Acta Biochim Biophys Sin 2010, 42: 847–853 | © The Author 2010. Published by ABBS Editorial Office in association with Oxford University Press on behalf of the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. DOI: 10.1093/abbs/gmq099.
Advance Access Publication 9 November 2010

Original Article

Development of retinol-binding protein 4 immunocolloidal gold fast test strip using high-sensitivity monoclonal antibodies generated by DNA immunization

Chao Bian[1*†], Fang Zhang[2†], Feng Wang[3†], Zhiyang Ling[4], Min Luo[1], Hongqiang Wu[1], Yizhuo Sun[1], Junhui Li[3], Bingnan Li[5], Jingyan Zhu[5], Linna Tang[5], Yanyan Zhou[5], Qunfang Shi[5], Yongyong Ji[1], Lin Tian[1], Guomei Lin[1], Yu Fan[2], Niansong Wang[3], and Bing Sun[1,4*]

[1]Key Laboratory of Molecular Cell Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China
[2]Shanghai First People's Hospital, Shanghai Jiaotong University, Shanghai 200080, China
[3]Shanghai Sixth People's Hospital, Shanghai Jiaotong University, Shanghai 200233, China
[4]Key Laboratory of Molecular Virology and Immunology, Institut Pasteur of Shanghai, Chinese Academy of Sciences, Shanghai 200025, China
[5]Shanghai Immune Biotech. Co. Ltd., Shanghai 200233, China
[†]These authors contributed equally to this work.
*Correspondence address. Tel: +86-21-54921312; Fax: +86-21-54921013; E-mail: bsun@sibs.ac.cn (B.S.)/cbian@sibs.ac.cn (C.B.)

DNA immunization is an efficient method for high-affinity monoclonal antibody generation. Here, we describe the generation of several high-quality monoclonal antibodies (mAbs) against retinol-binding protein 4 (RBP4), an important marker for kidney abnormality and dysfunction, with a combination method of DNA priming and protein boost. The mAbs generated could bind to RBP4 with high sensitivity and using these mAbs, an immunocolloidal gold fast test strip was constructed. The strip can give a result in <5 min and is very sensitive with a detection limit of about 1 ng/ml. A small-scale clinical test revealed that the result of this strip was well in accordance with that of an enzyme-labeled immunosorbent assay kit currently available on the market. Consequently, it could be useful for more convenient and faster RBP4 determination in the clinic.

Keywords DNA immunization; monoclonal antibody; retinol-binding protein 4; immunocolloidal gold; renal damage

Received: June 28, 2010 Accepted: September 14, 2010

Introduction

DNA immunization can effectively trigger an immune response *in vivo* [1], and is a promising vaccine approach for many diseases [2,3]. Preparing DNA for immunization is very convenient. More importantly, because heterogeneous DNA is processed in host muscular cells, the target antigen's natural conformation is generally preserved, though some post-transductional modifications might be different from the native antigen. Consequently, DNA immunization is an efficient way to generate high-affinity monoclonal antibodies (mAbs), especially when the protein antigen is difficult to prepare or when a traditional immunization procedure cannot be applied.

Moreover, besides DNA immunization, a protein (with its natural conformation preserved) boost is also recommended for hybridoma generation [4–6]. A final protein boost before cell fusion may facilitate the generation of more antibody-producing B cells in the spleen and result in a higher hybridoma recovery rate [5,6].

Retinol-binding protein 4 (RBP4) is a low-molecular-weight protein (about 21 kDa) predominantly synthesized in the liver [7]. In plasma, RBP4 binds to all-*trans*-retinol at 1:1 ratio and transports it from the liver to various target tissues [7,8]. Because of the small molecular size, RBP4 is easily filtered through the renal glomerular membrane. Plasma RBP4 thus binds to transthyretin (55 kDa, involved in thyroid hormones transportation) at 1:1 ratio to form a large protein complex to prevent renal clearance [8,9].

During renal failure and kidney abnormality, especially proximal tubule dysfunction, RBP4 level increases dramatically in urine and is widely accepted as a marker for renal damage and dysfunction [10–12]. Researches have revealed that RBP4 is very sensitive for early detection of proximal tubular impairment [10,11]. Compared with β_2-microglobulin, RBP4 is more stable in acid urine [10]. Consequently, it is a more practical and reliable marker for proximal tubular damage. Besides, RBP4 is also useful for detecting renal dysfunction in kidney transplant patients [13]. Fast, easily performed and highly sensitive antibody-based immunoassays for the detection of RBP4 are therefore needed for clinical use.

Downloaded from http://abbs.oxfordjournals.org/ by guest on May 30, 2016

The method of DNA immunization followed by protein boost allowed us to obtain a number of mAbs with high-sensitivity binding to RBP4 at a variety of different epitopes. Two mAbs among them were further selected and paired in a sandwich enzyme-labeled immunosorbent assay (ELISA) for detecting RBP4 quantitatively. The detection limit reached as far as 1 ng/ml. Based upon these results, an immunocolloidal gold test strip was also constructed. This strip could give a result in <5 min and is very sensitive with a RBP4 detection limit of about 1 ng/ml. In clinical sample testing, this strip showed results comparable with those of ELISA kits commercially available, indicating it has favorable potential clinical application.

Materials and Methods

RBP4 plasmid

The *RBP4* gene was obtained using RT–PCR from human liver tissues with the sense primer (5′-CGGGGTACC ATGATGAAGTGGGTGTGGGCGCT-3′) and the reverse primer (5′-CCGCTCGAGTCGCTACAAAAGGTTTCTTT CTGATC-3′). A plasmid for mouse immunization was constructed by inserting the *RBP4* gene into a pBudCE4.1 vector (Invitrogen, Carlsbad, USA).

Natural RBP4 purification

RBP4 protein was purified from urine of patients with renal damage using the AKTA explorer purification system (GE, Fairfield, USA). Briefly, urine was collected during a 24-h period, filtered through a 0.45 μm filtration membrane (Millipore, Billerica, USA) and diluted with an equal volume of double-distilled water. An anion exchange Q-FF column (20 ml volume), a hydrophobic Butyle-FF column (1 ml volume), and a Supperdex-75 column (24 ml volume) were used in tandem. The sample was loaded onto a Q-FF column (equilibrated with 0.02 M phosphate buffer, pH 8.0, buffer A) at 5 ml/min. After loading, the column was washed with buffer A until complete removal of all the non-binding fractions. Afterwards, an ion gradient (0–0.08 mM NaCl) elution was performed to elute RBP4 from the Q-FF column. Fractions were collected and assayed for RBP4 content using sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with Coomassie blue staining. Fractions that were rich in RBP4 were mixed and further loaded onto a Butyle-FF column (equilibrated with buffer A containing 1 M ammonium sulfate, buffer B) at 1 ml/min and washed with buffer B until non-binding fractions were completely removed. A linear ion strength gradient elution was carried out at 50–100% (*v*/*v*) of buffer A and fractions were collected and assayed for RBP4 content by SDS–PAGE. Fractions rich in RBP4 were finally loaded onto a Supperdex-75 column and RBP4 was separated by its molecular weight. After purification, RBP4 content could reach as high as 90%. Purified protein was then assayed for concentration and stored at −20°C.

Transfection of RBP4 plasmid

HEK293T cells purchased from ATCC (ATCC No. CRL-11268^{TM}) were cultured in Dulbecco's Modified Eagle Medium (Invitrogen) containing 10% fetal bovine serum (Invitrogen). Cells (6×10^5/well) were plated at ~60% confluence in 6-well plates. Twenty-four hours later, 3 μg/well of RBP4 plasmid or the vector was transfected into the HEK293T cells, respectively, using Lipofectamine (Invitrogen) according to the manufacturer's instruction. Forty-eight hours after transfection, the culture medium was removed and cells were washed with phosphate buffered saline (PBS; pH 7.4) once. After that, cells were lysed with 1× SDS–PAGE loading buffer (0.2 ml/well). Samples were then collected, heated at 100°C for 5 min and stored at −20°C for further analysis.

Animal immunization

Six-week-old female BALB/c mice ($n = 6$) of SPF class were purchased from Shanghai Laboratory Animal Center, Chinese Academy of Sciences. Animals were handled according to the Guidelines of Chinese Academy of Sciences for Animal Care and Use. The immunization method was previously described [14,15]. Briefly, mice were immunized three times with a 3-week interval between immunizations. For each immunization, 100 μg plasmid (in 100 μl sterile PBS, pH 7.4) per mouse was injected from the quadriceps muscles of both legs using a 29-gauge needle (BD, Franklin Lakes, USA) with each leg receiving 50 μg plasmid DNA. Immediately after injection, a pair of electrode needles, separated by 5 mm, was inserted into the muscle covering the DNA injection sites and electric pulses were delivered using an electric pulse generator ECM830 (BTX, Holliston, USA). Three square pulses of 100 V/50 ms each followed by three pulses of the opposite polarity were delivered into each injection site at the rate of one pulse per second. The cell fusion experiment was carried out 3 weeks (21 days) after the last DNA immunization. Three days before cell fusion, the mouse was given another intraperitoneal booster injection of 100 μg natural RBP4 protein. Mouse blood was collected 12 days after the third DNA immunization from the eye artery and sera were obtained by centrifugation (10,000 *g*, 10 min) to remove the blood clot after incubation at 37°C for 1–2 h and then at 4°C overnight.

Monoclonal antibody generation

Monoclonal antibody generation was conducted as previously described [16]. Briefly, at the day for cell fusion, one of the immunized mice, whose serum showed the highest antibody titer against RBP4 protein was sacrificed.

Downloaded from http://abbs.oxfordjournals.org/ by guest on May 30, 2016

The spleen cells were immediately collected and fused with murine myeloma cells SP2/0 using 50% (*w/v*) polyethylene glycol in 50 ml centrifugal tube (Corning, New York, USA). Cells were distributed into 96-well microplates (10^7 spleen cells before cell fusion per plate) and HAT (hypoxanthine, aminopterin, and thymidine; Sigma-Aldrich, St. Louis, USA) was added for hybridoma selection. About 10 days after cell fusion, the supernatants from 96-well plates were collected and assayed for antibody secretion by ELISA. A limited dilution method was adopted for positive-well hybridoma cloning.

HRP labeling of antibody

Anti-RBP4 mAbs were purified by a Protein G Sepharose 4 Fast Flow column (GE) according to the manufacturer's instruction. Purified antibodies were labeled with horseradish peroxidase (HRP; Sigma-Aldrich) by 1.25% glutaraldehyde. The reaction was stopped by incubation with 0.2 M lysine for 2 h and then dialyzed in PBS at 4°C overnight. After that, the HRP-conjugated antibody was precipitated by 33% saturated ammonium sulfate and then dialyzed again in PBS. The HRP-conjugated antibody was stored at −20°C in 50% glycerol.

Enzyme-labeled immunosorbent assay

Ninety-six-well microplates were coated with RBP4 protein or mAb in 0.1 M carbonate buffer (pH 9.6) at a concentration of 10 μg/ml and incubated at 4°C overnight. PBS containing 10% bovine serum albumin and 0.1% Tween-20 was used as blocking and dilution buffer. After blocking at 37°C for 2 h, the plates were washed, serial-diluted antibody or RBP4 (clinical sample or protein) was added and the plates were incubated at 37°C for 2 h. Subsequently, HRP-conjugated goat anti-mouse IgG antibody was added and the plates were incubated at 37°C for 1 h. Tetramethylbenzidine (TMB; Sigma-Aldrich) was used as the substrate and optical density was measured at 450 nm (OD_{450nm}) using a micro-plate autoreader (Thermo, Waltham, USA).

Western blot analysis

Samples were separated by SDS–PAGE and then transferred onto a polyvinylidene fluoride membrane. After blocking in 3% bovine serum albumin for 2 h, the membrane was incubated with an anti-His tag monoclonal antibody (Tiangen, Beijing, China) at room temperature for 2 h. The membrane was then incubated with HRP-conjugated goat anti-mouse IgG antibody at room temperature for another 1 h. The blots were developed using ECL detection reagents (Pierce, Rockford, USA).

Immunocolloidal gold technique

Briefly, the generation of RBP4 immunocolloidal gold fast test strip includes three steps: preparation of colloidal gold solution, preparation of colloidal gold-conjugated antibody, and production of a test strip. First, a colloidal gold solution was generated by constant mixing at boiling temperature of 0.01% (*w/v*) chloroauric acid hydrate (gold chloride) and 0.01% (*w/v*) sodium citrate for ~10 min. The pH of the solution was then adjusted to 8.5 with K_2CO_3. Second, anti-RBP4 antibody was dialyzed with 20 mM phosphate buffer (pH 7.4) and added to colloidal gold solution (pH 8.5) at 30 μg/ml. After 30 min of incubation at room temperature, the antibody was labeled with colloidal gold. Another 15 min incubation was carried out after bovine serum albumin was added into the solution at a final concentration of 2% (*w/v*). Centrifugations (500 *g*, 10 min) were then performed to remove large debris and enrich the labeled probe (6000 *g*, 20 min). The probe was re-suspended in blocking solution [1% (*w/v*) bovine serum albumin in PBS (pH 7.4)]. Test strips were produced using instruments HGS101 and HGS 201 (JN-Bio, Shanghai, China) according to the manufacturer's protocol.

Clinical samples

Urine was collected from patients with renal damage or after kidney transplantation. A total of 40 urine samples were collected and assayed for RBP4 concentration. Urine samples were aliquoted and stored at −20°C.

Results

Construction and verification of plasmid encoding RBP4 for immunization

The *RBP4* gene was inserted into a pBudCE4.1 vector (with a His tag gene following the multiple cloning site) and sequenced. Expression of the RBP4 plasmid was verified by *in vitro* transfection and western blot analysis. As shown in **Fig. 1**, RBP4 protein was detected by an anti-His tag monoclonal antibody in cells that were transfected with RBP4 plasmid but not the vector. This result demonstrates that the RBP4 plasmid is well constructed and can be used for animal immunization.

DNA immunization induces a strong antibody response against RBP4

Mouse sera were tested using ELISA for antibody titer against RBP4. As shown in **Fig. 2**, after DNA immunization the mice developed considerable antibody responses targeting RBP4. The serum titer reached 1:50,000 after the third immunization. The results demonstrate that DNA immunization is an effective method for triggering antibody response *in vivo*.

Downloaded from http://abbs.oxfordjournals.org/ by guest on May 30, 2016

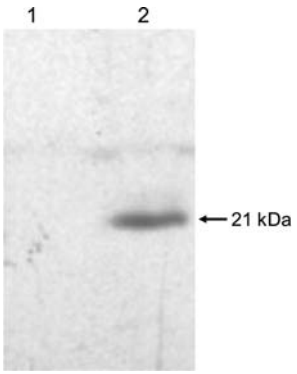

Figure 1 Western blot analysis of the expression of RBP4 plasmid in HEK293T cells Anti-His tag monoclonal antibody was used for detection of the RBP4 protein. The molecular weight of RBP4 is about 21 kDa. Lane 1, sample of cells transfected with vector (negative control), Lane 2, sample of cells transfected with plasmid encoding RBP4.

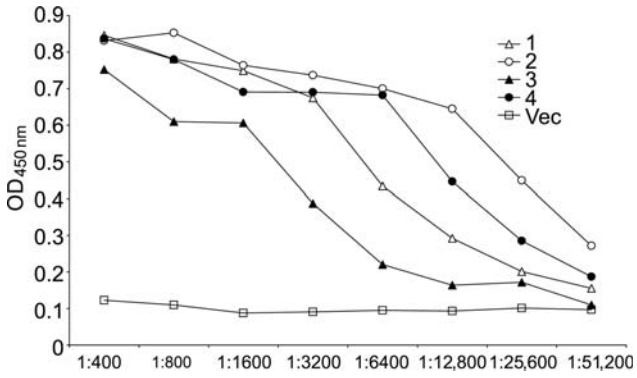

Figure 2 ELISA detection of antibody response in mice immunized with RBP4 DNA Urine-purified RBP4 (10 μg/ml) was coated on 96-well plate. After blocking by 10% bovine serum albumin (in PBS), a 2-fold dilution of mouse serum starting from 1:400 was set and added to the plates for binding activity determination. A HRP-conjugated goat anti-mouse IgG antibody was used for antibody detection and the OD_{450nm} was measured for each well. 1–4, four mice immunized with RBP4 DNA; vec, mouse immunized with the DNA vector as negative control.

mAbs raised against RBP4 have a high sensitivity

On the third day before cell fusion, mice were boosted with RBP4 protein purified from the urine of patients with kidney disease. **Figure 3** shows RBP4 protein purified from the urine of patients using three chromatography columns (Q-FF, Butyle-FF and Supperdex-75) in tandem were at about 21 kDa and 95% purity. The mice were sacrificed and spleen cells were collected for hybridoma generation. mAbs were generated by fusion of the mouse spleen cells and myeloma cell line SP2/0. After three rounds of cloning using a limiting dilution method and testing the antibody reactivity by ELISA, three hybridoma clones (S-17-4, S-113-7, and SA-64-11) were selected. All the three clones displayed high-sensitivity antibody-binding activity with naturally purified RBP4 protein. The antibody

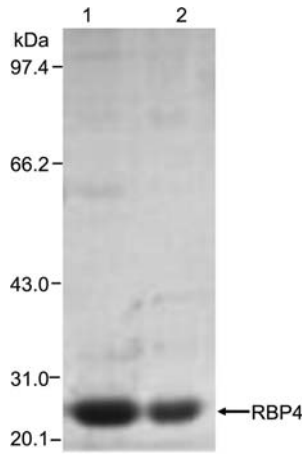

Figure 3 SDS–PAGE of the urine-purified RBP4 stained with Coomassie blue Lanes 1 and 2: RBP4 protein finally obtained after purification by the three chromatography columns (Q-FF, Butyle-FF and Supperdex-75) used in tandem. A high purity (>90%) could be achieved using this method.

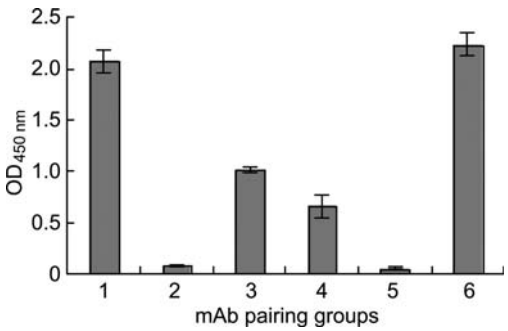

Figure 4 Pairing of the three mAbs to detect RBP4 As coating mAbs, S-17-4 was used in Group 1 and 2, SA-64-11 in Group 3 and 4, S-113-7 in Group 5 and 6; as detecting mAbs, S-17-4-HRP was used in Group 3 and 5, SA-64-11-HRP in Group 1 and 6, S-113-7-HRP in Group 2 and 4. mAbs were coated on 96-well plate at 10 μg/ml. The concentration of RBP4 added was 500 ng/ml. For detecting antibody, a 1:10,000 dilution was applied in each group. Group 1, S-17-4/SA-64-11-HRP; Group 2, S-17-4/S-113-7-HRP; Group 3, SA-64-11/S-17-4-HRP; Group 4, SA-64-11/S-113-7-HRP; Group 5, S-113-7/S-17-4-HRP; Group 6, S-113-7/SA-64-11-HRP.

titer in the ascites reached 1:1,000,000. The isotypes of the three mAbs were also determined to be either IgG1/κ or IgG2a/κ.

Development of paired mAbs to detect RBP4 using ELISA

The three mAbs were applied in ELISA to detect RBP4 after purification using Protein G Sepharose 4 Fast Flow columns and labeling with horseradish peroxidase. As shown in **Fig. 4**, two antibody pairs were identified for use in a sandwich ELISA for RBP4 detection. One pair is S-17-4 and SA-64-11, the other is S-113-7 and SA-64-11. S-17-4 and S-113-7 was unable to function as an antibody

Downloaded from http://abbs.oxfordjournals.org/ by guest on May 30, 2016

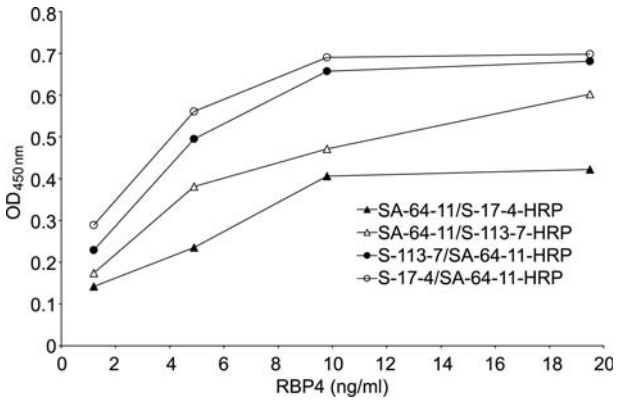

Figure 5 Detection of RBP4 with different coating and HRP-labeling mAb combinations Coating with S-113-7 or S-17-4 and using SA-64-11-HRP as the detection antibody could yield higher signal than the combinations of using SA-64-11 as the coating antibody. Moreover, coating with S-113-7 and S-17-4 were almost equivalent for RBP4 detection. A concentration of 10 μg/ml was used for mAb coating. HRP-labeling antibody was diluted 10,000-fold for use. RBP4 was determined in a concentration range from 1 to 20 ng/ml. S-17-4, SA-64-11 and S-113-7 were as the coating antibody. S-17-4-HRP, SA-64-11-HRP, and S-113-7-HRP were as the detection antibody after labeling with HRP.

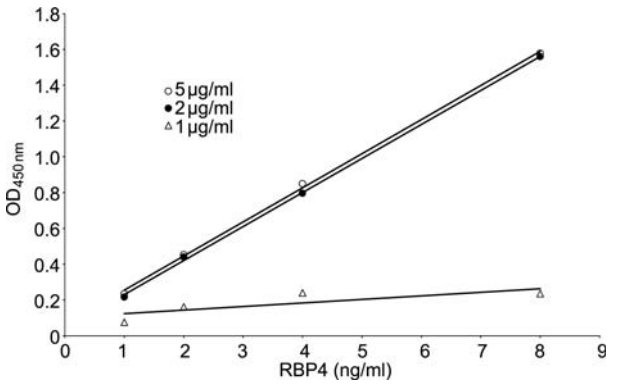

Figure 6 Modification of the sandwich ELISA using the S-113-7/SA-64-11-HRP pair S-113-7 was coated with concentrations of 1, 2, and 5 μg/ml, respectively; SA-64-11-HRP was diluted 10,000-fold as the detection antibody. RBP4 was diluted serially to 8, 4, 2, and 1 ng/ml for detection. Both of the 2 and 5 μg/ml (but not the 1 μg/ml) of S-113-7 showed an excellent linear detection range of RBP4 within 1–8 ng/ml ($R^2 > 0.99$).

pair, indicating that they may recognize the same epitope. In addition, coating S-113-7 or S-17-4 and using SA-64-11-HRP for detection yielded better signal than did other Ab combinations with SA-64-11 as the coating antibody (**Figs. 4 and 5**). The pairs of S-113-7/SA-64-11-HRP and S-17-4/SA-64-11-HRP were almost equivalent (**Figs. 4 and 5**). Consequently, we selected the pair of S-113-7/SA-64-11-HRP for further characterization. A series of experiments were carried out to optimize the working conditions of the sandwich ELISA. An S-113-7 coating concentration of 5 μg/ml or 2 μg/ml with an SA-64-11-HRP dilution rate of 1:10,000 (**Fig. 6**) was chosen. An antibody coating of S-113-7 at 1 μg/ml showed a weak OD_{450nm} signal and an impaired linear pattern (**Fig. 6**). The linear range of the sandwich ELISA was under 10 ng/ml (**Fig. 6**). Our results suggest that the selected antibody pair was optimal for the testing of RBP4 protein.

Development of an immunocolloidal gold fast test strip

On the basis of the primary data, we constructed an immunocolloidal gold fast test strip for RBP4 detection. S-113-7 was used as capture Ab in the test (T) line and SA-64-11 was used after colloidal gold labeling for reaction with RBP4 in test samples. The control (C) line was set as goat anti-mouse IgG antibody. After various parameters were optimized, samples with RBP4 concentration more than 1 ng/ml produced a generally visible red color on the T line and the more RBP4 present, the deeper the color produced (data not shown).

We collected 40 urine samples from patients with renal failure or after kidney transplantation and evaluated the clinical usage potential of our strips. The samples were first assayed for RBP4 concentration with commercial uRBP4 ELISA kit (Debo, Shanghai, China). As shown in **Table 1**, the concentrations of RBP4 in these samples ranged from 0 to 80936 ng/ml. Test samples were prepared by diluting the urine at 1:100 in 0.01 M PBS containing 2% Tween-20. The RBP4 concentration of 100 ng/ml was set as an example of distinguishing level for positive and negative samples. Our strip's detection limit is 1 ng/ml, so the 1:100 dilution of the urine could meet the criterion for distinguishing samples with RBP4 less or more than 100 ng/ml. The strips were soaked in diluted samples and after ~3 min of the reaction, the T line of the strips showed red color of different depths or remained blank (**Fig. 7**). All of the C line of the strips displayed clear red color indicating that the strips were in good condition and effective. Further analysis revealed that tests of urine samples (before dilution) with RBP4 concentration <100 ng/ml all displayed blank (no color visible) [**Fig. 7(C)**] on the T line and samples with RBP4 concentration more than 1000 ng/ml (including No. 15 sample with RBP4 concentration of 944 ng/ml) displayed a heavy red color on the T line [**Fig. 7(A)**]. As for samples with RBP4 concentration between 100–1000 ng/ml, generally, a moderate or light red color was shown on the strips' T line [**Fig. 7(B)**]. This result is comparable with that of the ELISA assay, except that No. 19 with a concentration of 173.8 ng/ml showed blank when tested on the strip (**Fig. 7** and **Table 1**). These data demonstrate that our immunocolloidal gold test strip is able to detect RBP4 in patient urine effectively and conveniently.

Downloaded from http://abbs.oxfordjournals.org/ by guest on May 30, 2016

Table 1 Concentration of urinary RBP4 from 40 patients with renal damage and comparison of the results of ELISA and immunocolloidal gold test strip for RBP4 determination

No.[a]	RBP4 (ng/ml)	ELISA[b]	IG[c]	No.	RBP4 (ng/ml)	ELISA	IG	No.	RBP4 (ng/ml)	ELISA	IG	No.	RBP4 (ng/ml)	ELISA	IG
1	1400.0	H	++	11	184.1	M	+	21	80936.6	H	++	31	6.8	L	−
2	218.8	M	+	12	125.3	M	+	22	12.8	L	−	32	7.0	L	−
3	29812.7	H	++	13	32.7	L	−	23	52.5	L	−	33	181.6	M	+
4	8969.6	H	++	14	26.9	L	−	24	39.4	L	−	34	43635.4	H	++
5	43179.7	H	++	15	944.3	H	++	25	5298.7	H	++	35	1960.5	H	++
6	32.3	L	−	16	49154.4	H	++	26	813.8	M	+	36	0	L	−
7	10.9	L	−	17	616.1	M	+	27	13.2	L	−	37	18237.8	H	++
8	13.8	L	−	18	54.2	L	−	28	26.6	L	−	38	305.5	M	+
9	6273.4	H	++	19	173.8	M	−	29	18800.0	H	++	39	189.4	M	+
10	52.9	L	−	20	1032.9	H	++	30	29.7	L	−	40	222.6	M	+

[a]Clinical number assigned to patients, [b]results of ELISA, [c]results of immunocolloidal gold test strip. H, RBP4 more than 1000 ng/ml (including No. 15 of 944.3 ng/ml); M, 100 ng/ml ≤ RBP4 ≤ 1000 ng/ml; L, RBP4 <100 ng/ml; ++, strong positive; +, weak positive; −, negative.

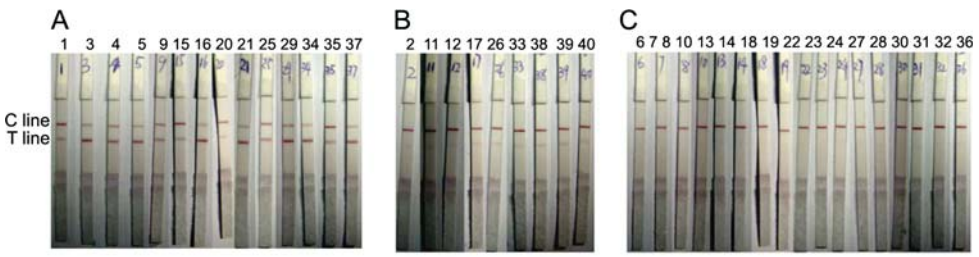

Figure 7 Clinical test of the RBP4 immunocolloidal gold fast test strip Forty urine samples of different RBP4 levels were collected. Test samples were prepared by diluting the urine at 1:100 in 0.01 M PBS containing 2% Tween-20. The strips were then soaked in the test samples. After ~3 min, when the C line of the strips turned red, the strips were taken out and their T line showed various depths of red color or blank. A 1:100 dilution of urine sample set the distinguishing concentration of urinary RBP4 to 100 ng/ml. (A) Deep red (strong positive). (B) Mild or moderate red (weak positive). (C) Blank (negative). The control line (C line) all demonstrated 'positive' indicating assurance of the strips' quality.

Discussion

As reflected by our primary experiments, it is very hard to obtain high-affinity mAbs that bind to natural RBP4 on different epitopes. In the beginning, we generated mAbs using *Escherichia coli* expressing recombinant RBP4 protein. We obtained two mAbs with the ability to recognize natural RBP4 but both bound to the same epitope. We then used different recombined overlapping fragments as antigens for immunization and screening, but still could not obtain another mAb that bound natural RBP4 on a different epitope. Next, we used natural RBP4 protein directly to immunize mice and generate mAbs. After being immunized with natural RBP4 following a conventional procedure (with Freund's complete and incomplete adjuvants), mice showed severe fatigue and great loss of weight. Further, the antibody responses were poorly induced.

DNA combined with protein immunization is an efficient method for high-affinity monoclonal antibody generation. Different immunization procedures have been applied such as DNA priming followed by protein boost [4–6] and DNA/protein co-immunization [17]. The protein antigen used for immunization is also in various forms such as antigen gene-transfected cells [4], recombinant proteins [17], and live viruses [6]. The critical point is to keep the protein's natural structure. Interestingly, unlike immunization with recombinant protein that expressed in *E. coli* only yielded mAbs binding to the same site, this DNA priming and naturally purified protein boost immunization method allowed us to obtain a number of high-affinity mAbs that could recognize different epitops on RBP4. This may be because protein boost promotes more antibody-producing cells accumulating in the spleen, and thus more antigen specific hybridomas are generated [5,6]. It should be noted that the immunization with the naturally purified RBP4 protein caused mouse death. This may be because RBP4 is very important for vitamin A transportation and too much RBP4 destroys the natural vitamin metabolic process *in vivo* [7–9].

The concentration of urinary RBP4 for distinguishing various situations of renal diseases is different. For example, Bernard *et al*. [11] detected RBP4 levels in 100

Downloaded from http://abbs.oxfordjournals.org/ by guest on May 30, 2016

healthy subjects and set the upper limit of normal to 308 ng/ml. In patients with different glomerulopathies, Kirsztajn *et al*. [18] used 1000 ng/ml to identify ones who progressed with loss of renal function. RBP4 concentration of 100 ng/ml is just an example of distinguishing concentration set for the strip. We can easily change the distinguishing RBP4 concentration by adjusting the dilution rate of the urine samples. For example, a 1:300 dilution of urine can lift the distinguishing RBP4 concentration to 300 ng/ml and a 1:1000 dilution can make the distinguishing concentration to 1000 ng/ml. This allows people to use this strip with more flexibility in case different RBP4 distinguishing concentrations are needed.

We noticed that patients tested in our work showed a broad range of urine RBP4 concentration. Because these patients were in different phases of renal disease and received the treatments, the RBP4 in their urine might have decreased to a very low level as the treatments took effect.

Our work reported here demonstrates that DNA immunization with a protein boost is very useful for high-quality mAb generation, especially under circumstances in which the mAbs are intended for diagnostic and/or therapeutic use or when there exist various antigen limitations. Moreover, we report here for the first time construction of an RBP4 immunocolloidal gold fast test strip. This strip, when compared with RBP4 ELISA/EIA kits currently available on the market, provides faster results and is more convenient to use. It could potentially be applied to clinical detection of RBP4 and for renal damage diagnosis especially suitable for large-scale physical examination and surveillance of patients with chronic renal diseases or after renal transplantation. Because the result of the strip test is visually judged and no numerical value is obtained, samples with an RBP4 concentration near the distinguishing concentration may give ambiguous results. Further confirmation thus may be required.

Acknowledgements

We thank Dr Sheri Skinner (NIH, USA) for reviewing the manuscript and providing language support.

Funding

This work was supported by grants from National 863 Project (2006AA02A247), National 973 Project (2007CB512404), National Science and Technology Major Project (2008ZX10002-014, 2008ZX10004-002, and 2009ZX10004-105), National Natural Science Foundation of China (30950002, 30623003, 30721065, 30801011, and 088014199), Science and Technology Commission of Shanghai Municipality (06DZ22032), Chinese Academy of Sciences (KSCX2-YW-R-161), and E-institutes of Shanghai Universities, Immunology Division.

References

1 Tang CK and Pietersz GA. Intracellular detection and immune signaling pathways of DNA vaccines. Expert Rev Vaccines 2009, 9: 1161–1170.
2 Garren H. DNA vaccines for autoimmune diseases. Expert Rev Vaccines 2009, 9: 1195–1203.
3 Kim JH and Jacob J. DNA vaccines against influenza viruses. Curr Top Microbiol Immunol 2009, 333: 197–210.
4 Tearina Chu TH, Halverson GR, Yazdanbakhsh K, Oyen R and Reid ME. A DNA-based immunization protocol to produce monoclonal antibodies to blood group antigens. Br J Haematol 2001, 113: 32–36.
5 Chen Z, Guo X, Ge X, Chen Y and Yang H. Preparation of monoclonal antibodies against pseudorabies virus glycoprotein gC by adenovirus immunization alone or as a boost following DNA priming. Hybridoma (Larchmt) 2008, 27: 36–42.
6 Puttikhunt C, Kasinrerk W, Srisa-ad S, Duangchinda T, Silakate W, Moonsom S and Sittisombut N, *et al*. Production of anti-dengue NS1 monoclonal antibodies by DNA immunization. J Virol Methods 2003, 109: 55–61.
7 Goodman DS. Plasma retinol-binding protein. Ann N Y Acad Sci 1980, 348: 378–390.
8 Goodman DS. Retinoid-binding proteins in plasma and in cells. Ann N Y Acad Sci 1981, 359: 69–78.
9 Zanotti G and Berni R. Plasma retinol-binding protein: structure and interactions with retinol, retinoids, and transthyretin. Vitam Horm 2004, 69: 271–295.
10 Bernard AM, Moreau D and Lauwerys R. Comparison of retinol-binding protein and beta 2-microglobulin determination in urine for the early detection of tubular proteinuria. Clin Chim Acta 1982, 126: 1–7.
11 Bernard AM, Vyskocil AA, Mahieu P and Lauwerys RR. Assessment of urinary retinol-binding protein as an index of proximal tubular injury. Clin Chem 1987, 33: 775–779.
12 Flynn FV. Assessment of renal function: selected developments. Clin Biochem 1990, 23: 49–54.
13 Câmara NO, Silva MS, Nishida S, Pereira AB and Pacheco-Silva A. Proximal tubular dysfunction is associated with chronic allograft nephropathy and decreased long-term renal-graft survival. Transplantation 2004, 78: 269–275.
14 Miyazaki J and Aihara H. Gene transfer into muscle by electroporation in vivo. Methods Mol Med 2002, 69: 49–62.
15 Chen J, Fang F, Li X, Chang H and Chen Z. Protection against influenza virus infection in BALB/c mice immunized with a single dose of neuraminidase-expressing DNAs by electroporation. Vaccine 2005, 23: 4322–4328.
16 Bian C, Zhang X, Cai X, Zhang L, Chen Z, Zha Y and Xu Y, *et al*. Conserved amino acids W423 and N424 in receptor-binding domain of SARS-CoV are potential targets for therapeutic monoclonal antibody. Virology 2009, 383: 39–46.
17 Xue T, Xue XP, Huang QS, Wei L, Sun K and Xue T. Monoclonal antibodies against human aspartyl (asparaginyl) beta-hydroxylase developed by DNA immunization. Hybridoma (Larchmt) 2009, 28: 251–257.
18 Kirsztajn GM, Nishida SK, Silva MS, Ajzen H, Moura LA and Pereira AB. Urinary retinol-binding protein as a prognostic marker in glomerulopathies. Nephron 2002, 90: 424–431.

Downloaded from http://abbs.oxfordjournals.org/ by guest on May 30, 2016