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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 1.0×10^6 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

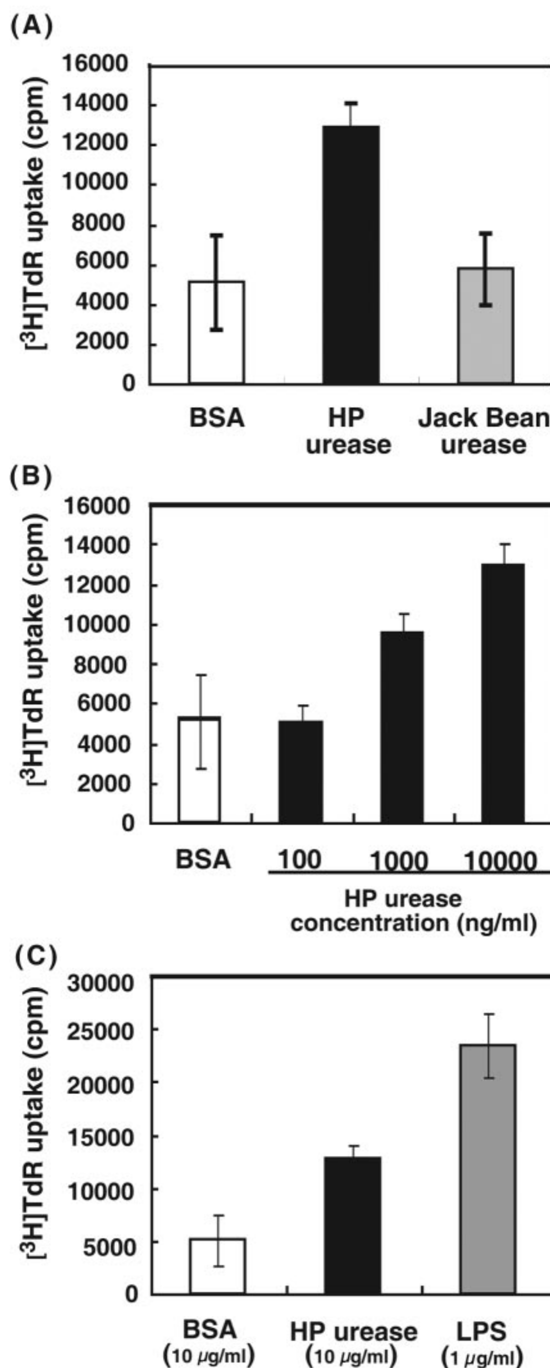

FIG. 1. Proliferative responses of naive splenic lymphocytes to purified *H. pylori* urease. (A) Significantly greater stimulatory capacity was observed when 10^6 naive splenocytes were cocultured with 10-μg/ml purified *H. pylori* urease than when they were cocultured with the same amount of BSA or 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.

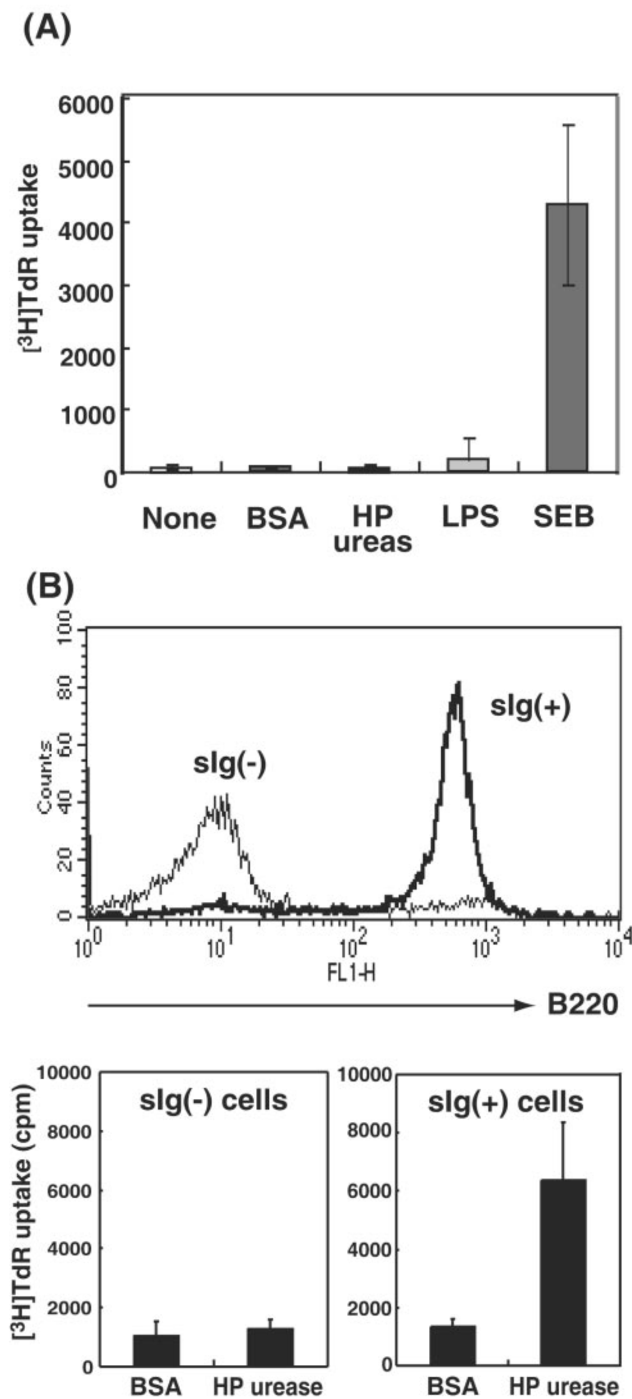

FIG. 2. sIg(+) B cells proliferated on stimulation with purified *H. pylori* urease. (A) When 10^6 naive thymocytes were cocultured with 10-μg/ml purified *H. pylori* urease, 10-μg/ml BSA, or 1-μg/ml *E. coli*-derived LPS for 3 days, no measurable proliferative responses were observed, although 1-μg/ml 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 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.

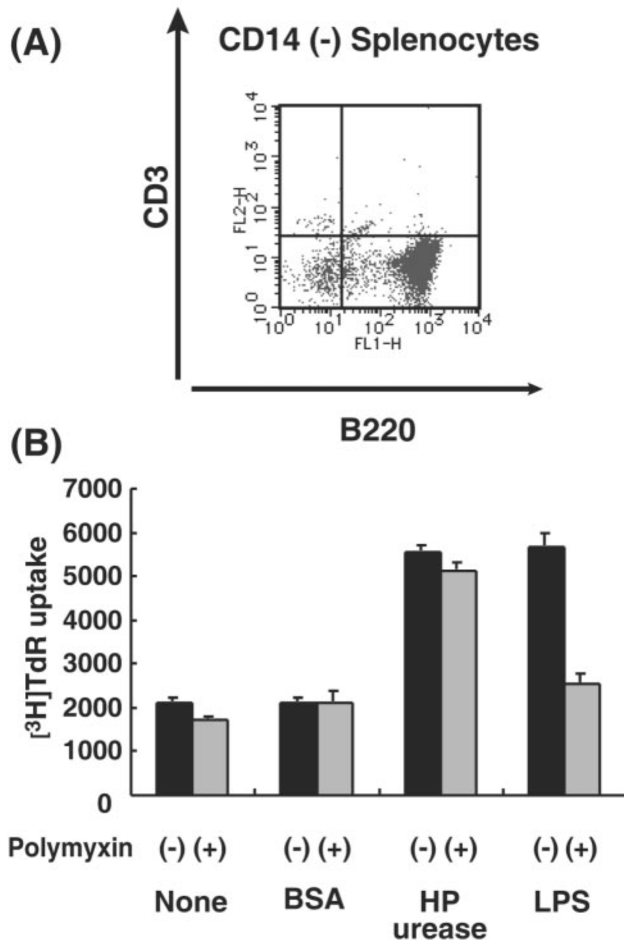

FIG. 3. Purified B lymphocytes were specifically stimulated by purified *H. pylori* urease. (A) Enriched $CD3^-$ $CD14^-$ $B220^+$ B lymphocytes with >90% purity were obtained reproducibly by the procedure described in Materials and Methods. (B) Using 10^6 of these purified B lymphocytes, we confirmed 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-

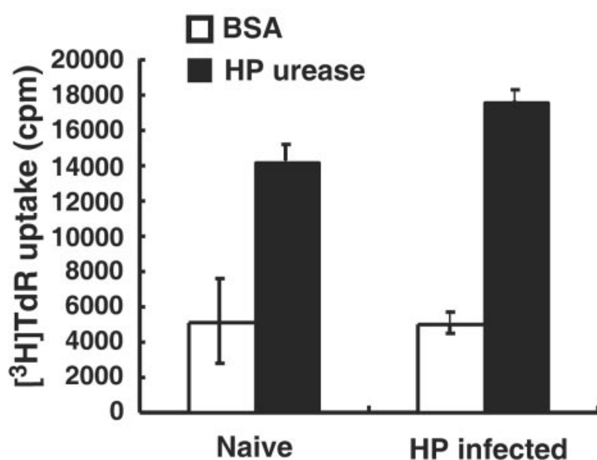

FIG. 4. Effects of in vivo priming with orally administrated *H. pylori* on B-cell stimulation by purified *H. pylori* urease. Mice were given three doses of 10^8 CFU of infectious *H. pylori* SS-1 orally, 6 to 8 weeks previously. When 10^6 of spleen cells from the primed mice were stimulated in vitro 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.

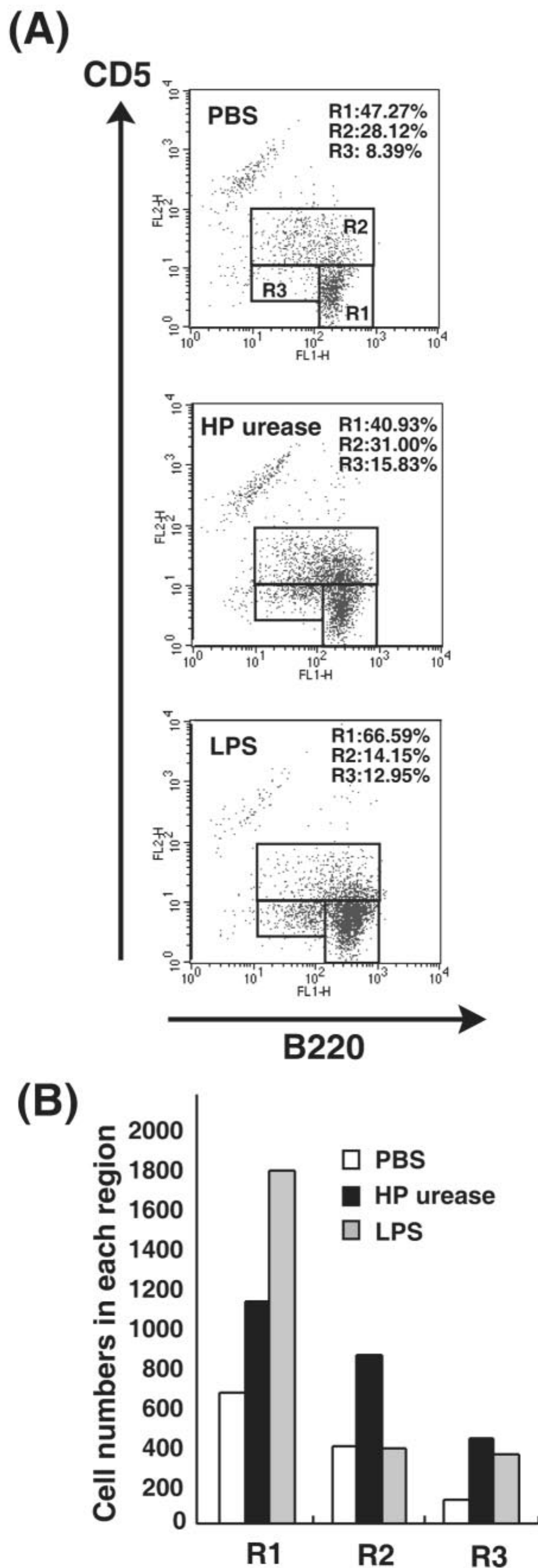

FIG. 5. CD5-positive B-1 cells are the major targets for purified *H. pylori* urease. (A) A total of 10^6 of purified B lymphocytes were cocultured with each reagent for 5 days, and all the cells were harvested for flow cytometric analysis. We divided the B220-positive B cells into three groups, R1, 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.

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-

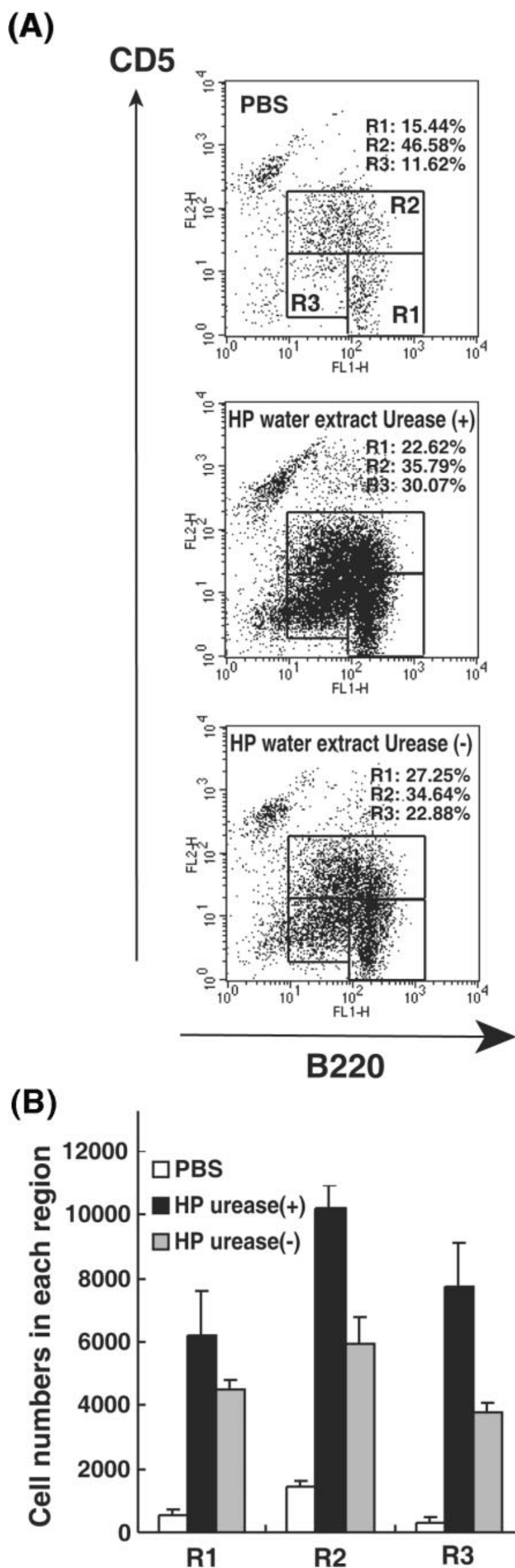

FIG. 6. Effects of urease depletion from *H. pylori* water extract on B-1-cell proliferation. (A) A total of 10^6 of purified splenic B cells were cultured with 5 μl of water extract of *H. pylori* for 5 days, and all the cells were harvested for flow cytometric analysis. As in the experiments shown 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.

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

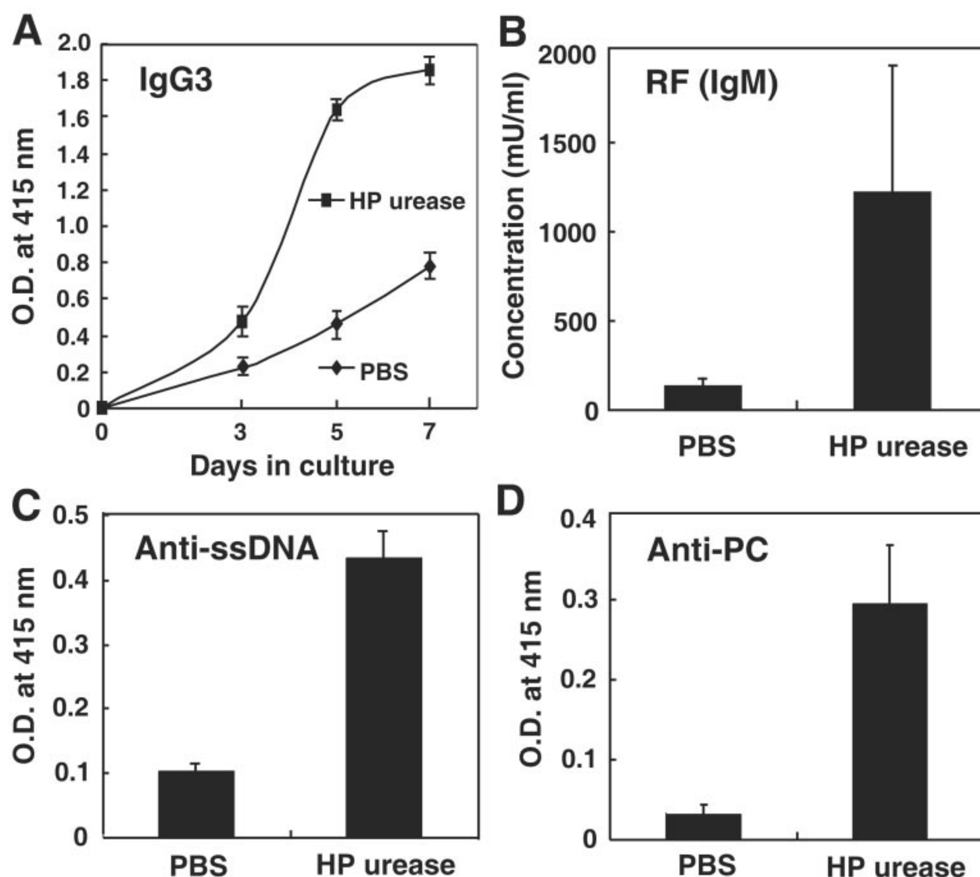

FIG. 7. Secretion of autoantibodies from purified B cells stimulated with purified *H. pylori* urease. A total of 10^6 of the purified splenic B cells were cultured with 10-μg/ml purified *H. pylori* urease or PBS for 3 to 7 days, and the supernatants were harvested to test autoantibody production by 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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