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Immunology



Production of polyclonal antibody against kidney antigens: a model for studying autoantibody in feline chronic kidney diseases

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ABSTRACT

Chronic kidney disease is considered to be most common in geriatric domestic cats. It has been reported that the feline viral rhinotracheitis, calicivirus, and panleukopenia (FVRCP) vaccine prepared from the Crandell-Rees feline kidney (CRFK) cell line can induce cross-reactions of antibodies with feline kidney tissues. As an anti-cat kidney antibody was not available commercially for this study of autoantibody in cats, the purpose of this study was to produce anti-cat kidney antibody in rabbits for further study of autoantibody in cats after FVRCP vaccination. Kidney proteins from cadaveric cats were extracted and immunized into rabbits using Montanide as the adjuvant. Based on enzyme-linked immunosorbent assay measurement, all immunized rabbits produced high levels of anti-cat kidney antibodies and some began to produce antibodies as early as 2 weeks after immunization. Immunofluorescence staining of rabbit sera showed kidney-bound antibodies in glomerulus, Bowman's capsule, apical surface of the proximal convoluted tubule, peritubular surface, and interstitial cells. Western blot analysis of cat kidney proteins revealed molecular weights (M.W.) of 72, 55, 47, and 31 kDa, while binding to the CRFK cell proteins was observed at M.W. of 43 and 26 kDa. The antibody that recognized the 47 kDa protein was similarly detected in cats with autoantibody presence after FVRCP vaccination. The kidney-bound antibody profile at different time points and its patterns in rabbits could be used as a model for the study of autoantibody to cat kidney in feline chronic kidney diseases.

Keywords: Chronic kidney disease; autoantibody; feline; vaccines; adjuvant

INTRODUCTION

Chronic kidney disease was reported to have a high incidence in geriatric domestic cats [1]. Several factors associated with the presence of this renal disease have been proposed, and the receipt of too many vaccinations has been considered as a risk factor [2]. An association between feline viral rhinotracheitis, calicivirus, and panleukopenia (FVRCP) vaccination

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Conflict of Interest

The authors declare no conflicts of interest.

Author Contributions

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and autoantibody to kidney has been demonstrated in some studies [3]. Feline vaccine viruses have been grown on Crandell-Rees feline kidney (CRFK) cells during FVRCP vaccine preparation [4]. However, proteins from the CRFK cell line can contaminate feline viral vaccines, thereby inducing autoantibody to kidney tissue [3]. Frequent FVRCP vaccination increased the risk of kidney inflammation in an experimental model; lymphocytic-plasmacytic interstitial nephritis was developed in a cat after immunizing and boosting with CRFK cell line lysates many times [5]. Identification of CRFK cell line antigens, recognized by FVRCP-vaccinated cats, was related to α -enolase [6]. The α -enolase autoantibodies have been shown to be able to induce autoimmune diseases, due to the antibody pathology being nephritogenic to the kidney [7,8]. A previous study demonstrated lesions in kidney, thus, the use of immunofluorescence to identify the localization of cat kidney-bound autoantibodies is very important when studying the pathogenicity of the autoantibody.

As no demonstration has been performed on the kidney bound antibody to cat kidney tissues, the production of antibody to cat kidney is a tool for studying the pathogenicity of cat autoantibody. In order to study kidney-bound autoantibody, rabbits are commonly chosen as the preferred laboratory animal for use in producing novel and desired antibodies. In such production, there are several protocols from simple to complicated and with short to long immunization periods. An adjuvant can be added to an antigen in order to enhance the antibody response. There are several adjuvants, for example, TiterMax, aluminum hydroxide and Montanide, as well as Freund's adjuvant, which is the most popular and widely used. However, the latter has been shown to induce severe inflammation, which is an animal welfare concern [9-11]. In contrast, Montanide provides an antibody response similar to that of Freund's adjuvant, but it has fewer side effects such as inflammatory lesions [12]. In addition to selecting an adjuvant with fewer side effects, it is important to consider choosing a short-term protocol that uses a minimal amount of antigen and few manipulations of the test animals, thereby reducing animal stress [13-15].

In this study, the cat kidney tissue antibody profile was investigated by producing a polyclonal antibody to cat kidney protein in rabbits that had been immunized with cat kidney tissue using Montanide as an adjuvant. Kinetic antibody responses in rabbits were monitored by performing enzyme-linked immunosorbent assay (ELISA)-, immunofluorescence-, and Western blot-based protein analyses.

MATERIALS AND METHODS

Animals

Three healthy New Zealand white rabbits, aged 3–6 months, with a bodyweight of approximately 2.5 kg, were purchased from the National Laboratory Animal Center, Mahidol University, Thailand, and housed under strict hygienic conditions at the animal facility at the Faculty of Veterinary Medicine, Chiang Mai University, Thailand. Protocols used in the animal experiments were approved by the Institutional Animal Care and Use Committee, Faculty of Veterinary Medicine, Chiang Mai University, Thailand (approval No. R25/2559).

Antigen preparation

Proteins from cat kidney tissue were obtained and purified by using the Qproteome mammalian protein preparation kit (Qiagen, USA) according to the manufacturer's instructions. Briefly, approximately 1 g of kidney tissue was placed in a Petri dish and the

sample was cut into smaller pieces. Then, 20 mL of cell lysis buffer (Qproteome) was added to the tissue sample and the combination homogenized by using a mortar and pestle. The obtained suspension was harvested and centrifuged for 10 min at $14,000 \times g$ at 4°C . The kidney protein supernatant was measured to determine protein concentration and kept at -20°C until use.

The CRFK cell line (kindly provided by Dr. Kakanang Piyarungsri from the Department of Companion Animal and Wildlife Clinic, Chiang Mai University, Thailand) was cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum at 37°C and 5% CO_2 . Approximately 1×10^6 cells in log phase were harvested by trypsinizing with a 0.25% trypsin-ethylenediaminetetraacetic acid solution. The cells were centrifuged at $450 \times g$ for 5 min and the obtained cell pellet was then washed twice with 5 mL of ice-cold phosphate buffer saline (PBS, pH 7.2). After centrifugation, the cell pellet was prepared for protein extraction using the same procedure as that described above for the cat kidney tissue.

Rabbit immunization protocol

The rabbits were immunized according to a previously reported protocol for polyclonal anti-CRFK antibodies, but Montanide was used as the adjuvant [6]. The cat kidney antigen used for the immunization was obtained from the mixture of cat kidney tissue extract and Montanide (Seppic, France) at a ratio of 50:50 by volume and by applying a T-connector emulsifying protocol. The rabbits were immunized subcutaneously (0.2 mL/site in 3 sites) with 500 μg of the cat kidney tissue/Montanide mixture on days 0, 14, 28, 42, and 56. Prior to each immunization, 2 mL of blood samples were collected on days 0, 14, 28, 42, and 56. Fifteen milliliter blood samples were collected on day 70 and sera samples were kept at -20°C until use.

ELISA for detecting antibody to kidney antigen

Cat kidney antigen at a concentration of 30 $\mu\text{g}/\text{mL}$ was diluted to 1:500 with carbonate coating buffer (pH 9.2). Then, 100 μL of the diluted antigen was added to the plate wells and incubated overnight at 4°C . The plate was then blocked with 100 μL of 3% bovine serum albumin (BSA) (Bio Basic, USA) in PBS and incubated at 37°C for 1 h. One hundred microliters of rabbit serum were diluted to 1:4,000, added to the plate wells and incubated at 37°C for 1 h. One hundred microliters of horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG; KPL, USA) was diluted to 1:80,000, added to the wells and incubated at 37°C for 1 h. Finally, 100 μL of SureBlue TMB Peroxidase Substrate (KPL, USA) was added to the wells and incubated at 37°C for 30 min. The enzyme reactions were then stopped by adding 50 μL of 1N H_2SO_4 . The plate was washed after every step 5 times with PBS/0.5% Tween-20, except for the last step (stop reaction step). The plate was measured at 450 nm absorbance by an ELISA reader (Synergy H4 Hybrid Reader, Biotek).

Immunofluorescence for kidney-bound antibody detection

Formalin-fixed, paraffin-embedded (FFPE) tissue processing and immunofluorescence testing were performed according to the methods described in a previous report [16]. Briefly, FFPE slides were deparaffinized and rehydrated before being placed in citric buffer (pH 6) and heated in a microwave at 800W for 30 min. Thereafter, they were washed 3 times (5 min each) with distilled water. The slides were then blocked with 1% BSA in Tris-buffered saline with Tween (TBST) for 30 min at room temperature (RT). Rabbit serum was diluted in 1% BSA in TBST to 1:200, dropped onto a slide, and incubated for 2 h at 37°C . In addition, normal goat serum diluted with TBS (1:5) was dropped onto the slide for 30 min at RT. After washing 3 times (5 min each) with TBST, fluorescein isothiocyanate-conjugated goat anti-rabbit IgG

(Thermo Scientific, USA), diluted to 1:200, was dropped onto the slide and incubated for 1 h at RT. The nuclei were then counterstained with 2-(4-amidinophenyl)-6-indolecarbamide dihydrochloride (Sigma Aldrich, USA) for 10 min at RT and the slides were observed under a fluorescence microscope (Zeiss, Germany).

Western blot analysis of antibody recognition of kidney protein

The cat kidney and CRFK cell proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Twenty-five micrograms of cat kidney proteins or 100 µg of CRFK cell line protein were loaded into the wells of a 12.5% gel, adjacent to a molecular weight (M.W.) marker in a parallel well, and the gel electrophoresis was then run for 90 min at 100 V. Proteins from the SDS-PAGE gel were blotted to a nitrocellulose membrane (Thermo Scientific) by passing a current of 15 V through the membrane for 1 h. The membrane was then blocked for staining with 5% BSA in TBST for 1 h at RT. Rabbit sera diluted to 1: 1,000 and 1:100 were added to membrane blotted with cat kidney and CRFK cell line proteins, respectively, and the membranes were then incubated overnight at 4°C. The membranes were then incubated with HRP-conjugated goat anti-rabbit IgG for 1 h at RT. Every step was followed by washing 5 times (5 min each) with TBST. After that, 3,3'-diaminobenzidine tetrahydrochloride (Thermo Scientific) substrate was added to the membrane for 30 sec until bands appeared. Image analysis software (GeneTools, USA) was used to determine an M.W. of protein bands.

RESULTS

Kinetic antibody responses in rabbits after immunization with cat kidney antigen

An antibody response was detected as soon as 2 weeks (day 14) after the first immunization in rabbits B and C with an optical density (OD) value of 0.169 ± 0.001 and 0.104 ± 0.001 , respectively, while rabbit A still had a low OD, which was similar to that in its pre-immune sera. Two weeks after the 1st booster, all 3 rabbits produced high antibody titers to the cat kidney antigen (Fig. 1). The antibody response slope increased with the 1st and 2nd booster and then plateaued after the 3rd booster. The 4th booster did not improve the antibody response further and, therefore, 3 or 4 immunizations were deemed sufficient to produce a

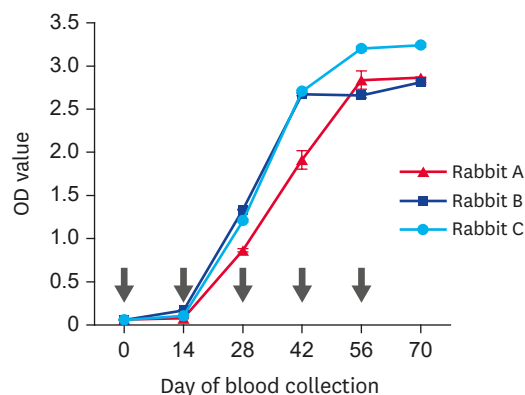


Fig. 1. Kinetic antibody responses in rabbits after immunization with cat kidney antigen. Three rabbits (A, B, and C) showed antibody responses, as detected by enzyme-linked immunosorbent assay, at different blood collection times. The arrows indicate the time of immunization or booster. OD, optical density.

high antibody titer. Thus, the immunization protocol using 4 immunizations with Montanide as the adjuvant was successful for producing anti-cat kidney antibodies in rabbits. The rabbits had no skin wounds, scars, or inflammation and they were deemed to be healthy.

Immunofluorescence pattern of cat kidney-bound antibodies from immunized rabbit sera

The cat kidney sections underwent immunofluorescence staining with rabbit sera at different time points after immunization. In general, cat kidney-bound antibody profiles in rabbits were observed at the glomerulus, Bowman's capsule, the apical surface of the proximal convoluted tubule, peritubular surface, and interstitial cells. Different patterns of antibody-binding locations were observed at different times after immunization (**Fig. 2**). Rabbit anti-cat kidney antibody was produced initially on days 14 and 28 after immunization, and green fluorescence signals were detected at interstitial cells and the peritubular surface of the kidney tissue. Two of the rabbits (B and C) showed green fluorescence at the Bowman's capsule, peritubular surface, and interstitial cells on days 42, 56, and 70, whereas rabbit A showed green fluorescence at the proximal convoluted tubule and glomerulus on day 70. In addition, rabbit A showed antibodies against different tissue locations than those observed in rabbits B and C.

Western blot analysis of antibody recognition of cat kidney and CRFK cell line proteins

On day 14 after primary immunization, a 55 kDa M.W. band of cat kidney protein appeared in all 3 rabbits, and more cat kidney proteins appeared with M.W. of 72, 47, and 31 kDa between days 28 and 70 (**Fig. 3**). Regarding the CRFK cell line proteins, the preimmunized rabbits showed no protein bands, but 2 protein bands (M.W. 43 and 26 kDa) did appear on day 28 after the rabbits received initial immunization. The antibody recognized that the kidney tissue and CRFK cell line proteins were different, and the Western blot results suggest that the 72, 55, 47, and 31 kDa M.W. cat kidney proteins might have stronger immunogenicity, as they could be detected as early as 2 weeks after initial immunization, while the 43 and 26 kDa CRFK cell line proteins were not detected until later (day 28 after initial immunization).

DISCUSSION

The association between FVRCP vaccination and presence of autoantibody to cat kidney was previously demonstrated and the presence of that autoantibody was due to the existence of contaminated proteins from a CRFK cell line used for growing viruses in vaccine preparation [3]. Accordingly, the pattern of autoantibody response to kidney tissue was investigated by producing the antibody to the cat kidney antigen in rabbits. That production allows the study of the proteins recognized by the autoantibody.

Regarding the immunization protocol, Freund's adjuvant is widely used as an immunopotentiator for the production of polyclonal antibodies. It is used commonly in animal models, for example, rabbits, but it can produce inflammation and pain. Therefore, a balance between the welfare of animals and the goal of producing antibodies efficiently needs to be considered [17]. A previous study assessed the Gerbu and Montanide adjuvants, which are available commercially, as potential alternatives to Freund's adjuvant in veterinary use. The authors reported that Montanide adjuvant caused no adverse effects, and had adequate antibody sensitivity when compared to that from immunized rabbits when Freund's adjuvant

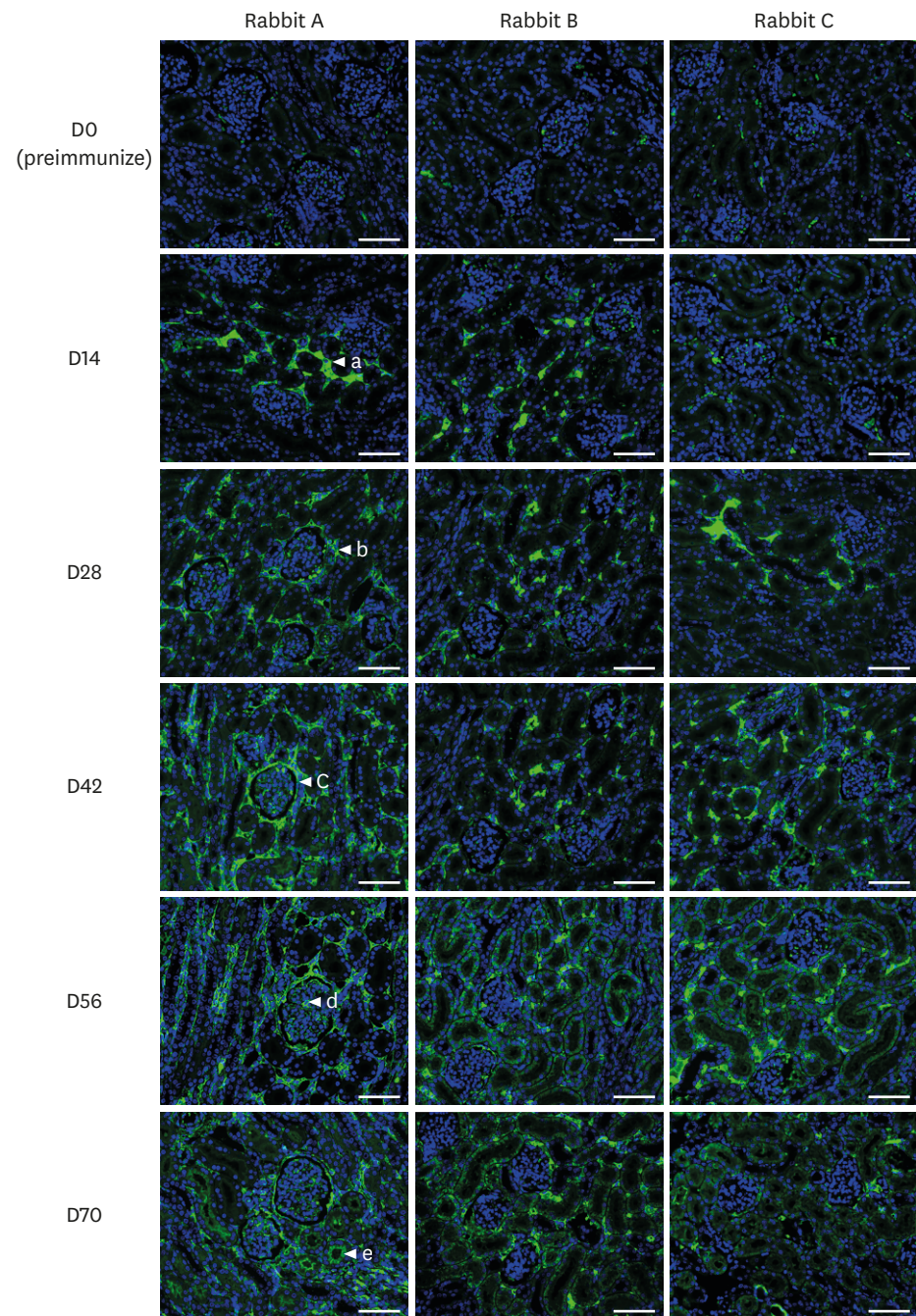


Fig. 2. Immunofluorescence profile of kinetic antibody responses in rabbits after immunization with cat kidney antigen. Cat kidney tissues were stained with rabbit immune sera (rabbits A–C) collected at different time points. Green fluorescence signals indicate stained kidney-bound antibody. Nuclei were counterstained with 2-(4-amidinophenyl)-6-indolecarbamide dihydrochloride and are shown in blue color. Arrowheads indicate kidney-bound antibodies at the peritubular surface (a), interstitial cells (b), Bowman's capsule (c), and glomerulus (d), and at the apical surface of the proximal convoluted tubule (e). Scale bar = 200 μ m. D, day of blood collection.

was used [18]. On that basis, the immunization protocol in this study used Montanide as the adjuvant. In this study, we used a simple and short protocol, and all 3 study rabbits produced high antibody titer to cat kidney antigen as quickly as day 28 after initial immunization. This

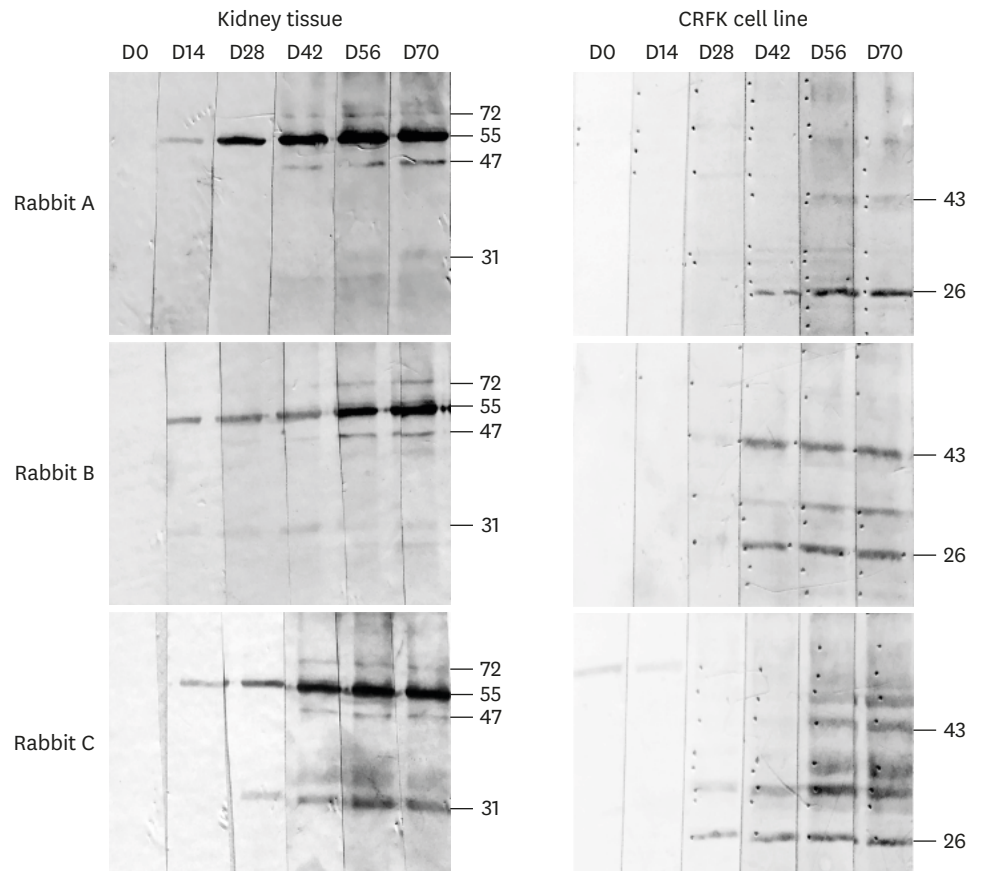


Fig. 3. Results of western blot analysis of kinetic antibody responses to cat protein recognition. Rabbits A, B, and C were immunized with cat kidney tissue proteins, and antibody responses to cat kidney tissue proteins and CRFK cell line proteins are shown on blood collection days 0, 14, 28, 42, 56, and 70. Day 0 represents the immune serum level before immunization. Molecular weights are shown on the right side of the images. CRFK, Crandell-Rees feline kidney; D, day of blood collection.

study confirmed that Montanide can be considered a suitable alternative adjuvant for the production of high titer polyclonal antibodies in rabbits.

The presence of rabbit antibody to cat kidney antigen was detected at the glomerulus, Bowman's capsule, the apical surface of the proximal convoluted tubule, peritubular surface, and interstitial cells. The observed rabbit immunofluorescence profile was similar to that observed in humans with kidney anti-brush border antibodies and in renal failure [19,20]. The autoantibodies to kidney in these patient profiles were similar to the anti-kidney antibody profiles in rabbits immunized with cat kidney proteins in this study. The use of the rabbit antibody response to cat kidney antigen as a model and the examination of the autoantibody profiles in cats with kidney diseases are of interest when planning further investigation of cats undergoing frequent FVRCP vaccination.

Rabbits and cats immunized with CRFK antigens have been shown to have antibodies to CRFK cell proteins at M.W. of 47, 40, and 38 kDa [6]. In this study, the rabbits produced antibodies against cat kidney proteins at M.W. of 72, 55, 47, and 31 kDa, while they produced antibodies against CRFK cell proteins with M.W. of 43 and 26 kDa. The protein band with an M.W. of 47 kDa, recognized by an autoantibody found in cats, was identified as α -enolase, which has been shown to have the greatest concentrations in thymus and kidney [6,8].

The 72 kDa protein was reported to be a heat shock protein that increased expression in kidney tubular cells under various stress conditions, and a suggested role of this protein is to protect renal tubular cells against injury from oxidation and cisplatin toxicity [21]. Another protein, kidney injury molecule-1 (KIM-1) is expressed in the kidney of many species such as cats, dogs, humans, and mice. Kim-1 has several isoforms within an M.W. range of approximately 40–80 kDa and has been suggested as a potential biomarker of kidney injury [22]. Another protein, transglutaminase-2 (M.W. of 75 kDa), has been proposed as a mediator of tubulointerstitial fibrosis in the kidney [23]. Further study to identify the bands that were detected in this study is needed to determine whether they are related to the proteins described in these previous reports.

In conclusion, this study showed, for the first time, that the production of antibodies against cat kidney proteins is feasible in rabbits; moreover, such production may be useful as an alternative tool in studies into the pathogenesis of kidney diseases.

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