Acta Biochimica Polonica

Vol. 52 No. 1/2005

261–266

QUARTERLY

Communication

Genetic immunization of ducks for production of antibodies specific to *Helicobacter pylori* UreB in egg yolks✪

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Received: 12 January, 2005; accepted: 10 February, 2005

Key words: antibody, antigen, DNA immunization, *Helicobacter pylori*, IgY, urease

Following genetic immunization of laying ducks with a plasmid expressing *Helicobacter pylori* UreB (large subunit of urease), IgY against UreB were obtained from egg yolks. These polyclonal and monospecific IgY antibodies are of higher-titer and specifically recognize recombinant *H. pylori* urease purified from *Escherichia coli*. To our knowledge this is the first report describing generation of IgY antibodies directed against antigens of *H. pylori* by DNA-based immunization.

Helicobacter pylori is a Gram-negative, spiral, microaerophylic bacterium that infects the stomach of more than 50% of the human population worldwide. It is associated with gastritis, peptic ulcer and gastric cancer and it has been classified as a category 1 carcinogen by WHO (Ruggiero *et al.*, 2002; Prinz *et al.*, 2003). Several existing detection methods such as Western blots or ELISA are useful to identify antibodies against *H. pylori* in blood, saliva or urine of the infected individuals. Other tests consist of the revealing of the urease activity (urea breath test) or detection of *H. pylori* antigens in biopsies or stools. Cur-

✪This work was partially supported by the program POLONIUM (Project No. 4244).

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Abbreviations: BSA, bovine serum albumin; PBS, phosphate-buffered saline; UreB, large subunit of urease.

rent therapies are not effective in 100% and fail due to antibiotic resistance. No effective vaccine against *H. pylori* exists, although several vaccine candidates including an oral whole-cell vaccine (Helivax, Antex Biologicals) and an intramuscular trivalent acellular vaccine (Chiron Vaccines) have been tested in clinical trials (for a recent review: Ruggiero *et al.*, 2002). Antibodies directed against specific *H. pylori* targets could be used either for diagnosis or as therapeutic agents.

Only few recent reports exist about generation of antibodies against *H. pylori* antigens. These reports include monoclonal antibodies isolated from an immune phage display library constructed from peripheral blood lymphocytes of infected patients (Reiche *et al.*, 2002), bovine colostral antibodies isolated from colostrums of cows immunized with killed *H. pylori* bacteria (Casswall *et al.*, 2002; Marnila *et al.*, 2003) and chicken yolk-derived antibodies obtained, as in the previous case, against the whole-cell lysate of *H. pylori* (Shimamoto *et al.*, 2002; Shin *et al.*, 2002; 2003). The results achieved by the above authors suggest that passive immunization upon oral administration of antibodies can be effective. Urease is the major antigen of *H. pylori* that is essential for bacteria to survive within the acidic environment of the stomach. Effective blocking of urease activity seems to be a good strategy to prevent colonization of the stomach by *H. pylori.* Significant research efforts concerning vaccine development concentrate around this protein, including our attempts to obtain effective production of UreB in plant tissues (Brodzik *et al.*, 2000a; 2000b). An interesting observation is that antibodies raised against entire urease do not neutralize its enzymatic activity; however, even in such a case their protective potential has been shown (Blanchard *et al.*, 1995). Monoclonal antibodies capable not only of binding but also of inhibiting *H. pylori* urease have been identified and shown to recognize a short linear epitope (Hirota *et al.*, 2001).

In the present study we took advantage of a novel approach of "DNA designed" egg yolk antibody production by DNA immunization of ducks (Rollier *et al.*, 2000). As these authors described, following genetic immunization of breeding ducks with a plasmid encoding a given antigen, specific and biologically active IgY antibodies are transmitted vertically from their serum into the egg and accumulate in egg yolk from which they can be extracted and purified.

The aim of this work was to use the above approach for generation and preliminary characterization of polyclonal, monospecific antibodies against the catalytic subunit (UreB) of *H. pylori* urease.

MATERIALS AND METHODS

DNA manipulation. All manipulations were performed according to standard techniques (Sambrook *et al.*, 1989). The *ureB* gene of *H. pylori* was amplified using the oligonucleotides UREBPCI1 (5′ gcgaattccgccatggaaaagatta 3′) and UREBPCI2 (5′ gcgctctagactagaaaatgctaaag 3′) as primers, and pAR3 (Brodzik *et al.*, 2000a) as a template. The 1.7-kb DNA fragment was cloned into the *Nco*I and *Xba*I sites of pCI vector (Promega). The resulting plasmid pAR72 was purified from DH5α using commercial plasmid purification systems (Qiagen).

Immunization of ducks. Immunization of ducks was performed in animal facilities of INSERM U271 (Lyon, France) with a total of 500 μg of plasmid DNA per animal by intramuscular injection in multiple sites, followed by two subsequent boosts (Rollier *et al.*, 2000). Eggs collection and IgY purification from the eggs were performed as previously described (Rollier *et al.*, 2000).

Dot-blot. Aliquots of 0.5 μl of partially purified recombinant antigens of *H. pylori* or bovine serum albumin (BSA) were spotted on Nylon membranes. Each dot contained ap-

proximately 125 ng of protein. The spots were allowed to dry and the membranes were blocked for 1 h in phosphate-buffered saline (PBS) with 5% skim milk, incubated for 1 h with IgY diluted to 10 μg/ml, washed (3 × 10 min) and incubated with anti-duck-IgG, AP conjugated (Kirkegaard & Perry Laboratories) used as secondary antibodies (5 000 fold diluted, 1 h). After four washes of 5 min each in PBS the NBT/BCIP detection system (Promega) was applied.

ELISA. Ninety-six-well ELISA plates (EIA/RIA high binding, Costar) were coated O/N with 0.5 μg of either recombinant UreB protein purified from *Escherichia coli* or BSA in 100 μl of PBS. The coating was followed by 60-min incubation with the tested IgY antibodies (dilutions 1:500 to 1:16 000 were used), 5-min washing in PBS, 60-min blocking with 0.5% BSA in PBS and 60-min incubation with secondary antibodies (goat derived anti-duck- IgG, HRP conjugated, Kirkegaard & Perry Laboratories). For the detection TMB Peroxidase EIA Substrate Kit (BioRad) was used and absorbance was determined at either 655 nm (reactions not stopped) or at 450 nm (reactions stopped with 2 M sulfuric acid).

Preparing E. coli extract containing active recombinant urease of H. pylori. Crude protein extract containing active *H. pylori* urease was prepared from *E. coli* strain transformed with two plasmids: pHP902 and pHP808 (Hu & Mobley, 1993), harboring *ureAB* and the cluster of genes (*ureCD ureAB ureIEFGH*), respectively. As a negative control served a protein extract prepared from *E. coli* transformed with pHP902 and pACYC184. The bacteria were grown (until A_{600} reached 0.8–1.2) in minimal M9 medium (Sambrook *et al.*, 1989) supplemented with glucose (0.4%), tryptophan (20 mg/l), $NiCl_2$ (1 μM), and ampicillin (100 μg/ml) and chloramphenicol (30 μg/ml) as selection markers. Alternatively, LB medium (Sambrook *et al.*, 1989) with ampicillin and chloramphenicol as above additionally supplemented with 700 μM $NiCl_2$ was used for extract preparation. Bacterial cells were centrifuged, suspended in 0.1 vol. of 20 mM sodium phosphate buffer, pH 6.8, and sonicated (6 × 10 s at 40% Duty Cycle, 5^{th} level Output Control, on ice. Model 250 Sonifier, Branson Ultrasonics Corporation). The supernatant obtained after centrifugation (10 000 × ***g***, 10 min, 4°C) was treated as a crude extract for urease activity assays.

Urease activity assay. The final volume of the reaction was always 1 ml. Each sample contained 2.6 μl of 1 M Na_2HPO_4, 6.5 μl of 1 M KH_2PO_4 and 10 μl of enzyme extract. Reaction was started by addition of 50 μl of 4.16 M urea and incubated for 15 min at 37°C. For testing of the neutralizing activities, antibodies were added before urea and incubated with the enzymatic extract for 10 or 30 min. Then, urea was added and incubation continued as above. Aliquots of 100 μl were taken and added to the freshly prepared mixture containing 500 μl of solution A (1% phenol, 0.05% sodium nitroprusside) and 500 μl of solution B (0.5% NaOH and 0.05% NaOCl). A_{650} was read after 5 min at 37°C. A control extract without active urease was used as a negative control. The activity of urease is presented as the difference in absorbance at 650 nm between the extracts containing active urease and the control extracts that were identically treated.

Cultures of H. pylori. A strain of *H. pylori* ($CagA^+$ $VacA^+$ $urease^+$ $catalase^+$) previously isolated from the stomach of an infected patient and stored at –80°C was used for verification of the neutralizing potential of the tested IgY. For the solid media test, antibodies were spotted on small filter discs, placed on plates (Columbia with 5% Horse Blood, BioMerieux) containing the bacteria spread and incubated for 48 h at 37°C in an atmosphere with 5% CO_2. For the liquid culture tests, bacteria were grown in Brucella Broth (Becton Dickinson) supplemented with fetal bovine serum in the presence or absence of antibodies.

RESULTS AND DISCUSSION

UreB-specific IgY antibodies can be obtained from eggs of ducks immunized with plasmid DNA expressing ureB of *H. pylori*

Plasmid pAR72, containing *H. pylori ureB* gene was used for DNA immunization of two ducks. Eggs were collected and large amounts of IgY antibodies (about 50 mg/egg) were purified from their yolks. Preliminary characterization of the obtained IgY using dot-blot technique indicated that IgY from one of the immunized ducks (No. J435) specifically recognized partially purified recombinant *H. pylori* UreB (Fig. 1). The same antibodies bound neither recombinant *H. pylori* HspB nor BSA, both used as controls. In addition, IgY purified from eggs of another duck (duck No. J511), which was immunized with a plasmid expressing *hspB* of *H. pylori* and which has not mounted a specific anti-HspB response, were used as an additional negative control. The IgY from the duck No. J511 were not able to detect any of the three proteins tested (Fig. 1).

Figure 1. Dot-blot experiment with IgY antibodies purified from eggs of duck immunized with plasmid pAR72 (J435) and a control duck (J511).

Positions of partially purified proteins is marked in the box; H, recombinant *H. pylori* HspB purified from *E. coli* cells; U, recombinant *H. pylori* UreB purified from *E. coli* cells; B, bovine serum albumin (BSA) from commercial source.

Detailed characterization of IgY specificity was performed by ELISA. The IgY from duck No. J435 specifically recognized UreB in this test. Importantly, the titer of these IgY was high since a 8000-fold dilution was still able to recognize UreB (Fig. 2).

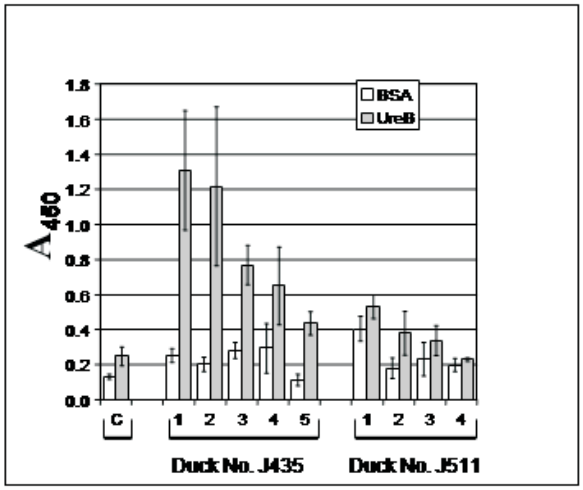

Figure 2. Specific activities of anti-UreB IgY antibodies tested in ELISA experiments.

IgY antibodies were purified from eggs obtained either from duck No. J435 (immunized with pAR72 plasmid) or from duck No. J511 (control). Dilutions of stock preparations of IgY indicated below were tested in triplicates for their ability to recognize either BSA or recombinant *H. pylori* UreB used to coat the ELISA plates: 1, 1:500; 2, 1:1000; 3, 1:2000; 4, 1:4000; 5, 1:8000. C, denotes the control (buffer without any antibodies).

UreB-specific IgYs do not have neutralizing properties nor inhibit growth of *H. pylori*

Bacterial extracts containing catalytically active recombinant *H. pylori* urease were prepared from *E. coli* cells co-transformed with two plasmids: pHP902 encoding two subunits of *H. pylori* urease and pHP808 containing not only the structural genes for *H. pylori* urease (*ureAB*) but also genes necessary for production of catalytically active urease, which encode accessory proteins involved in incorporation of nickel into the active center of the enzyme (Hu & Mobley, 1993). IgY from ducks No. J435 and J511 were tested for their ability to inhibit the activity of *H. pylori* urease. The lack of influence of preincubation of the bacterial extract with anti-UreB IgY antibodies from duck or with polyclonal anti-urease IgG antibodies from rabbit (not

shown) on the urease activity clearly demonstrated the absence of any neutralizing activity of the tested immunoglobulins. This is not surprising since such activity is detected only rarely when antibodies are raised against entire UreB protein (Thomas *et al.*, 1992). Most anti-UreB antibodies with urease-inhibiting activity were raised against well-defined short epitopes of UreB protein (Hirota *et al.*, 2001; Houimel *et al.*, 2001). Additional experiments performed with bacterial cells grown either on solid media or in liquid cultures failed to demonstrate any influence of IgY on growth parameters of the tested *H. pylori* strain (not shown).

In summary, highly specific anti-UreB IgY antibodies were generated by genetic immunization of ducks with a plasmid encoding the entire UreB protein. Similarly to the rabbit antibodies raised against the entire UreB, the obtained IgY antibodies were not neutralizing *in vitro,* although this does not exclude the possibility that these antibodies might be protective *in vivo* (Blanchard *et al.*, 1995). Whether genetic immunization of ducks with plasmids encoding selected UreB epitopes may generate neutralizing and protective anti-UreB IgY antibodies is not known and warrants further studies.

The anti-UreB IgY antibodies obtained by us using this new approach of "DNA-designed" antibody production in egg yolk may be of particular value for diagnostic purpose due to: (i) large yield of IgY generated in the "egg yolk factory"; (ii) non invasive production of such antibodies; (iii) no need of purified protein for immunization; (iv) high affinity of antibodies; (v) absence of cross reactivity with mammalian immunoglobulins and complement.

We gratefully acknowledge Prof. Włodzimierz Zagórski-Ostoja, without whom this project could never be started, for his constant interest and support. The expert assistance of Catherine Jamard (INSERM Unit 271, Lyon, France) with animals is gratefully acknowledged.

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