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# Th1-type immune responses by Toll-like receptor 4 signaling are required for the development of myocarditis in mice with BCG-induced myocarditis

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**Abstract**

The immunological aspects of autoimmune myocarditis are difficult to understand because of the existence of many infectious agents and animal models suggesting different mechanisms in autoimmune myocarditis. To overcome these difficulties, two strains of mice, C3H/HeN and C3H/HeJ, showing different immune responses to mycobacteria, were immunized with myosin mixed with BCG. The C3H/HeN mice with a wild-type Toll-like receptor 4 (TLR4) showed severe myocarditis, whereas the C3H/HeJ mice with nonfunctional mutated TLR4 did not. $CD4^+$ cells from both strains of mice exhibited appreciable proliferative responses following myosin stimulation; however, the cytokines from these cells differed between these two strains. The C3H/HeN mice showed T helper (Th)1-type cytokine responses, whereas the expressions of mRNA in C3H/HeJ mice were Th2-type cytokine. When both of these strains of immunized mice were inoculated with a plasmid encoding cDNA of interleukin (IL)-4 or agonistic IL-4, the development of myocarditis was inhibited in C3H/HeN mice. Moreover, C3H/HeJ mice, in which development of myocarditis was not induced by immunization of myosin mixed with BCG, showed myocarditis after injection of IL-4 antagonistic mutant DNA for the induction of Th1-type immune responses. The results suggested that the induction of autoimmune myocarditis by myosin is affected by Th1-type immune responses.


*Keywords:* Autoimmunity; Bacillus Calmette–Guérin; Myocarditis; Th1/Th2; TLR4

## 1. Introduction

Myocarditis is a potentially lethal disorder of various etiologies for which no treatment is currently satisfactory [1]. Although the etiology of dilated cardiomyopathy is unknown, more than 10% of cases are associated with a previous virus infection, such as Coxsackievirus B3 [2]. Since heart failure generally occurs long after infection with autoimmune responses, autoimmunity is thought to play an important role in myocarditis as well as contributing to the progression to cardiomyopathy and heart failure [3]. To explore the mechanisms

*Abbreviations:* DC, dendritic cell; DTH, delayed-type hypersensitivity; EAM, experimental autoimmune myocarditis; TLR, Toll-like receptor.

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of such immune system-mediated damage to the heart in this disease, various animal models have been established by infection of various pathogens and immunization of cardiac myosin (reviewed in [4]). Although animal models of experimental autoimmune myocarditis (EAM) have provided information on pathogenesis that is valuable for the prevention and treatment of myocarditis, an understanding of the pathogenesis of EAM in animal models is difficult to apply to human myocarditis. Animal models of EAM have been established in various species and strains of animals using various types of infectious pathogens and immunization of cardiac myosin, and the pathogenic mechanisms in these models have not shown identical immune responses [5,6]. To overcome these difficulties, animal models of EAM that are established for understanding immune response to myocytes should allow us to identify several factors that induce EAM such as pathogens and the genetic basis of animals.

T helper (Th) cells are thought to have crucial roles in both autoimmune diseases and immunological disorders. Th cells are identified by functions as Th1 or Th2 subsets secreting distinct cytokine patterns that demonstrate effector functions and cross inhibition [7]. Cytokines are important for controlling the response of Th cells to self antigens (Ags) and they play a critical role in shifting the immune response toward a Th1 or Th2 pattern. A Th1 response shifts the cytokine profile toward delayed-type hypersensitivity (DTH), macrophage activation, and proinflammatory T-cell response associated with interferon (IFN)-γ and interleukin (IL)-2 and -12, whereas a Th2 response is associated with B cell activation and humoral immunity and with IL-4, -5, -9, -13 and IgE production. As a result, understanding the Th cell responses to auto-Ags is important for the prevention and treatment of autoimmune diseases such as autoimmune myocarditis in human patients.

Mice with a C3H/He lineage were originally established in 1941, and two laboratories have maintained this strain as C3H/HeN and C3H/HeJ since 1947 and 1951, respectively. These two strains of mice showed different responses to some strains of bacteria, and the differences in the responses to some strains of bacteria have been thought to be caused by Toll-like receptor (TLR) 4 [8]. C3H/HeJ mice have an unfunctional TLR4 [9], and these mice are more susceptible to mycobacteria infection than are TLR4 wild-type mice [10–12]. TLR activation elicits adaptive immune responses with a bias towards Th1 T-cell response. It has also been reported that TLR4 wild-type C3H/HeN mice, but not mutated TLR4 C3H/HeJ mice, showed typical Th1-type immune responses to mycobacteria infection [10,12,13], although both strains of mice have the wild-type Bacillus Calmette–Guérin (BCG) resistant gene *(N-ramp)*.

To elucidate the immunological mechanisms by which cardiac myosin is recognized without various factors, we tried to establish an animal model of EAM by using the responses to mycobacteria in the present study. Two strains of mice, mycobacteria-susceptible C3H/HeJ mice and mycobacteria-resistant C3H/HeN mice, were immunized with porcine cardiac myosin mixed with BCG. Interestingly, mycobacteria-resistant C3H/HeN mice, but not mycobacteria-susceptible C3H/HeJ mice, developed myocarditis. We herein report the differences in immune responses to myosin in the development of an animal model of EAM in mice with a close genetic background.

## 2. Materials and methods

### *2.1. Mice*

Six- to eight-week-old C3H/HeN (TLR4 wild type) and C3H/HeJ (TLR4 mutated) female mice were purchased from CLEA Japan (Osaka, Japan) and housed in the Laboratory Animal Center of Mie University School of Medicine.

### *2.2. Immunization of myosin*

Each mouse was immunized with 100 μg of porcine cardiac myosin (Sigma) mixed with 1 mg of BCG Tokyo strain (Japan BCG Laboratory, Tokyo, Japan) in IFA into the footpad on day 0 and day 14. The BCG used for immunization was well-ground and killed. This myosin and BCG mixture was completely emulsified with IFA. The injection site (footpad) and degree of emulsification are very important for the development EAM. The control mice were injected with the same amount of BCG alone in emulsified IFA. All mice were sacrificed on day 21 for pathological observations (Fig. 1a).

### *2.3. Administration of DNA*

The plasmids encoding cDNA of antagonistic interleukin (IL)-4 double mutant (Q116D/Y119D) (IL-4DM) and agonistic IL-4 single mutant (Q116D) (IL-4SM) have been described previously [14]. The mice were intraperitoneally administered 100 μg of plasmid DNA encoding IL-4, IL-4SM or IL-4 DM on days −7, 0, 7 and 14 to regulate the Th balances. An empty plasmid (pcDNA 3.1) vector was used as a control (Fig. 1b).

### *2.4. Proliferative responses of spleen cells to porcine myosin*

The responding spleen cells obtained from the immunized mice were depleted of $CD4^+$ or $CD8^+$ cells using a commercially available system of magnetic bead-coupled specific antibodies (Abs) to confirm the subset of effector cells. The purity of cells (CD4 or CD8 cells) was confirmed by FACS analysis. The proportion of targeted cells did not exceed 0.01%, and dead cells were removed after cell washing (viability >90%). The cells were resuspended in complete medium and cultured at a concentration of $2 \times 10^5$ cells per culture well in a total volume of 0.2 ml with 10 μg/ml of myosin. The same amount of OVA was used as a control Ag. Each culture was performed in triplicate in 96-well microculture plates and was then maintained in a humidified atmosphere of 5% $CO_2$ at 37 °C. The cultures were harvested using a cell harvester at 96 h after a 6-h pulse with 18.5 kBq/well of [$^3$H]thymidine. The results were calculated from the uptake of [$^3$H]thymidine and expressed as the mean uptake in cpm ± SD of triplicate cultures.

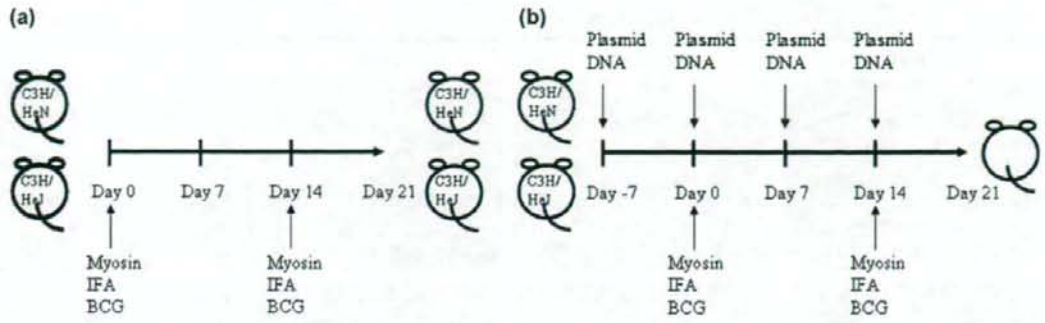


Fig. 1. Experimental design in this study. (a) Each mouse was immunized with 100 μg of porcine cardiac myosin mixed with 1 mg of BCG in IFA into the footpad on day 0 and day 14 (see Section 2). (b) Plasmid DNA of IL-4, IL-4SM, IL-4DM or control was intraperitoneally injected once on days −7, 0, 7 and 14.

### 2.5. Detection of cytokine mRNA from lymphocytes using RT–PCR

Total RNA was purified from the OVA (control)- or myosin-stimulated spleen cells using Isogen (Nippongene, Japan) following the manufacturer's instructions. For the RT reaction, a reverse transcription system (Promega, WI, USA) was used. PCR was performed in a total volume of 50 μl of 1× PCR buffer (Takara Shuzo, Japan) containing 0.5–1.0 μg of cDNA, 0.25 mM of each dNTP, 2 μM of each primer, and 2.5 U of *Taq* DNA polymerase (Takara Shuzo, Japan). The specific primer pairs used were as follows: IL-2, 5′-AAGAT GAACTTGGACCTCTGCGG-3′ (sense) and 5′-CCTTATG TGTTGTAAGCAGGAGG-3′ (antisense); IL-4, 5′-ATGGGT CTCAACCCCCAGCTAGT-3′ (sense) and 5′-GCTCTTTAG GCTTTCCAGGAAGTC-3′ (antisense); IL-12p40, 5′-TCC TGCACTGCTGAAGACATC-3′ (sense) and 5′-TCTCGCCA TTATAGATTCAGAGAC-3′ (antisense); IL-13, 5′-GACCCA GAGGATATTGCATG-3′ (sense) and 5′-CCAGCAAAGTCT GATGTGAG-3′ (antisense); and mouse HPRT, 5′-GATAC AGGCCAGACTTTGTTGG-3′ (sense) and 5′-GAGGGTA GGCTGGCCTATAGG-3′ (antisense). The samples were amplified for 30–35 cycles under the following conditions: annealing for 30 s at 56 °C, extension for 1 min at 73 °C, and denaturation for 30 s at 93 °C. The reaction products were analyzed on 2% agarose, Tris-buffered EDTA TBE gel.

### 2.6. Measurement of interferon-γ (IFN-γ)

Spleen cells from immunized mice ($5 \times 10^6$) were cultured with 10 μg/ml of myosin in 24-well culture plates at a volume of 2 ml. After incubation at 37 °C in a humidified incubator (5% $CO_2$) for 96 h, culture supernatants were quantified by using a standard ELISA kit (BioSource International, CA, USA).

### 2.7. Statistical analysis

Statistical analysis was performed using the Mann–Whitney *U*-test and the Kruskal–Wallis test. The values are expressed as means ± SD. A 95% confidence limit was considered to be significant ($p < 0.05$).

## 3. Results

### 3.1. Development of EAM

Although rodent models of EAM have been established in various species and strains, it is not easy to understand the mechanisms underlying the development of EAM by recognition of autologous myosin Ags because of the effects of genetic backgrounds. To determine whether cardiac myosin immunization can induce EAM in strains of mice with the same genetic background except for TLR4, C3H/HeN and C3H/HeJ mice were immunized with cardiac myosin mixed with BCG. The histological findings were classified into severe (>50%), moderate (10–49%) and mild (<9%) depending on the ratio of affected area to total myocardium. The C3H/HeN mice developed mild (2/20), moderate (14/20) and severe (4/20) myocarditis, whereas only one C3H/HeJ mouse showed mild myocarditis (1/20) by histopathological observations on day 21 (Fig. 2). Two C3H/HeN mice died before sacrifice on day 18 (severe and moderate myocarditis). Moreover, EAM did not develop in either strain of mice immunized with myosin and IFA without BCG, and the control mice immunized with IFA emulsion containing BCG or myosin alone did not show any abnormalities (data not shown). These results demonstrated that immunization of an emulsion containing myosin and BCG induced the development of EAM in wild-type C3H/HeN mice but not in TLR4-mutated C3H/HeJ mice.

### 3.2. Myosin-specific spleen cell proliferative responses in myosin-immunized mice

We next confirmed the presence of effector cells that recognize the myosin induced by the immunization of myosin mixed with BCG in *in vivo* experiments. Spleen cells from both strains of mice immunized with myosin mixed with BCG were assessed for their proliferative responses after stimulation *in vitro* with myosin. Spleen cells from both C3H/HeN mice with myocarditis and C3H/HeJ mice without myocarditis exhibited proliferative responses after *in vitro* stimulation with myosin (Fig. 3). These proliferative responses were not observed in the case of stimulation with an irrelevant Ag

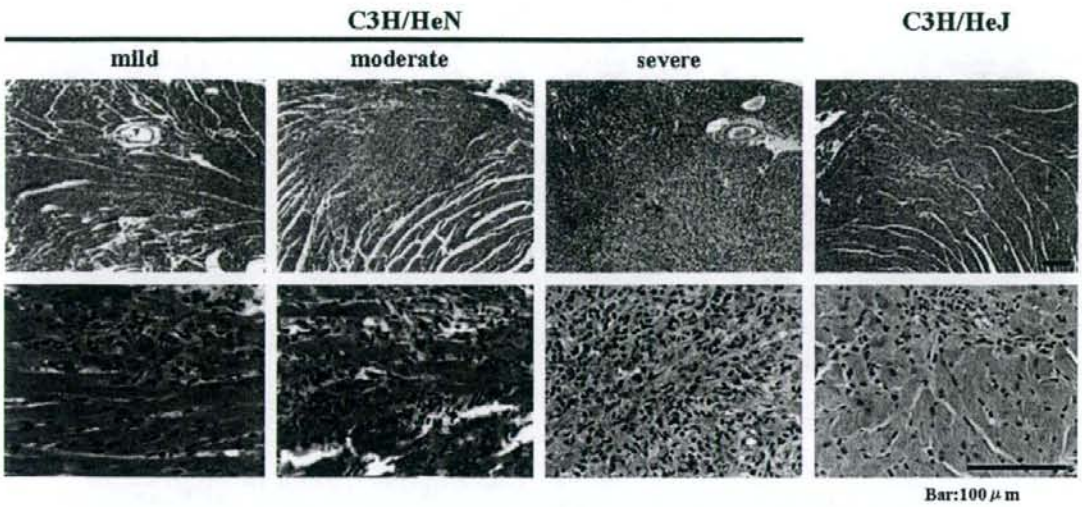


Fig. 2. Results of histopathological examination of hearts from mice that had been immunized with cardiac myosin mixed with BCG in IFA. The histological findings were classified into severe (>50%), moderate (10–49%) and mild (<9%) depending on the ratio of affected area to total myocardium. All tissue specimens were obtained 21 days after the first myosin immunization. The tissue specimens were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. These results are representative of five independent experiments. Bars represent 100 μm.

(OVA). A significant component of this proliferative response was attributed to the presence of $CD4^+$ cells, because $CD4^+$ cell-depleted spleen cells exhibited a substantially reduced myosin-specific proliferative response. Moreover, the C3H/HeN mice did not develop myocarditis when they received a monoclonal Ab to CD4 for depleting $CD4^+$ cells (data not shown). The observation that immunization of myosin mixed with BCG in both strains of mice elicited a $CD4^+$ proliferative T-lymphocyte response suggested that myosin mixed with BCG induces a myosin-specific Th cell response in not only mice with myocarditis but also in mice without myocarditis.

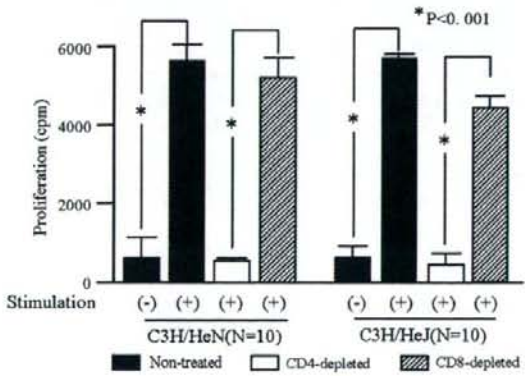


Fig. 3. Myosin-immunized mice develop $CD4^+$, myosin-specific spleen cell proliferative responses. Responding spleen cells were depleted of $CD4^+$ or $CD8^+$ cells using a commercially available system of magnetic bead-coupled specific Abs and co-cultured with myosin. Each value is the mean cpm ± SE of ten mice/group. $*p < 0.001$.

### *3.3. Myosin-specific cytokine responses of spleen cells from immunized mice*

To elucidate the immunological qualities of myosin-specific $CD4^+$ T cells, myosin-specific cytokine responses were analyzed in experimental mice. The responses of myosin-specific cytokines in spleen cells obtained from the experimental mice on day 21 were examined by two different methods. The production of IFN-γ from spleen cells after stimulation *in vitro* with myosin or OVA (control) was assessed by ELISA. Spleen cells from C3H/HeN mice with myocarditis immunized with myosin mixed with BCG produced a significantly larger amount of IFN-γ in the supernatant of the culture than did spleen cells from C3H/HeJ mice without myocarditis after stimulation *in vitro* with myosin (Fig. 4a). We next assessed the mRNA expression levels of Th1-type cytokines (IL-2 and -12) and Th2-type cytokines (IL-4 and -13) in spleen cells after *in vitro* stimulation with myosin or OVA (control). Spleen cells from C3H/HeN mice with myocarditis showed strong IL-2 and -12 expression and weak IL-4 and -13 expression of mRNA, whereas completely opposite results were obtained for spleen cells from C3H/HeJ mice without myocarditis. Spleen cells from C3H/HeJ mice without myocarditis showed strong expression of mRNA of Th2-type cytokines (IL-4 and -13) and weak expression of Th1-type cytokines (IL-2 and -12). Moreover, spleen cells from C3H/HeN mice treated with myosin and IFA without BCG showed Th2-type immune responses (data not shown). Upstream of the release of some Th1-type cytokines are the TLRs, and C3H/HeN mice with wild-type TLR4 developed EAM while also showing a Th1-type immune response to myosin. Although induction of another T-cell linage, Treg, was also assessed by the expression

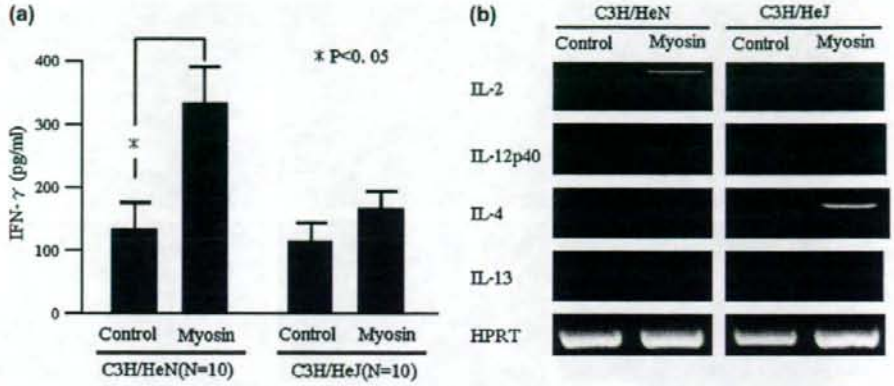


Fig. 4. Cytokine production in culture supernatant and expression of mRNAs of cytokines from spleen cells. (a) The amount of IFN-γ in the culture supernatant was measured by ELISA 21 days after the first myosin immunization. Each value shown is the mean and SD of ten mice per group. (b) Spleen cells were stimulated *in vitro* with myosin for 1 day in culture. Spleen cells stimulated with OVA were used as controls. The reaction products were analyzed on 2% agarose, Tris-buffered EDTA TBE gels. The profiles are representative of three independent experiments.

of mRNA of IL-10 and transforming growth factor-β, these cytokines were not different in C3H/HeN mice with myocarditis and C3H/HeJ mice without myocarditis (data not shown). These results indicated that the $CD4^+$ cells of C3H/HeN mice (TLR4 wild type) and C3H/HeJ mice (TLR4 unfunctional mutated) were polarized toward different Th responses after immunization by an emulsion of myosin mixed with BCG followed by proliferation.

### *3.4. Effects of IL-4, IL-4SM or IL-4DM DNA administration on the development of myocarditis in mice immunized with myosin mixed with BCG*

To examine the effects of regulating Th responses using IL-4, IL-4SM (agonistic IL-4 single mutant) and IL-4DM (antagonistic IL-4 double mutant) DNA on the development of myocarditis in both strains of mice immunized with myosin mixed with BCG, the mice were intraperitoneally administered 100 μg of DNA vaccine or control plasmid on days −7, 0, 7 and 14 (Fig. 1b). The IL-4 mutant Q116D/Y119D, which forms unproductive complexes with the IL-4Rα-chain, acts as an antagonist by inhibiting the formation of heterodimers with other receptors [15]. These IL-4-binding inhibitors act not only by inhibiting IL-4 binding to its receptor but also by preventing IL-13 from eliciting its activity, since the IL-4Rα-chain also forms a functional signaling component of the IL-13R heterodimer [16,17]. We previously reported that such plasmid administration can regulate the systemic Th immune responses in autoimmune and allergic diseases by only a single injection [14]. C3H/HeN mice, in which the development of myocarditis was induced by immunization of myosin mixed with BCG, did not develop myocarditis when they were injected with the IL-4 and IL-4SM (agonistic IL-4 mutant) DNA vaccines to inhibit the Th1-type immune response ($n = 10$ respectively) (Fig. 5). On the other hand, C3H/HeJ mice, in which the development of myocarditis was not induced by the same immunization, were not affected by the injection of IL-4 and IL-4SM DNA vaccines ($n = 10$ respectively). However, surprisingly, C3H/HeJ mice developed myocarditis (mild in 3/10 and moderate in 7/10) when they were injected with antagonistic IL4 mutant, IL-4DM, DNA vaccine for inhibition of Th2-type immune responses by prevention of IL-4 signaling (Fig. 5). The injection of control plasmid did not influence the development of myocarditis in either strain of mice ($n = 10$ respectively) (Fig. 5). Mice did not develop myocarditis without BCG in any plasmid DNA injected (data not shown). These results were derived by four injections of DNA, although the experimental model of allergic inflammation was inhibited by only a single injection of DNA. We previously reported that administration of IL-4DM DNA did not change the Ag-specific Th responses in cytokine production by *in vitro* stimulation of Ag without the presence of IL-4DM protein [14]. In fact, the results for myosin-specific cytokine production from spleen cells of both strains of mice administered IL-4, IL-4SM or IL-4DM DNA vaccines were the same as the results shown in Fig. 4 (without injection of DNA vaccines) after *in vitro* stimulation of myosin (data not shown). The existence of a small amount of IL-4 or IL-4 mutant for a long time *in vivo* might have played a role in the development of EAM. Since pharmacokinetic half-lives of IL-4 and IL-4 mutant proteins are very short *in vivo* ($t_{1/2} = 0.83$ h), a high concentration of these molecules in plasma must be maintained for a long period in order for effects on various phenotypes to be obtained. These effects usually disappeared immediately after discontinuing administration of these proteins. The antagonistic IL-4 is more effective than neutralizing Ab to IL-4, and commercially available Abs to IL-4 do not have sufficient effects to inhibit the activity of IL-4. These results indicated that the regulation of functions of IL-4 played an important role in the development of myocarditis induced by the immunization of myosin mixed with BCG in the strains of C3H/He mice. These results also indicated the possibility

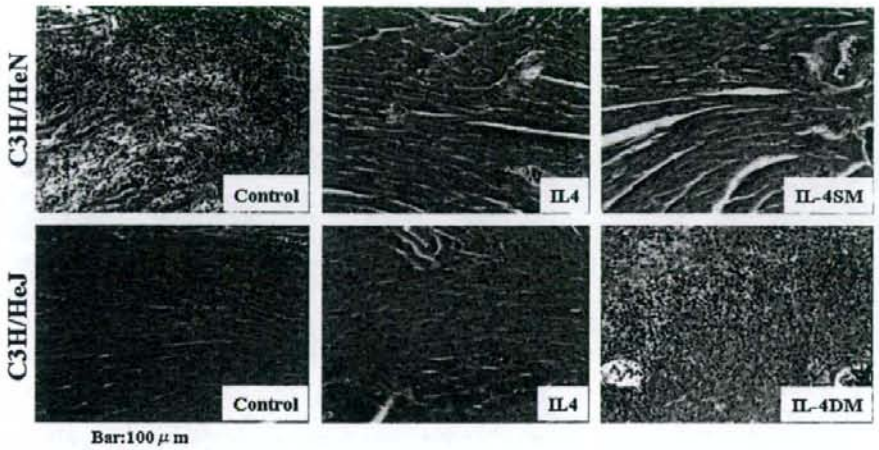


Fig. 5. Results of histopathological examination of hearts from myosin-immunized mice that had been administered IL-4, IL-4SM, IL-4DM or control. All tissue specimens were obtained 21 days after the first myosin immunization. The tissues specimens were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Mice that had been immunized with myosin mixed with BCG in IFA were each injected with 100 μg of IL-4, IL-4SM, IL-4DM or control plasmid DNA four times on days −7, 0, 7 and 14. These results are representative of three independent experiments. Bars represent 100 μm.

that myocarditis is associated with Th1-type $CD4^+$T-cells in these strains of mice.

## 4. Discussion

$CD4^+$ T cells have been reported to be required for the induction of EAM in mice. The development of EAM in A/J mice was prevented by the depletion of $CD4^+$ T cells, and disease severity was reduced by depleting $CD8^+$T cells (reviewed in [18,19]). A widely held belief is that when the cytokine profile of autoreactive T cells shifts toward an inflammatory Th1 type, the result is pathogenicity and autoimmune diseases (reviewed in [20,21]). Autoimmune myocarditis in the Lewis rat model was promoted by Th1-type immune responses in the same manner as that seen in our experiments [22]. On the other hand, it is difficult to understand the development of myocarditis in the EAM mouse model based on the Th1/Th2 paradigm. Although Th2-type immune responses played a critical role in the development of myocarditis in the mouse model of EAM using A/J and BALB/C strains [23,24], Th1-type immune responses were also suggested to participate in the development of EAM. Moreover, it has been reported that another novel population of T cells, regulatory T cell (Treg), are also important for controlling development of EAM as well as other disease [25–30]. These reports suggested that loss of immune tolerance regulated by Treg cells are one of the mechanisms of development of EAM. It has been reported that EAM did not develop in mice deficient in the Th1-type cytokine IL-12 or its receptor after administration of myosin or myosin Ag peptide, and that the CC-chemokine secreted by Th1-type T cells mediates EAM [31–33]. These differences in immune responses in EAM models are thought to be due to the correlation between mouse strain and mycobacteria species. The EAM model of A/J was established by using a large amount of virulent mycobacteria (*Mycobacterium tuberculosis*), and a small amount of the same bacteria was used for BALB/c mice. We used avirulent *Mycobacteria bovis* BCG (vaccine strain) for establishment of EAM. The A/J mouse strain is susceptible to *Mycobacterium tuberculosis* and resistant to BCG. C3H/HeN mice are resistant to both strains of mycobacteria with the same responses as those in humans, and BALB/c mice show responses opposite to those of A/J mice (resistant to *Mycobacterium tuberculosis* and susceptible to BCG) [34–36]. These differences are dependent on various genetic factors such as *Nramp* gene.

Some studies have suggested a relationship between TLR4 and myocarditis. Infection with a Coxsackievirus, which is a well-known agent of myocarditis, was found to upregulate TLR4 on mast cells and macrophages immediately following infection. TLR4 signaling also increases the occurrence of acute myocarditis and production of proinflammatory cytokines in the heart [37]. Moreover, the critical requirement of TLR4 signaling in dendritic cells (DCs) for myocarditis induction was genetically proven by the fact that myosin Ag peptide (MYHC-α)-pulsed and TLR4/CD40-activated DCs isolated from TLR4-deficient mice did not induce myocarditis in wild-type recipients when DCs isolated by the same procedure from TLR4-wild type transfer elicited myocarditis in a wild-type recipient [38]. In our system, two important points regarding the establishment of EAM were observed. Porcine myosin must be mixed well in IFA, and EAM was only observed by immunization of this emulsion into the footpad. These observations suggest that myosin Ag is incorporated in the same APCs such as macrophages or DCs together with BCG for a long period of time as oil particles, and then myosin-specific immune responses are induced by the influence of the characteristic immune responses of a large quantity of BCG associated with TLR4.

Relationships between TLRs and mycobacteria have been reported. TLR2, TLR4 and TLR1/TLR6 heterodimers with

TLR2 have been implicated in the recognition of mycobacterial Ags [39]. The emerging concept of TLRs as key molecules for shaping the quality of immune responses against microbes is further supported by results of experiments showing that mice lacking MyD88 are incapable of developing Ag-specific Th1 responses after immunization with OVA mixed with CFA (containing dead mycobacteria as an active component) [40]. These results are thought to be due to a mechanism involving both TLR2 and TLR4, since CFA contains a complex mixture of mycobacterial components, some of which are recognized by different members of the Toll family, TLR2 and TLR4. The EAM established in our system utilized these TLRs by immunization with extremely large amounts of BCG and cardiac myosin as a mixed emulsion, thus suggesting the importance of TLR4 for the induction of Th1-type immune responses related to EAM. The C3H/HeJ mouse, which has unfunctional TLR4, showed Th2-type immune responses to myosin after immunization of cardiac myosin mixed with BCG (Fig. 4). Mycobacteria induce Th1-type immune responses through TLR2 and TLR4 stimulation; however, our results showed Th2-type immune responses through TLR2 stimulation without TLR4 by BCG as an adjuvant. Similar results have also been reported by other investigators. Th2-type cytokines were induced from DCs by mycobacteria dependent on TLR2-mediated recognition but not TLR4-mediated recognition [41]. In our experiment, TLR4 mutant C3H/HeJ and wild-type C3H/HeN mice were used for analysis of Th responses in EAM. Unfunctional TLR4 mutations in humans have also been reported (reviewed in [42]). Studies using TLR4 knockout mice are needed to clarify this. Since the commonly used mouse background to generate knockout mice is associated with an increased susceptibility to mycobacteria, extensive backcrossing of such mice is required [43].

Many animal models of myocarditis are available to investigate the optimal therapy for myocarditis. However, the establishment of new animal models of myocarditis is still necessary to better understand myocarditis, because the understanding of myocarditis in humans is still insufficient. In the present study, we utilized two strains of C3H/He mice, which showed different susceptibilities to BCG, for the establishment of EAM involving Th1-type immune responses. The results of this study provide evidence of the potential utility of studying immunological mechanisms in order to both treat and prevent myocarditis.

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# Implications for Induction of Autoimmunity via Activation of B-1 Cells by *Helicobacter pylori* Urease

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**Besides various gastroduodenal diseases, *Helicobacter pylori* infection may be involved in autoimmune disorders like rheumatoid arthritis (RA) or idiopathic thrombocytopenic purpura. Such autoimmune disorders are often associated with autoreactive antibodies produced by B-1 cells, a subpopulation of B lymphocytes. These B-1 cells are mainly located in the pleural cavity or mucosal compartment. The existence of *H. pylori* urease-specific immunoglobulin A (IgA)-producing B cells in the mucosal compartment and of their specific IgM in the sera of acutely infected volunteers suggests the possibility that urease stimulates mucosal innate immune responses. Here, we show for the first time that purified *H. pylori* urease predominantly stimulates the B-1-cell population rather than B-2 cells, which produce antigen-specific conventional antibodies among splenic B220$^+$ B cells. The fact that such stimulation of B-1 cells was not affected by the addition of polymyxin B indicates that the effect of purified *H. pylori* urease was not due to the contamination with bacterial lipopolysaccharide. Furthermore, the production of various B-1-cell-related autoreactive antibodies such as IgM-type rheumatoid factor, anti-single-stranded DNA antibody, and anti-phosphatidyl choline antibody was observed when the splenic B cells were stimulated with purified *H. pylori* urease in vitro. These findings suggest that *H. pylori* components, urease in particular, may be among the environmental triggars that initiate various autoimmune diseases via producing autoreactive antibodies through the activation of B-1 cells. The findings shown here offer important new insights into the pathogenesis of autoimmune disorders related to *H. pylori* infection.**

*Helicobacter pylori*, a gram-negative, spiral-shaped bacterium living in the acidic stomach, causes chronic gastritis and ulcers on the gastroduodenal tract, and it is linked with the development of gastric cancer and mucosa-associated lymphoid tissue (MALT) lymphoma (6, 35). In addition to such gastroduodenal disorders, *H. pylori* infection is associated with various autoimmune diseases such as rheumatoid arthritis (22), Sjögren's syndrome (12), and idiopathic thrombocytopenic purpura (ITP) (17). In the case of ITP, the binding ability of anti-platelet-specific immunoglobulin G (IgG) is enhanced by rheumatoid factors (RFs) that may sequester IgG (26). The marked improvement in platelet counts after *H. pylori* eradication (14) indicates a direct correlation between the pathogenicity of ITP and *H. pylori* infection. Although the precise mechanism by which *H. pylori* infection generates autoimmune disorders remains to be elucidated, the production of RFs seems to be a key event in initiating the autoimmunity.

There are two distinct types of murine B-cell lineages: one is made up of conventional B cells (now called B-2 cells), which reside predominantly in the adult spleen and lymph nodes to form systemic acquired immunity, and the other is made up of CD5$^+$ B cells (now called B-1 cells), which localize mainly in the peritoneal and pleural cavities or the mucosal compartment (23). Several lines of evidence suggest that the B-1 cells generally produce low-affinity and less-mutated antibodies (7). Their repertoire is skewed toward reactivity with T-cell-independent (TI) antigens such as phosphatidyl choline (3) and polyvinyl pyrrolidinone (39), and they dominantly produce IgM and IgG3 antibodies containing little or no somatic mutations caused by gene rearrangements for the establishment of memory and specificity (30). Thus, in contrast to conventional B-2 cells, they do not usually create long-term memory for secondary responses. Moreover, such B-1-cell-derived antibodies are often autoreactive, like the RFs that react with the Fc portion of self-IgG (2). Furthermore, the disappearance of B-1 cells markedly reduces the serum level of IgG3 but not of other IgG subclasses (38), indicating that IgG3 is the dominant subclass of IgG produced by innate B-1 cells.

We have reported previously that the major antigenic component for antibody production against *H. pylori* is its urease (16), and urease-specific IgA antibody is seen in both the sera and gastric juices of *H. pylori*-infected patients (15, 18), indicating that *H. pylori* urease can stimulate mucosal immune responses. We have also observed the close relationship between *H. pylori* urease-specific IgA antibody production and gastric mucosal damage, and such urease-specific IgA-producing B cells are actually found in the mucosal compartment of

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the duodenum (15). Moreover, as an acute infection model, production of *H. pylori* urease-specific IgM antibodies in the sera of *H. pylori*-naive volunteers challenged with *H. pylori* has recently been reported (33). These findings suggest that *H. pylori* urease may stimulate mucosal innate B lymphocytes.

We thus speculated that *H. pylori* urease might have the capacity to activate mucosal B-1 cells and initiate various autoimmune diseases via the production of autoreactive antibodies. Here, we show for the first time that purified *H. pylori* urease does predominantly stimulate the B-1-cell population among splenic B cells, whereas lipopolysaccharide (LPS), the known B-cell stimulus, mainly activates B-2 cells. We also demonstrated the active production of various B-1-cell-associated autoreactive antibodies, such as IgM-type RF, anti-single-stranded DNA (anti-ssDNA) antibody, and anti-phosphatidyl choline (anti-PC) antibody, as well as IgG3, in the culture supernatant of splenic B cells stimulated with purified *H. pylori* urease. These findings suggest that *H. pylori* components, in particular its urease, may be one of the key factors in initiating various autoimmune disorders via the production of autoreactive antibodies through the activation of B-1 cells.

## MATERIALS AND METHODS

**Mice.** Six- to 8-week-old female BALB/c mice were purchased from Nisseizai (Tokyo, Japan) and maintained in microisolator cages under pathogen-free conditions. The animals were fed autoclaved laboratory chow and water. All animal experiments were performed according to the guidelines of the National Research Council *Guide for the Care and Use of Laboratory Animals* and approved by the Review Board of Nippon Medical School.

**Bacterial strains and growth conditions.** The bacterium used in the present study was wild-type *H. pylori* strain, Sydney strain 1 (SS-1), which is a mouse-virulent isolate originally isolated from a human patient (27). To obtain a large amount of bacterial cells, we used the following methods as described previously (21). SS-1 was cultured on brain heart infusion (BHI) agar (Oxoid, Hampshire, United Kingdom) containing 7% defibrinated horse blood (Nisseizai) at 37°C under microaerophilic conditions (5% $O_2$, 15% $CO_2$, and 80% $N_2$) with Anaero-Pack Campylo (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan). After being cultured for 2 days, the colonies were harvested by being scraped with a sterile metal spatula, transferred to 50 ml of BHI broth, and further cultured for 24 h at 37°C in a bidirectional shaker at 80 rpm (Takasaki Scientific Instruments Corp., Takasaki, Japan). Then, 500 μl of cell-containing medium was plated on BHI agar for an additional 3 days at 37°C, and the grown bacterial cells were harvested and washed twice with cold phosphate-buffered saline (PBS) at pH 7.0. The cells were sedimented by centrifugation (10,000 × *g* for 10 min at 4°C), and the cell pellet was stored at −80°C.

**Preparation of water extract.** Based on a previously described procedure (20), the stored cell pellet containing about 1 g of *H. pylori* cells (wet weight) was thawed at room temperature and then vortexed with 6.5 ml of sterile distilled water per tube for a total of 20 s, with brief stops every 5 s. The cells were removed from the mixture by centrifugation at 15,000 × *g* for 30 min, and the supernatant was filtered with a 0.22-μm filter (Millipore, Billerica, MA). The filtered supernatant was added to a 10× concentration of PBS at a volume ratio of 1:10 to the total supernatant volume and stored as water extract.

**Purification of *H. pylori* urease.** *H. pylori* urease was purified biochemically as described previously (20). Briefly, to obtain purified *H. pylori* urease, the column containing Cellufine sulfate (Millipore) was first equilibrated with PE65 buffer (20 mM phosphate buffer and 1 mM EDTA at pH 6.5). About 6.5 ml of prepared water extract was then applied to the column and eluted with the PE65. Urease-containing fractions were harvested by measuring enzyme activity, adjusted to pH 5.5, and adsorbed to the second-step column that had been preequilibrated with another buffer, termed PO55 (20 mM phosphate buffer at pH 5.5), for washing. Gel-bound urease was also eluted with PO74 buffer (20 mM phosphate buffer and 0.15 M NaCl at pH 7.4). Each eluted fraction was quantitatively analyzed for its enzyme activity, and the positive fractions were collected into a single tube. The collected sample was also confirmed to contain *H. pylori* urease by Western blot analysis as described below. The purity of the eluted urease was examined by silver staining with a Silver Staining kit (Amersham Bioscience, Uppsala, Sweden), and the purified urease protein concentration was estimated with a Micro BCA Protein Assay Reagent kit (Pierce Co., Inc., Rockford, IL).

**Western blotting.** Purified urease was loaded onto a sodium dodecyl sulfate-polyacrylamide gel for electrophoresis and then transferred to nitrocellulose-polyvinylidene difluoride (Atto Co., Inc., Tokyo, Japan). The nitrocellulose blots were blocked with 25% Block Ace (Dainihon Seiyaku, Osaka, Japan) in Tris-buffered saline (2 M Tris [pH 8.0], 5 M NaCl, 10% Tween 20) and incubated with two murine *H. pylori* urease-specific monoclonal antibodies (MAbs), termed L2 (19) and S2 (32). The blots were washed three times with blotting buffer (2 M Tris [pH 8.0], 1.43% glycine, 5% methanol) and incubated with biotinylated goat anti-mouse Ig (PharMingen, San Diego, CA) at 1:100 in PBS for 2 h at room temperature. After being washed three times, the blots were incubated with Horseradish Peroxidase Avidin D (Vector Laboratories, Burlingame, CA) diluted 1:2,000 in PBS for 30 min at room temperature. Then, the blots were detected with a ProtoBlot NBT and the BCIP Color Development system (Promega Corporation, Madison, Wis.).

**Measurement of *H. pylori* urease enzymatic activity.** Ten microliters of the collected fractions was incubated with 100 μl of 50 mM phosphate buffer (pH 6.8) containing 500 mM urea and 0.02% phenol red in flat-bottomed 96-well plates. The color development was monitored at 550 nm with a microplate reader (model 3550; Bio-Rad, Hercules, CA) at room temperature.

**Lymphocyte proliferation assay.** Cellular proliferative responses were measured by incubating $1.0 \times 10^6$ splenic lymphocytes with various mitogenic reagents in 200 μl of RPMI 1640-based medium (culture medium) (36) containing 10% heat-inactivated fetal calf serum, 20 mM HEPES (GIBCO BRL, Grand Island, NY), 10 μM 2-mercaptoethanol (Sigma Chemical, St. Louis, Mo.), 100-U/ml penicillin, 0.1-mg/ml streptomycin, and 50-μg/ml gentamicin for 3 days at 37°C in a 5% $CO_2$ atmosphere. Samples were cultured in triplicate on 96-well U-bottom plates. In certain experiments, mouse lymphocyte responses to LPS and *H. pylori* urease were tested in the presence of 20 μg of the lipid A antagonist polymyxin B/ml (8). The cells were then labeled for 16 h with 1 μCi/well of tritiated thymidine (MP Biomedicals, Morgan, CA), harvested in an automated plate harvester (TomTech, Orange, CT), and counted in a 1450 Micro Beta TRILUX scintillation spectrometer (Wallac, Gaithersburg, MD). Data are expressed as the mean counts per minute ± the standard error of the mean (SEM).

**B-cell purification.** After red blood cells were depleted with ammonium chloride (34), the remaining splenic lymphocytes were incubated in a dish coated with anti-mouse Ig (Dako A/S, Glostrup, Denmark) at 4°C for 30 min. More than 80% of the Ig-positive cells were confirmed as B cells by flow cytometric analysis using fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse B220 MAb (RA3-6B2; PharMingen) and phycoerythrin-conjugated hamster anti-mouse CD3 MAb (145-2C11; PharMingen). To obtain B cells of higher purity, naive spleen cells were incubated in a plastic dish with the culture medium at 37°C for 1 h, and nonadherent splenic lymphocytes were further incubated with anti-Thy-1.2 MAb (Serotec, Ltd., Oxford, United Kingdom) for 30 min at 4°C, followed by the addition of rabbit complement (Cederane, Ontario, CA) at 37°C for 1 h to deplete T lymphocytes as described previously (37). Then, the live cells were harvested and confirmed as B cells of >90% purity by flow cytometry.

**Fluorescence-activated cell sorter analysis of purified B cells stimulated with *H. pylori* urease.** A total of $10^6$ purified B cells were cultured in 200 μl of culture medium containing 10-μg/ml *H. pylori* urease or 1-μg/ml *Escherichia coli*-derived LPS at 37°C in a 5% $CO_2$ atmosphere for 5 days in triplicate on 96-well U-bottom plates. After incubation, the cells were harvested and analyzed with a FACScan cytometer with CellQuest soft ware (BD Bioscience, Mountain View, CA) using FITC-conjugated rat anti-mouse B220, phycoerythrin-conjugated rat anti-mouse CD5 (53-7.3; PharMingen), or biotinylated rat anti-CD9 (KMC8; PharMingen) MAbs for staining. Negative controls were incubated with irrelevant, isotype-matched MAbs.

***H. pylori* infection.** The mice were infected with *H. pylori* was done according to the following recently established procedure (21). Three hundred microliters of the bacterial solution containing about $10^8$ CFU of *H. pylori* (SS-1) was orally administered to each mouse on three successive days.

**Depletion of urease from water extract.** Thirty microliters of protein G beads (Sigma) was incubated with 300 μg of *H. pylori* urease-specific MAb (S2) (32) in a 1.5-ml tube at 4°C overnight. After incubation, the protein G beads were washed with PBS and incubated with 100 μl of urease-positive water extract at 4°C overnight to specifically deplete *H. pylori* urease and to create a urease-negative water extract. After this procedure was carried out twice, the obtained extract was confirmed as urease negative by the Western blotting analysis described above.

**Enzyme-linked immunosorbent assay.** Purified B cells ($10^6$ cells) were cultured with 10-μg/ml *H. pylori* urease or PBS for 3 to 7 days in vitro. The culture supernatants were harvested and stored at −20°C for further analysis.

**Detection of IgG3.** A 50-μl aliquot of affinity purified rabbit anti-mouse IgG3 (Rockland, Gilbertsville, PA) (10 μg/ml in PBS) was added to flat-bottomed Immulon 2 plates (Dynatech Laboratories, Inc., Alexandria, Va.), and incubated at 4°C. After overnight incubation, the antigen-coated plates were blocked with 1% bovine serum albumin (BSA) in PBS, and then a 50-μl aliquot of the culture supernatant was plated for an additional 60 min at room temperature. After the plate was washed three times with PBS containing 0.05% Tween 20, a 100-μl aliquot of diluted biotinylated goat anti-mouse Igs (Amersham Bioscience) (1:5,000) was added for 60 min at room temperature, followed by Horseradish Peroxidase Avidin D (1:2,000; Vector Laboratories) binding. The activity of peroxidase was determined by measuring the hydrolysis of ABTS [2,2'-amino-bis (3-ethylbenzothiazoline-6-sulfonic acid) di-ammonium salt] (Sigma) to the green product, which was quantitated by absorbance at 415 nm with a microplate reader (Bio-Rad).

**Detection of ssDNA.** Stock solution containing calf thymus DNA, type I (1 mg/ml in $H_2O$) (Sigma) was boiled for 10 min in a 1/10 volume of 1 N NaOH. The boiled solution was immediately put on ice for 10 min and diluted to 3 μg/ml with cold borate-buffered saline. A 100-μl aliquot of prepared ssDNA was added to flat-bottomed Immulon 2 plates and incubated at 4°C. After being blocked with BBT (0.5% BSA and 0.04% Tween 20 in borate-buffered saline), a 100-μl aliquot of diluted (1:10) culture supernatant was plated and incubated overnight at 4°C. Then, a 100-μl aliquot of diluted biotinylated goat anti-mouse Igs (1:5,000) was added. Bound Igs were detected with Horseradish Peroxidase Avidin D using ABTS as a substrate, and the activity was determined by absorbance at 415 nm.

**Detection of phosphatidyl choline.** A 100-μl aliquot of phosphatidyl choline (50 μg/ml in ethanol) was added to flat-bottomed Immulon 2 plates and incubated overnight at 4°C. After being blocked, a 50-μl aliquot of the culture supernatant was plated, followed by biotinylated goat anti-mouse Igs. Bound Igs were detected with Horseradish Peroxidase Avidin D using ABTS as a substrate.

**Detection of IgM type rheumatoid factor (RF IgM).** RF IgM was detected with an LBIS RF IgM (mouse) ELISA kit (Shibayagi, Gunma, Japan). In brief, after the antigen-coated plate in the kit was washed, a 100-μl aliquot of the diluted (1:2) culture supernatant or prepared RF standard solution was added and incubated for 120 min at room temperature. Then, a 100-μl aliquot of the diluted (1:2,000) peroxidase-conjugated antibody was added, followed by a 100-μl aliquot of the color development solution. The activity of peroxidase was determined by quantifying the yellow product by absorbance at 450 nm. A standard curve was made by the RF standard solution to determine the actual concentration.

**Statistical analysis.** All values are expressed as the mean ± SEM. Student's *t* test was employed to test the levels of significance among the experimental groups.

## RESULTS

**Purification of *H. pylori* urease and its enzymatic activity.** To examine the lymphoproliferative capacity of *H. pylori* urease, we first carried out intensive purification of urease from *H. pylori* as described in Materials and Methods, and we were able to purify *H. pylori* urease quite effectively by the procedure reported previously (20). Silver staining and immunoblots using specific MAbs (19) confirmed excellent purity (data not shown). The purified *H. pylori* urease had strong enzymatic activity to hydrolyze urea and release basic ammonia, as measured by the procedure described in Materials and Methods (data not shown).

**Effects of purified *H. pylori* urease on lymphocyte proliferation.** Next, we examined the effect of *H. pylori* urease on lymphocyte proliferation using murine splenocytes as responders. As shown in Fig. 1A, >2.5-times-higher stimulatory capacity was observed when $10^6$ responder naive splenocytes were cocultured with 10-μg/ml purified *H. pylori* urease than when they were cocultured with the same amount of BSA or Jack Bean urease. This stimulatory effect of purified *H. pylori* urease was confirmed in a dose-dependent manner (Fig. 1B). It should be noted that <1-ng/ml of *H. pylori*-derived LPS could be detected in the 10-μg/ml purified *H. pylori* urease. So far as

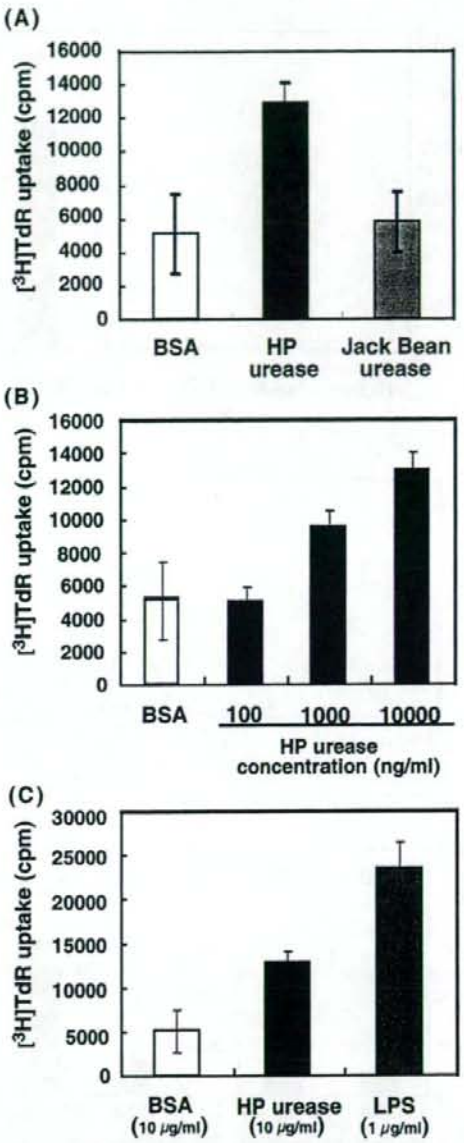



FIG. 1. Proliferative responses of naive splenic lymphocytes to purified *H. pylori* urease. (A) Significantly greater stimulatory capacity was observed when $10^6$ naive splenocytes were cocultured with 10-μg/ml purified *H. pylori* urease than when they were cocultured with the same amount of BSA or Jack Bean urease for 3 days at 37°C in a 5% $CO_2$ atmosphere. (B) The stimulatory effect of purified *H. pylori* urease was observed to be dose dependent. (C) One microgram of *E. coli*-derived LPS/ml showed much stronger proliferative responses than 10-μg/ml purified *H. pylori* urease. Data are expressed as the mean counts per minute ± SEM of three independent experiments.

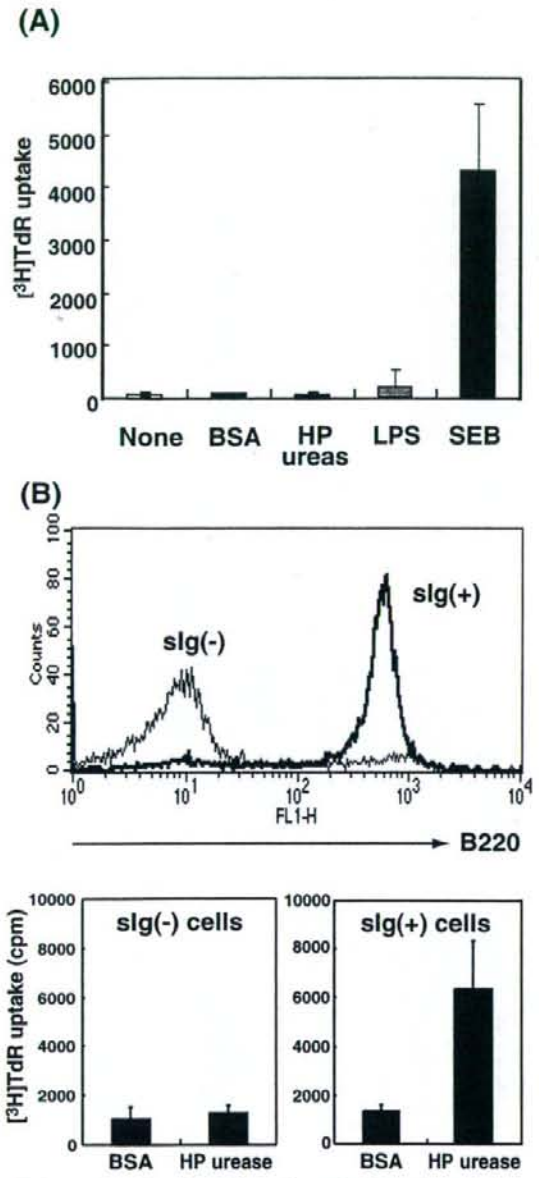



FIG. 2. sIg(+) B cells proliferated on stimulation with purified *H. pylori* urease. (A) When $10^6$ naive thymocytes were cocultured with 10-μg/ml purified *H. pylori* urease, 10-μg/ml BSA, or 1-μg/ml *E. coli*-derived LPS for 3 days, no measurable proliferative responses were observed, although 1-μg/ml staphylococcus enterotoxin B induced a remarkable proliferation of naive thymocytes. (B) To enrich B cells, naive spleen cells were incubated in a plastic dish with the culture medium at 37°C for 1 h, and the nonadherent splenic lymphocytes were further incubated in a dish coated with anti-mouse Ig at 4°C for 30 min. The adherent cells [sIg(+)] and nonadherent cells [sIg(−)] were then harvested, and the B-cell ratio of the adherent cells was confirmed by flow cytometric analysis using a FITC-conjugated rat anti-mouse B220 MAb. The boldface line in the top panel represents the sIg(+) cells, and the thin line represents the sIg(− cells. The

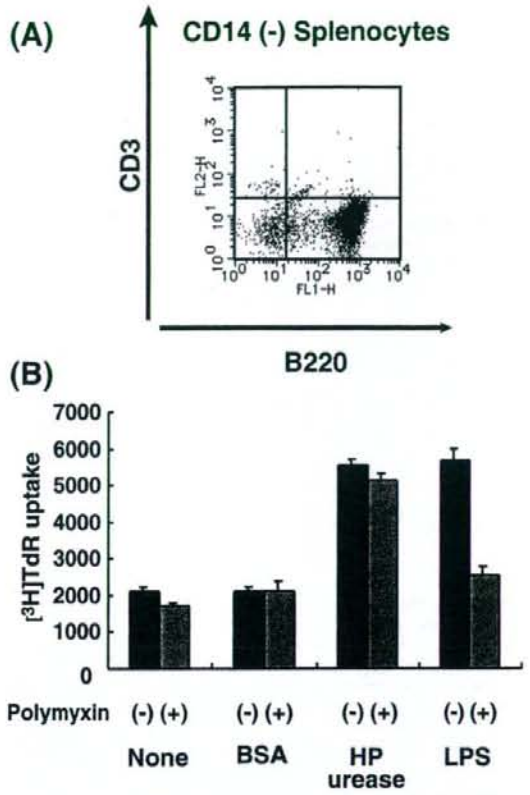



FIG. 3. Purified B lymphocytes were specifically stimulated by purified *H. pylori* urease. (A) Enriched $CD3^-$ $CD14^-$ $B220^+$ B lymphocytes with >90% purity were obtained reproducibly by the procedure described in Materials and Methods. (B) Using $10^6$ of these purified B lymphocytes, we confirmed their significant proliferative responses when the cells were cocultured with 10-μg/ml purified *H. pylori* urease, whose stimulatory activity was not blocked at all by the addition of 20-μg/ml polymyxin B, a known lipid A antagonist. In contrast, when the B cells were stimulated by 10-ng/ml *E. coli*-derived LPS, the stimulatory activity was specifically abrogated by the addition of 20-μg/ml polymyxin B. Data are expressed as the mean counts per minute ± SEM of three independent experiments.

our investigations go, 1-ng/ml commercially available *E. coli*-derived LPS did not induce any measurable proliferation of the same number of naive splenocytes (data not shown). In addition, it has been reported that *H. pylori*-derived LPS has much weaker mitogenic activity than *E. coli*-derived LPS (31). Therefore, the stimulatory capacity of *H. pylori* urease was not due to the contaminated *H. pylori*-derived LPS. However, 1-μg/ml *E. coli*-derived LPS did induce much stronger proliferative re-

bottom panel indicates the proliferative responses of $10^6$ cells when cocultured with 10-μg/ml purified *H. pylori* urease or 10-μg/ml BSA for 3 days. The purified *H. pylori* urease showed a good stimulatory capacity against sIg(+) B lymphocytes but not against sIg-negative cells. Data are expressed as the mean counts per minute ± SEM of three independent experiments.

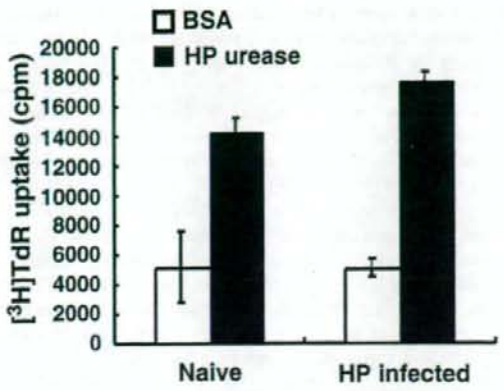



FIG. 4. Effects of in vivo priming with orally administrated *H. pylori* on B-cell stimulation by purified *H. pylori* urease. Mice were given three doses of $10^8$ CFU of infectious *H. pylori* SS-1 orally, 6 to 8 weeks previously. When $10^6$ of spleen cells from the primed mice were stimulated in vitro with 10-μg/ml purified *H. pylori* urease, we saw no significant proliferative enhancement in comparison with naive B cells obtained from uninfected controls. Data are expressed as the mean counts per minute ± SEM of three independent experiments.

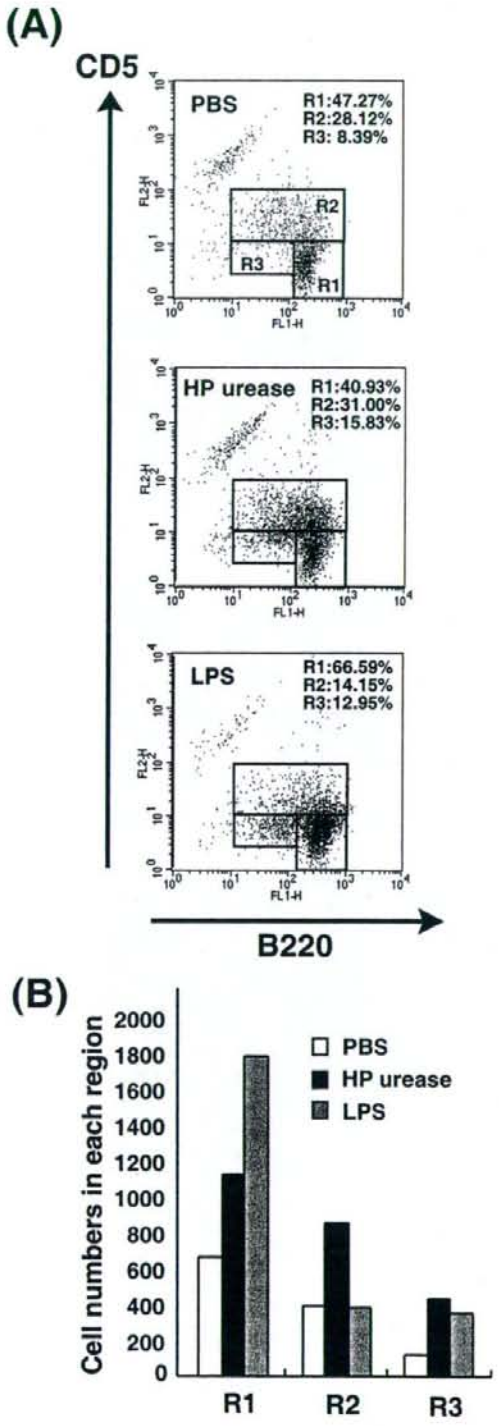
sponses in the naive splenocytes than 10-μg/ml of purified *H. pylori* urease (Fig. 1C).

**B lymphocytes were stimulated by the purified *H. pylori* urease.** To determine the actual target cells stimulated by the purified *H. pylori* urease, we first tested the effect of *H. pylori* urease on naive thymocytes. There were no detectable responses when we used naive thymocytes as responders, although 1-μg/ml of a known T-cell superantigen, staphylococcus enterotoxin B, showed strong proliferation against the thymocytes (Fig. 2A), indicating that the purified *H. pylori* urease might be a B-cell mitogen like LPS. Then, we tested urease activity against purified B cells. Using panning methods with anti-mouse Ig-coated plates, we divided the splenocytes into two groups: surface Ig-positive B [sIg(+)] cells and surface Ig-negative [sIg(−)] cells (Fig. 2B). Compared with the control BSA, the purified *H. pylori* urease showed a good stimulatory capacity against Ig-bearing B lymphocytes but not against sIg-negative cells (Fig. 2B). Since the purity of the B lymphocytes obtained by the panning method was around 70 to 80% at most, we tried to increase the purity further by treating the nonadherent splenocytes with anti-$CD3^+$ complement to delete the T cells. As demonstrated in Fig. 3A, purified $CD3^-$ $CD14^-$ $B220^+$ B lymphoctes with >90% purity were obtained. Using those purified B lymphoctes, we confirmed good proliferation when they were cocultured with 10 μg of purified *H. pylori* urease/ml (Fig. 3B). Therefore, the actual target cells stimulated by purified *H. pylori* urease turned out to be B lymphocytes. Also, such B cells are similarly stimulated by 10-ng/ml of *E. coli*-derived LPS (Fig. 3B), whose stimulatory activity was specifically cancelled by the addition of 20-μg/ml polymyxin B, a known lipid A antagonist (8). In contrast, the same amount of polymyxin B did not affect the B-cell proliferation generated by the purified *H. pylori* urease, again indicating that the effect of purified *H. pylori* urease on B-cell proliferation was not due to contamination with LPS.

**Effect of in vivo priming with orally administrated *H. pylori* on B-cell stimulation by purified *H. pylori* urease.** The next question was whether immunological memory against the purified *H. pylori* urease could be established within B cells in vivo when the mice were given $10^8$ CFU of infectious *H. pylori*, SS-1, orally (27). In general, antigen-specific B-cell-proliferative responses require both antigen-presenting cells and T-cell help in the in vitro culture system if the antigens are conventional T cell-dependent ones (10). Therefore, whole splenocytes from mice given SS-1 three times orally 6 to 8 weeks previously were stimulated in vitro with purified *H. pylori* urease. Unexpectedly, we could see no enhancement of the proliferation in the primed B cells in comparison with the uninfected naive controls (Fig. 4). The results suggests that the target B cells cannot be primed to establish immunological memory by oral *H. pylori* infection.

**B-1 cells are the major targets for purified *H. pylori* urease.** There are two distinct types of murine B-cell lineages: one is the conventional B-2-cell lineage and the other is the B-1-cell lineage. The B-1 cells usually express detectable levels of CD5, but some do not. The CD5-positive B-1 cells are called B-1a cells, and the CD5-negative B-1 cells are called B-1b cells (25). We carried out further analysis to determine which type (i.e., B-1a or B-1b) was stimulated by the purified *H. pylori* urease. As indicated in Fig. 5A, top, we divided B220-positive B cells into three groups (R1, R2, and R3), corresponding to B-2, B-1a, and B-1b, respectively. Interestingly, although the majority of the proliferated B cells stimulated by LPS appeared to be B-2 cells, both B-1a and B-1b cells seemed to be strongly stimulated to proliferate by *H. pylori* urease, compared with the PBS-stimulated controls (Fig. 5A). This was confirmed by direct counting of the proliferated cells in each region; the $CD5^+$ B-1a cells seemed to be more specifically stimulated by *H. pylori* urease (Fig. 5B). Therefore, the major targets for *H. pylori* urease stimulation turned out to be B-1 cells, particularly B-1a cells.

**Effect of depletion of urease from *H. pylori* water extract on B-1-cell proliferation.** We then tried to confirm whether urease was the critical stimulant of B-1 cells among the components of *H. pylori*. First, we made a water extract from *H. pylori* containing various bacterial components including urease and tested its ability to stimulate purified murine splenic B lymphocytes. As indicated in Fig. 6A, in comparison with the PBS control, the *H. pylori* water extract appeared to stimulate both B-1 (R2 plus R3) and B-2 (R1) cells to proliferate during a 5-day culture period. The depletion of the *H. pylori* urease with *H. pylori* urease-specific MAb-conjugated protein G beads, as described in Materials and Methods, appeared to reduce this stimulation. Moreover, we counted all the viable remaining proliferated cells and estimated the number of cells for each region, confirming a significant reduction in B-1-cell proliferation in the R2 and R3 regions in comparison with B-2 cells in the R1 region (Fig. 6B). Therefore, urease seems to be the principal component of *H. pylori* water extract for stimulating the B-1 cells that might generate autoimmune disorders. Also, the data shown in Fig. 6A and B suggest the possibility that other *H. pylori*-derived factors also stimulate B-1 cells if the urease is completely depleted from the extract.

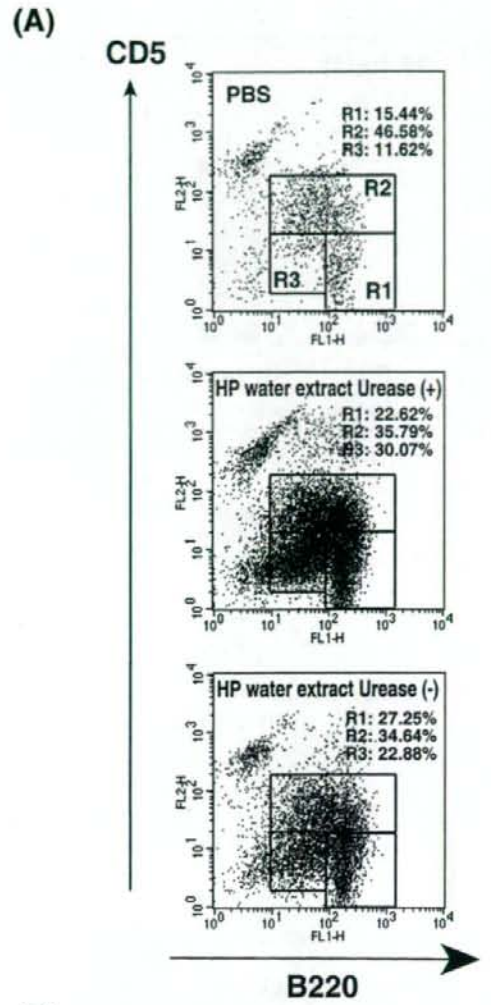



**Secretion of autoantibodies from purified B cells stimulated with purified *H. pylori* urease.** Finally, we tried to detect the production of autoreactive antibodies from purified splenic B cells when they were stimulated in vitro with purified *H. pylori* urease. As shown in Fig. 7A, we observed a good amount of IgG3 antibody production in the culture supernatant of the stimulated B cells. Because such IgG3 is the dominant subclass of IgG produced by innate B-1 cells (38), this indicates that B-1 cells must be stimulated by the purified *H. pylori* urease in vitro without the requirement of T-cell help. Moreover, we also detected a considerable amount of IgM type RF (B), as well as anti-ssDNA antibody (C) and anti-PC antibody (D), in the culture supernatant. Therefore, various types of autoreactive antibodies related to innate B-1 cells seem to be produced when B cells are stimulated with *H. pylori* urease.

## DISCUSSION

Urease is the most prominent component of *H. pylori* and is expressed on the surface of the bacterial membrane. It is critical for attachment to the gastric mucosa (13) and may thus initiate the primary immune response to innate mucosal immunity when the bacterium orally enters the human body. In the present study, by making a comparison with purified Jack Bean urease, we demonstrated that purified *H. pylori* urease had a strong capacity to stimulate Ig-bearing B lymphocytes in particular innate B-1 cells rather than in acquired B-2 cells. The depletion of *H. pylori* urease from water extract induced a significant reduction in B-1-cell proliferation, indicating that the principal bacterial component for stimulating B-1 cells in *H. pylori* is its urease and not other B-cell mitogens like LPS. In addition, it has been reported that soluble *H. pylori* surface components enriched with urease do not usually contain detectable levels of LPS (28) and that *H. pylori* LPS shows lower levels of mitogenic activity than other enterobacterial LPSs (31). Also, as shown in the present study, polymyxin B did not affect B-cell proliferation generated by purified *H. pylori* urease. These findings indicate that the effect of purified *H. pylori* urease on B-cell proliferation was not due to contamination with LPS, which mainly stimulates the B-2-cell population. Therefore, compared to other gram-negative bacteria existing in the gastrointestinal tract, *H. pylori*, bearing less LPS with weaker mitogenic activity for B-2-cell proliferation, may dom-

FIG. 5. CD5-positive B-1 cells are the major targets for purified *H. pylori* urease. (A) A total of $10^6$ of purified B lymphocytes were cocultured with each reagent for 5 days, and all the cells were harvested for flow cytometric analysis. We divided the B220-positive B cells into three groups, R1, R2, and R3, corresponding to B-2, B-1a, and B-1b, respectively. The $CD5^+$ B-1a cells were proliferated by 10-μg/ml *H. pylori* urease stimulation compared with the PBS-stimulated controls. In contrast, the majority of the proliferated B cells induced by 1-μg/ml *E. coli*-derived LPS were B-2 cells. (B) The number of viable cells in each region among 10,000 cultured cells in total was counted. The $CD5^+$ B-1a cells in the R2 region predominantly proliferated by purified *H. pylori* urease stimulation, whereas the B-2 cells in the R1 region mainly proliferated by LPS stimulation. Also, the B-1b cells in region R3 seemed to be stimulated to some extent by both purified *H. pylori* urease and LPS. Data shown are representative of three distinct experiments.




inantly activate B-1 cells via long-term *H. pylori* infection through constant exposure to its urease.

Such B-1 cells have the capacity to respond to TI antigens and produce IgM and IgG3 antibodies containing few or no somatic mutations. Typical immunoglobulin genes in B-1 cells have fewer N insertions than those in B-2 cells (24) and will not, therefore, usually create antigen-specific long-term memory similar to innate immune system-competent cells. Also, B-1 cells are thought to be the primary source of natural IgM antibodies, which are usually polyreactive and autoreactive against bacterial polysaccharide, lipids, and proteins, as well as autoantigens such as ssDNA and IgG-like RFs (5). These self-antigen-reactive antibodies may bind to their own components, initiate an inflammatory response, and contribute to the pathogenesis of various autoimmune disorders. Indeed, elevated numbers of $CD5^+$ B-1 cells producing a variety of self-reactive antibodies have been reported in patients suffering from Sjögren's syndrome (11) and rheumatoid arthritis (42). Also, the close association of *H. pylori* infection with several autoimmune diseases such as rheumatoid arthritis (22), Sjögren's syndrome (12), and ITP (17), has been shown. In this study, we demonstrated that when purified B lymphocytes were stimulated in vitro with purified *H. pylori* urease, IgG3, IgM-type RFs, and anti-ssDNA and anti-PC antibodies were actually produced in the culture supernatant. These findings clearly indicate that *H. pylori* urease has the capacity to stimulate B-1 cells to produce those self-reactive antibodies in a TI manner. Moreover, the fact that spleen cells from *H. pylori*-infected animals did not show any enhancement of their proliferative responses against purified *H. pylori* urease stimulation suggests that the major targets for that urease are not conventional B-2 cells with antigen-specific long-term memory, but rather innate B-1 cells. Taken together, these findings suggest that the activation of B-1 cells by some pathogen-derived substance like *H. pylori* urease shown here could lead to autoimmunity via breaking negative regulation of B-1 cells and that this may be why

FIG. 6. Effects of urease depletion from *H. pylori* water extract on B-1-cell proliferation. (A) A total of $10^6$ of purified splenic B cells were cultured with 5 μl of water extract of *H. pylori* for 5 days, and all the cells were harvested for flow cytometric analysis. As in the experiments shown in Fig. 5, we divided the B220-positive B cells into three groups, R1, R2, and R3, corresponding to B-2, B-1a, and B-1b, respectively. Both the B-1 cells and B-2 cells were markedly proliferated (middle) in comparison with the PBS-stimulated controls (top). In contrast, when $10^6$ purified splenic B cells were cocultured with 5 μl of urease-depleted water extract, although the percentage of cells in the R1 region was elevated, the percentages in both the R2 and R3 regions were decreased (bottom). (B) The number of viable cells in each region was counted. Although the number of proliferated cells in the R1 region was around 6,100 when stimulated with water extract of *H. pylori*, the number in the R2 region was around 11,000 and the number in the R3 region was around 7,800. In contrast, while the number of cells in the R1 region stimulated with the urease-depleted component was around 4,400 (27.9% reduction), the number in the R2 region was around 5,900 (46.4% reduction), and in the R3 region the number was around 3,700 (52.6% reduction). Thus, the B-1 cells in the R2 and R3 regions appeared to be dominantly stimulated by *H. pylori* urease, although the B-1 cells must also have been stimulated by some other factors in the bacterial components. Data shown are representative of five independent experiments.

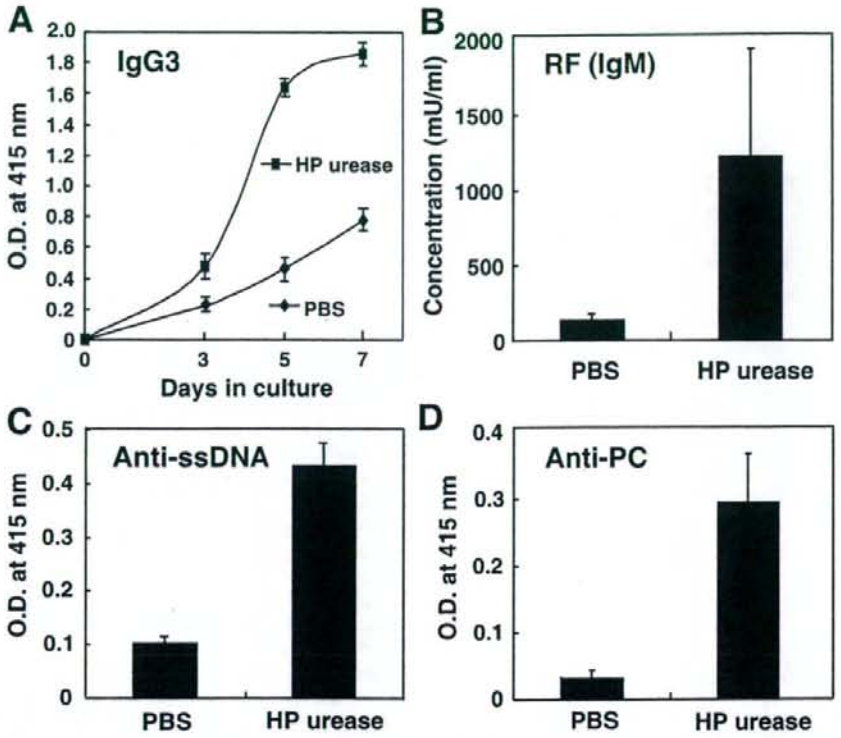



FIG. 7. Secretion of autoantibodies from purified B cells stimulated with purified *H. pylori* urease. A total of $10^6$ of the purified splenic B cells were cultured with 10-μg/ml purified *H. pylori* urease or PBS for 3 to 7 days, and the supernatants were harvested to test autoantibody production by enzyme-linked immunosorbent assay. (A) A significant amount of IgG3, the dominant substance of B-1 cells, was produced in the culture supernatants compared with PBS. In comparison with the control supernatants stimulated with PBS, >1,000 mU of IgM type RFs/ml (B), as well as anti-ssDNA antibody (C) and anti-PC antibody (D), were detected in the culture supernatants stimulated with *H. pylori* urease for 7 days. Data shown are representative of three independent experiments.

there is a link between various autoimmune diseases and *H. pylori* infection.

In the present study, we observed B-1-cell proliferation not only in CD5-positive B-1a cells but also in CD5-negative B-1b cells by stimulation with purified *H. pylori* urease. Recently, B-1b cells were demonstrated to be the progenitors of marginal zone B (MZB) lymphocytes (29), which dominantly express CD9 molecules (40). In addition, the architectural and immunophenotypic properties of gastric MALT lymphoma suggest that they originate from MZB cells (41), and autoreactive B-cell clones have been detected in the MZB cells of MALT lymphoma (43). Such MALT cells may accumulate within the gastric mucosa as a result of long-standing *H. pylori* infection and thus may eventually develop into low-grade B-cell MALT lymphoma (4). We confirmed the proliferative responses of $CD9^+$ B-1 cells among B lymphocytes stimulated with purified *H. pylori* urease (data not shown). Moreover, using confocal laser microscopic analysis, we observed the remarkable infiltration of B-1 cells within the gastric mucosa of BALB/c mice chronically infected with SS-1 for about 1 year (S. Yamanishi. and H. Takahashi, unpublished observations). Collectively, our present study shows that cells activated by purified *H. pylori* urease did express CD9 molecules and might thus affect MZB cells. Therefore, *H. pylori* urease might contribute to the development of low-grade MALT lymphoma.

If continuous exposure to some bacterial components like *H. pylori* urease is required to maintain B-1 cell activation, the easiest way to stop that activation is to eliminate the bacterium from the body. Hence, eradication of *H. pylori* from the gastric mucosa can significantly improve various autoimmune diseases (1), as well as low-grade MALT lymphomas in cases (9) in which B-1 cells are intact and newly activated. However, once the B-1 cells gain the ability to activate themselves uncontrollably, eradication is no longer sufficient to cease the activation. Further precise analysis of the two distinct statuses of the B-1 cells associated with *H. pylori* infection will reveal other strategies for controlling disorders caused by it.

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# Importance of gastrointestinal ingestion and macromolecular antigens in the vein for oral tolerance induction

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## Summary

Oral administration of a certain dose of antigen can generally induce immunological tolerance against the same antigen. In this study, we showed the temporal appearance of ovalbumin (OVA) antigens in both portal and peripheral blood of mice after the oral administration of OVA. Furthermore, we detected 45 000 MW OVA in mouse serum 30 min after the oral administration of OVA. Based on this observation, we examined whether the injection of intact OVA into the portal or peripheral vein induces immunological tolerance against OVA. We found that the intravenous injection of intact OVA did not induce immunological tolerance but rather enhanced OVA-specific antibody production in some subclasses, suggesting that OVA antigens via the gastrointestinal tract but not intact OVA may contribute to establish immunological tolerance against OVA. Therefore, we examined the effects of digesting intact OVA in the gastrointestinal tract on the induction of oral tolerance. When mice were orally administered or injected into various gastrointestinal organs, such as the stomach, duodenum, ileum, or colon and boosted with intact OVA, OVA-specific antibody production and delayed-type hypersensitivity (DTH) response were significantly enhanced in mice injected into the ileum or colon, compared with orally administered mice. These results suggest that although macromolecular OVA antigens are detected after oral administration of OVA in tolerant-mouse serum, injection of intact OVA cannot contribute to tolerance induction. Therefore, some modification of macromolecular OVA in the gastrointestinal tract and ingestion may be essential for oral tolerance induction.

**Keywords:** gastrointestinal ingestion; macromolecular antigen; oral tolerance; ovalbumin

## Introduction

Although the gastrointestinal tract is incessantly exposed to dietary antigens and commensal micro-organisms, the antigens are not only eliminated, but immunological unresponsiveness to the antigens is also acquired. When an antigen is orally administered to animals, antigen-specific immune responses are suppressed after systemic immunization of the antigen, and this phenomenon is called oral tolerance.[1,2] The development of food hypersensitivity is related to the failure of oral tolerance induction.[3] Food allergy is categorized as class 1 food allergy, which might result from a breach in oral tolerance to foods, or class 2 food allergy, which might

Abbreviations: ABTS, 2,2′-azino-bis diammonium salt; APC, antigen-presenting cell; BSA, bovine serum albumin; CT, cholera toxin; CTM, complete T-cell medium; DC, dendritic cell; DTH, delayed-type hypersensitivity; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; IL, interleukin; i.p., intraperitoneally; IP, immunoprecipitated; M cell, microfold cell; MHC, major histocompatibility complex; IEC, intestinal epithelial cell; MLN, mesenteric lymph node; OVA, ovalbumin; PBS, phosphate-buffered saline; PP, Peyer's patch; SE, standard error; SRBC, sheep red blood cell; TGF, transforming growth factor; Th, T helper; TMB, tetramethyl benzidine.

 

result from sensitization to respiratory allergens or other sensitization not via gastrointestinal mucosa.[4,5] Class 1 food allergy typically occurs with food proteins, such as eggs or peanuts that are generally stable in digestion, in infants or children.[5] In typical class 2 food allergy, immunoglobulin E (IgE) antibody against respiratory allergens such as pollens recognizes homologous epitopes in food proteins of some fruits or vegetables.[5] In particular, to elucidate the pathogenesis of class 1 food allergy, it is fundamental to clarify mechanisms in the development and failure of oral tolerance. Moreover, the constructive induction of immunological suppression by oral tolerance is expected to contribute to prevent allergy[6] or autoimmune diseases[7,8] in which antigen-specific immune responses are pathologically enhanced.

When a dietary protein antigen is ingested, it is treated by digestive enzymes in the stomach and small intestine. Generated amino acids and small peptides are absorbed via the small intestinal lumen, and enter the portal vein through capillary vessels in the small intestine.[9] However, an antigen that escapes digestion can also enter the body via the intestinal surface. Microfold cells (M cells) over Peyer's patches (PPs) of the intestines take up soluble macromolecule proteins[10–12] as well as viruses[13–15] and bacteria.[16–18] After uptake via M cells, the antigens are processed and presented by dendritic cells (DCs) in PPs.[19] In addition, DCs under intestinal epithelia send dendrites between epithelial cells and directly acquire antigens over epithelial cells.[20] PPs are shown to be inductive sites for oral tolerance where T cells secreting regulatory cytokines, including interleukin (IL)-10[21] and transforming growth factor-β (TGF-β)[22] are induced; however, it is reported that oral tolerance can be induced in mice lacking PPs and mesenteric lymph nodes (MLNs).[23] On the other hand, the liver is shown to be crucial to tolerance induction because the intraportal injection of allogeneic donor cells,[24,25] eggs of a parasite[26] or insoluble protein[27] induces immunological tolerance against the corresponding antigen.

Ovalbumin (OVA) from chicken eggs is a dietary protein antigen that frequently causes food allergy.[28,29] After the oral administration of intact OVA, OVA antigens are known to be detected in peripheral blood and are suggested to contribute to the induction of immunological tolerance against OVA.[30–32] In this study, we attempted to examine OVA antigens in both portal and peripheral blood after the oral administration of OVA and tried to induce tolerance by intraportal and intravenous injection of intact OVA. Furthermore, to investigate the effects of digestion in the gastrointestinal tract on oral tolerance induction, intact OVA molecules were directly injected into the gastrointestinal tract and then the induction profile of tolerance against OVA was assessed.

## Materials and methods

### *Mice*

Female BALB/c, C57BL/6 or $BDF_1$ mice were used between the ages of 6 and 12 weeks. Mice were purchased from Charles River (Tokyo, Japan) or Sankyo Labo Service Co. (Shizuoka, Japan) and maintained in a specific pathogen-free environment.

### *Oral administration of OVA*

OVA, chicken egg, grade V (Sigma, St. Louis, MO) were dissolved in sterilized phosphate-buffered saline (PBS, pH 7·4). Mice were orally administered with 250 ng, 250 μg, 2·5 mg, 25 mg or 250 mg of OVA once. As a control, mice were orally treated with PBS in the same way. In some experiments, mice were orally administered with 1 or 100 mg of OVA or the same dose of OVA plus 10 μg of cholera toxin (CT; List Biological Laboratory Inc., Campbell, CA) once weekly for 4 weeks.

### *Intraportal or intravenous injection of OVA*

Intraportal injection was performed as described previously.[27] Mice were anaesthetized and underwent an abdominal operation. Filtered 2·5 mg or 250 mg of OVA in 250 μl of 0·03% trypan blue-PBS was injected into the portal vein using a 29G needle-tipped syringe. As a control, filtered 0·03% trypan-blue PBS was injected in the same way. In this case, OVA solution was coloured by adding trypan blue to confirm that it was really injected into the liver through the portal vein. After the injection, bleeding from the portal vein was stopped with thrombin (Mochida Pharmaceutical Co., LTD, Tokyo, Japan) and then the peritoneum and skin were sutured.

For intravenous injection, mice were anaesthetized and injected with filtered 2·5 mg or 250 mg of OVA in 250 μl of PBS into the tail vein. As a control, PBS was injected in the same way.

### *Injection of OVA into the digestive tract*

Mice were anaesthetized and underwent an abdominal operation. They were injected with 25 mg of OVA in 250 μl of PBS into the stomach, duodenum, ileum or colon using a 29G needle-tipped syringe, respectively, and then the peritoneum and skin were sutured.

### *Intraperitoneal immunization of OVA*

Mice were intraperitoneally (i.p) injected with 50 μg of OVA and 4 mg of alum, $Al(OH)_3$, in 0·5 ml of PBS. Two weeks later, the second immunization was performed in the same manner. In some experiments, a third boost



 

was performed by i.p. injection of 0·5 mg of OVA in 0·5 ml of PBS.

*Gut content collection*

The stomach and small intestine were removed from mice and washed with PBS. Supernatants were collected from the wash fluid and stored frozen at −80° until assay.

*Collection of portal or peripheral plasma and faecal samples*

For portal blood collection, mice were anaesthetized and underwent an abdominal operation. Portal blood was collected from the portal vein using a 24G catheter and heparinized capillary tubes, and then the peritoneum and skin were sutured. Peripheral blood was collected from anaesthetized mice using heparinized capillary tubes. The blood was centrifuged for 10 min at 6000 ***g***, and plasma was collected and stored frozen at −80° until assay.

Faecal extracts were prepared by the method described previously.[33] Fresh faeces were collected and weighed, and PBS containing 0·01% sodium azide was added to the faeces (100 mg/ml). The faeces in PBS were homogenized by continuous shaking for 10 min with a Vortex, and centrifuged for 10 min at 12 000 ***g*** at 4°. Supernatants were collected and stored frozen at −80° until assay.

*Enzyme-linked immunosorbent assay (ELISA)*

OVA antigen levels in the gut contents or the plasma and anti-OVA antibody levels in the plasma or the faecal extracts were determined by ELISA as described previously.[34,35] For the assay of OVA antigen levels, 96-well flat-bottomed microtitre plates were coated with rabbit anti-OVA IgG (Rockland, Gilbertsville, PA) in carbonate buffer (pH 9·6) at 4° overnight. Wells were blocked with 1% bovine serum albumin (BSA) in PBS at 37° for 1 hr. Gut contents or plasma samples diluted appropriately in PBS were added to the wells in duplicate, and incubated at 37° for 1 hr. Biotinylated anti-OVA IgG (Rockland) was added to the wells and incubated at 37° for 1 hr. Horseradish peroxidase-conjugated streptavidin (Caltag Laboratory, Burlingame, CA) was then added and incubated at 37° for 30 min. Enzyme reaction was performed with 1 mM 2,2′-azino-bis diammonium salt (ABTS, Sigma) in sodium citrate buffer (pH 5·4) in the presence of 0·01% $H_2O_2$. The reaction was interrupted by the addition of 2 mM $NaN_3$ in PBS, and absorbance was measured at 415 nm. To draw a standard curve, various quantities of OVA were added to the part of the plate coated with anti-OVA IgG and blocked. The wells were added to biotinylated anti-OVA IgG and coloured in the same manner as the sample wells. For the assay of anti-OVA immunoglobulin, plates were coated with OVA (100 μl of 1 mg/ml) in carbonate buffer. After blocking, diluted plasma or faecal extract samples were added to the wells and incubated. Biotinylated anti-mouse immunoglobulins (Amersham Life Science, Amersham, UK), IgA (Sigma), IgG1, IgG2a, IgG2b, IgM or IgE (BD PharMingen, San Diego, CA) were added to the wells and incubated. Horseradish peroxidase-conjugated streptavidin was then added and incubated. Enzyme reaction was performed with ABTS, and absorbance was measured at 415 nm. To draw a standard curve, part of the assay plate was coated with various quantities of purified mouse IgG1, IgG2a (BD PharMingen) or IgA (ICN/Cappel, Aurora, OH). After blocking, biotinylated antimouse IgG1, IgG2a or IgA were added to the wells and coloured in the same manner as the sample wells.

*Immunoprecipitation and immunoblotting*

For immunoprecipitation, 0·1 mg of rabbit anti-OVA IgG (Rockland) was incubated with 20 μl of protein-G coupled sepharose (Sigma) at 4° on an orbital shaker overnight. After washing with PBS three times, the treated sepharose was incubated with 1 ml of mouse serum or 100 ng of OVA mixed with 1 ml of untreated mouse serum at 4° on an orbital shaker overnight. After washing with PBS twice and 0·05 M Tris buffer once, the sepharose was resuspended in 20 μl of sample buffer (Invitrogen, Carlsbad, CA) including sample reducing agent (Invitrogen) and heated at 70° for 10 min. The supernatants were collected and diluted fivefold with the sample buffer. Five μl of the diluted samples were loaded on 4–12% Bis-Tris gel (Invitrogen). Proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Invitrogen). The membranes were blocked in 1% BSA 0·1% Tween-20 PBS and incubated with rabbit anti-OVA polyclonal IgG (Rockland) at 4° overnight. This was followed by incubation with peroxidase-conjugated anti-rabbit IgG (Seikagaku Corporation, Tokyo, Japan). The tetramethyl benzidine (TMB) substrate kit for peroxidase (Vector Labotarories, Inc., Burlingame, CA) was used for detection.

*OVA-specific T cell proliferation*

The spleens or MLNs were removed and crushed in RPMI-1640 medium (Sigma). Red blood cells in spleen cells were depleted by cell lysis. Single spleen cells or MLN cells were suspended in complete T-cell medium (CTM) composed of RPMI-1640 medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 0·1 mM nonessential amino acid, a mixture of vitamins, 1 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-mercaptoethanol, and 10% heat-inactivated fetal calf serum (FCS). OVA-specific T-cell proliferation was analysed by the modified method described previously.[36] Spleen or MLN T cells were taken using a nylon wool column and a single cell suspension was