Childhood *Helicobacter pylori* infection in a murine model: maternal transmission and eradication by systemic immunization using bacterial antigen–aluminium hydroxide

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SUMMARY

In humans, transmission of *Helicobacter pylori* is thought to occur largely during childhood. Infected mothers are generally considered to be the main source of the pathogen. However, little is known about when and how often maternal transmission of *H. pylori* occurs during childhood. In the present study, we examined these issues in an experimental murine model. Pregnant C57BL/6 mice, infected experimentally with *H. pylori*, delivered and nursed their litters. The stomachs of the infants were isolated and assessed for transmission of *H. pylori*. We also investigated the effect of systemic immunization using *H. pylori* antigen–aluminium hydroxide (AlOH) with regard to providing anti-*H. pylori* immunity and eradicating the maternally transmitted bacteria in infants. Polymerase chain reaction (PCR) was used to examine the presence of transmitted bacteria and their eradication. Maternal transmission of *H. pylori* varied widely during the nursing period, but almost all litters showed bacterial transmission at 2 weeks postpartum. Systemic immunization with bacterial antigen–AlOH eradicated the bacteria in most litters; this immunization induced a local decrease of Th2 cytokines and a local increase of Th1 cytokines in the gastric tissue, as determined by ELISA. Our results indicate that our *H. pylori* vaccine provides not only protection, but also eradication of the already transmitted *H. pylori*.

Keywords aluminium hydroxide (AlOH)   experimental murine model   *Helicobacter pylori*   maternal transmission   systemic immunization

INTRODUCTION

Helicobacter pylori is a Gram-negative, spiral-shaped, microaerophilic bacterium that infects the human gastric mucosa [1]. Chronic infection is thought to be associated with chronic active gastritis, peptic ulcer and gastric malignancies, such as mucosa-associated B cell lymphoma and adenocarcinoma [2]. In particular, this organism has been categorized as a class I carcinogen by the World Health Organization [3] and previous studies have confirmed that long-term infection with *H. pylori* induces adenocarcinoma in Mongolian gerbils [4,5].

Young children are particularly vulnerable to infection by transmission of *H. pylori* from their infected parents, especially infected mothers [6,7], and it is generally believed that such transmission is influenced by socio-economic status. However, little is known about how and when maternal transmission occurs during childhood, especially whether this occurs before or after weaning.

If prolonged and chronic *H. pylori* infection causes gastric cancer, an *H. pylori* vaccine should be targeted at children as a prophylaxis against cancer. Gottwein *et al*. [8] noted that prophylactic vaccination of infants might prevent *H. pylori* infection and the associated long-term consequences that occur in adults, and confirmed that systemic immunization with aluminium hydroxide (AlOH), already approved for human children, is effective at inducing protective anti-*H. pylori* immunity in mice [8]. However, these investigators did not examine the effects of transmission of *H. pylori* before immunization and did not determine whether such immunization can eradicate previously transmitted bacteria.

The present study was designed to examine the incidence and mechanisms of maternal transmission of *H. pylori* in an experimental murine model. Furthermore, we evaluated the effects of systemic immunization using AlOH to eradicate maternally transmitted *H. pylori* through the stimulation of production of cytokines by gastric tissues.

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Table 1. PCR primers used in the present study and derived from the following species-specific genes

Ure A gene		
First primer	Sense	5′-ATATTATGGAAGAAGCGAGAGC-3′
	Antisense	5′-ATGGAAGTGTGAGCCGATTTG-3′
Second primer	Sense	5′-CATGAAGTGGGTATTGAAGC-3′
	Antisense	5′-AAGTGTGAGCCGATTTGAACC-3′
26 KDa gene		
First primer	Sense	5′-TGGCGTGTCTATTGACAGCGAGC-3′
	Antisense	5′-CCTGCTGGGCATACTTCACCATG-3′
Second primer	Sense	(same as the first sense primer)
	Antisense	5′-GATCACTGCATGTCTTACTTTCATGTTTTT-3′

MATERIALS AND METHODS

Mice

Specific-pathogen-free (SPF) C57BL/6 mice were purchased from Seac Yoshitomi (Yoshitomi-cho, Fukuoka, Japan). Mice were housed under SPF conditions and provided with food and water *ad libitum*. All procedures described in the present study were conducted in accordance with the guidelines of the Ethical Committee for Animal Experiments of Oita Medical University, Oita, Japan.

Bacteria

H. pylori (Sydney strain 1) stocked in our laboratory was used for *H. pylori* infection and immunogen preparation. It was grown at 37°C for 48 h with continuous shaking in Brucella broth supplemented with 10% horse serum in microaerobic conditions (5% O_2, 10% CO_2, 85% N_2). For quantitative analysis, *H. pylori* was cultured on M-BHM pylori agar medium (Nikken Biomedical Laboratory, Kyoto, Japan) under the same conditions for 5 or 6 days. The detection limit of the quantitative assay was 1×10^2 colony forming units (CFU)/g gastric tissue.

Mice were inoculated twice at 3-day intervals by oral administration of 1×10^8 CFU of *H. pylori* suspended in 0·5 ml of Brucella broth. At the final examination, the challenged mice were sacrificed and confirmed to be *H. pylori*-positive by culture of gastric tissue. *H. pylori*-negative mice were excluded from the following study.

Adult-adult transmission of H. pylori

Each animal group consisted of six mice. One mouse in each group was challenged with *H. pylori*, housed separately for 4 weeks and then co-raised with five unchallenged mice in a laboratory cage. Mice were sacrificed after co-habitation for 3 weeks. The stomach of each mouse was dissected out and homogenized in 0·5 ml sterile saline.

Maternal transmission of H. pylori

One week after challenge with *H. pylori*, three female infected mice and one male mouse in each group were transferred to one cage for mating. As soon as a female mouse was confirmed to be pregnant, one was separated from the group and cared for until delivery. After birth at full term, the families were divided into four maternal-transmission groups and three immunization groups. In the maternal-transmission groups, the mother and litters in each group were sacrificed at 2 days, 1 week, 2 weeks and 3 weeks postpartum. The gastric specimen was isolated as described above. In the immunization groups, litters were weaned after 3 weeks of breastfeeding, and then the mother was

Table 2. Amplification cycles in the first and second PCR using the primers listed in Table 1

First PCR				Second PCR
Ure A gene				
First cycle		First cycle		
94°C	2·0 min	94°C		2·0 min
40 cycles		40 cycles		
94°C	1·0 min	94°C		1·0 min
54°C	1·5 min	58°C		1·5 min
72°C	1·5 min	72°C		1·5 min
Final cycle		Final cycle		
72°C	5·0 min	72°C		5·0 min
26 KDa gene				
First cycle		First cycle		
94°C	2·0 min	94°C		2·0 min
40 cycles		40 cycles		
94°C	1·0 min	94°C		1·0 min
54°C	1·5 min	56°C		1·5 min
72°C	1·5 min	72°C		1·5 min
Final cycle		Final cycle		
72°C	5·0 min	72°C		5·0 min

sacrificed. In the negative control group, the litters were nursed by an uninfected mother mouse, and they were sacrificed 3 weeks postpartum.

Isolation of H. pylori

Aliquots (100 μl) of homogenate were cultured on M-BHM pylori agar medium plates and the plates were incubated under the aforementioned conditions. Isolated colonies of *H. pylori* were examined by a standard RUT assay as described previously [9] to confirm *H. pylori* infection. The remainder of the homogenate was used for the following polymerase chain reaction (PCR) procedure.

PCR

Bacterial DNAs were extracted from the above homogenate by the SDS-proteinase K method using cetyltrimethylammonium bromide (CTAB) [10]. The PCR primers used in the present study were derived from the *Ure A* and *26 kDa* genes (Table 1), both of which have been used previously [11–13]. *Taq* polymerase (1·25 U; Promega, Madison, WI, USA) was added and mixed to give a final 50-μl reaction mixture. PCR (first and nested) was performed with an automatic thermal cycler (PC-700, Astec Inc., Fukuoka, Japan) (Table 2). The PCR products were visualized by

gel electrophoresis. Mice were considered to have been colonized with *H. pylori* via transmission when PCR (by at least one kind of primer) or culture was positive.

Immunization

H. pylori antigen (Hp), which we have demonstrated to be an effective experimental vaccine antigen by the oral route, was generated in our laboratory, as described previously [14]. Ovalbumin (OVA) (Sigma Chemical Co., St Louis, MO, USA) was used as a control antigen. Aluminium hydroxide (AlOH) (Imject Alum, Pierce, IL, USA) was used as the adjuvant. Antigen (100 μg) in aqueous solution (50 μl), mixed with 50 μl of adjuvant, was injected intraperitoneally. Hp–AlOH was injected into half of the litters from each family in the immunization groups, while OVA–AlOH was injected into the remaining members. The injection was performed twice: at 3 and 4 weeks of age. In addition, one control group that was not vaccinated was included for cytokine measurements.

Determination of vaccine efficacy

Mice were sacrificed 2 weeks after the last vaccination. One half of the stomach, to be prepared for cytokine measurement, was frozen immediately in liquid nitrogen. The other half, to be prepared for the determination of vaccine efficacy, was treated in a manner similar to that described in the section 'Isolation of *H. pylori*'. Eradication of *H. pylori* was considered successful when both PCR and culture were negative.

Cytokine measurement

The concentrations of interleukin [IL]-4, IL-10, interferon [IFN]-γ and IL-12 in the gastric tissue of the above mice were measured by enzyme-linked immunosorbent assay (ELISA). First, each frozen specimen was homogenized with 2 ml of phosphate-buffered saline (PBS) containing 0·1% sodium dodecylsulphate (SDS) and 0·1% Tween 20 [15]. After overnight storage at −20°C, the sample was centrifuged at 400 *g* for 15 min at 4°C. The supernatant was analysed with mouse IFN-γ ELISA kit (MedSystems Diagnostics GmbH, Vienna, Austria) and the OptEIA™ set for mouse IL-4, IL-10, and IL-12 (BD Biosciences Pharmingen, San Diego, CA, USA). The results were expressed as concentration per mg protein. Protein levels were measured using *Dc* protein assay reagents (Bio-Rad Technologies, Richmond, CA, USA).

Statistical analysis

Statistical analysis was carried out using Statview software. The χ^2 test was used to assess differences in the transmission rate between maternal-transmission groups. The eradication rate in the Hp–AlOH-vaccinated group was compared to the OVA–AlOH-vaccinated group using the Fisher's exact test. The unpaired *t*-test was used to analyse differences in cytokine production. A *P*-value of <0·05 was considered to be statistically significant.

RESULTS

The mouse strain C57BL/6 has often been used as a murine model of *H. pylori* immunization. In the present study, we used the same mouse strain not only as a vaccination but also as a transmission model. First, we investigated the stage at which maternal transmission occurs, and compared its frequency with that of adult–adult transmission. While culture of the

bacterium is one of the gold standards in the diagnosis of *H. pylori* infection, in the present study it was not considered to be ideal for determination of *H. pylori* transmission for two reasons: (i) only small amounts of bacteria were suspected to colonize the stomach and (ii) the detection limit of the quantitative culture assay was 1×10^2 CFU/g gastric tissue. Therefore, we also used the PCR method along with the culture method to detect small numbers of *H. pylori*. Maternal transmission was examined at 2 days, 1 week, 2 weeks and 3 weeks postpartum (corresponding to colostrums, transitional milk, mature milk and weaning stage, respectively). Each stage group was composed of three or four families.

The frequency of maternal transmission fluctuated during the nursing period, but it was noteworthy that *H. pylori* was detected in almost all (15/16) 2-week-old-mice (Table 3). The transmission rate at 2 weeks postpartum was significantly higher than at 1 week postpartum ($P < 0.001$). Furthermore, the rate at 3 weeks postpartum (44%) was significantly lower than at 2 weeks postpartum ($P = 0.002$), but the 3-week rate tended to be higher than the adult–adult transmission rate after the same co-habitation period (20%), albeit statistically insignificant ($P = 0.16$). *H. pylori* was not detected in any litters of the negative control group.

Because these experiments suggested frequent maternal transmission, we next determined whether systemic immunization with AlOH, which has been approved for use in human children, is efficacious in treating these litters. Gottwein *et al.* [8] showed that immunization of C57BL/6 mice with this adjuvant can confer protection against *H. pylori* infection of the gastric mucosa, and that immunity with this adjuvant can induce splenocytes in these mice to specifically produce Th2 cytokines (IL-5), as measured by ELISPOT assay. In our next series of experiments, we tested whether immunization using this adjuvant could eradicate already transmitted *H. pylori*. We systemically immunized C57BL/6 litters, weaned from *H. pylori*-infected mothers, with *H. pylori* antigens emulsified in AlOH. Two weeks later, the presence or absence of *H. pylori* was examined by culture and PCR. It was found that systemic immunization with AlOH resulted in significant eradication of the pathogen, as determined by the almost complete absence of *H. pylori* (Table 4). While six of eight control

Table 3. Transmission rate of *H. pylori* in respective transmission groups

Group	Co-raising period	Culture	Ure A	26 KDa	Total[d]	P[e]
			PCR			
MT[a] (0)	2 days	0/19	12/19	0/19	12/19	⎫ f
MT (1)	1 week	0/25	9/25	0/25	9/25	⎬ g
MT (2)	2 weeks	0/16	15/16	13/16	15/16	⎬ h
MT (3)	3 weeks	0/18	4/18	6/18	8/18	⎬ i
AT[b]	3 weeks	0/20	2/20	4/20	4/20	
NC[c]	3 weeks	0/7	0/7	0/7	0/7	

[a]MT, maternal transmission group. [b]AT, adult-adult transmission group. [c]NC, negative control group. [d]Mice with positive transmission of *H. pylori* as confirmed by culture or PCR (*Ure A* or *26 KDa*). [e]Differences in the transmission rate between MT groups were evaluated by the χ^2 test. Differences in the transmission rate between MT (3) group and AT group were evaluated by Fisher's exact test. [f]$P = 0.074$; [g]$P < 0.001$; [h]$P = 0.002$; [i]$P = 0.16$.

mice (75%) continued to exhibit gastric colonization with *H. pylori*, eight of nine mice (89%) immunized with Hp–AlOH showed eradication of the pathogen. The difference in the cure rate between the Hp–AlOH immunized and OVA–AlOH immunized litters was significant (*P* = 0·015).

Table 4. Detection rate of *H. pylori* in immunization groups

Group	Immunization[a]	Culture	PCR		Total[e]	*P*[f]
			Ure A	*26 kDa*		
A	Hp[b]: AlOH[d]	0/9	1/9	0/9	1/9	⎱ [g]
B	OVA[c]: AlOH	0/8	6/8	2/8	6/8	⎰

[a]A total of 100 μg of Hp or OVA with AlOH, as specified; [b]Hp, *Helicobacter pylori* antigen; [c]OVA, ovalbumin; [d]AlOH, aluminium hydroxide. [e]Eradication was defined as the complete absence of detectable bacteria. Two weeks after the second vaccination, litters of the immunization groups were examined for the eradication of *H. pylori* by culture and PCR. [f]Differences in postimmunization detection rates were evaluated by Fisher's exact test. [g]*P* = 0·015.

To explore the mechanism(s) for the anti-*H. pylori* effects of the Hp–AlOH vaccine, we measured the production of Th1 (IFN-γ, IL-12) and Th2 cytokines (IL-4, IL-10) by gastric tissue using ELISA (Fig. 1) and evaluated the balance of Th1 and Th2 immunity in the stomach, but not in the spleen. There was no significant difference in the production of IL-4 and IFN-γ between Hp–AlOH-vaccinated, OVA–AlOH-vaccinated and non-vaccinated mice, although the production of IFN-γ in Hp–AlOH-vaccinated mice tended to be higher than in the non-vaccinated mice (*P* = 0·079). In contrast, the production of IL-10 in Hp–AlOH-vaccinated mice was significantly lower than in non-vaccinated mice (*P* = 0·026), and production of IL-12 in Hp–AlOH-vaccinated mice was significantly higher than in non-vaccinated mice (*P* = 0·0067), although the production of both cytokines in OVA–AlOH-vaccinated mice was almost similar to that in Hp–AlOH-vaccinated mice. Furthermore, only one mouse (Fig. 1, open square) failed to show complete eradication of *H. pylori* by Hp–AlOH-immunization and, interestingly, production of IL-4 and IFN-γ in this mouse was the lowest among the Hp–AlOH-immunized mice. Thus, in the present study, a specific induction of Th2 immunity by vaccination with AlOH, as detected by Gottwein *et al.* [8] using the ELISPOT assay, was not observed when using ELISA and gastric tissues here. In contrast,
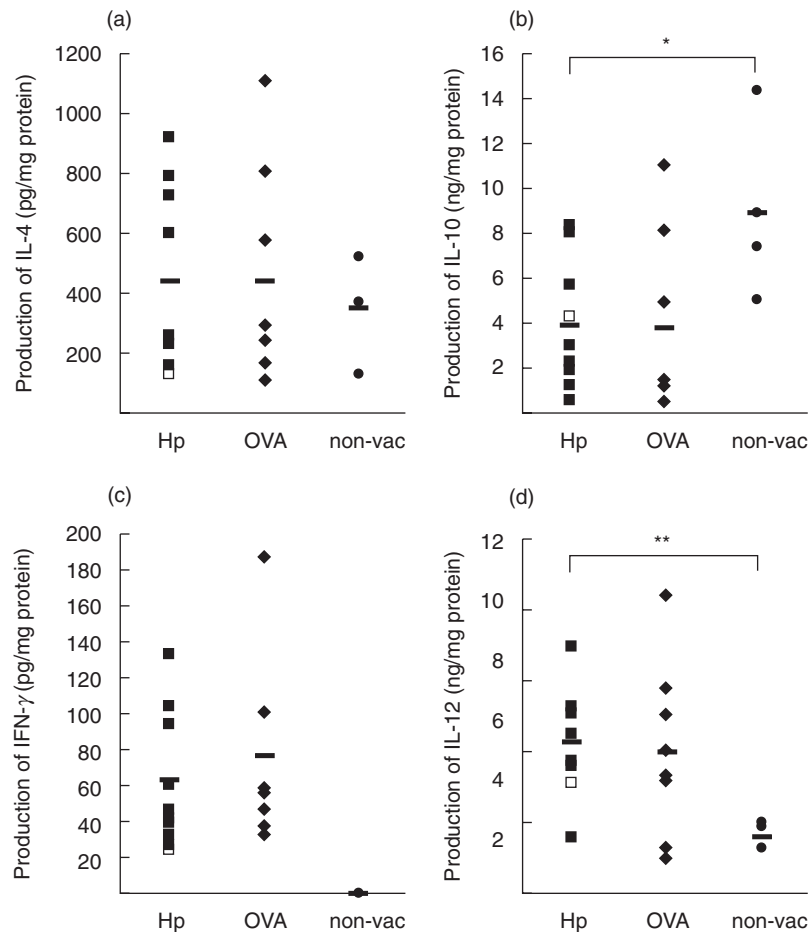

Fig. 1. Production of (a) interleukin (IL)-4, (b) IL-10, (c) interferon (IFN)-γ and (d) IL-12 in the gastric tissue of *H. pylori*-A1OH-inmmunized (Hp, ■), OVA-A1OH-immunized (OVA, ◆) and non-vaccinated (non-vac, ●) mice. Open squares (□): a single mouse that failed to show a positive response to *H. pylori*-A1OH immunization. Horizontal bars indicate mean value of each cytokine. *P = 0·026; **P = 0·0067.

immunization with AlOH resulted in induction of Th1 immunity in the gastric tissue.

DISCUSSION

The mouse is not the natural host for *H. pylori* so the results obtained in this study may not apply to humans. On the other hand, data from human children are limited, because most *H. pylori*-infected children have no symptoms and it is difficult for a paediatrician to examine such asymptomatic children invasively. In the present study, we analysed the stomachs of many infant mice directly and we believe that it is meaningful to use our results to speculate when *H. pylori* infection occurs in human children.

The five major findings of the present study are the following: (1) maternal transmission of *H. pylori* is almost complete during the breastfeeding period; (2) the incidence of maternal transmission of *H. pylori* varied during the nursing period; (3) the incidence of maternal transmission tended to be higher than that of adult–adult transmission; (4) *H. pylori* vaccine not only protected against infection but also eradicated already transmitted bacteria; (5) parenteral immunization with AlOH resulted in a lowered production of Th2 cytokines and a higher production of Th1 cytokines in the stomach.

The results of the maternal-transmission experiment indicated that maternal transmission of *H. pylori* was almost complete at a certain stage (mature milk) of the breastfeeding period. Recent epidemiological studies in humans suggest that the acquisition of *H. pylori* occurs during childhood. For example, Rothenbacher *et al.* [6] reported that *H. pylori* acquisition seems to occur mainly between the first and second year of life: that is, after the age of weaning. This conclusion conflicts with our results, although our study was conducted in a mouse model, not in humans. We consider that the cause of this discrepancy is as follows: Rothenbacher *et al.* [6] applied an *H. pylori* antigen enzyme immunoassay using stool samples (HpSA test; Meridian Diagnostic Inc.) to define current infection status, while we used a PCR method and gastric tissue for the same purpose. Although the HpSA test is thought to have higher sensitivity, especially in young children, than serological methods [16,17], our method is more sensitive for the detection of bacteria. On the other hand, a number of studies have indicated that lactoferrin [18], sialyllactose [19], oligosaccharides [19] and secretory IgA antibody [20], present naturally in milk, have a bacteriostatic effect on *H. pylori* infection. Thus, we hypothesize that maternal transmission is often established within the breastfeeding period, but the bacterial burden is below the level of detection of the HpSA test because of the bacteriostatic effect of milk. Presumably in humans, especially individuals who live in better sanitary conditions, maternal transmission before the weaning period is not as frequent as in mice. However, in the presence of poor sanitary conditions a more frequent maternal transmission is likely to occur during the nursing period.

Our results also showed that the maternal-transmission rate of *H. pylori* fluctuated during the observation period, that is it decreased from 2 days to 1 week postpartum (albeit insignificantly), increased from 1 to 2 weeks postpartum and decreased from 2 to 3 weeks postpartum. We think that the first decrease is due to spontaneous eradication of *H. pylori* by the increased immunity of the neonatal stomach. The middle increase was probably due to the longer period of maternal–neonate contact, while

the last decrease could be explained by the reduced maternal–newborn contact during the weaning stage combined with spontaneous eradication of *H. pylori*. Perri *et al.* [21] reported that eradication of *H. pylori* occurs spontaneously during human childhood, and that *H. pylori* infection shows a fluctuating pattern. Our results are in agreement with this report.

The results of our control studies, i.e. adult–adult transmission studies, did not indicate any significant difference in the rates of adult–adult transmission and maternal transmission. It is possible that these results were due to the experimental design: we compared the rates in the two groups three weeks after co-habitation, i.e. at the time of reduced maternal transmission. However, the high maternal-transmission rate at 2 weeks postpartum (94%) suggests that the incidence of mother–child transmission could be higher, at least occasionally, than that of adult–adult transmission. Rothenbacher *et al.* [7] reported that infected parents, especially infected mothers, play a key role in the transmission of *H. pylori* within families. Maternal contact behaviour during the breastfeeding period may be responsible for the high frequency of maternal transmission.

It is not known whether infant mice develop gastric inflammation, and this is technically difficult to investigate because the stomachs of infant mice are too small to prepare histological specimens. We have some preliminary data on the histology of infant mice right after weaning, i.e. 3 weeks postpartum mice, and they show no inflammation. We conjecture that infant mice (<3-week mice) also do not have gastritis.

The above results indicate that an *H. pylori* vaccine should be considered not only as protective against *H. pylori* infection, but also able to eradicate the already transmitted pathogen. Our vaccination studies showed that systemic immunization with AlOH resulted in eradication of maternally-transmitted *H. pylori*. These results are compatible with those of Gottwein *et al.* [8], who reported that AlOH-induced systemic immunity could induce protective immunity in mice. In this regard, AlOH is the only adjuvant available for use in human children, and it is a very promising vaccine for this situation. However, we have reported recently that vaccination using AlOH did not reduce bacterial density in *H. pylori*-infected adult mice that had been inoculated with a bacterial suspension and confirmed the presence of *H. pylori* in cultured tissues from these mice [22]. The same result was also noted in young mice in preliminary studies conducted in our laboratories (unpublished results). Considered together, these results suggest that, with regard to eradication of *H. pylori,* the AlOH vaccine is useful only for eliminating a very low load of the bacteria. Furthermore, Lee *et al.* [23] reported that monkeys receiving only systemic immunization of urease plus AlOH failed to respond with respect to protection against the infection, compared with sham-immunized controls. The mechanisms underlying the complex relationship between host and bacteria may function differently in mice and primates. Thus, several questions remain unanswered regarding the effects of the AlOH vaccine, emphasizing the need for further studies before widespread use of this vaccine.

Finally, we examined the mechanisms underlying the beneficial effects of the vaccine seen in our mice. Gottwein *et al.* [8] conjectured that the local release of Th2 cytokines in the stomach after parenteral immunization with AlOH might be effective in recruiting or activating the effector cells that eliminate the bacteria. However, they did not evaluate the dynamics of cytokine production in the stomach, although they did investigate the profile

of cytokines released by splenocytes. In our study, parenteral immunization with AlOH induced a local decrease of the Th2 cytokine IL-10 and local increase of the Th1 cytokine IL-12 in the stomach. These results were quite the opposite to the speculation of Gottwein *et al.* [8]. Our results also indicated that the polarized Th1 immunity was not antigen-specific. One mouse immunized with Hp–AlOH that failed to show eradication of *H. pylori* showed the lowest production of IL-4 and IFN-γ, which are downstream cytokines of IL-10 and IL-12, respectively. These results suggest that antigen-specific immunity followed by minimum production of both types of downstream cytokines in the stomach may play an important role in the eradication of *H. pylori*, while a change in the local cytokine profile induced by immunization with AlOH is not very important.

In conclusion, the present study provides new and important information on maternal transmission of *H. pylori* and systemic immunity with AlOH. Because acquisition of *H. pylori* infection during childhood seems to be a critical risk factor for the later development of gastric cancer, the elucidation of the mechanisms involved in transmission of *H. pylori* during childhood and subsequent improvement of the vaccine that can provide both protective and therapeutic immunity against *H. pylori* are essential.

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