uremic toxins with PS structure, as uremic toxins elevated in CKD are not restricted to PS. Simple and rapid comprehensive prescreening for elevated blood
concentrations of PS analogs could have merit for identifying potential CKD patients, who would need to receive precise medical exams and treatment depending on their pathology. We hope that the anti-PS mAbs generated in this study represent preliminary diagnostic agents for such prescreening and provide a way to investigate a pathological role of PS on the progression of CKD and the potential for an early diagnostic marker for CKD in future clinical study. For the purpose of clinical use, elucidation of the mechanism of increased Ab specificity in human blood is required.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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