International Journal of Infectious Diseases 17 (2013) e723–e728

Contents lists available at SciVerse ScienceDirect

International Journal of Infectious Diseases

journal homepage: www.elsevier.com/locate/ijid

A novel nanobody against urease activity of *Helicobacter pylori*

Leila Safaee Ardekani [a], Seyed Latif Mousavi Gargari [a,*], Iraj Rasooli [a], Masoumeh Rajabi Bazl [b], Mohammad Mohammadi [c], Walead Ebrahimizadeh [a], Hamid Bakherad [a], Hamed Zare [a]

[a] *Department of Biology, Faculty of Basic Science, Shahed University, Tehran, Iran*
[b] *Department of Clinical Biochemistry, Faculty of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran*
[c] *Department of Biology, Faculty of Basic Science, Chamran University, Ahvaz, Iran*

ARTICLE INFO

Article history:
Received 4 September 2012
Received in revised form 3 December 2012
Accepted 16 February 2013

Corresponding Editor: Eskild Petersen, Aarhus, Denmark

Keywords:
Helicobacter pylori
Phage display
Camelid
Heavy chain antibody
Nanobody

SUMMARY

Background: *Helicobacter pylori* infection is associated with gastritis and in some cases with gastric and duodenal ulcers, and even adenocarcinoma. Antibiotic therapy has significant limitations, such as the high cost and the emergence of antibiotic-resistant strains, generating the need for new treatments. The administration of antibody against *H. pylori* is a new effective therapeutic strategy. In this study, we successfully developed a single-variable domain of heavy chain antibody against recombinant UreC.
Methods: A VHH phagemid library was constructed from immune camel heavy chain antibodies. The nanobodies were displayed on M13 phage. Library selection was performed against UreC recombinant protein. A specific single-variable domain of heavy chain antibody against UreC was screened in five rounds of panning. The nanobody with the highest score in the phage ELISA was selected for soluble expression. The nanobody was purified with a nickel–nitrilotriacetic acid (Ni–NTA) column and confirmed with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. Affinity, specificity, and urease inhibitory properties of the nanobody were assayed.
Results: Here we showed the isolation and purification of a specific nanobody with high affinity against UreC recombinant protein that can inhibit urease activity.
Conclusions: The isolated UreC nanobody can specifically detect and bind to UreC and inhibit urease activity. This nanobody could be a novel class of treatment measure against *H. pylori* infection.

1. Introduction

Helicobacter pylori is considered a major health threat. It is estimated that almost half of the world's population is infected with this bacterium.[1,2] *H. pylori* causes gastritis in most people, and in some cases can further induce more severe diseases such as gastric and duodenal ulcers, and even gastric adenocarcinoma.[3–5] The relevance of *H. pylori* infection to other disorders such as cardiovascular, neurologic, ocular, and dermatological diseases has recently been reported.[6] Treatment of *H. pylori* infection usually consists of antibiotics along with a proton pump inhibitor.[7–9] The emergence of antibiotic resistance,[8,10] the high cost of the currently available treatment measures, and the increase in the number of reported relapses[11] highlight the need for new alternative therapeutic approaches.

UreC is one of the urease enzyme subunits showing great potential in vaccine production. UreC is an antigenic protein that can stimulate a specific and innate response and contains an enzyme active site.[12] Urease was found in all clinical isolates and was highly conserved among different strains.[13] Furthermore, antibodies against UreC were found in all patients suffering from stomach ulcer and *H. pylori* infections.[14] Based on our previous research,[15] UreC-specific antibodies can neutralize *H. pylori* urease enzyme and reduce bacterial colonization in an in vitro environment. Antibodies, unlike antibiotics, can recognize certain antigens on the microorganism and neutralize virulence factors that enable the host immune system to interact with the microorganism, and furthermore prevent relapses.[16] Antibodies can bind to a wide spectrum of antigens with versatile mechanisms and be useful in the treatment of cancer, autoimmune, inflammatory, and infectious diseases.[17,18] However, classic antibodies have functional limitations including interaction with the patient immune system and inadequate pharmacokinetics or tissue accessibility.[19,20]

Heavy chain antibodies (HCAbs) – antibodies lacking the light chain and CH1 domain – were discovered in the serum of camelids in the early 1990s.[21] These antibodies have a single variable domain referred to as VHH, sdAb, or nanobody. Nanobodies have better tissue penetration and effective pharmacodynamics with

* Corresponding author.
E-mail addresses: slmousavi@shahed.ac.ir, mlatifm@yahoo.com (S.L.M. Gargari).

1201-9712/$36.00 – see front matter
http://dx.doi.org/10.1016/j.ijid.2013.02.015

less interference with the host immune system.[22,23] The greater therapeutic value of nanobodies over conventional antibodies is due to their small size (2.5 nm in diameter and nearly 4 nm high),[24] high stability at extreme temperatures and pH,[23,25] physical stability, capability of refolding,[26] and binding to unique epitopes inaccessible to conventional antibodies.[27] Recently, antibody administration against urease enzyme for the treatment of *H. pylori* infection has been studied as a new effective therapeutic strategy.[28–32] Nanobodies have also been utilized for the treatment of several intestinal infections such as retroviral intestinal infections.[33] In this study was aimed to produce a nanobody against the recombinant UreC subunit of urease enzyme of *H. pylori*.

2. Materials and methods

2.1. Preparation of antigen

The pET28a vector carrying the UreC gene used in this study was from our earlier work.[15] The expression and purification of recombinant UreC protein was carried out as described previously.[15]

2.2. Immunization of Camelus dromedaries

The purified protein concentration was estimated by the Bradford method.[34] After collection of normal serum from a non-immune camel, the dromedary was subcutaneously injected with 1, 1.5, 2, 2.5, and 3 mg UreC at 2-week intervals. Freund's complete adjuvant (Razi Institute, Iran) was used for the first injection, followed by injection of the protein mixed with an equal volume of Freund's incomplete adjuvant for the booster doses. Serum was taken 1 week after each booster injection for measurement of the produced antibody level.

2.3. ELISA

A 96-well microplate (JET BIO FIL, Canada) was coated with 10 μg/ml of purified UreC recombinant protein per well and incubated at 4 °C overnight. After washing with phosphate buffered saline (PBS)–T (PBS 0.05% Tween-20), the wells were blocked with 150 μl of 5% skim milk in PBS-T and incubated for 1 h at 37 °C. One hundred microliters of serum at different dilutions was added and incubated for 1 h at 37 °C. One hundred microliters of a 1/16 000 dilution of rabbit anti-camel antibody in PBS-T was added to each well and the microplate was incubated for 1 h at 37 °C. One hundred microliters of horseradish peroxidase (HRP)-conjugated mouse anti-rabbit antibody (Abcam, UK) was added to the wells at a final concentration of 1/5000 and incubation was performed at 37 °C for 1 h. One hundred microliters of tetramethylbenzidine (TMB) (Bangalore GeNei, India) was used as substrate buffer. The reaction was incubated at 37 °C for 15 min and was then stopped with 100 μl/well of 3 N H_2SO_4 and the optical density (OD) was measured at 450 nm (OD_{450}). Wells were washed with 200 μl of PBS-T after each step.

2.4. VHH amplification and library construction

After confirming immunization, camel blood was taken in an ethylenediaminetetraacetic acid (EDTA) coated tube, and peripheral blood mononuclear lymphocytes (PBMCs) were isolated using a Ficoll gradient. Total RNA was extracted from isolated lymphocytes with the High Pure RNA Isolation Kit (Roche, USA). cDNA was generated with a RevertAid First Strand cDNA Synthesis Kit (Fermentase, Germany) using Oligo dT primer. A nested PCR was used for VHH amplification. In the first PCR, fragments between framework 1 and CH2 regions were amplified with two sets of specific primers: CALL001: 5′-GTC CTG GCT CTC TTC TAC AAG G-3′; CALL002: 5′-GGT ACG TGC TGT TGA ACT GTT CC-3′; VHBACKA6: 5′-GAT GTG CAG CTG CAG GCG TCT GG(A/G) GGA GG-3′; and CH2FORTA4: 5′-CGC CAT CAA GGT ACC AGT TGA-3′.[23,35–37] Fragments driven from heavy chain antibody (600 and 700 bp) were purified from agarose gel with a Bioneer AccuPrep Gel Purification Kit and were then used as templates for the second PCR. VHH was amplified using a specific degenerated VHH F primer: 5′-CTGGCCCAGGCGGCCGAGGTGCAGCTG(C/G)(A/T)G(C/G)A(G/T)TC(G/T)G-3′; and VHH R primer: 5′-ACTGGCCGGCCTGGCCTGAGGAGACGGTGATGACC(A/T)GGGTC-3′. These primers attach to framework 1 and framework 4 regions. VHH genes were purified from agarose (Bioneer, Korea) and cloned into an *sfi*I digested pcomb3x phagemid vector. Recombinant phagemids were transformed into XL1-Blue *Escherichia coli* electrocompetent cells. The transformed bacteria were then sub-cultured on Luria-Bertani (LB)–ampicillin (80 μg/ml) agar plates.

2.5. Panning of the VHH library

VHH fragments were displayed on a phage after infecting the host bacteria with M13K07 helper phage (Amersham-Pharmacia-Biotech, Vienna, Austria). Briefly, a phage library was grown in 300 ml of SB (Super Broth) medium containing 80 μg/ml ampicillin. Bacteria at mid-log phase at OD_{600} of 0.5–0.7 were infected with 3 ml of M13K07 helper phage with 10^{11} pfu/ml. Infected bacteria were incubated stationary for 30 min and then with shaking for 30 min at 37 °C. Kanamycin 70 μg/ml was added to the culture and incubated at 37 °C for 16 h with shaking at 250 rpm. The culture was centrifuged for 20 min at 4000 × *g* at 4 °C. PEG 6000 (20%) and 2.5 M NaCl was added to the supernatant and this was incubated for 60 min on ice. After centrifugation for 15 min at 15 000 × *g* at 4 °C the pellet was resuspended in 2–3 ml of 1% bovine serum albumin (BSA). The suspension was centrifuged for 5 min at 12 000 × *g* and phage-containing supernatant was collected for biopanning.

Specific VHHs for UreC were propagated by five rounds of biopanning. The microplate was coated overnight at 4 °C with UreC recombinant protein with Tris-buffered saline (TBS). One hundred microliters of concentrated phage library was added to the wells. The washing intensity and frequency were increased throughout the panning process with ascending amounts of Tween-20 in PBS-T from 0.1% to 0.5%. The bound phages were eluted with glycine–HCl 0.2 M pH 2.2 and neutralized in 1 M Tris buffer pH 9. Half of the eluted phages were used to infect *E. coli* XL1-Blue in the mid-log phase and the other half were used for propagation and polyclonal phage ELISA. Polyclonal phage ELISA was used to assess the panning process. Colonies were randomly selected from the third round of panning and screened for VHH gene by PCR. VHH affinity towards UreC protein in positive clones was assessed by phage ELISA. UreC proteins, 10 μg/ml, were coated for 16 h at 4 °C in microplate wells with TBS. The wells were blocked with 150 μl of 5% skim milk in PBS-T for 1 h and were then washed with PBS-T. The purified phages from selected clones were then added to the wells and incubated for 2 h at 37 °C. After washing with 200 μl of PBS-T, 100 μl anti-M13 monoclonal antibody conjugated to HRP (Amersham-Pharmacia-Biotech, Vienna, Austria) was added at a final concentration of 1/8000 and the microplate was incubated for 1 h at 37 °C. TMB substrate was added after washing and the reaction was stopped with H_2SO_4 (3 N) after 15 min. Color intensity was measured at 450 nm by ELISA reader.

2.6. Expression of VHH fragment

The C5 clone that scored the highest OD in monoclonal phage ELISA was selected for expression of soluble periplasmic VHH and was transformed into *E. coli* Top10F′. Expression was induced with

1 mM isopropylthio-β-galactoside (IPTG; Bangalore GeNei, India) at OD_{600} of 0.5 at 28 °C. Cell pellets were collected by centrifugation at 5000 × *g* at 4 °C for 20 min. Pellets were sonicated in lysis buffer containing PBS with protease inhibitor phenyl methyl sulfonyl fluoride at a final concentration of 1.0 mM. After centrifugation the supernatant was collected and production of nanobody was analyzed by 14% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

2.7. Over-expression of nanobody

The selected nanobody was subcloned into pET28a expression vector using a pair of PCR primers, Fr4-*EcoR*1 5′-ACTTCAGAATTCGAGGTGCAGCTGSWGSAKTCKG-3′ and Fr1-*Hind*III 5′-ACTACAAAGCTTTTAGGAGACGGTGACCWGGGTC-3′, with *EcoR*I and *Hind*III restriction sites.[23] The construct was transformed into *E. coli* BL21(DE3). VHH expression was then induced with 1 mM IPTG for 16 h at 28 °C at OD_{600} of 0.5. Bacteria were harvested by centrifugation at 4000 × *g* at 4 °C for 10 min and then resuspended in lysis buffer. The cells were sonicated on ice. After centrifugation, the cleared supernatant was loaded onto a nickel–nitrilotriacetic acid (Ni–NTA) agarose column (Qiagen, Valencia, CA, USA). The bound proteins were eluted with 250 mM imidazole.

2.8. SDS-PAGE and Western blotting

The purified nanobody was studied on 14% SDS-PAGE under reducing conditions. The gel was stained with Coomassie brilliant blue. For Western blotting the gel was run at a constant voltage of 100 V for 45 min. The protein bands were then transferred to the nitrocellulose membrane using a Mini Protein Tetra System (Bio-Rad). The nitrocellulose membrane was blocked with 5% skim milk in PBS-T for 16 h at 4 °C. The membrane was washed and detection was done with 1/5000 dilution of HRP conjugated anti-His Tag antibody (Abcam, UK) and diaminobenzidine (DAB) (Bangalore GeNei, India) as substrate.

2.9. Sequencing

The VHH sequence of C5 clone was performed with VHH F and VHH R primers and the result was compared with the NCBI database.

2.10. Assessment of nanobody binding affinity to UreC

Affinity of the purified nanobody was determined using ELISA. UreC at 1.25, 2.5, 5, and 10 μg/ml concentrations were coated in microplate wells. After washing and blocking as described earlier, the nanobody was added at 0, 1.25, 2.5, 5, and 10 μg/ml concentrations. HRP-conjugated anti-His antibody was added and the immune reactivity was developed with TMB substrate. The reaction was stopped with 3 N H_2SO_4 and OD_{450} was measured.[38]

2.11. Binding specificity

Various antigens produced and standardized in our biotechnology laboratory such as UreC, *H. pylori*, recombinant C-terminal fragment of UreC protein, BSA, *Vibrio cholerae* lipopolysaccharide, *Salmonella typhimurium* lipopolysaccharide, Acinetobacter biofilm-associated protein, and *Clostridium botulinum* neurotoxin type E, were incubated in microplate ELISA at 10 μg/ml concentrations for 16 h at 4 °C. ELISA was performed as described in the section on ELISA.

2.12. Evaluation of nanobody inhibitory effect

H. pylori (10^9 cfu) was mixed with different concentrations of nanobody and incubated in microplate wells for 16 h at 4 °C. One hundred microliters PBS containing 500 mM urea and 0.2 g/l phenol red was added to the wells and incubated for 3 h at 37 °C. Three controls including the culture media, *H. pylori*, and nanobody were used. Colorimetric measurement at OD_{550} was done every 30 min during a 3-h period. The inhibition percentage was calculated as: inhibition percentage = [(the enzymatic activity of bacteria without nanobody − the enzymatic activity of bacteria with nanobody)/the enzymatic activity of bacteria without nanobody] × 100.[12]

2.13. In vitro proteolytic stability of VHHs

Proteolytic stability of VHHs was analyzed using porcine pepsin and bovine trypsin. Purified nanobody, 5 and 10 μg/ml, was incubated with different concentrations of pepsin in10 mM HCl (pH 2) and trypsin in 1 mM Tris–Cl and 20 mM $CaCl_2$ (pH 8. 0) for 1 h at 37 °C prior to the test. For the control test, PBS was used instead of proteolytic enzymes. The affinity of nanobody was determined by antigen-specific ELISA as described in the section on ELISA.

2.14. Temperature treatment

Nanobody, 5 and 10 μg/ml, in PBS was incubated for 2 h at various temperatures (4 °C, 25 °C, 60 °C, 80 °C, and 90 °C), followed by a 30-min incubation at room temperature (RT), and then stored at 4 °C. The ELISA was developed as described in the section on ELISA.

2.15. Binding in the presence of urea

Different concentrations of nanobody (5 and 10 μg/ml) were incubated overnight at room temperature in 0–8 M urea in PBS. The ELISA was carried out as mentioned earlier, using urea-treated nanobodies.

3. Results

3.1. Camel immune response

Immune camel serum at 1/1000, 1/3000, and 1/10 000 dilutions, and non-immune camel serum as a negative control, were assessed by indirect ELISA using UreC recombinant protein as an antigen. The antibody titer of the immune serum against UreC reached a maximum of about 0.7 after the fifth injection, as against 0.1 of the normal serum.

3.2. Construction and panning of the nanobody library

Lymphocytes were isolated from anti-coagulated blood of an immunized camel. Total RNA were purified from 10^6 cells (Figure 1a). cDNA was prepared from purified RNA. The amplified fragments are derived from classical antibody (900 bp) and heavy chain antibody (700 600 bp). The regions between CH2 and framework 1 were amplified (Figure 1b). In the second PCR, the VHH fragments (400 bp) were generated with specific primers that attach to framework 1 and framework 4 and amplify the variable region (Figure 1c). The nanobody library was prepared with 3.2×10^7 members. Phages binding to UreC recombinant protein were selected by five consecutive rounds of biopanning against recombinant UreC. The third round of panning showed the highest immunoactivity in the polyclonal phage ELISA (Figure 2).

In the monoclonal phage ELISA, 18 clones were screened from the third round of panning that bound strongly to UreC recombinant protein and showed minimal affinity towards control BSA. The C5 clone showing the highest OD was selected for further

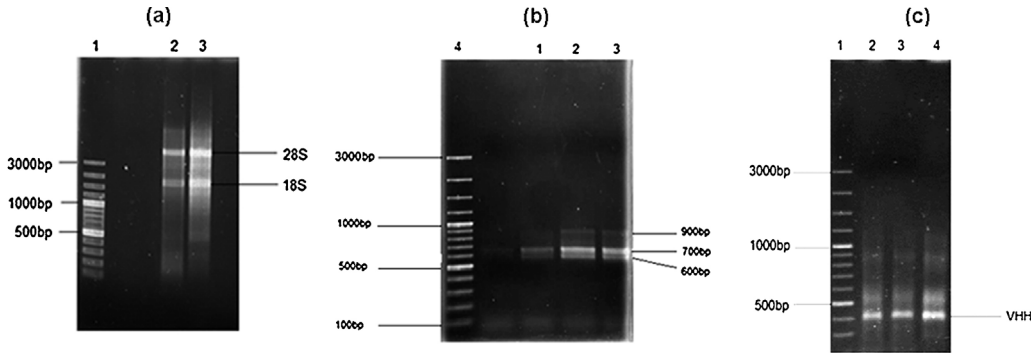

Figure 1. Library construction. (a) Clear definition of the ribosomal RNA 28S and 18S demonstrates the integrity of the samples. (b) Analysis of the first PCR product with agarose gel electrophoresis (1%); lanes 1, 2, and 3: the PCR product with different sizes 900, 700, and 600 bp, respectively; lane 4: molecular weight marker. (c) Analysis of the second PCR product by gel electrophoresis (1%); the VHH fragment with 400 bp size is marked.

characterization. Sequence analysis of this clone exhibited high homology (92%) with the VHH sequence of Camelus dromedaries in the NCBI database.

3.3. SDS-PAGE and Western blot

For production of soluble nanobody, the C5 phagemid was transferred into non-suppressor *E. coli* Top10F′. The nanobody was expressed with an OmpA leader signal for periplasmic secretion. SDS-PAGE analysis showed an 18-kDa band (Figure 3a). For characterization of nanobody, the anti-UreC-VHH gene was subcloned into pET28a expression vector. Nanobody was expressed in fusion with N-terminal His-tag and purified by NTA-affinity chromatography. The nanobody was eluted by an imidazole buffer 250 mM (Figure 3b) and was confirmed by Western blotting. The purified nanobody showed an 18-kDa band (Figure 3c).

3.4. Nanobody affinity determination

Affinity of the purified nanobody measured by ELISA was 5×10^{-8} M.

3.5. Cross-reactivity of the nanobody

The nanobody was specific for UreC and no cross-reactivity was observed with BSA, *V. cholerae* lipopolysaccharide, *S. typhimurium* lipopolysaccharide, Acinetobacter biofilm-associated protein, or *C. botulinum* neurotoxin type E.

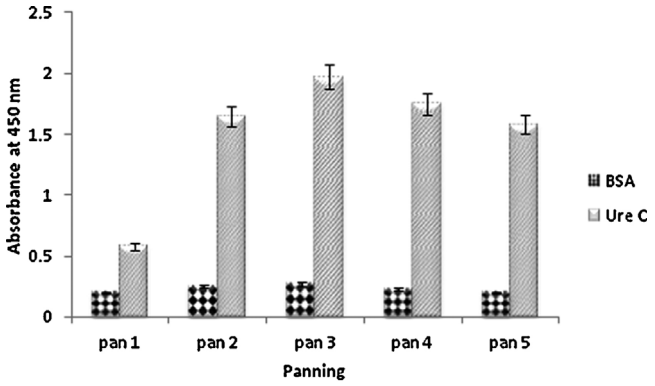

Figure 2. Assessment of the panning process with phage ELISA. The absorbance and enrichment against UreC was increased and the highest enrichment was obtained in the third panning. The absorbance against BSA (negative control) remained constant.

3.6. Urease inhibitory effect

A serial dilution of nanobody (from 0 to 100 μg/ml) incubated with *H. pylori* showed elevated urease inhibition with the increasing concentration of the nanobody. The minimum amount of nanobody required to inhibit urease activity was calculated as 70 μg/ml and the maximum urease inhibition was calculated as 35% (Figure 4).

3.7. Proteolytic stability

The nanobodies retained their full activity even when incubated with 1 mg of proteolytic enzymes.

3.8. Temperature stability

Nanobodies preserved functional activity even after incubation at 90 °C. The results demonstrate that antigen binding in nanobodies is unaffected by the temperature.

3.9. Binding in the presence of urea

Nanobody had preserved binding when tested in an ELISA experiment in the presence of increasing amounts of Urea. This result suggests that the urea-treated VHHs retained function and antigen binding ability even in denaturing conditions.

4. Discussion

The prevalence of antibiotic-resistant *H. pylori* strains, the high cost of treatment, and the risk of relapse, have led to the need for a new approach to the treatment of *H. pylori*-related diseases.[11] The effects of various inhibitors such as L-ascorbic acid, copper ions, and acetohydroxamic acid on *H. pylori* urease activity have been investigated in previous studies. However, the use of such compounds in vivo causes problems such as toxicity and instability.[13] So far, several monoclonal and single chain variable fragment (scFv) antibodies against urease enzyme have been produced.[28–32] Due to problems such as low stability and low yield of production and immunogenicity, the need for a new generation of antibodies seems necessary.[19]

The variable domain of camel heavy chain antibody has phenomenal features such as a small size and high stability and affinity, which makes it suitable for biotechnological applications.[22,24–27] Because of the problems mentioned above, our aim was to produce a phage displayed VHH library against UreC recombinant protein and to screen for a nanobody with high affinity and specificity. In this study UreC was successfully

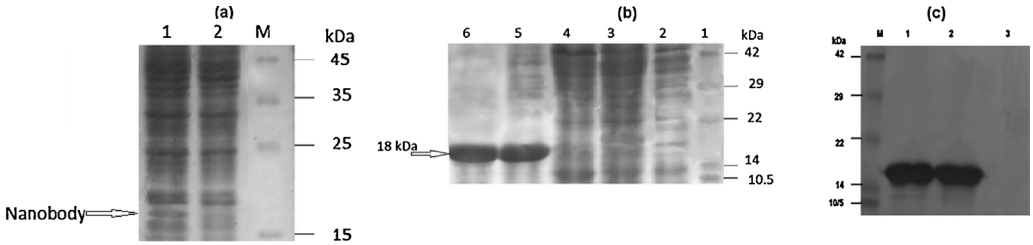

Figure 3. SDS-PAGE and Western blotting. (a) SDS-PAGE gel stained with Coomassie blue; lane M: molecular weight marker; lane 1: the expressed VHH after 1 mM IPTG induction; lane 2: cell lysate before induction. (b) SDS-PAGE analysis of nanobody purified by Immobilized metal affinity chromatography (IMAC); lane 1: molecular weight marker; lanes 2–4: the fractions after washing with 20, 50, and 100 mM imidazole, respectively; lanes 5 and 6: the nanobody eluted by 250 mM imidazole buffer. (c) Western blot analysis of VHH binding to UreC; lane M: molecular weight marker; lanes 1 and 2: the specific reaction of the HRP-conjugated anti-His Tag antibody with nanobody after induction; lane 3: before induction.

produced and purified. The highest titer of the antibody confirmed by ELISA was achieved after three booster injections. The 3.2×10^7 member nanobody phage library constructed in this work indicates its adequate variety.

Urease is an important pathogenic factor that helps *H. pylori* colonize the epithelium in the acidic environment of the stomach.[29] This enzyme exists in two forms of cytoplasmic and surface protein and releases NH_3 by hydrolyzing urea.[14] The urease enzyme was selected for its unique role in *H. pylori* colonization and survival.

To date, many VHH phage libraries have been reported. In these publications the affinities of selected antibodies have been calculated to be in the range of 10^{-9} to 10^{-10}.[39,40] The affinity of monoclonal antibody and scFv fragments against urease has ranged from 1.7×10^{-8} to 3×10^{9}.[28,30,31] In the present work the affinity of nanobody against UreC recombinant protein was 5×10^{-8} M. This is high in comparison with the previous reports. VHHs, unlike conventional antibodies, can recognize epitopes such as cavity and cleft in the active sites as they have a convex paratope.[27] Our nanobody has advantages over those of previous studies in this respect. Furthermore the VHH stability tests revealed high thermostability and resistance to denaturing agents and proteolytic enzymes. The resistance to proteolytic enzymes is of significant importance in the oral administration of anti-*H. pylori* antibodies. This characteristic allows the oral administration of VHH in the treatment of stomach ulcer without any loss of binding activity. Resistance to thermal denaturation and the retention of full activity after incubation at high temperatures increases the antibody's shelf-life. The results of the urease inhibitory test showed that nanobody can successfully inhibit the surface urease activity of *H. pylori*. This can significantly reduce bacterial survival in acidic environments such as the stomach.

The results suggest that single domain antibodies with high affinity and specificity could be a novel class of treatment measure in *H. pylori* infection. Implementing our previous successful in vivo study[15] to the present work would add practical dimensions to our findings.

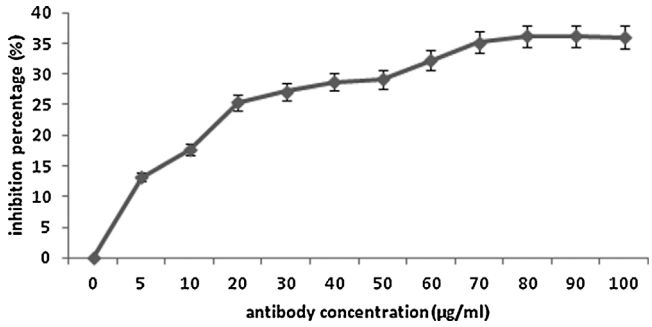

Figure 4. Neutralization of *Helicobacter pylori* urease activity by the nanobody. *H. pylori* was treated with nanobody and the optical density of the mixture was determined at 550 nm by phenol red indicator showing 35% inhibition.

Conflict of interest: No conflict of interest to declare.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijid.2013.02.015.

References

1. Petersen AM, Krogfelt KA. *Helicobacter pylori*: an invading microorganism? A review. *FEMS Immunol Med Microbiol* 2006;**36**:117–26.
2. Sougioultzis S, Lee CK, Alsahli M, Banerjee S, Cadoz M, Schrader R, et al. Safety and efficacy of *E. coli* enterotoxin adjuvant for urease-based rectal immunization against *Helicobacter pylori*. *Vaccine* 2002;**21**:194–201.
3. Hatzifoti C, Bajaj-Elliott M, Dorrell N, Anyim M, Prentice MB, Nye KE, et al. A plasmid immunization construct encoding urease B of *Helicobacter pylori* induces an antigen-specific antibody response and upregulates the expression of β-defensins and IL-10 in the stomachs of immunized mice. *Vaccine* 2004;**22**:2651–9.
4. Ito Y, Shibata K, Hongo A, Kinoshita M. Ecabet sodium, a locally acting antiulcer drug, inhibits urease activity of *Helicobacter pylori*. *Eur J Pharmacol* 1998;**345**:193–8.
5. Lee MH, Roussel Y, Wilks M, Tabaqchali S. Expression of *Helicobacter pylori* urease subunit B gene in *Lactococcus lactis* MG1363 and its use as a vaccine delivery system against *H. pylori* infection in mice. *Vaccine* 2001;**19**:3927–35.
6. Izzotti A, Durando P, Ansaldi F, Gianiorio F, Pulliero A. Interaction between *Helicobacter pylori*, diet, and genetic polymorphisms as related to non-cancer diseases. *Mutat Res* 2009;**667**:142–57.
7. Graham DY, Shiotani A. New concepts of resistance in the treatment of *Helicobacter pylori* infections. *Nat Clin Pract Gastroenterol Hepatol* 2008;**5**:321–31.
8. Malfertheiner P, Megraud F, O'Morain C, Bazzoli F, El-Omar E, Graham D, et al. Current concepts in the management of *Helicobacter pylori* infection: the Maastricht III Consensus Report. *Gut* 2007;**56**:772–81.
9. Gisbert J, Gisbert JL, Marcos S, Jimenez-Alonso I, Moreno-Otero R, Pajares J. Empirical rescue therapy after *Helicobacter pylori* treatment failure: a 10-year single-centre study of 500 patients. *Aliment Pharmacol Ther* 2008;**27**:346–54.
10. DeLoney CR, Schiller NL. Characterization of an in vitro-selected amoxicillin-resistant strain of *Helicobacter pylori*. *Antimicrob Agents Chemother* 2000;**44**:3368–73.
11. Trust TJ, Alm RA, Pappo J. *Helicobacter pylori*: today's treatment, and possible future treatment. *Eur J Surg* 2003;**167**:82–8.
12. Li HX, Mao XH, Shi Y, Ma Y, Wu YN, Zhang WJ, et al. Screening and identification of a novel B-cell neutralizing epitope from *Helicobacter pylori* UreB. *Vaccine* 2008;**26**:6945–9.
13. Houimel M, Mach JP, Corthésy-Theulaz I, Corthesy B, Fisch I. New inhibitors of *Helicobacter pylori* urease holoenzyme selected from phage-displayed peptide libraries. *Eur J Biochem* 2001;**262**:774–80.
14. Rokita E, Makristathis A, Hirschl AM, Rotter ML. Purification of surface-associated urease from *Helicobacter pylori*. *J Chromatogr B Biomed Sci Appl* 2000;**737**:203–12.
15. Malekshahi ZV, Gargari SL, Rasooli I, Ebrahimizadeh W. Treatment of *Helicobacter pylori* infection in mice with oral administration of egg yolk-driven anti-UreC immunoglobulin. *Microb Pathog* 2011;**51**:366–72.
16. Hudson PJ, Souriau C. Engineered antibodies. *Nat Med* 2003;**9**:129–34.
17. Berghman L, Abi-Ghanem D, Waghela S, Ricke S. Antibodies: an alternative for antibiotics? *Poult Sci* 2005;**84**:660–6.
18. Enever C, Batuwangala T, Plummer C, Sepp A. Next generation immunotherapeutics—honing the magic bullet. *Curr Opin Biotechnol* 2009;**20**:405–11.

19. Chames P, Van Regenmortel M, Weiss E, Baty D. Therapeutic antibodies: successes, limitations and hopes for the future. *Br J Pharmacol* 2009;**157**:220–33.
20. Kuus-Reichel K, Grauer L, Karavodin L, Knott C, Krusemeier M, Kay N. Will immunogenicity limit the use, efficacy, and future development of therapeutic monoclonal antibodies? *Clin Diagn Lab Immunol* 1994;**1**:365–72.
21. Hamers-Casterman C, Atarhouch T, Muyldermans S, Robinson G, Hammers C, Songa EB, et al. Naturally occurring antibodies devoid of light chains. *Nature* 1993;**363**:446–8.
22. Kolkman JA, Law DA. Nanobodies—from llamas to therapeutic proteins. *Drug Discov Today Technol* 2010;**7**:e139–46.
23. Ebrahimizadeh W, Mousavi Gargari S, Rajabibazl M, Safaee Ardekani L, Zare H, Bakherad H. Isolation and characterization of protective anti-LPS nanobody against *V. cholerae* O1 recognizing Inaba and Ogawa serotypes. *Appl Microbiol Biotechnol* 2012 Nov 8 [Epub ahead of print].
24. Spinelli S, Frenken LG, Hermans P, Verrips T, Brown K, Tegoni M, et al. Camelid heavy-chain variable domains provide efficient combining sites to haptens. *Biochemistry* 2000;**39**:1217–22.
25. Van der Linden R, Frenken L, De Geus B, Harmsen M, Ruuls R, Stok W, et al. Comparison of physical chemical properties of llama V_{HH} antibody fragments and mouse monoclonal antibodies. *Biochim Biophys Acta* 1999;**1431**:37–46.
26. Alvarez-Rueda N, Ladjemi MZ, Béhar G, Corgnac S, Pugnière M, Roquet F, et al. A llama single domain anti-idiotypic antibody mimicking HER2 as a vaccine: immunogenicity and efficacy. *Vaccine* 2009;**27**:4826–33.
27. Lauwereys M, Ghahroudi MA, Desmyter A, Kinne J, Hölzer W, De Genst E, et al. Potent enzyme inhibitors derived from dromedary heavy-chain antibodies. *EMBO J* 1998;**17**:3512–20.
28. Ikeda Y, Fujii R, Ogino K, Fukushima K, Hifumi E, Uda T. Immunological features and inhibitory effects on enzymatic activity of monoclonal antibodies against *Helicobacter pylori* urease. *Journal of Fermentation and Bioengineering* 1998;**86**:271–6.
29. Nagata K, Mizuta T, Tonokatu Y, Fukuda Y, Okamura H, Hayashi T, et al. Monoclonal antibodies against the native urease of *Helicobacter pylori*: synergistic inhibition of urease activity by monoclonal antibody combinations. *Infect Immun* 1992;**60**:4826–31.
30. Wu Y, Zou Q, Luo P, Guo G, Wang X. Preparation of anti–{\sl *Helicobacter pylori*} UreB monoclonal antibody and the detection of the ability of inhibition the urease activity. *Mian yi xue za zhicehsp sp025/cehsp sp025/Journal of Immunology* 2005;**21**:393.
31. Houimel M, Corthesy-Theulaz I, Fisch I, Wong C, Corthesy B, Mach JP, et al. Selection of human single chain Fv antibody fragments binding and inhibiting *Helicobacter pylori* urease. *Tumor Biol* 2001;**22**:36–44.
32. Reiche N, Jung A, Brabletz T, Vater T, Kirchner T, Faller G. Generation and characterization of human monoclonal scFv antibodies against *Helicobacter pylori* antigens. *Infect Immun* 2002;**70**:4158–64.
33. Van der Vaart J, Pant N, Wolvers D, Bezemer S, Hermans P, Bellamy K, et al. Reduction in morbidity of rotavirus induced diarrhoea in mice by yeast produced monovalent llama-derived antibody fragments. *Vaccine* 2006;**24**: 4130–7.
34. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* 1976;**72**:248–54.
35. Thys B, Schotte L, Muyldermans S, Wernery U, Hassanzadeh-Ghassabeh G, Rombaut B. In vitro antiviral activity of single domain antibody fragments against poliovirus. *Antivir Res* 2010;**87**:257–64.
36. Saerens D, Stijlemans B, Baral TN, Nguyen Thi GT, Wernery U, Magez S, et al. Parallel selection of multiple anti-infectome nanobodies without access to purified antigens. *J Immunol Methods* 2008;**329**:138–50.
37. Arbabi Ghahroudi M, Desmyter A, Wyns L, Hamers R, Muyldermans S. Selection and identification of single domain antibody fragments from camel heavy-chain antibodies. *FEBS Lett* 1997;**414**:521–6.
38. Beatty JD, Beatty BG, Vlahos WG. Measurement of monoclonal antibody affinity by non-competitive enzyme immunoassay. *J Immunol Methods* 1987;**100**:173–9.
39. Tremblay JM, Kuo CL, Abeijon C, Sepulveda J, Oyler G, Hu X, et al. Camelid single domain antibodies (VHHs) as neuronal cell intrabody binding agents and inhibitors of *Clostridium botulinum* neurotoxin (BoNT) proteases. *Toxicon* 2010;**56**:990–8.
40. Ahmadvand D, Rasaee MJ, Rahbarizadeh F, Kontermann RE, Sheikholislami F. Cell selection and characterization of a novel human endothelial cell specific nanobody. *Mol Immunol* 2009;**46**:1814–23.