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ORIGINAL ARTICLE

Application of *Dolichos biflorus* in immunoassay detection of kidney collecting duct biomarkers

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Abstract

Currently there are no biomarkers for detecting collecting duct damage in man. Antibodies to several collecting duct-specific antigens exist but sandwich assays have been difficult to establish due to the need for two different antibodies to the same protein. We hypothesized that a collecting duct-specific lectin could be used in combination with a collecting duct-specific antibody to negate the need for two different antibodies. The collecting duct specificity of selected antibodies (NiCa II 13C2, Pap XI 3C7, HuPaP VII 2B11 and aquaporin 2), was verified by immunohistochemistry. Aquaporin 2 and Pap XI 3C7 were used successfully in setting up assays with the lectin *Dolichos biflorus*, using the Meso Scale Discovery (MSD) platform. Antigen expression was highest in the papillae of rat and human kidney (corresponding to the greatest density of collecting ducts) and was also present in normal urine. We propose that further qualification and validation would lead to an assay for detecting collecting duct damage in man.

Keywords: *Renal papillary necrosis; renal papillary antigen 1; aquaporin 2; Meso Scale Discovery (MSD)*

Introduction

Renal papillary necrosis (RPN) is a toxicity observed with several classes of drug including non-steroidal anti-inflammatory drugs (NSAIDS) and analgesics (Bach 1998). It is a common cause of renal failure and is characterized by functional lesions in the collecting duct (Sabatini 1984). RPN can be induced experimentally with compounds such as 2-bromoethanamine (BEA), propyleneimine (PI) and *N*-phenylanthranilic acid (NPAA). It is observed relatively frequently in preclinical drug safety testing, particularly with compounds in the tyrosine kinase inhibitor class. Urinary enzymes, such as *N*-acetyl glucosaminidase, can be used to detect RPN following administration of toxins such as BEA (Stonard et al. 1987). However, this enzyme has highest activity in the S3 proximal tubule (Le Hir et al. 1979) and therefore is not specific to papillary damage. An early urinary biomarker for RPN would be extremely useful in both preclinical drug safety testing and for monitoring renal toxicity in the clinic. Recently renal papillary antigen-1 (RPA-1) has been identified as a urinary biomarker of collecting duct damage in the rat (Falkenberg et al. 1996, Price et al. 2010). A commercially available enzyme-linked immunosorbent assay (ELISA) kit is available and the US Food and Drug Administration is currently considering this biomarker for use in preclinical safety testing. Unfortunately preliminary studies have shown that this antibody does not detect a similar antigen in the human kidney. Therefore, alternative biomarkers need to be investigated. A panel of antibodies exist that have been produced in a similar manner to RPA-1 – by injecting mice with material prepared from either rat or human kidney papilla (Falkenberg et al. 1981, 1996, Hildebrand et al. 1999). These include an IgG – NiCa II 13C2 (13C2 – human renal carcinoma antigen) and two IgMs: Pap XI 3C7 (3C7 – rat papilla antigen) and HuPap VII 2B11 (2B11 – human papilla antigen). Initial data using immunofluorescence suggested that these antibodies may have collecting duct selectivity (unpublished data). However, in order to develop a sandwich assay, a pair of antibodies to different

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regions of the same protein is usually required (one for capture and one for detection). This is difficult if only one antibody exists to an unknown antigen. The RPA-1 assay, in which the antibody is an unknown antigen, is thought to work because RPA-1 is presumed to possess a repeated epitope as the commercially available sandwich assay uses the same antibody for capture and detection (Hildebrand et al. 1999). Unless the other antibodies possess a similar property, the identity of the antigens would need to be determined and a partner antibody would also need to be raised. An alternative would be to use a known collecting duct-specific protein such as aquaporin 2 (AQP2). However, this still requires two different antibodies that detect different regions of the protein.

RPA-1 is known to be a heavily glycosylated protein, and preliminary work with 13C2, 3C7 and 2B11 also suggests these recognize heavily glycosylated proteins. Therefore, we hypothesized that a collecting duct-specific lectin could be used instead of a partner antibody. *Dolichos biflorus* (DB) is an *N*-acetylgalactosamine-specific lectin that has been reported to be collecting duct-specific in rodents (Carter & Etzler 1975, Holthofer et al. 1987, Michael et al. 2007). Assay specificity would be achieved by capturing with the putative collecting duct-specific antibodies (RPA-1, 13C2, 3C7 and 2B11) and using DB to detect these, presuming an *N*-acetylgalactosamine group is present on the antigen. To test this hypothesis we used the Meso Scale Discovery (MSD) platform. The application of this platform would enable the future analyses of multiple kidney biomarkers simultaneously. The aim of these experiments was therefore to set up an MSD assay for urine that would detect RPN in humans using a collecting duct-specific antibody and DB lectin.

Methods

Antibodies

Mouse monoclonal antibodies to collecting duct antigens, RPA-1 (IgG), 13C2 (IgG), 3C7 (IgM) and 2B11 (IgM) were kindly provided by Argutus Medical (Dublin, Ireland). Mouse IgM MOPC-104E (Sigma, Poole, UK) was used as an IgM negative control. Three different AQP2 antibodies were tested: one antibody against AQP2 amino acids 254–271 (A7310; Sigma, St Louis, MO, USA) and two anti-N-terminal-specific antibodies (ab55989, Abcam, Cambridge, UK; LS-C3800, LifeSpan Biosciences, Seattle, WA, USA).

Sample preparation

Kidney extracts

Rat kidney extracts were obtained from normal male Wistar rats. Kidneys were either dissected into cortex, medulla and papilla regions, or whole kidneys were used for preparing extracts. Morphologically normal human kidney tissue samples were obtained from surgically resected nephrectomies. Specimens were delivered to the laboratory within 1 h of removal and dissected fresh. Samples of cortex, medulla and papilla were dissected from non-neoplastic kidney tissue by a pathologist and examined to confirm that the tissue was morphologically normal kidney tissue. Kidney samples were chopped into small cubes (approximately 1 × 1 × 1 mm) and homogenized in RIPA buffer (New England Biolabs, Hitchin, UK) containing Halt protease inhibitors (Pierce, Rockford, IL, USA) using a Polytron (Kinematica, Bohemia, NY, USA) homogenizer. Samples were homogenized for two bursts of 30 s and placed on ice for 1 min in between bursts. Samples were then centrifuged at 10 000 *g* for 5 min to remove insoluble debris.

Rat urine

RPN was induced in male Wistar rats using BEA. Five animals received a single intraperitoneal injection of 100 mg kg^{-1} and five animals (controls) received vehicle only. Necropsies were carried out on the 15th day after injection. Urine samples were taken 4 days before the start of treatment and 3 and 11 days after treatment. All samples were collected in metabolism cages on ice for 18 h overnight.

Human urine

Urine samples from healthy volunteers were purchased from The Binding Site (San Diego, CA, USA). Briefly, urine collection was performed as follows. Mid-stream spot-urine was collected, ~10 ml of each sample was transferred to a separate tube, mixed by inversion and thereafter briefly centrifuged to pellet cellular components. The resulting supernatant was stored at -80°C within 2 h after collection.

Immunohistochemistry

For rat kidney samples, kidneys were fixed for 24–48 h in 10% neutral-buffered formalin, embedded in paraffin and 4 μm sections were cut. Sections were de-waxed using xylene and rehydrated using 100% and 95% ethanol. Endogenous peroxidase activity was blocked with 3% (aqueous) hydrogen peroxide for 10 min. Immunostaining was carried out using a Labvision autostainer (Labvision, Fremont, CA, USA). Non-specific binding of the antibody was prevented by incubating slides with normal goat serum (1:20; Dako, Cambridgeshire, UK) for 20 min. The primary antibodies, RPA-1 (mouse IgG; 1:50), 3C7 (mouse IgM; 1:650), AQP2 (rabbit IgG; 1:500) and HRP-conjugated DB lectin (1:200) were incubated on individual sections for 1 h. Dual Link HRP Labelled Polymer (Dako) supplied with the kit was incubated on the sections of the

unconjugated antibodies for 30 min and the immunoreactivity was visualized using diaminobenzidine (DAB) as the chromogenic peroxidase substrate. All the slides were counterstained with haematoxylin and mounted in DPX Mountant (Sigma). A mouse and rabbit isotype control (Dako) was used to replace the primary antibody on the two negative control slides. All immunoreactions were carried out at room temperature. Image capturing of the slides was carried out using a Scanscope® scanner (Aperio Technologies Inc., Vista, CA, USA).

For dual staining with RPA-1 and DB, sections were prepared as above. RPA-1 antibody, diluted 1:50 in tris buffered saline containing 0.1% Tween (TBST), was applied for 1 h at room temperature. Following a wash in TBST, sections were treated for 30 min with prediluted mouse alkaline phosphatase (AP)-labelled polymer (K4018; Dako), which had been previously incubated with levamisole solution to block all endogenous AP activity in sections. After washing in TBST, sections were treated with liquid permanent red solution (K0640; Dako) for 10 min, and rinsed in distilled water. Following a further wash in TBST, sections were again protein blocked by application of prediluted serum free protein block (X0909; Dako) for 20 min at room temperature. Sections were then treated with DB lectin, diluted to 1:4000 in TBST. Following washing in TBST, sections were treated with streptavidin–horse radish peroxidase (PK-4000; Vector Laboratories, Peterborough, UK) for 30 min, prepared following manufacturer's instructions. Subsequently, sections were washed in TBST and incubated with DAB (K3468; Dako) for 10 min. Sections were rinsed in distilled water and counterstained with Carazzi's haematoxylin for 1 min. They were then washed in tap water, dehydrated, cleared and cover-slipped with mountant. For the negative control, TBST was applied (in place of antibodies) to a section from one of the animals previously positively stained by the method described above. In addition, mouse IgG1 isotype (X0931; Dako) was utilized as a further negative control for RPA-1 antibody specificity in corresponding rat kidney.

For human kidney immunohistochemistry, samples of cortex, medulla and papilla tissues were dissected from non-neoplastic kidney tissue, as described above. Half the samples were fixed in buffered formalin and routinely processed into paraffin wax as described for the rat kidneys and the remainder of the samples were snap-frozen in liquid nitrogen and mounted in optimal cutting temperature compound (OCT) prior to sectioning. Immunohistochemistry on paraffin sections was carried out exactly as for the rat immunohistochemistry.

For frozen human kidney samples, 4-μm sections were cut from the respective frozen OCT blocks onto strongly adhesive slides (Superfrost Plus; Thermo Scientific, Waltham, MA, USA). The sections were blocked using serum-free Protein Block (Dako) for 10 min. The primary antibody, RPA-1 (1:100), 13C2 (1:650) or 2B11 (1:650) was incubated on the sections for 1 h. Mouse fluorescently conjugated secondary antibodies (goat antimouse IgG 1:400, goat antimouse IgG3 1:400 and goat antimouse IgM 1:400; Molecular Probes, Invitrogen Ltd, Paisley, UK) were incubated on the sections for 1 h at room temperature in the dark. All the slides were counterstained with a fluorescent nuclear stain (Hoechst; 1:10 000) and mounted using ProLong Gold mounting medium (Molecular Probes). A mouse IgG/IgM isotype control (Dako) was used to replace the primary antibody on three negative control slides. All immunoreactions were carried out at room temperature.

Meso Scale Discovery immunoassay

The different antibodies described above were tested on the MSD platform using MSD high-binding plates (MSD, Gaithersburg, MD, USA). Each antibody was diluted to 5 μg ml^{-1} using Tris-buffered saline (TBS) buffer and 25 μl was added per well. The plate was incubated overnight at 4°C and each well was blocked with 25 μl of 5% Blocker-A (MSD) in TBS. After 1 h of incubation in blocking buffer, the plate was washed three times with 0.1% Tween-containing TBS (TBST, wash-buffer). All incubations in the MSD immunoassay were done on an orbital shaker at 650 rpm. Throughout the MSD immunoassay a buffer containing 2.5% Blocker-A dissolved in TBS was used as a dilution buffer. A dilution series of rat papillary extract was used for the standard curve. A single batch was prepared, aliquoted and frozen at -20°C. For each experiment, an aliquot was diluted 1:5 and used as the highest concentration of the standard curve. A 1:4 dilution series of this 1:5 standard was then prepared and used as a standard for the RPA-1, 3C7 and AQP2 immunoassays. Because it is not possible to measure the exact concentration of the standard protein, U ml^{-1} was used as an arbitrary unit. Duplicates of both standard and samples were analysed and 25 μl was pipetted per well. Rat urines analysed were diluted 1:4 by adding the samples to 75 μl of dilution buffer added in each well. After incubating standard and sample for 1 h, the plate was washed three times with wash-buffer and 25 μl of sulfo-tagged detection agent (2 μg ml^{-1}; see below), was added per well. The plate was then covered from light while incubating for 45 min and then washed three times with wash-buffer. Finally, 150 μl of 2x MSD read buffer T was added per well and the plate was measured on the MSD Sector Imager 6000. The raw data were analysed using the default setting of the Discovery Workbench 3.0 software (MSD).

Protein Sulfo-tagging

Lyophilized DB lectin (Sigma L6533) was dissolved in TBS buffer to a final concentration of 1 mg ml^{-1} and 100 μl

was used for labelling. Sulfo-tag (MSD) was dissolved in ddH_2O to a concentration of 3 nmol μl^{-1} and the manufacturer's instructions were followed. For the comparative analysis of RPA-1 antibody and DB detection of RPA-1, both the RPA-1 antibody and the lectin were labelled at a ratio of five sulfo-tags per protein molecule. The labelling reaction was incubated for 2 h, protected from light and then terminated by adding 1 µl sodium azide (10%). DB used in the 3C7 and AQP2 immunoassays was labelled at a ratio of 12 sulfo-tags per protein molecule and then treated as previously described. In immunoassays where anti-AQP2 or 3C7 antibodies were used for detection, the antibody was labelled in the same manner as the DB used in the AQP2 immunoassay.

DB immunoassay inhibition analysis

N-acetyl-D-galactosamine (Sigma) was dissolved in ddH_2O to a final concentration of 100 mM and was used to investigate the specificity of the 3C7 and DB signal. A 2.5% Blocker A-containing TBS buffer was used to dilute *N*-acetyl-D-galactosamine at concentrations of 10, 1 and 0.1 mM, respectively, which were then used as dilution buffers in the 3C7/DB immunoassay. In one set of experiments, the kidney extract dilution series were diluted in each of the different *N*-acetyl-D-galactosamine-containing dilution buffers. In another set of experiments, the same buffers were used for diluting DB. The remaining steps of the immunoassay were performed as described above.

Results

Immunohistochemistry on rat tissues

RPA-1 antibody, an AQP2 C-terminus antibody, 13C2, 3C7 and 2B11 were all tested using immunohistochemistry to determine their tissue localization in rat kidney. Consistent with previous data, immunohistochemistry showed that RPA-1 was selectively expressed in collecting duct cells in the rat kidney (Figure 1A–C). Immunohistochemistry also revealed that DB stained collecting duct cells in rat kidney with weak staining in the proximal tubule cells (Figure 1D–F). Negative controls did not exhibit any immunoreactivity and therefore staining in the proximal tubules was considered to be specific, despite the much lower levels of expression compared with collecting ducts. Dual staining with the RPA-1 antibody and DB showed that overlapping staining only occurred in collecting ducts (Figure 1G–I). Both 3C7 and AQP2 stained collecting ducts in the papilla and also in the medulla and cortex although there were fewer positive cells in the collecting ducts in these regions (Figure 1J–L and M–O, respectively). In addition, 3C7 stained distal tubules in the cortex. Isotype controls were all negative. Neither 13C2 nor 2B11 produced any positive staining (not shown).

Immunohistochemistry on human tissue

The same panel of antibodies (RPA-1, 13C2, 2B11, AQP2 and 3C7) were also screened using immunohistochemistry to determine their expression in human kidney sections (Figure 2). The antibodies were initially tested on formalin-fixed paraffin-embedded tissue but if this staining was negative (RPA-1, 13C2 and 2B11) then the antibodies were also tested on frozen sections of human kidney. The results show that AQP2, 13C2, 2B11 and 3C7 all recognized collecting ducts in human kidney. In addition, immunoreactivity for 3C7, DB and 2B11 was also detected within the loop of Henle. RPA-1 detected weak (although specific) immunoreactivity in the human loop of Henle (Figure 2A). Such a signal was specific for this nephron segment, and was absent from the collecting ducts and only visible upon using the RPA-1 antibody at high concentrations and by exposing the slide for a longer time.

RPA-1 immunoassay

The principle of the MSD assay is described in Figure 3. MSD plates were coated with RPA-1 antibody, thereafter RPA-1 was detected either with labelled RPA-1 antibody or labelled DB. Both assays detected an RPA-1 signal when rat renal papillary extract was used as a standard. Despite the higher background observed with the use of labelled DB, the maximum signal of the assay was increased threefold compared with detection with the RPA-1 antibody (Table 1). The coefficient of variation (CV) value in both assays was <10% and the RPA-1 % recovery was ± 20% of the expected values.

To confirm the specificity of the DB in the RPA-1 immunoassay, extracts from different regions of rat kidney were analysed (Table 2). The signal detected from each region corresponded well with the immunohistochemistry for RPA-1. The immunoassay detected most RPA-1 in rat papilla extract and lesser amounts in the medullary and cortical extracts, respectively. Analysis of human kidney extracts demonstrated low affinity to the three different regions, confirming the results from RPA-1 immunohistochemistry staining of the human kidney (Figure 2).

Urinary RPA-1 analysis

To assess the sensitivity of DB in detecting changes in urinary RPA-1 expression in rats with RPN, urine samples were analysed before and after treatment with BEA and compared with untreated rats. Histopathology on the kidneys from BEA-treated rats confirmed that four out of

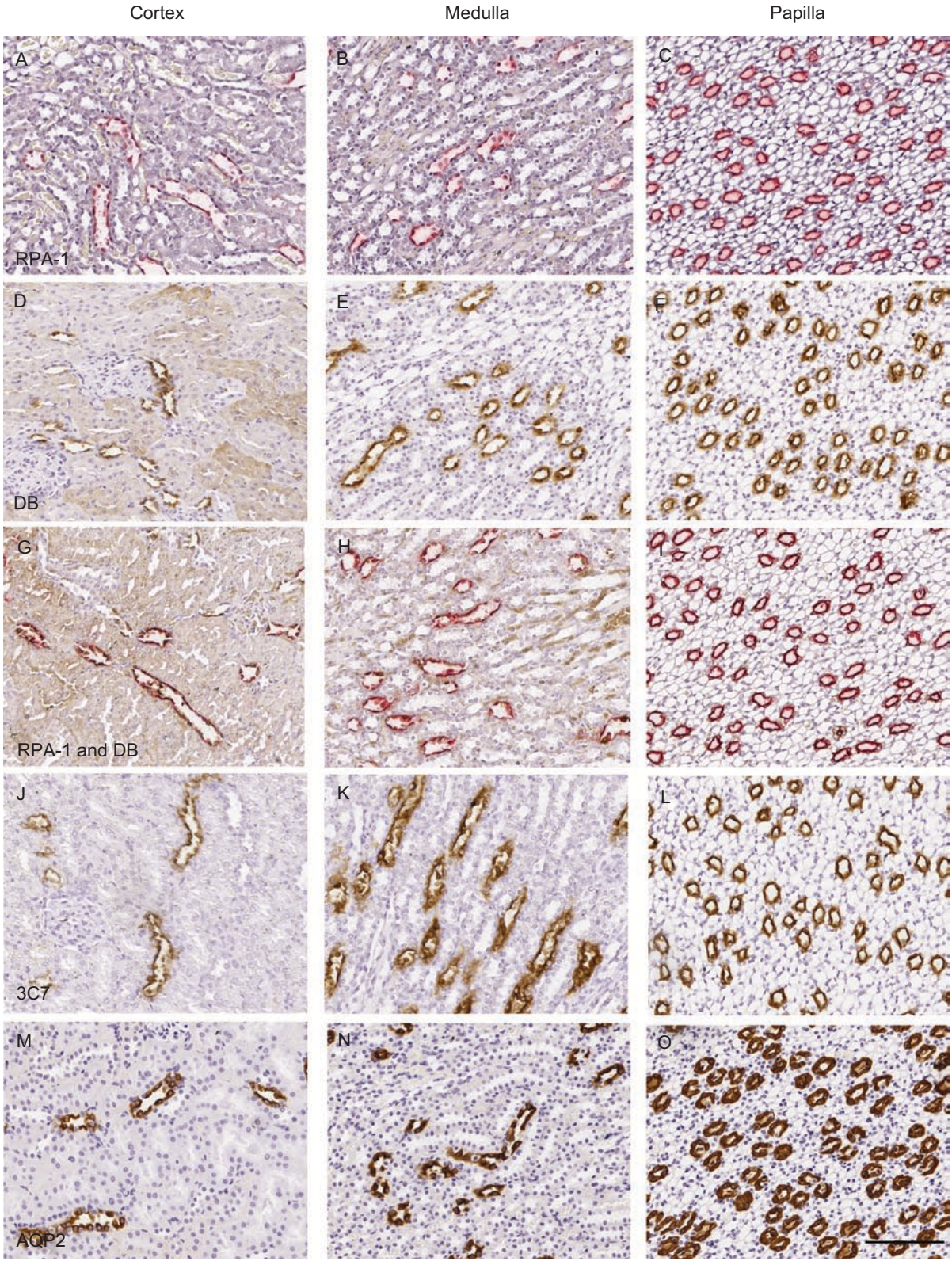


Figure 1. Immunohistochemistry on formalin-fixed paraffin-embedded normal rat kidney with renal papillary antigen-1 (RPA-1) (A–C), *Dolichos biflorus* (DB) (D–F), RPA-1 (red) and DB (brown) (G–I), 3C7 (J–L) and aquaporin 2 (AQP2) (M–O) on control rat kidney sections. Left panel (A, D, G, J and M) shows typical staining from the cortex, middle panel (B, E, H, K and N) shows typical staining from the medulla and right panel (C, F, I, L and O) shows typical staining from the papilla. Scale bar = 100 μm.

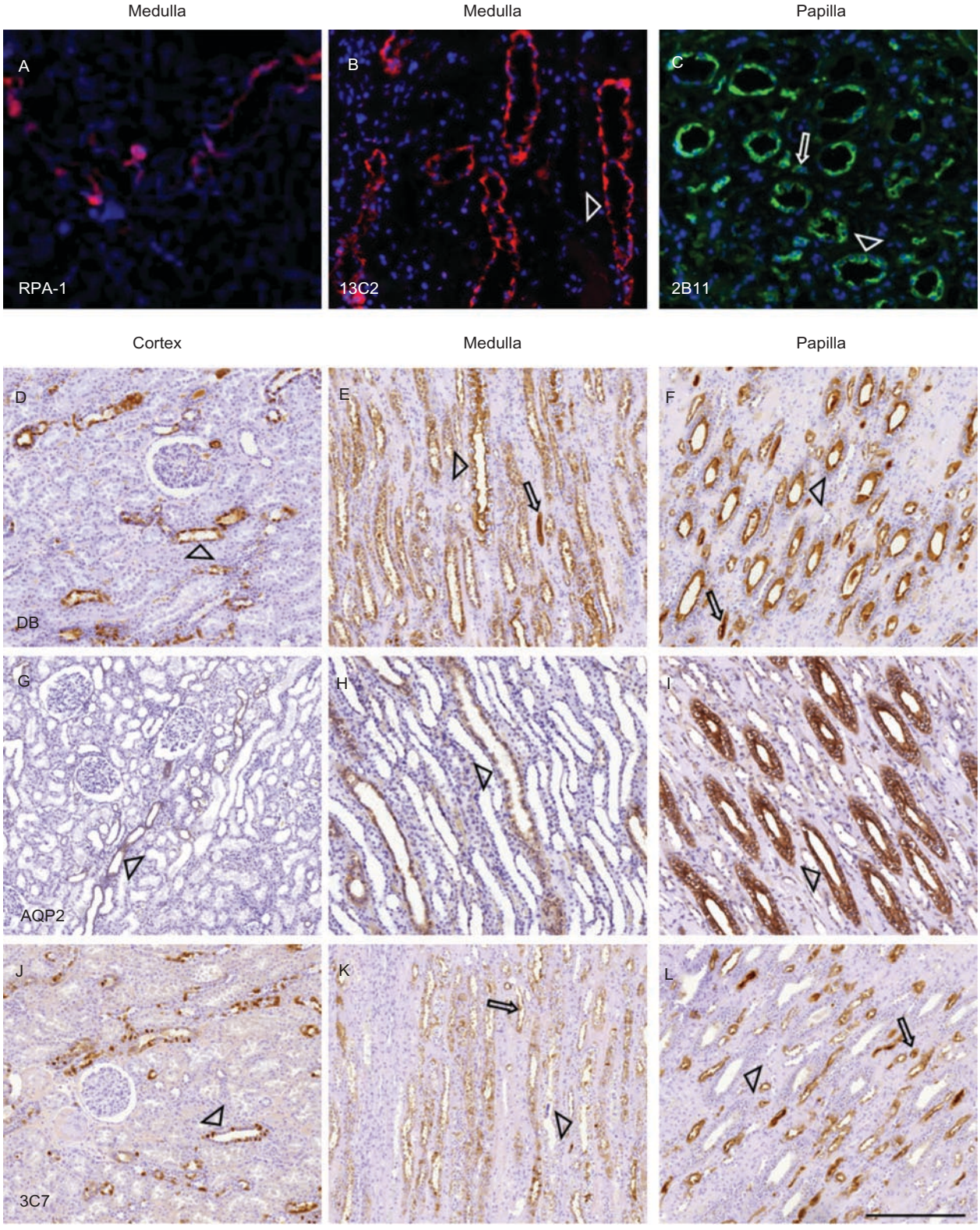


Figure 2. Expression of renal papillary antigen-1 (RPA-1), 13C2, 2B11, *Dolichos biflorus* (DB), aquaporin 2 (AQP2) and 3C7 in morphologically normal adult kidney tissue. RPA-1 detected weak immunostaining in the loop of Henle (A, inner medulla) whereas 13C2 (B, inner medulla) and 2B11 (C, papilla) both demonstrate specific immunostaining in the collecting ducts. 2B11 also shows positive immunoreactivity in the loop of Henle. DB immunostaining is positive in about 70–80% of collecting ducts as well as the loop of Henle (D–F). AQP2-specific immunoreactivity is identified in the collecting ducts of human kidney tissue (G–I), 3C7-specific immunoreactivity is observed in the intercalated cells of the collecting ducts and the loop of Henle (J–L). Scale bar: 500 μm (A–C) or 1000 μm (D–L). Arrow heads, collecting ducts; block arrows, loop of Henle.

five rats had renal papillary necrosis and all rats showed moderate to severe changes in the kidney including: increased interstitial matrix in the papilla, papillary congestion/haemorrhage, cortical tubular protein casts, focal corticomedullary interstitial nephritis, papillary collecting tubule hypertrophy, corticomedullary and cortical collecting tubule dilation and corticomedullary collecting tubular basophilia. Similar findings were not seen in the control rats. The results from urine demonstrated an increase in RPA-1 detection on days 3 and 11 after treatment compared with 4 days before treatment (Table 3). A similar change in RPA-1 expression was not detected in the control animals, although expression levels were comparable to the expression in the BEA group before treatment. The CV values of the detected urinary RPA-1 were <20% and the RPA-1 level in some of the BEA-treated animals was above the assays detection range. Analysis of human urine samples indicated that RPA-1 could not be detected by DB (data not shown). This was consistent with the lack of

Table 1. Comparative analysis of renal papillary antigen-1 (RPA-1) in rat renal papilla extract detected by the RPA-1 antibody versus *Dolichos biflorus* (DB).

	RPA-1				DB			
Concentration (U ml $^{-1}$)	Signal	CV %	Cal. Conc. (U ml^{-1})	% Recovery	Signal	CV %	Cal. Conc. (U ml^{-1})	% Recovery
10000	9480	6.6	8946	89	28682	6.3	9888	99
2500	6520	3.2	3055	122	15047	16	2579	103
625	2782	2.4	607	97	5801	0.5	644	103
156	1121	9.1	146	93	1859	2.1	147	94
39	491	2.6	39	99	754	4.5	40	102
10	264	1.6	12	120	397	6.8	11	111
2.4	151	14	2.1	88	271	10	2	85

Table 2. Renal papillary antigen-1 (RPA-1) analysis of rat kidney extract and human kidney extract using the RPA-1/*Dolichos biflorus* Meso Scale Discovery assay.

	Papilla		Medulla		Cortex	
Dilution factor	Signal	CV %	Signal	CV %	Signal	CV %
Rat kidney extract						
5	50338	2.35	9081	3.69	1797	2.16
20	27370	0.62	4056	2.09	1546	0.46
80	11180	0.25	2253	0.57	1165	0.67
320	3351	0.65	1280	2.16	673	1.16
1280	1263	1.68	656	19.4	426	3.32
5120	679	11.4	527	0.13	384	0.55
20480	450	1.26	401	0	330	4.51
Human kidney extract						
5	569		473		515	
80	397		392		447	
1280	358		389		415	
20480	306		310		353	

Table 3. Renal papillary antigen-1 (RPA-1) analysis of urine from 2-bromoethanamine-treated and control rats using the RPA-1/*Dolichos biflorus* Meso Scale Discovery immunoassay.

	Day -4		Day 3		Day 11	
Animal	RPA-1 (U ml^{-1})	CV %	RPA-1 (U ml^{-1})	CV %	RPA-1 (U ml^{-1})	CV %
Control						
1	403	1.7	509	13		
2	555	1.4	522	0.8		
3	204	1.8	557	13		
Treated						
4	319	3.3	3485	11	>10000	3.2
5	501	5.0	>10000	9.4	>10000	9.2
6	248	5.2	2847	16	2551	5.9
7	221	12	1155	17	1492	2.2

RPA-1 staining in collecting ducts in human kidney by immunohistochemistry.

AQP2 immunoassay

The above results were 'proof of principle' that DB can be used in combination with collecting duct-specific antibodies to detect collecting duct antigens in rat tissue and urine. However, the RPA-1 antibody does not recognize collecting duct antigens in humans. Therefore, we looked at the other collecting duct-specific antigens: AQP2, 13C2, 3C7 and 2B11 for use in an MSD assay for humans. Initially we looked at AQP2 as expression in human urine has previously been reported. MSD plates were coated with anti-AQP2 C-terminus antibody and labelled DB was used to detect AQP2. The assay was tested on rat kidney papilla extract first, as there is a more plentiful supply of rat tissue. Figure 4 depicts the standard curve for this assay. AQP2 percentage recovery was ± 20% of the expected value, with the exception of the measurement in the highest extract dilution. The CV values of the measured AQP2 were <10% throughout the different extract dilutions.

As the assay could detect AQP2 in rat kidney extract, the specificity of the AQP2 immunoassay was investigated using extracts prepared from different regions of human kidney. The signal in human kidney was weaker than in rat but there was still a good dynamic range (Table 4). The results confirmed the immunohistochemistry staining where a greater expression of AQP2 was observed in the renal papilla compared with the medulla and cortex. This was also similar to the expression pattern in rat.

Other immunoassays were tested for the detection of AQP2, none of which yielded positive results. In one attempt, a labelled anti-AQP2 N-terminus antibody was tested for detecting the protein captured by an anti-AQP2 C-terminus antibody. In another attempt, the same anti-AQP2 N-terminus antibody was used for capturing the AQP2 protein and labelled DB was used for detection. Using a different anti-AQP2 N-terminus antibody for capturing antigens and labelled DB for detection was equally unsuccessful.

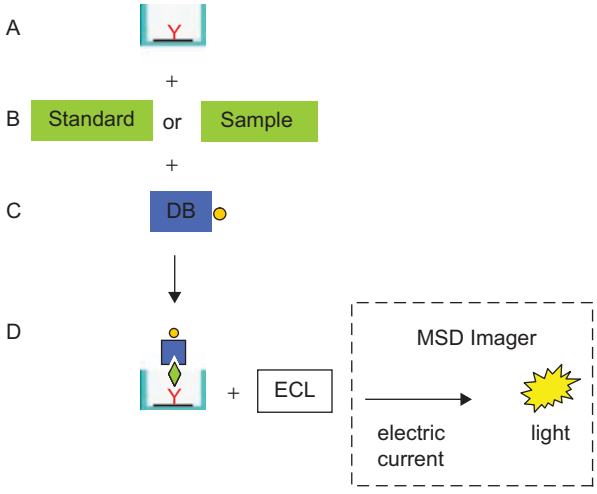


Figure 3. Meso Scale Discovery (MSD) assay schematic. (A) MSD plates have carbon electrodes integrated into the bottom of their surface. The carbon surface at the bottom of each well is coated overnight with collecting duct-specific antibody (renal papillary antigen-1 (RPA-1), 3C7 or aquaporin 2 (AQP2)), by passive absorption. (B) The antibody-coated wells were blocked with blocking buffer and a dilution series of rat papilla extract was used as standard. Each standard and sample tested was added separately in duplicates and incubated. (C) After washing the wells, sulfo-tagged *Dolichos biflorus* (DB) was added to detect the proteins captured by the collecting duct-specific antibody. The sulfo-tag emits light when activated enabling the quantification of the captured proteins detected by DB. (D) Excess sulfo-tagged DB is washed and electrochemiluminescence (ECL) buffer is added. The plate is then analysed through ECL detection using the MSD Imager. In the presence of the ECL buffer, the sulfo-tag emits light when the MSD Imager passes an electric current through the plate electrodes. The ECL buffer contains co-reactants that enhance the ECL signals through multiple excitation cycles. The light emitted is at 620 nm and is measured by the MSD Imager.

3C7 immunoassay

As with AQP2, immunoassays were set up for the analysis of the 3C7 antigen. In an initial attempt, an immunoassay was tested using 3C7 as both capture and detection antibody. Unlike with RPA-1, no signal was detected when rat renal papilla was analysed in this assay (data not shown).

Table 4. Comparison of the aquaporin 2/*Dolichos biflorus* Meso Scale Discovery immunoassay in rat and human kidney extract.

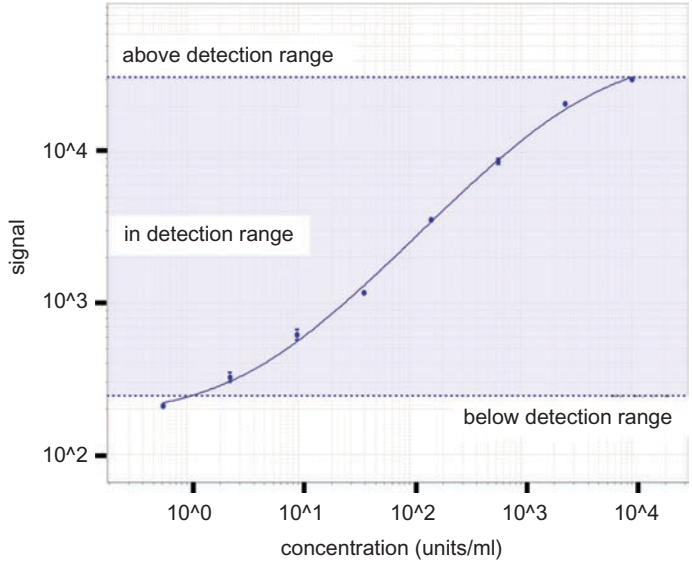
	Papilla		Medulla		Cortex	
Dilution factor	Signal	CV %	Signal	CV %	Signal	CV %
Rat						
5	30049	0.2	668	1.4	626	0.6
20	18188	2.1	447	5.4	503	1.9
80	8652	4.6	310	3.7	404	2.8
320	2953	2.2	228	4.1	282	2.8
Human						
5	3767	3.4	487	4.2	375	4.2
20	1661	4.5	343	0.4	360	7.9
80	828	1.5	241	2.7	293	6.8
320	427	5.3	212	0.3	229	9.2

Instead, MSD plates were coated with 3C7 and labelled DB was used to successfully detect the 3C7-captured antigens. Figure 5 depicts a standard curve for the 3C7 antigen analysis, using rat renal papillary extract. Analysis of rat renal medullary and cortical regions indicated a lower expression of the 3C7 antigen compared with the papilla (Table 5). The assay was then tested on human kidney extracts. As with AQP2, the signal for the 3C7 antigen was lower in human renal papilla compared with rat.

3C7/DB immunoassay control experiments

As IgMs are immature antibodies that have broader specificity, the investigation of 3C7 specificity was essential. To do this, the assay was run with a non-specific IgM and also with wells just blocked with BSA (Table 6). There was no difference in the signal observed with the non-specific IgM compared with the BSA-blocked wells. Using the non-specific IgM, the maximum signal detected with the lowest dilution of rat papilla extract was <10% than the signal observed for 3C7. Furthermore, the first three dilutions of the standard curve are similar using the non-specific IgM, suggesting that the signal observed is not specific to a particular antigen. The results from the non-specific IgM and the BSA-blocked wells indicate a background signal in the DB assay, but more importantly they confirm the specificity of the signal observed for 3C7.

The specificity of DB was also confirmed in another control experiment. DB binds specifically to *N*-acetyl-D-



Concentration U/ml	Signal	CV %	Cal. Conc. U/ml	% Recovery
10000	30049	0.2	9199	92
2500	18188	2.1	2762	110
625	8652	4.6	716	115
156	2953	2.2	142	91
39	1174	0.8	35	90
10	619	7.4	11	110
2.4	324	7.7	2.8	117
0.6	210	1.7	0.4	73

Figure 4. Standard curve for the aquaporin 2 (AQP2)/*Dolichos biflorus* (DB) assay using different concentrations of rat kidney papilla extract.

Table 5. Comparison of the 3C7/*Dolichos biflorus* Meso Scale Discovery immunoassay in rat and human kidney extract.

	Papilla		Medulla		Cortex	
Dilution factor	Signal	CV %	Signal	CV %	Signal	CV %
Rat						
5	4011369	2.6	42379	18	40404	2.1
20	273699	4.8	14906	8.6	15689	8.9
80	122507	1.5	5964	2.2	5789	2.5
320	42514	0.3	3261	0.1	3443	3.1
1280	14608	2.5	2177	6.9	2274	0.5
5120	5301	4.5	1614	1.8	1700	0.5
20480	2453	2	1394	0.2	1459	1.4
Human						
5	47208	1.6	12980	2.1	5652	0.1
20	16413	6.4	4866	0.5	3187	0.4
80	5914	5.4	2164	1.8	2121	5.1
320	2954	7.9	1582	0.4	1634	1.2
1280	1934	3.8	1375	0.9	1473	3.2
5120	1512	2.3	1359	1.4	1444	0.1
20480	ND	ND	1355	4.8	1445	9.8

galactosamine; therefore, *N*-acetyl-D-galactosamine was used to inhibit DB binding to the proteins captured by 3C7. It was possible to inhibit DB binding using *N*-acetyl-D-galactosamine, but not 3C7 (Table 7). The inhibition of DB using *N*-acetyl-D-galactosamine was concentration dependent. Interestingly, *N*-acetyl-D-galactosamine appeared to increase the 3C7 binding to its antigen at the higher dilution of rat renal papilla. It may be that the lack of protein concentration in these samples allowed *N*-acetyl-D-galactosamine to bind non-specifically to the carbon surface of each well. This sugar may have then been detected by DB.

2B11 and 13C2 assays

The application of DB in detecting other collecting duct antigens was tested using 2B11 or 13C2 as capture antibodies. In immunoassays where MSD plates were coated with either antibody, no signal was detected with the analysis of human kidney extracts (data not shown).

Human urine analysis

Of all the antibodies analysed, it was possible to set up immunoassays for the detection of AQP2 and the 3C7 antigen in human kidney extracts. To determine whether these assays could also detect their antigens in human urine, the assays were carried out on samples from 20 healthy individuals (Table 8). The CV values for AQP2 analysis were lower than for 3C7, both of which were <20%. A higher variation in the AQP2 values was observed between the different individuals compared with 3C7. However, creatinine normalization increased both AQP2 and 3C7 variation between different individuals. There appeared to be no gender-specific or body mass index-dependent effect on the AQP2 and 3C7 values, even though the number of samples analysed is relatively small.

Table 6. Control experiment for the 3C7/*Dolichos biflorus* Meso Scale Discovery assay: comparison of the assay using 3C7, a non-specific IgM or BSA.

	Signal		
Dilution factor	3C7	Non-specific IgM	BSA
5	87674	3783	3391
20	59862	3873	3698
80	33421	3160	4424
320	13559	1629	2077
1280	5593	646	716
5120	2485	340	415
20480	1398	230	253
81920	1108	206	199

Discussion

This study has developed MSD assays for rat and human that can detect biomarkers of collecting duct damage in urine. While RPA-1 is being validated as a biomarker for collecting duct damage in rat, there are no equivalent biomarkers for detecting renal papillary necrosis in man. Therefore, this study might provide a major breakthrough in developing tools that have the potential to assess renal toxicity in man. This could be particularly useful for pharmaceuticals moving from preclinical to clinical studies where drug-induced kidney injury has been detected preclinically with unknown human relevance. In addition the method described improves the sensitivity of the assay for RPA-1 in rats.

This study describes the use of a lectin in partnership with an antibody to detect a specific antigen by immunoassay. DB has previously been shown to be selective for collecting ducts (Carter & Etzler 1975, Holthofer et al. 1987, Michael et al. 2007). However, our work also showed weak immunoreactivity in rat proximal tubules. This was considered to be specific as negative controls were all negative. In humans, where the specificity of DB is less certain (Engel et al. 1997, Laitinen et al. 1990, Truong et al. 1988), staining was found in the collecting ducts and also in the loop of Henle. The advantage of using an antibody/lectin combination to assay the same molecule is that specificity is achieved by the overlap in the binding. Therefore, the additional staining in proximal tubules with DB in rat was not a problem because RPA-1 is collecting duct specific. In humans, 3C7 and DB both recognized thin limbs of the loop of Henle in addition to collecting duct cells; however, this also was not a problem as both tubule types are found in the papilla, where RPN occurs. The use of a lectin also avoids the requirement for two different antibodies to the same protein, which is a problem for antigens like 3C7 where the molecular identity is largely unknown.

RPA-1 was used as an antibody to determine the utilization of the lectin approach because it is currently the only biomarker for collecting duct damage and it has been shown to increase in urine following treatment of rats with the nephrotoxins NPAA, BEA and indomethacin (Falkenberg et al. 1996, Price et al. 2010). While our data with the RPA-1/DB assay showed comparable increases in RPA-1 expression in urine from BEA-treated rats, there was an increase in the dynamic range of the immunoassay, increasing the sensitivity and generating a higher signal level at high concentrations of kidney extract. As RPA-1 detects a repeated epitope (Hildebrand et al. 1999), there is the possibility that any molecules containing a single epitope are not detected with the current commercial assay. Therefore, it may be that the RPA-1/DB assay is detecting monomeric and oligomeric forms of RPA-1,

whereas the RPA-1/RPA-1 assay detects only oligomeric forms of RPA-1. Alternatively, the epitope recognized by DB may be less sterically hindered, allowing for the increased detection of RPA-1.

We were unable to develop an assay for detecting collecting duct damage using two different AQP2 antibodies in a sandwich immunoassay. This was largely due to the limited choice of suitable antibodies for detecting different regions of the protein. However, using an anti-AQP2 antibody as a capture antibody and DB for detection, we detected a signal in urine from healthy individuals. This is consistent with data suggesting approximately 3% of AQP2 in collecting duct cells is excreted in urine (Ishikawa 2000). Currently there are no sandwich immunoassays available for AQP2. In a recent AQP2 immunoassay, urine samples were directly immobilized on plates and captured AQP2 was detected by anti-AQP2 antibody (Umenishi et al. 2002). However, differences in urine content may cause variation in the immobilization efficiency of AQP2. There are two alternative methods for detection of urinary AQP2 (Umenishi 2002). However, these either require the use of radioisotopes (Kanno et al. 1995) or immunoblotting (Baumgarten et al. 2000, Martin et al. 1999) and are therefore less practical for multiple urine samples.

Due to their pentameric structure, IgM antibodies are not normally used in immunoassays. Nevertheless, the application of DB as a detection agent has enabled the use of 3C7 as a capture antibody. Unlike the RPA-1 antibody, it was not possible to use 3C7 as both a capture and detection antibody. This would suggest that the antigen does not express multiple 3C7 epitopes. Whereas it was possible to set up an immunoassay for 3C7, the application of DB could not detect antigens captured by 2B11 and 13C2. It may be that both antibodies recognize epitopes that prevents DB from binding to their captured antigens.

The 3C7/DB assays also detected a signal in healthy individual samples. Data from this study suggest that 3C7

Table 7. Control experiment for the 3C7/*Dolichos biflorus* (DB) Meso Scale Discovery assay: comparison of the assay after inhibiting either 3C7 or DB with *N*-acetyl-D-galactosamine.

	% Recovery							
	3C7 + *N*-acetyl galactosamine				DB + *N*-acetyl galactosamine			
Extract (U ml^{-1})	10 mM	1 mM	100 μM	10 μM	10 mM	1 mM	100 μM	10 μM
10000	90	102	84	95	2	28	89	115
625	98	108	101	117	2	40	82	95
40	115	108	113	178	5	29	84	85
2.5	1600	560	80	480	0	0	63	112

Table 8. Human urinary analysis of aquaporin 2 (AQP2) and 3C7 antigens using the AQP2/*Dolichos biflorus* (DB) and 3C7/DB Meso Scale Discovery assays.

				3C7			AQP2		
Age (years)	Sex	BMI	Creatinine (g ml^{-1})	U ml−1	CV %	U g−1 Cre	U ml−1	CV %	U g−1 Cre
20	M	26.8	38	228	17	5.95	65	1	1.7
31	F	24.6	21	53	4	2.47	65	8	3.03
38	M	25.5	44	30	12	0.68	246	3	5.6
39	F	20.8	41	22	11	0.54	6	2	0.15
40	M	31.6	12	81	3	6.88	58	6	4.93
43	F	24.6	29	77	15	2.61	28	6	0.95
46	F	22.1	7	244	2	36.39	485	12	72.34
46	M	28.1	18	92	19	5.06	22	12	1.21
50	F	30.4	23	55	2	2.35	24	12	1.02
50	M	25	43	213	15	4.98	30	5	0.7
52	F	22.8	9	217	10	23.88	63	2	6.93
50	M	21.2	22	46	1	2.08	6	2	0.27
55	F	24.7	13	67	2	4.97	3	3	0.22
54	M	25.9	21	34	5	1.65	6	6	0.29
59	F	32.7	31	59	3	1.89	8	6	0.26
60	M	30.9	13	31	4	2.41	8	4	0.62
60	F	30.4	6	119	3	18.97	19	5	3.03
60	M	26.6	12	281	6	24.22	21	6	1.81
74	F	30.3	16	144	8	8.98	2	1	0.12
75	M	25	8	92	19	11.87	22	12	2.84

BMI, body mass index; Cre, creatine.

may be a better urinary biomarker for collecting duct damage in man compared with AQP2. Detection of urinary AQP2 displayed higher variation between the different healthy individuals compared with the 3C7 antigen. This may be due to the fact AQP2 urinary expression is affected by water as well as mineral (salt) consumption (Buemi et al. 2007, Kanno et al. 1995, Saito et al. 1997). Both AQP2 and 3C7 gave lower signal intensities in human kidney compared with rat, but this is consistent with previous observations made for other renal proteins (Sourial et al. 2009). AQP2 and 3C7 antibodies have the advantage over RPA-1 in that they recognize both rat and human antigens; therefore, the same assay could be used both preclinically and clinically.

The use of DB on the MSD platform allows the identification of putative novel collecting duct damage biomarkers, which were not previously quantifiable. These could provide the basis for the first immunoassay for detecting RPN in man. The MSD platform also allows multiplex analysis of biomarkers. Therefore, these markers could form part of a wider nephrotoxicity assay plate. Further work validating these assays and qualifying the potential biomarkers in different patient populations is now essential.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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