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The role of outer inflammatory protein A (OipA) in vaccination of the C57BL/6 mouse model infected by *Helicobacter pylori*

Mohaddese MAHBOUBI[1], Tahereh FALSAFI[1,*], Majid SADEGHIZADEH[2], Fatemeh MAHJOUB[3]

[1]Department of Microbiology, Faculty of Biological Sciences, Alzahra University, Vanak, Tehran, Iran
[2]Department of Genetics, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran
[3]Department of Pathology, Children's Medical Center of Tehran, Tehran, Iran

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Background/aim: Outer inflammatory protein A (OipA) is an important adhesin of *Helicobacter pylori*. Our goal was to assess the role of OipA in protection of C57BL/6 mice against *H. pylori*.

Materials and methods: C57BL/6 mice were mucosally immunized with recombinant OipA protein, OipA + propolis, propolis, and phosphate-buffered saline. After vaccination, anti-OipA IgA was measured. Mice were challenged three times with 5×10^7 CFU of the *H. pylori* B19 strain. Two weeks later, bacterial colonization and inflammation in the stomach was analyzed using standard methods.

Results: The CFU number in the OipA group was significantly ($P < 0.05$) lower than that of the control. The CFU number in the OipA + propolis group was higher than those of the OipA and propolis groups. IgA titers were significantly higher ($P < 0.0001$) in the OipA group compared to the control and OipA + propolis groups. Propolis did not play an adjuvant effect but it interfered with the efficient vaccine effect of OipA.

Conclusion: Results show the effect of vaccination by OipA in protection of the mouse model and the importance of OipA in *H. pylori* pathogenesis. OipA may be proposed as a suitable oral vaccine candidate against *H. pylori* infection; however, further study is required to determine adjuvant or adverse effects of propolis toward OipA.

Key words: *Helicobacter pylori*, OipA, vaccine, adjuvant, propolis, humoral immunity

1. Introduction

Stomach colonization by *Helicobacter pylori* is associated with gastritis, gastric ulcers, gastric adenocarcinoma, and mucosa-associated lymphoid tissue lymphoma (1). Treatment of *H. pylori* infections consists of combination therapy involving two or three antibiotics, plus a proton pump inhibitor (2–4). However, the rate of unsuccessful eradication due to the emergence of antibiotic-resistant strains is growing (5,6). Furthermore, in regions with high rates of *H. pylori* infection, recurrent infection is more frequent (7). Hence, prevention would be an ideal strategy to resolve the problems associated with *H. pylori* infections, especially in regions with a high incidence of infection (8).

Adherence to the gastric mucosa is the first step in successful colonization of the stomach by *H. pylori*, and among the bacterial factors essential for specific attachment to gastric epithelial cells, the outer membrane proteins (OMPs) play an important role (9).

OipA, with a molecular weight of 33–35 kDa, may be especially important in the initiation of inflammatory response to *H. pylori* via induction of proinflammatory cytokine interleukin (IL)-8 (10–12). Consistent with this important role, an association has been observed between the "on" status of the *oipA* gene conducting expression of a 34-kDa full-length protein and duodenal ulcers as well as gastric cancer (13).

In the present study, the OipA protein was selected as a suitable candidate for vaccine development since this OMP plays an important role in the first steps of *H. pylori* pathogenesis. The immunogenicity of the full-length protein of OipA was previously examined in an animal model (17). Experimentation with this important immunogenic protein as a vaccine candidate may be important in protection of the host against *H. pylori*.

Furthermore, utilization of natural adjuvants instead of the traditional chemical ones to avoid their side effects may be promising in the vaccination process. Among diverse

* Correspondence: falsafi.tahereh@yahoo.com

natural products, the compounds that are used as food and demonstrate adjuvant activities towards immunogenic proteins might be more important in vaccination processes. Among them, propolis may be a suitable candidate since its adjuvant activities in vaccination of mice models have been demonstrated (11,14–16).

By experimentation with the OipA protein as a vaccine candidate, we evaluated its role in protection of C57BL/6 mice against *H. pylori* infection by using propolis as a natural adjuvant.

2. Materials and methods

2.1. Characteristics of recombinant OipA antigen

A full-length *oipA* gene was obtained from a clinical *H. pylori* strain (S15) as previously described (17). This strain was isolated from a patient with severe gastritis and has demonstrated high expression for an OMP with an apparent MW of 33–34 kDa corresponding to the OipA protein. The *oipA* gene from the S15 strain was cloned and subsequently expressed in *Escherichia coli* BL21 (DE3), as previously described (17). The sequence of the *oipA* gene consisted of 924 bp (GenBank: KJ816695). The SDS-PAGE profile of the purified recombinant OipA protein as well as its western blot results with a specific anti-*H. pylori* effect demonstrated a band with an apparent MW of 33–34 kDa (17). This purified recombinant protein was used as an antigen in the vaccination of the mouse model.

2.2. Adjuvant preparation and bacterial growth condition

An Iranian propolis sample, prepared by Sepahan ASAL, Isfahan (Iran), from colonies of honeybees located in Isfahan, was used as the natural adjuvant. For adjuvant preparation, 1 g of propolis was ground and mixed with 50 mL of ethanol (70%), then stirred at room temperature for 24 h; the extract was filtered and the solvent was evaporated under vacuum at 50 °C until it was dried.

A clinical *H. pylori* isolate, B19, with status/genotype of *cagA/vacAs1m2* associated with a moderate chronic gastritis, was selected for challenging the mice. Three-day fresh culture of *H. pylori* was obtained on *Brucella* agar (Biolife; Albimi, Milan, Italy) supplemented with 7% sheep blood and antibiotics as previously described (17).

2.3. Mice immunization and challenge procedures

All experiments with mice were in accordance with the UK Animals Scientific Procedures Act of 1986 (86/609/EEC). Pathogen-free 5-week-old female C57BL/6 mice (Razi Institute, Iran) were housed in a clean environment with a constant temperature of 21 ± 2 °C, 55 ± 5% humidity, and a 12-h light/dark cycle as well as free access to food and water. The study was performed in an animal house from the Barij Essence Pharmaceutical Company, Kashan, Iran, in August 2014.

For vaccination of mice with the OipA protein, a dose of 100 μg was selected. This antigenic dose has been used for successful mice immunization (18).

Mice were divided into four groups (10 each). Mice respectively received the recombinant OipA (100 μg/dose), OipA (100 μg/dose) plus propolis (10 mg/dose), propolis (10 mg/dose), or phosphate-buffered saline (PBS) as a control three times (with intervals of 1 week). In each case, the vaccine emulsion was orally administered in a total volume of 200 μL per animal by gavage. One week after the last immunization and before bacterial challenge, blood samples (100 μL) were taken from the tail vein to measure the antibody responses. Groups were challenged three times on three continuous days with 0.2 mL of live *H. pylori* 19B strain (1×10^8 CFU/mL). At the end point of the challenge, the mice were anesthetized by peritoneal injection of 1.43 mg/kg diazepam (Khemidaru, Iran) and 13 mg/kg ketamine 10% (Alfasan, Woerden, the Netherlands). The abdominal cavities of mice were opened and their stomachs were collected, weighed, and divided into two samples. One part was used for histopathological examination and the other was used for determining the CFU number of *H. pylori* and for measurement of urease activity (18).

2.4. Histopathological examination of stomach samples

A longitudinal segment including the antrum and corpus was fixed in 10% neutral buffered formalin and embedded in paraffin. Thereafter it was sectioned by standard methods and stained with hematoxylin and eosin (H&E) to score inflammation and stained with Giemsa stain to visualize *H. pylori* (19,20). The samples were graded according to two methods: the modified Sydney system protocol (protocol I) and a protocol (protocol II) adopted from Chen et al (21). Protocol I consisted of evaluation of chronic inflammatory cell infiltration density, classifying *H. pylori*-related gastritis into none (0), mild (1), moderate (2), and severe (3). Protocol II consisted of classifying the presence of inflammatory cells as follows; 0: none, 1: less than 10 in each high power field, 2: >10 cells/high power field, 3: some areas with thick cell infiltration, 4: diffuse and dense cell infiltration, 5: presence of dense chronic inflammatory cells in nearly all parts of the entire mucosa such that they separate the gastric glands, and 6: entire mucosa contains a dense chronic inflammatory cell infiltrate.

2.5. Measurement of the anti-OipA IgA response

A 96-microwell plate (Nunc GmbH, Germany) was coated with 100 μL of recombinant OipA (50 μg/mL in 0.05 M carbonate buffer, pH 9.6) by incubation at 4 °C overnight. The wells were washed 3 times with PBS + Tween 20 (0.05 v/v). A blocking solution (200 μL) containing 1% w/v bovine serum albumin (BSA) was added and incubated at 37 °C for 1 h. After washing, 50 μL of serially diluted

mouse serum (1:100 in PBS + BSA) was added to the wells and incubated at 37 °C for 1 h. Wells were washed 5 times and then diluted (1/4000) peroxidase-conjugated goat antimouse IgA (Sigma-Aldrich) was added to the wells and they were incubated at room temperature for 1.5 h. After washing, 100 µL of OPD hydrogen peroxidase substrate (Sigma-Aldrich) was added to the wells and they were incubated in the dark. After 15 min of incubation, the reaction was stopped by sulfuric acid (2.5 N) and the absorbance was measured at 490 nm.

2.6. Statistics

The significance of difference between the number of bacteria (enumerated by CFU/g) as well as the score of inflammation obtained for the four groups of challenged mice were analyzed by SPSS 17 (Chicago, IL, USA). The Student t-test was used to compare the differences between mouse groups and the P-values were calculated. The graphs were drawn with GraphPad software (GraphPad Prism 6).

3. Results

3.1. Bacterial load in mouse stomachs

Bacterial loads in the stomach of mice vaccinated with OipA, propolis alone, or OipA + propolis and the nonvaccinated mice were determined by CFU enumeration (Table 1).

Comparison of bacterial loads in the stomachs of vaccinated mice with those of the controls (nonvaccinated) showed protection against *H. pylori* colonization. Highest protection was observed for the OipA group, followed by propolis. The least protection was observed for the mice vaccinated with propolis + OipA.

The statistical comparison of various groups (Figure 1) confirmed the protective effect of vaccination with OipA against *H. pylori* colonization. While propolis had a partial protective effect on *H. pylori* colonization, its combination with OipA decreased the effective vaccine effect of OipA.

3.2. Inflammation scores in mouse stomachs

The level of chronic inflammatory infiltrates in histopathological sections of gastric mucosa was scored using two protocols (Table 2). A correlation was observed between the results of the two protocols for evaluation of inflammation. Statistical comparison of inflammation scores among the four groups is demonstrated in Figure 2. This comparison confirms the protective effect of vaccination with OipA against *H. pylori*-related inflammation. A significant difference ($P < 0.05$) was also observed between the score of the control (nonvaccinated) group and the propolis as well as the propolis + OipA groups. Consistent with the results of bacterial load, vaccination of mice with OipA + propolis reduced the most efficient effect of OipA in diminishing the inflammation.

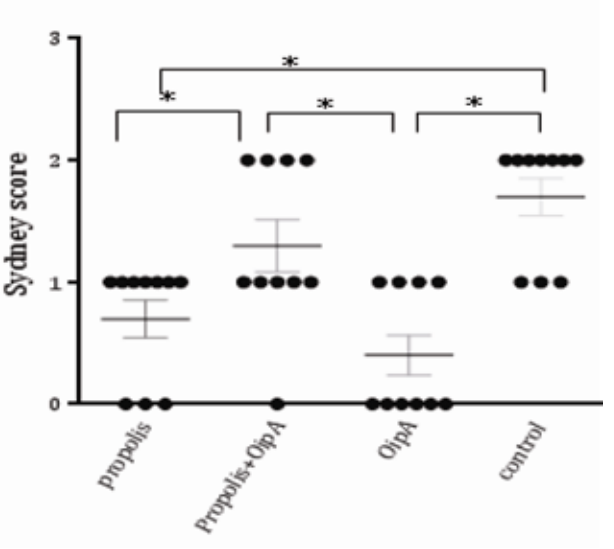

Figure 1. Statistical comparison of bacterial load in stomachs of infected mice among four groups. *: Reduction in bacterial load (CFU/g) was significant ($P < 0.05$).

3.3. Measurement of IgA amount in mice groups

A significant ($P < 0.0001$) difference was observed between the anti-OipA IgA titers produced in mice vaccinated with OipA and the control. A significant difference was also observed between the anti-OipA IgA titers produced in

Table 1. Bacterial load in three groups of mice compared to the control group.

Antigen	Mean CFU/g
Propolis	$6.8 \times 10^5 \pm 432232$
Propolis + OipA	$6.4 \times 10^6 \pm 4.257e{+}006$
OipA	$2.6 \times 10^3 \pm 1941$
Control	$7.7 \times 10^7 \pm 2.680e{+}007$

Mean results were obtained for four groups (each = 10) with standard errors.

Table 2. Inflammation scores determined by two protocols (I and II).

Groups	Protocol II	Protocol I
Propolis	1.6 ± 0.6	0.6 ± 0.2
Propolis+OipA	2.0 ± 0.36	1.3 ± 0.3
OipA	1.1 ± 0.3	0.5 ± 0.18
Control	3.7 ± 0.6	1.5 ± 0.2

Mean results were obtained for four groups (each = 10) with standard errors.

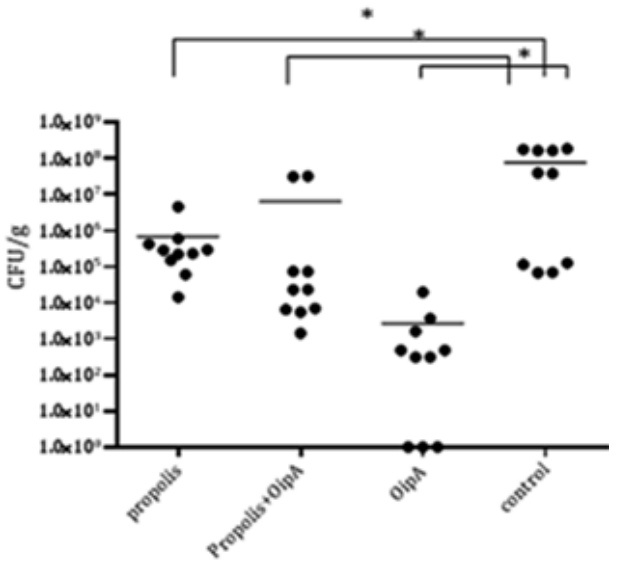

Figure 2. Statistical comparison of inflammation score among four groups by protocol I. *: The reduction in inflammation score was significant ($P < 0.05$).

the mice vaccinated with OipA + propolis and the control (Figure 3). No significant difference was observed between the titers of anti-OipA IgA between either the propolis + OipA and propolis groups or between the propolis and control groups ($P > 0.05$).

3.4. Microscopic observation of stained sections

Microscopic evaluation of H&E-stained and Giemsa-stained sections (Figure 4) showed a correlation between the rates of *H. pylori* colonization and inflammation scores. Higher colonization of *H. pylori* was associated with higher inflammation and vice versa. Densities of bacteria were evaluated microscopically in the stomach sections stained with Giemsa stain and were correlated with CFU enumeration.

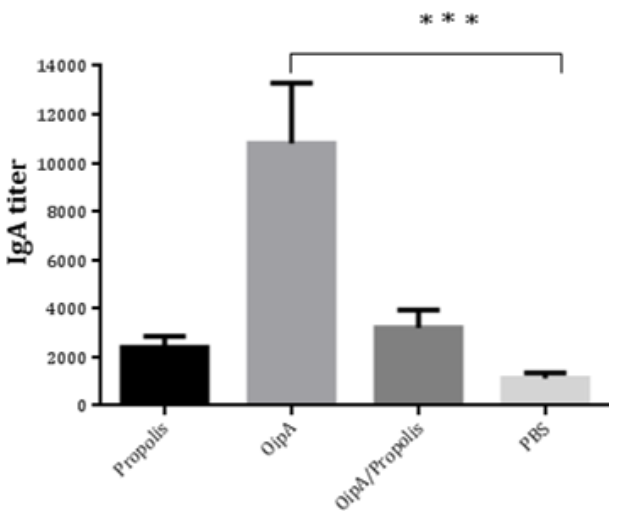

Figure 3. Quantitative evaluation of anti-OipA IgA in serum of vaccinated mice by ELISA method. ***: Significant at a level of 0.0001.

4. Discussion

The first consequence of interaction between *H. pylori* and the host's natural immune system is chronic inflammation of the gastric mucosa. Both cellular and humoral adaptive immune responses may be developed against *H. pylori* infection (22). It was proposed that efficient protection against *H. pylori* infection following immunization is mediated by Th1 effector cells via production of proinflammatory cytokines: interferon gamma (IFN-γ) and tumor necrosis factor (TNF)-α and -β (23,24).

Multiple efforts have been performed for obtaining an efficient vaccine candidate against *H. pylori* infection. They include whole cells or antigens such as urease, catalase, VacA, CagA, NapA, GroES, AlpA, BabA, and HpaA (25–31). Vaccination of mice with VacA induced erosions and it may not be an ideal vaccine candidate. Furthermore, there is concern about CagA, since it can affect a multitude of host cellular pathways, which may activate nondesirable host cell signaling cascades (32–34). Urease alone as a vaccine candidate may not be favorable, since *H. pylori* possesses other ammonia-producing enzymes, including two aliphatic amidases, AmiE and AmiF (35). Furthermore, nonurease-producing *Helicobacter pylori* strains have been isolated from chronic gastritis cases (36). Moreover, immunization of human volunteers with urease has shown that oral administration of urease alone did not modify *H. pylori*-mediated gastric mucosal inflammation (37).

In accordance with our results concerning the protective effects of OipA, three studies evaluated its vaccine potency in a mouse model under DNA vaccine (38–40). In the first study, the investigators used an *oipA* gene encoded DNA construct for vaccination of C57BL/6 mice and observed efficient results including less bacterial colonization of *H. pylori* after vaccination (38). They also examined the effects of IL-2 and the B subunit heat-labile toxin *Escherichia coli* gene encoded DNA constructs as adjuvants plus the *oipA* gene and observed a positive modulation of immune response to the Th1 effector immune response in mice. The second study, investigating the effect of OipA as a vaccine in mice, described usage of *Salmonella typhimurium* for expressing an optimized *oipA* gene for vaccination (39). In the last study by the same group, they studied the efficiency of a novel DNA vaccine based on an attenuated *Salmonella typhimurium* bacterial ghost (SL7207-BG) delivering *H. pylori oipA* DNA. They observed that oral administration of the *oipA* DNA vaccine to mice caused significantly higher levels of IgG2a/IgG1 antibodies and IFN-γ/IL-4

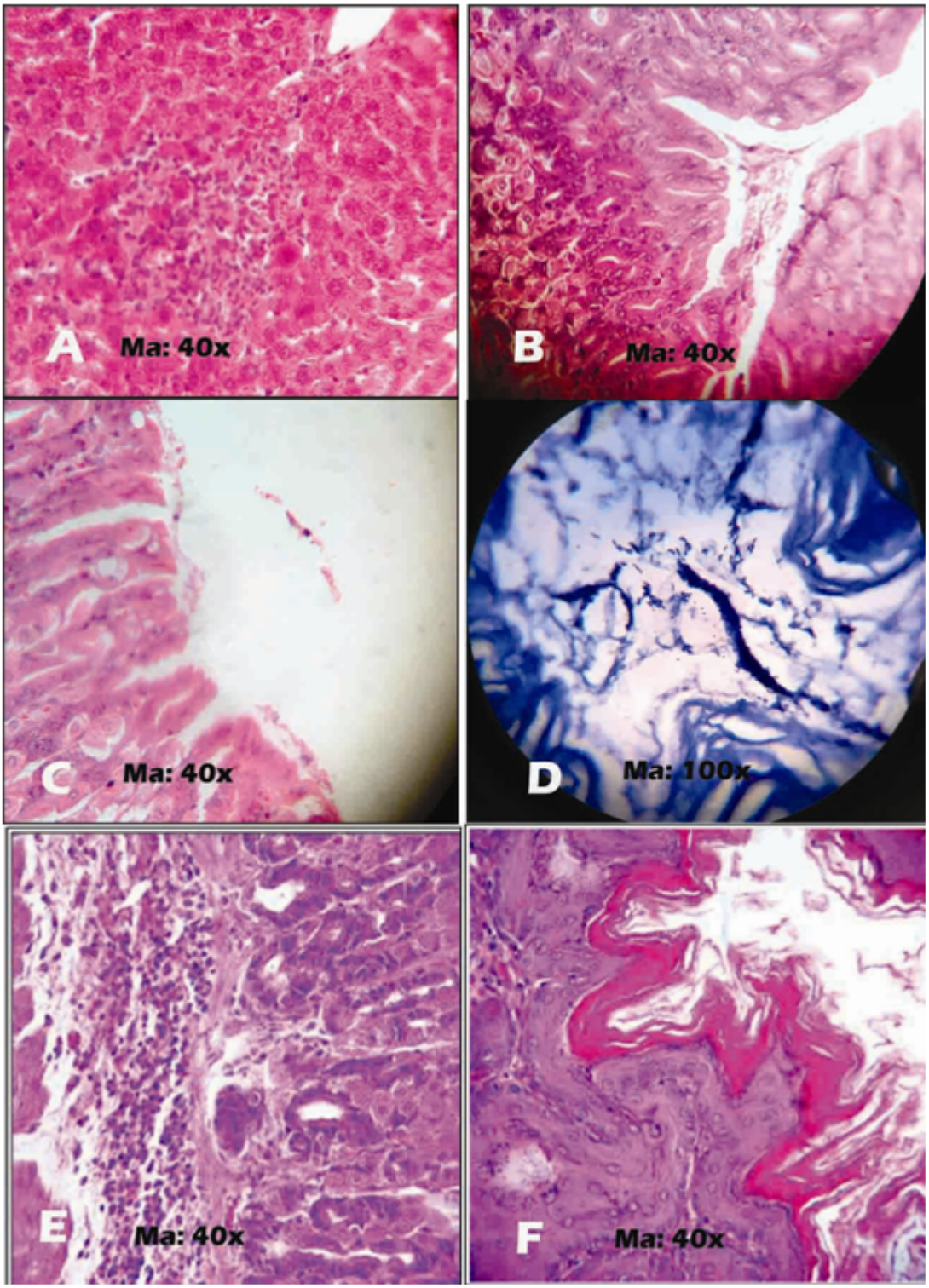

Figure 4. Microscopic evaluation of H&E- and Giemsa-stained sections. A) Mild infiltration of lymphoplasmocytes (propolis + OipA); B and C) no inflammation (mice vaccinated with OipA, propolis); D and E) bacterial colonization and infiltration of lymphoplasmocytes in nonvaccinated mice (control), respectively; F) normal mucosa observed in the mice vaccinated with OipA.

cytokines, indicating a mixed Th1/Th2 immune response and decreased bacterial colonization in the vaccinated mice (40). Although their results showed some protection against *H. pylori* in vaccinated mice, there may be some concerns related to the use of pathogenic bacteria such as *S. typhimurium*.

We observed that vaccination of mice with OipA significantly diminished the number of colonized bacteria in C57BL/6 mice and also diminished the *H. pylori*-related inflammation. Reduction of the bacterial load in mouse stomachs correlated with a significant difference ($P < 0.0001$) in the amount of anti-OipA IgA titers between the OipA-vaccinated and control groups. However, a lower difference was observed between the anti-OipA IgA titers in mice vaccinated with OipA + propolis and the controls, suggesting that propolis affected the induction of the most effective immune response towards the OipA antigen.

The dominant role of Th1-related immune response via production of IL-12, IFN-γ, and IL-18 in protection of the host against *H. pylori* infections has been recognized (23,41–43). The presence of high titers of IgA in mucosally vaccinated animals in this work may explain the role of anti-OipA IgA in protection of mice against *H. pylori* infection. Although IgA was measured in mouse serum, its significant increase in mouse serum may correlate with its significant presence in mouse stomach mucosa.

Usage of propolis in this work was based on its potential adjuvant activity since it was proposed that propolis improves humoral and cellular immune responses, especially Th1-related immune response (14,44,45). Observation of unexpected adverse effects of propolis towards OipA in this work (Tables 1 and 2; Figures 1 and 2) may be due to two things. The first possibility is that its phenolic, flavonoid, or other compounds attach to OipA and partially affect the antigenic structure of OipA, and the second possibility is that propolis affected the induction of appropriate cytokines and thereby prevented the effective immune response (16,46,47). Evaluation of the anti-OipA adverse effects of propolis from various fractions can help to clarify theses hypotheses.

The results of the present work support the choice of OipA as a component of oral vaccine candidates against *H. pylori* infection. It also indicates the importance of mucosal immunity in protection of the host against *H. pylori* infection.

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