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

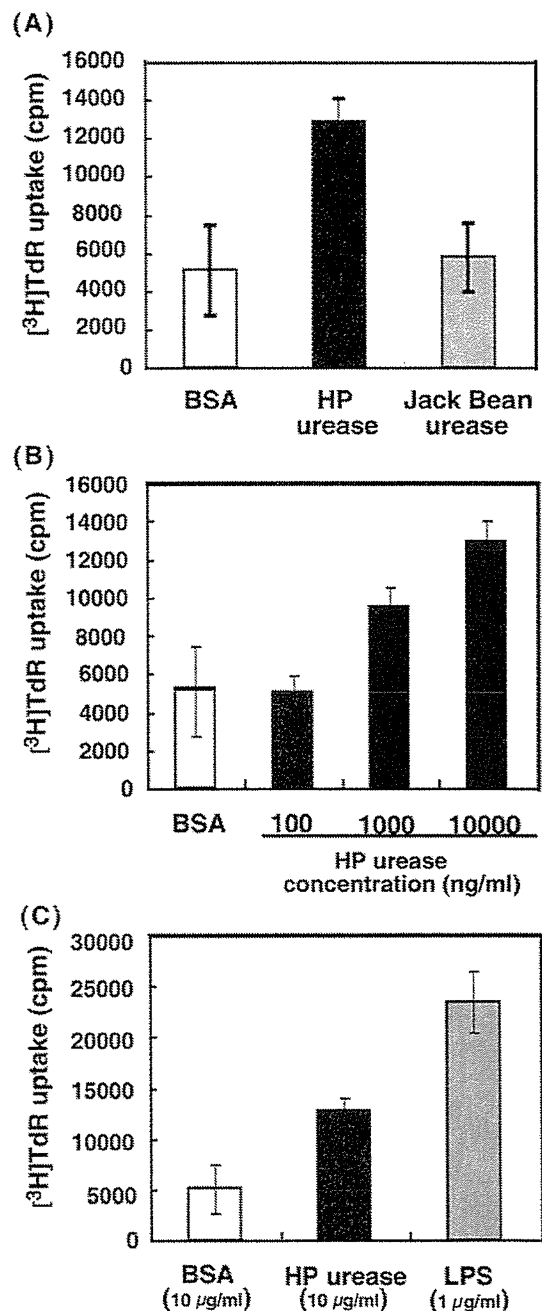


FIG. 1. Proliferative responses of naive splenic lymphocytes to purified *H. pylori* urease. (A) Significantly greater stimulatory capacity was observed when 10^6 naive splenocytes were cocultured with 10-μg/ml purified *H. pylori* urease than when they were cocultured with the same amount of BSA or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

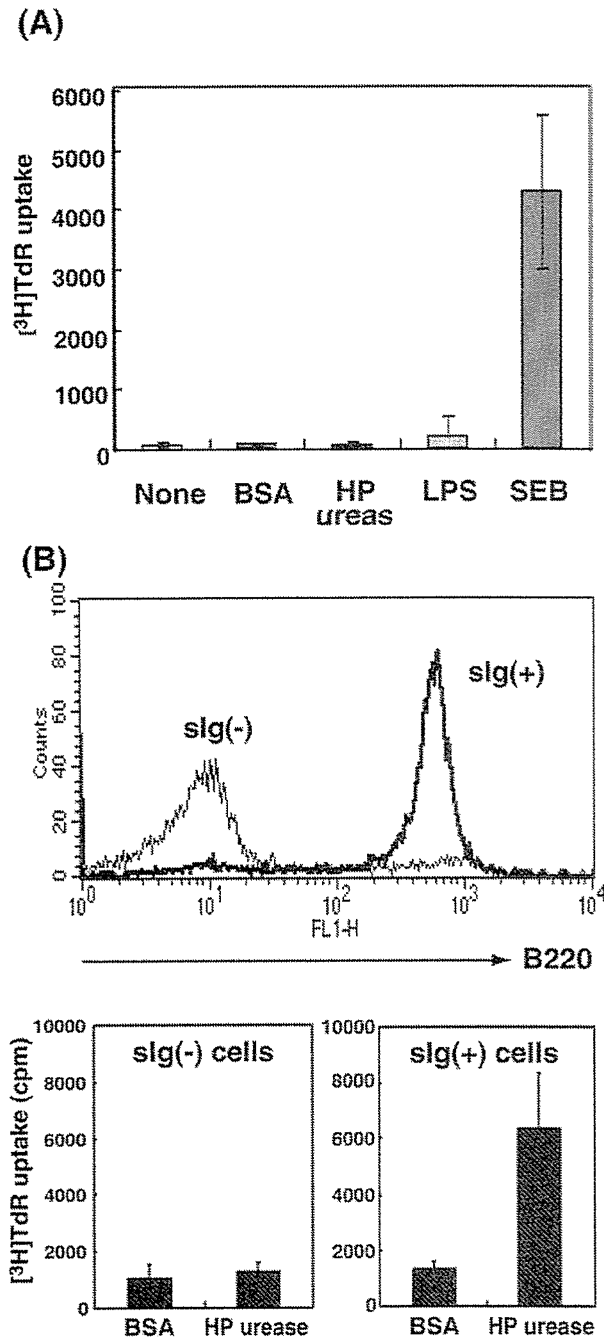


FIG. 2. sIg(+) B cells proliferated on stimulation with purified *H. pylori* urease. (A) When 10^6 naive thymocytes were cocultured with 10-μg/ml purified *H. pylori* urease, 10-μg/ml BSA, or 1-μg/ml *E. coli*-derived LPS for 3 days, no measurable proliferative responses were observed, although 1-μg/ml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

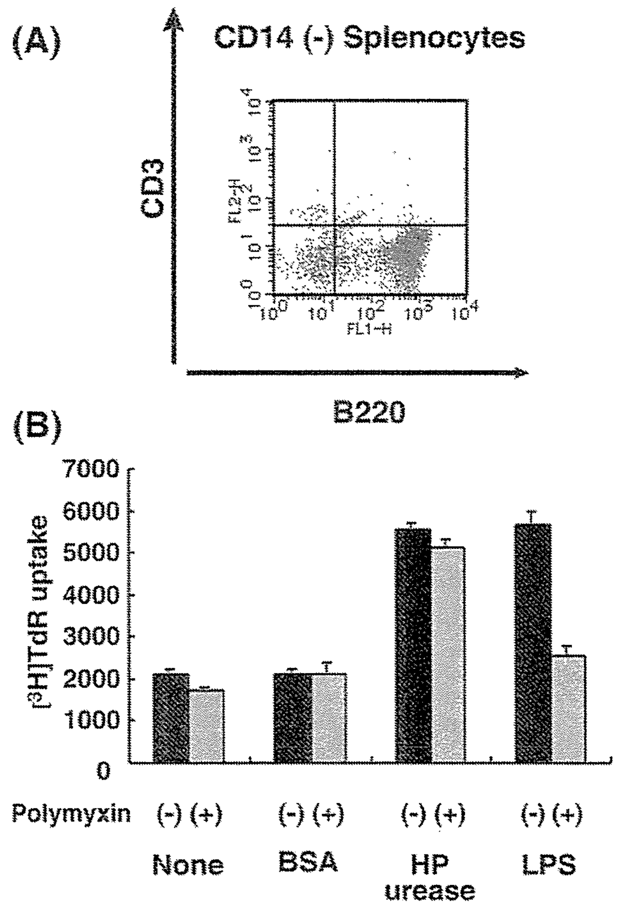


FIG. 3. Purified B lymphocytes were specifically stimulated by purified *H. pylori* urease. (A) Enriched $CD3^-$ $CD14^-$ $B220^+$ B lymphocytes with >90% purity were obtained reproducibly by the procedure described in Materials and Methods. (B) Using 10^6 of these purified B lymphocytes, we confirmed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

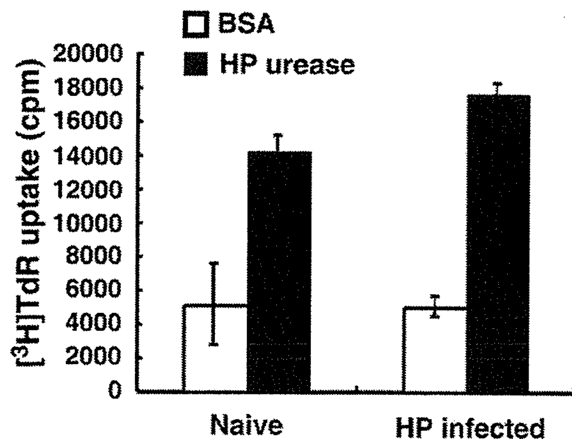


FIG. 4. Effects of in vivo priming with orally administrated *H. pylori* on B-cell stimulation by purified *H. pylori* urease. Mice were given three doses of 10^8 CFU of infectious *H. pylori* SS-1 orally, 6 to 8 weeks previously. When 10^6 of spleen cells from the primed mice were stimulated in vitro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

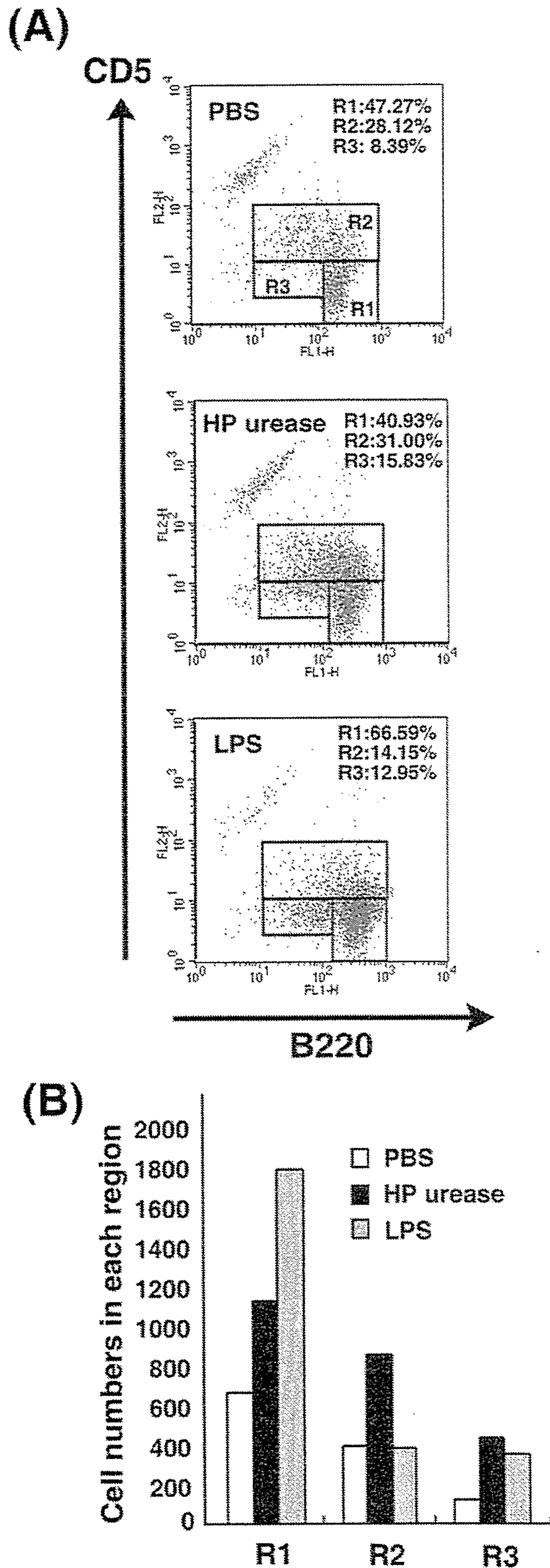
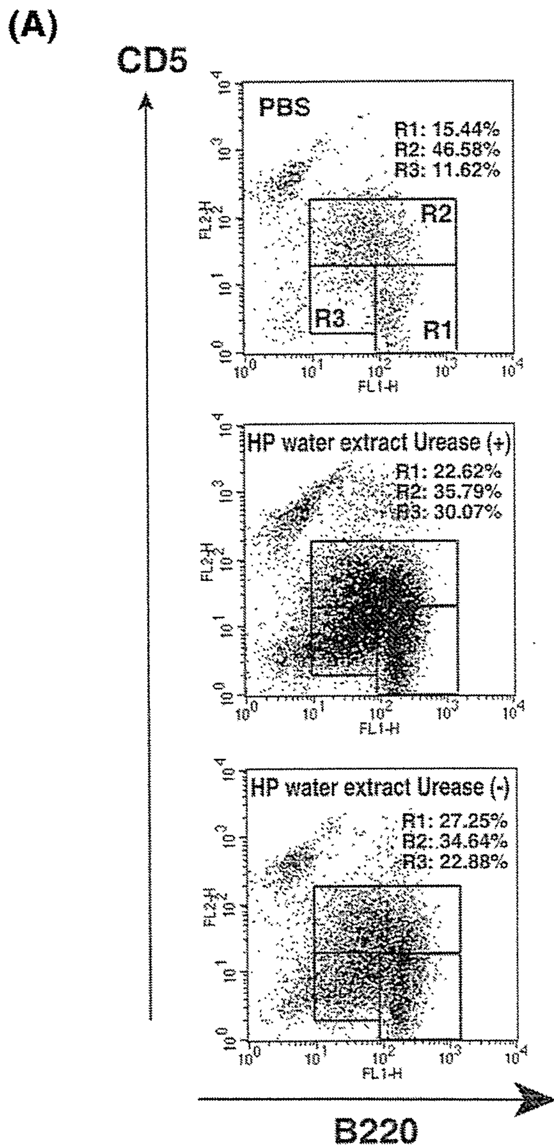


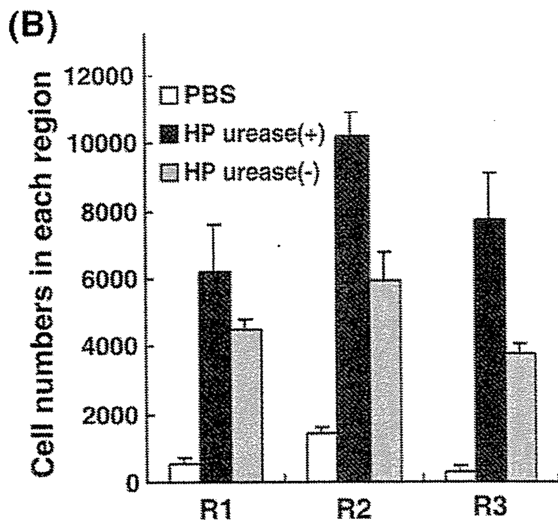
FIG. 5. CD5-positive B-1 cells are the major targets for purified *H. pylori* urease. (A) A total of 10^6 of purified B lymphocytes were cocultured with each reagent for 5 days, and all the cells were harvested for flow cytometric analysis. We divided the B220-positive B cells into three groups, R1,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





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.

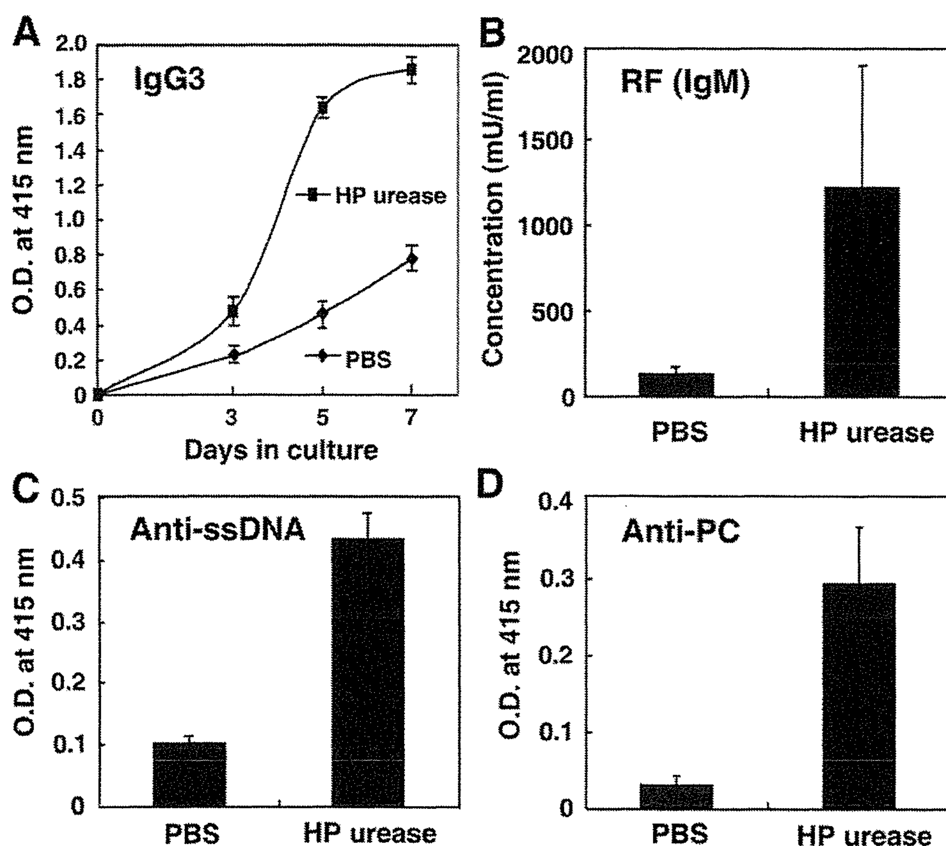


FIG. 7. Secretion of autoantibodies from purified B cells stimulated with purified *H. pylori* urease. A total of 10^6 of the purified splenic B cells were cultured with 10-μg/ml purified *H. pylori* urease or PBS for 3 to 7 days, and the supernatants were harvested to test autoantibody production by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

ACKNOWLEDGMENTS

This work was supported in part by grants from the Ministry of Education, Science, Sport, and Culture and from the Ministry of Health and Labor and Welfare, Japan; from the Japanese Health Sciences Foundation; and from the Promotion and Mutual Aid Corporation for Private Schools of Japan.

We are grateful to Timothy D. Minton for proofreading the manuscript.

REFERENCES

1. **Ando, T., T. Tsuzuki, T. Mizuno, M. Minami, K. Ina, K. Kusugami, J. Takamatsu, K. Adachi, E. El-Omar, M. Ohta, and H. Goto.** 2004. Characteristics of *Helicobacter pylori*-induced gastritis and the effect of *H. pylori* eradication in patients with chronic idiopathic thrombocytopenic purpura. Helicobacter **9:**443–452.
2. **Araujo, P. M., D. Holmberg, A. C. Martinez, and A. Coutinho.** 1987. Idiotypic multireactivity of 'natural' antibodies. 'Natural' anti-idiotypes also inhibit helper cells with cross-reactive clonotypes. Scand. J. Immunol. **25:**497–505.
3. **Arnold, L. W., C. A. Pennell, S. K. McCray, and S. H. Clarke.** 1994. Development of B-1 cells: segregation of phosphatidyl choline-specific B cells to the B-1 population occurs after immunoglobulin gene expression. J. Exp. Med. **179:**1585–1595.
4. **Banerjee, S. K., A. P. Weston, M. N. Zoubine, D. R. Campbell, and R. Cherian.** 2000. Expression of cdc2 and cyclin B1 in *Helicobacter pylori*-associated gastric MALT and MALT lymphoma: relationship to cell death, proliferation, and transformation. Am. J. Pathol. **156:**217–225.
5. **Berland, R., and H. H. Wortis.** 2002. Origins and functions of B-1 cells with notes on the role of CD5. Annu. Rev. Immunol. **20:**253–300.
6. **Blaser, M. J., and J. C. Atherton.** 2004. *Helicobacter pylori* persistence: biology and disease. J. Clin. Investig. **113:**321–333.
7. **Brezinschek, H.-P., S. J. Foster, R. I. Brezinschek, T. DöRner, R. Domiati-Saad, and P. E. Lipsky.** 1997. Analysis of the human VH gene repertoire. Differential effects of selection and somatic hypermutation on human peripheral $CD5^+/IgM^+$ and $CD5^-/IgM^+$ B cells. J. Clin. Investig. **99:**2488–2501.
8. **Brubaker, J. O., Q. Li, A. O. Tzianabos, D. L. Kasper, and R. W. Finberg.** 1999. Mitogenic activity of purified capsular polysaccharide A from *Bacteroides fragilis*: differential stimulatory effect on mouse and rat lymphocytes in vitro. J. Immunol. **162:**2235–2242.
9. **Caletti, G., T. Togliani, P. Fusaroli, E. Sabattini, E. Khodadadian, B. Gamberi, M. Gobbi, and S. Pileri.** 2003. Consecutive regression of concurrent laryngeal and gastric MALT lymphoma after anti-*Helicobacter pylori* therapy. Gastroenterology **124:**537–543.
10. **Cong, Y., and H. Braley-Mullen.** 1995. Proliferation and autoantibody production by mouse thyroglobulin (MTg)-specific B cells activated in vitro by MTg and MTg-specific T cells. Immunol. Lett. **45:**189–193.
11. **Dauphinee, M., Z. Tovar, and N. Talal.** 1988. B cells expressing CD5 are increased in Sjogren's syndrome. Arthritis Rheum. **31:**642–647.
12. **De Vita, S., G. Ferraccioli, C. Avellini, D. Sorrentino, R. Dolcetti, C. Di Loreto, E. Bartoli, M. Boiocchi, and C. A. Beltrami.** 1996. Widespread clonal B-cell disorder in Sjogren's syndrome predisposing to *Helicobacter pylori*-related gastric lymphoma. Gastroenterology **110:**1969–1974.
13. **Eaton, K. A., C. L. Brooks, D. R. Morgan, and S. Krakowka.** 1991. Essential role of urease in pathogenesis of gastritis induced by *Helicobacter pylori* in gnotobiotic piglets. Infect. Immun. **59:**2470–2475.
14. **Emilia, G., G. Longo, M. Luppi, G. Gandini, M. Morselli, L. Ferrara, S. Amarri, K. Cagossi, and G. Torelli.** 2001. *Helicobacter pylori* eradication can induce platelet recovery in idiopathic thrombocytopenic purpura. Blood **97:**812–814.
15. **Futagami, S., H. Takahashi, Y. Norose, and M. Kobayashi.** 1998. Systemic and local immune responses against *Helicobacter pylori* urease in patients with chronic gastritis: distinct IgA and IgG productive sites. Gut **43:**168–175.
16. **Futagami, S., H. Takahashi, Y. Norose, K. Nagata, M. Kobayashi, and T. Nomura.** 1994. Analysis of immune response to *Helicobacter pylori*-identification of the protein recognized by anti-*Helicobacter pylori* antibodies from sera of patients with gastroduodenal diseases. Nippon Shokakibyo Gakkai Zasshi **91:**2202–2213.
17. **Gasbarrini, A., F. Franceschi, R. Tartaglione, R. Landolfi, P. Pola, and G. Gasbarrini.** 1998. Regression of autoimmune thrombocytopenia after eradication of *Helicobacter pylori*. Lancet **352:**878.
18. **Hayashi, S., T. Sugiyama, K. Yokota, H. Isogai, E. Isogai, K. Oguma, M. Asaka, N. Fujii, and Y. Hirai.** 1998. Analysis of immunoglobulin A antibodies to *Helicobacter pylori* in serum and gastric juice in relation to mucosal inflammation. Clin. Diagn. Lab. Immunol. **5:**617–621.
19. **Hirota, K., K. Nagata, Y. Norose, S. Futagami, Y. Nakagawa, H. Senpuku, M. Kobayashi, and H. Takahashi.** 2001. Identification of an antigenic epitope in *Helicobacter pylori* urease that induces neutralizing antibody production. Infect. Immun. **69:**6597–6603.
20. **Icatlo, F. C., Jr., M. Kuroki, C. Kobayashi, H. Yokoyama, Y. Ikemori, T. Hashi, and Y. Kodama.** 1998. Affinity purification of *Helicobacter pylori* urease. Relevance to gastric mucin adherence by urease protein. J. Biol. Chem. **273:**18130–18138.
21. **Iizumi, T., S. Yamanishi, Y. Kumagai, K. Nagata, S. Kamiya, K. Hirota, E. Watanabe, C. Sakamoto, and H. Takahashi.** 2005. Augmentation of *Helicobacter pylori* urease activity by its specific IgG antibody: implications for bacterial colonization enhancement. Biomed. Res. **26:**35–42.
22. **Ishikawa, N., T. Fuchigami, T. Matsumoto, H. Kobayashi, Y. Sakai, H. Tabata, N. Takubo, S. Yamamoto, M. Nakanishi, K. Tomioka, and M. Fujishima.** 2002. *Helicobacter pylori* infection in rheumatoid arthritis: effect of drugs on prevalence and correlation with gastroduodenal lesions. Rheumatology **41:**72–77.
23. **Kantor, A.** 1991. A new nomenclature for B cells. Immunol. Today **12:**388.
24. **Kantor, A. B., C. E. Merrill, L. A. Herzenberg, and J. L. Hillson.** 1997. An unbiased analysis of V(H)-D-J.(H) sequences from B-1a, B-1b, and conventional B cells. J. Immunol. **158:**1175–1186.
25. **Kantor, A. B., A. M. Stall, S. Adams, and L. A. Herzenberg.** 1992. Differential development of progenitor activity for three B-cell lineages. Proc. Natl. Acad. Sci. USA **89:**3320–3324.
26. **Karpatkin, S., M. A. Nardi, and K. B. Hymes.** 1995. Sequestration of anti-platelet GPIIIa antibody in rheumatoid factor immune complexes of human immunodeficiency virus 1 thrombocytopenic patients. Proc. Natl. Acad. Sci. USA **92:**2263–2267.
27. **Lee, A., J. O'Rourke, M. C. De Ungria, B. Robertson, G. Daskalopoulos, and M. F. Dixon.** 1997. A standardized mouse model of *Helicobacter pylori* infection: introducing the Sydney strain. Gastroenterology **112:**1386–1397.
28. **Mai, U. E., G. I. Perez-Perez, L. M. Wahl, S. M. Wahl, M. J. Blaser, and P. D. Smith.** 1991. Soluble surface proteins from *Helicobacter pylori* activate monocytes/macrophages by lipopolysaccharide-independent mechanism. J. Clin. Investig. **87:**894–900.
29. **Martin, F., and J. F. Kearney.** 2001. B1 cells: similarities and differences with other B cell subsets. Curr. Opin. Immunol. **13:**195–201.
30. **Martin, F., and J. F. Kearney.** 2000. B-cell subsets and the mature preimmune repertoire. Marginal zone and B1 B cells as part of a "natural immune memory." fsImmunol. Rev. **175:**70–79.
31. **Muotiala, A., I. M. Helander, L. Pyhala, T. U. Kosunen, and A. P. Moran.** 1992. Low biological activity of *Helicobacter pylori* lipopolysaccharide. Infect. Immun. **60:**1714–1716.
32. **Nagata, K., T. Mizuta, Y. Tonokatu, Y. Fukuda, H. Okamura, T. Hayashi, T. Shimoyama, and T. Tamura.** 1992. Monoclonal antibodies against the native urease of *Helicobacter pylori*: synergistic inhibition of urease activity by monoclonal antibody combinations. Infect. Immun. **60:**4826–4831.
33. **Nurgalieva, Z. Z., M. E. Conner, A. R. Opekun, C. Q. Zheng, S. N. Elliott, P. B. Ernst, M. Osato, M. K. Estes, and D. Y. Graham.** 2005. B-cell and T-cell immune responses to experimental *Helicobacter pylori* infection in humans. Infect. Immun. **73:**2999–3006.
34. **Simon, M. R., L. D. Roi, S. Desai, D. J. Salberg, and N. R. Rose.** 1983. Comparison of cultures of human lymphocytes obtained following NH_4Cl induced red blood cell lysis and Ficoll-Hypaque density gradient centrifugation. Immunol. Commun. **12:**301–314.
35. **Suerbaum, S., and P. Michetti.** 2002. *Helicobacter pylori* infection. N. Engl. J. Med. **347:**1175–1186.
36. **Takahashi, H., K. B. Cease, and J. A. Berzofsky.** 1989. Identification of proteases that process distinct epitopes on the same protein. J. Immunol. **142:**2221–2229.
37. **Takahashi, H., Y. Nakagawa, K. Yokomuro, and J. A. Berzofsky.** 1993. Induction of $CD8^+$ cytotoxic T lymphocytes by immunization with syngeneic irradiated HIV-1 envelope derived peptide-pulsed dendritic cells. Int. Immunol. **5:**849–857.
38. **Takeshita, H., I. Taniuchi, J. Kato, and T. Watanabe.** 1998. Abrogation of autoimmune disease in Lyn-deficient mice by the mutation of the Btk gene. Int. Immunol. **10:**435–444.
39. **Whitmore, A. C., G. Haughton, and L. W. Arnold.** 1996. Phenotype of B cells responding to the thymus-independent type-2 antigen polyvinyl pyrrolidinone. Int. Immunol. **8:**533–542.
40. **Won, W. J., and J. F. Kearney.** 2002. CD9 is a unique marker for marginal zone B cells, B1 cells, and plasma cells in mice. J. Immunol. **168:**5605–5611.
41. **Wotherspoon, A. C.** 1996. Gastric MALT lymphoma and *Helicobacter pylori*. Yale J. Biol. Med. **69:**61–68.
42. **Youinou, P., L. Mackenzie, P. Katsikis, G. Merdrignac, D. A. Isenberg, N. Tuaillon, A. Lamour, P. Le Goff, J. Jouquan, A. Drogou, et al.** 1990. The relationship between CD5-expressing B lymphocytes and serologic abnormalities in rheumatoid arthritis patients and their relatives. Arthritis Rheum. **33:**339–348.
43. **Zucca, E., F. Bertoni, E. Roggero, G. Cazzaniga, G. Bosshard, A. Biondi, and F. Cavalli.** 1998. Autoreactive B cell clones in marginal-zone B cell lymphoma (MALT lymphoma) of the stomach. Leukemia **12:**247–249.

Editor: J. D. Clements

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

Ayako Wakabayashi,[1] Yoshihiro Kumagai,[1] Eiji Watari,[1] Masumi Shimizu,[1] Masanori Utsuyama,[2] Katsuiku Hirokawa[2] and Hidemi Takahashi[1]

[1]*Department of Microbiology and Immunology, Nippon Medical School, Tokyo, Japan and* [2]*Department of Pathology and Immunology, Graduate School, Tokyo Medical and Dental University, Tokyo, Japan*

doi:10.1111/j.1365-2567.2006.02418.x
Received 3 January 2006; revised 15 May 2006; accepted 15 May 2006.
Correspondence: Dr Y. Kumagai, Department of Microbiology and Immunology, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113–8602, Japan.
Email: ykumagai@nms.ac.jp
Senior author: Hidemi Takahashi,
email: htkuhkai@nms.ac.jp

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

© 2006 Blackwell Publishing Ltd, *Immunology*, **119**, 167–177

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

prepared in the CTM. The spleen or MLN T cells (5×10^5) were cultured with 0, 111, 333 or 1000 mg/ml of OVA in the presence of $2{\cdot}5 \times 10^5$ of irradiated (3000 rad) spleen cells from naive C57BL/6 mice in 96-well flat-bottomed culture plates at 37° in 5% CO_2 for 4 days. During the last 18 hr of the 4-day culture, 0·5 μCi of tritiated [^{3}H]thymidine was added to each well. The plates were harvested and counted using a β counter (1450 Microbeta Trilux; Wallac, Gaithersburg, MD).

Cytokine analysis

Levels of IL-4 or IL-2 in the spleen T-cell culture supernatants were analysed by an IL-4-dependent cell line, CT.4S cells (kindly gifted by Prof. William E. Paul, National Institutes of Health, Bethesda, MD), or an IL-2-dependent cell line, CTLL-2 cells (American Type Culture Collection, ATCC, Manassas, VA). T-cell culture supernatants were collected on day 3 and stored frozen at −80° until assay. CT.4S or CTLL-2 cells (5×10^3) were cultured in the presence of the supernatant in 96-well flat-bottomed culture plates at 37° in 5% CO_2 for 3 or 2 days, respectively. To draw a standard curve, CT.4S or CTLL-2 cells were cultured with various quantities of recombinant mouse IL-4 (rIL-4) or rIL-2 (Genzyme, Cambridge, MA), respectively. During the last 18 hr of incubation, 0·5 μCi of [^{3}H]thymidine was added to each well. The plates were harvested and counted using the β counter.

Delayed-type hypersensitivity (DTH) response

Mice were anaesthetized and intradermally injected 20 μg of OVA in 20 μl of saline into the right ears. As negative controls, saline was injected into the left ears. Ear thickness was measured using a dial thickness gauge (Ozaki MFG. Co., LTD. Tokyo, Japan) before and 24 hr after the injection. The swelling rate of the ear was calculated as follows: [(thickness of the ear 24 hr after challenge − thickness of the ear before challenge)/thickness of the ear before challenge] × 100%.

Statistical analysis

Student's *t*-test was used to determine the statistical significance of differences between groups. Data were considered significant at $P < 0{\cdot}05$.

Results

Appearance of detectable OVA antigens in both portal and peripheral blood after oral administration of OVA

The oral administration of OVA induces oral tolerance against OVA, and it has been shown that OVA antigens

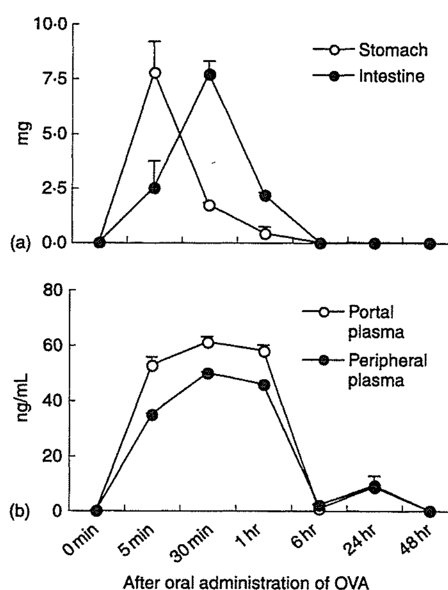


Figure 1. Kinetics of OVA antigens detected in gut contents and plasma after oral administration of OVA. BALB/c mice were orally administered with 25 mg of OVA. At various times after oral administration, the stomach or small intestine contents were collected (a). Portal and peripheral blood was collected from mice at various times after oral administration (b). Levels of OVA antigens in the gut contents or plasma were assessed by ELISA. The data are expressed as the mean + standard error (SE) of three mice.

are detected in peripheral blood after the oral administration of OVA.[30,31] First, we attempted to detect OVA antigens in the digestive tract and in blood after absorption via the guts.

When 25 mg of OVA was orally administered to mice, many OVA antigens in the gastric contents and fewer OVA antigens in the small intestinal contents were detected at 5 min after oral administration (Fig. 1a). While the amount of detectable OVA antigens was reduced at 30 min in the stomach, they reversely increased in the small intestine at the same time. OVA antigens in the guts could not be detected at 6 hr and subsequently (Fig. 1a).

OVA antigens were observed in both portal and peripheral blood at 5 min after the oral administration of OVA (Fig. 1b). They reached a peak at 30 min and became undetected at 6 hr after oral administration (Fig. 1b). Thus, kinetics of OVA antigens in the portal and peripheral blood corresponded to that in the small intestinal contents. The amount of OVA antigens detected in the portal and peripheral blood was dependent on the dose of orally administered OVA (Fig. 2). Levels of OVA antigens in the portal blood were more than in the peripheral blood (Fig. 1b and Fig. 2). OVA antigens were observed in the blood from all strains used in this study, BALB/c, C57BL/6, and BDF_1 mice (data

© 2006 Blackwell Publishing Ltd,

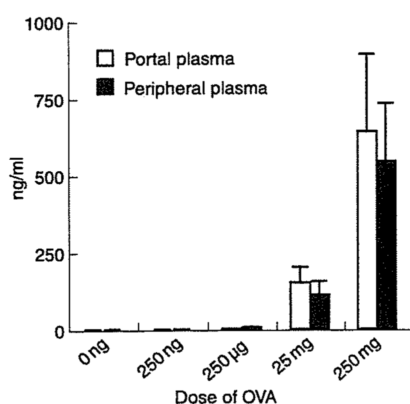


Figure 2. Level of OVA antigens in plasma is dependent on the dose of orally administered OVA. BALB/c mice were orally administered with various doses of OVA. Portal and peripheral blood was collected from mice at 30 min after oral administration. Levels of OVA antigens in plasma were assessed by ELISA. The data are expressed as the mean + SE of three mice.

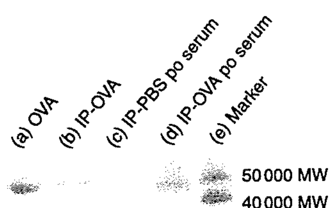


Figure 3. Immunoblot of OVA antigens in the serum after the oral administration of OVA. C57BL/6 mice were orally administered with 250 mg of OVA or PBS. Blood was collected from the heart 30 min after the oral administration (po) of OVA or PBS. OVA antigens in the serum were detected by immunoprecipitation and immunoblotting. Immunoprecipitation was performed using a rabbit anti-OVA polyclonal antibody on serum from mice or OVA mixed with serum from untreated mice. Immunoprecipitated (IP) samples or 2·5 ng of OVA were loaded. Proteins were transferred to a PVDF membrane and detected using the anti-OVA polyclonal antibody. (a) 2·5 ng of OVA, (b) IP sample of OVA mixed with untreated mouse serum, (c) IP sample of the serum after oral administration of PBS, (d) IP sample of the serum after the oral administration of OVA, (e) molecular markers.

not shown). To examine the molecular weights of OVA antigens in the serum, we performed immunoprecipitation and immunoblotting in serum samples using an anti-OVA polyclonal antibody. Surprisingly, 45 000 MW OVA was clearly detected in the serum 30 min after the oral administration of OVA (Fig. 3). In our immunoprecipitation and immunoblotting system using an anti-OVA polyclonal antibody, digested OVA fragments could not be detected in the serum. These results suggest that the macromolecules, 45 000 MW OVA antigens, were absorbed via the small intestines, transferred to the portal vein and circulated in the bloodstream in normal mice, in which oral tolerance was induced.

OVA antigens become undetected in the blood with an increase in mucosal and systemic OVA-specific immunoglobulin

Next, we attempted to analyse OVA antigens in blood from mice induced with OVA-specific immune responses by the oral administration of OVA plus CT adjuvant. Mice were orally administered with 1 or 100 mg of OVA or the same dose of OVA plus CT every week. Peripheral blood samples from mice were collected 30 min after oral administration every week and levels of OVA antigens and OVA-specific IgG in the blood were assessed by ELISA. The production of OVA-specific faecal IgA, OVA-specific T-cell proliferation, and secretion of cytokines were also analysed in mice.

The production of OVA-specific plasma IgG1 and faecal IgA was enhanced after the oral administration of 1 or 100 mg of OVA plus CT; however, the oral administration of OVA without CT did not induce OVA-specific systemic and mucosal antibody production (Fig. 4). OVA-specific serum IgG2a, T helper (Th)1-type antibody, could not be detected by oral administration with and without CT during the experimental period. As shown in Fig. 5, OVA-specific T-cell proliferation was also observed in spleen T cells and MLN T cells from mice orally administered with OVA plus CT (Fig. 5a). IL-4 secretion was observed in culture supernatants from proliferated spleen T cells; however, IL-2 was not

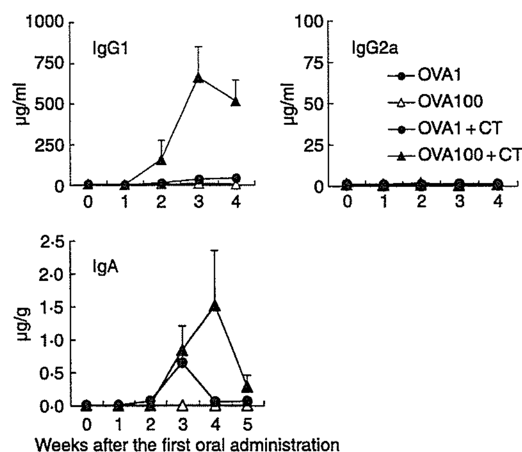


Figure 4. Production of OVA-specific systemic IgG and faecal IgA by oral administration of OVA plus CT. C57BL/6 mice were orally administered with 1 or 100 mg of OVA, or the same dose of OVA plus CT once weekly for 5 weeks. Peripheral blood and faeces were collected from the mice every week. OVA-specific plasma IgG1 and IgG2a and faecal IgA were assessed by ELISA. The data are expressed as the mean anti-OVA immunoglobulin in plasma (µg/ml) or faeces (µg/g) + SE of four mice.

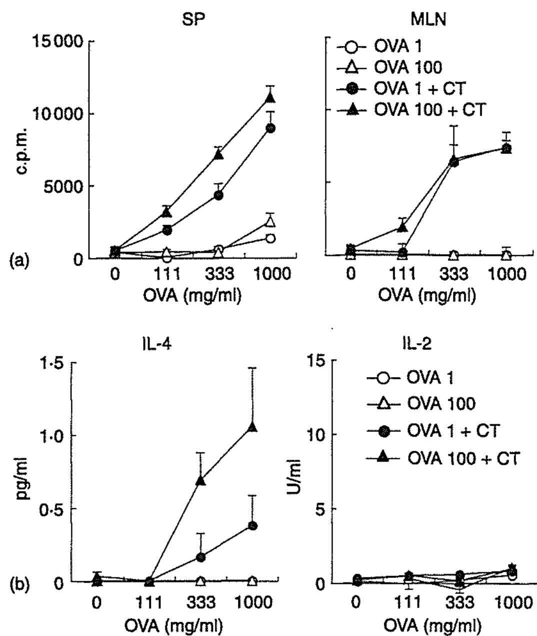


Figure 5. OVA-specific T cell proliferation and secretion of Th2-type cytokine by oral administration of OVA plus CT. C57BL/6 mice were orally administered with 1 mg or 100 mg of OVA, or the same dose of OVA plus CT once weekly for 3 weeks. Spleens and MLNs were removed 2 weeks after the third administration and T cells were isolated. T cells from the spleen or MLN were cultured with 0, 111, 333 or 1000 mg/ml of OVA in the presence of irradiated spleen cells from naive C57BL/6 mice for 4 days. OVA-specific T cell proliferation was assessed as described in Materials and methods (a). Splenic T cell culture supernatants were collected on day 3. IL-4-dependent cell line, CT-4S, or IL-2-dependent cell line, CTLL-2, were cultured in the presence of the supernatant for 3 or 2 days, respectively. Cytokine levels were assessed as described in Materials and methods (b). The results are expressed as the level of orally administered mice minus the level of normal mice. The results are shown as the mean + SE in triplicate. Data are representative of two separate experiments.

detected (Fig. 5b). These results demonstrate that the oral administration of OVA plus CT induces mucosal IgA and Th2-type systemic immune responses.

Although OVA antigens in the blood were sufficiently detected at 1 week after the oral administration of 100 mg of OVA plus CT, they were remarkably reduced at 2 weeks and subsequently remained reduced (Fig. 6), while OVA-specific systemic IgG1 and mucosal IgA were produced (Fig. 4). On the other hand, in mice orally administered with 100 mg of OVA, OVA antigens were detected every week. When 1 mg of OVA or the same dose of OVA plus CT was orally administered, OVA antigens were undetectable in the blood (Fig. 6).

These results show that in immunized mice, mucosal anti-OVA IgA may bind OVA antigens in the gastrointestinal tract and block OVA from entering into the mucosal tissues. Furthermore, OVA antigens may be caught by

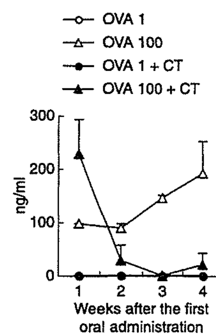


Figure 6. OVA antigens become undetectable after oral administration of OVA plus CT. C57BL/6 mice were orally administered with 1 or 100 mg of OVA or the same dose of OVA plus CT once weekly for 4 weeks. Peripheral blood was collected from the mice 30 min after oral administration every week. OVA antigens in plasma were assessed by ELISA. The data are expressed as the mean + SE of three mice.

anti-OVA IgG and immune complexes might be undetected in ELISA.

Oral administration of intact OVA suppresses OVA-specific immune responses whereas intraportal or intravenous injection cannot induce immunological suppression

The results indicate that OVA antigens appear in the blood from mice in which oral tolerance is induced, although they are markedly reduced in mice in which OVA-specific immune responses are induced. Therefore, we attempted to induce immunological tolerance against OVA by the intravenous injection of OVA. Mice were orally administered or injected into the portal or peripheral vein with 2·5 mg or 25 mg of OVA, and then i.p. immunized with OVA plus alum. OVA-specific systemic immunoglobulin production and DTH response were assessed in the mice.

The oral administration of a high dose of intact OVA significantly suppressed DTH response against OVA ($P < 0{\cdot}005$), compared with that in the oral treatment of PBS (Fig. 7a). The DTH response, however, was not significantly suppressed by the intraportal or intravenous injection of intact OVA (Fig. 7b, c). When control PBS was injected into ears, DTH response was not induced (Fig. 7).

Oral administration of OVA also significantly suppressed the production of anti-OVA immunoglobulins (Fig. 8a), whereas the production was significantly enhanced rather than suppressed at 1 week after the intraportal or intravenous injection of OVA (Fig. 8b, c). All immunoglobulin subclasses, IgG1, IgG2a, IgG2b, IgM and IgE, were significantly suppressed by the oral administration of OVA (Fig. 8a). On the other hand, in mice injected with OVA intraportally or intravenously, anti-OVA IgG1

© 2006 Blackwell Publishing Ltd, *Immunology*, **119**, 167–177

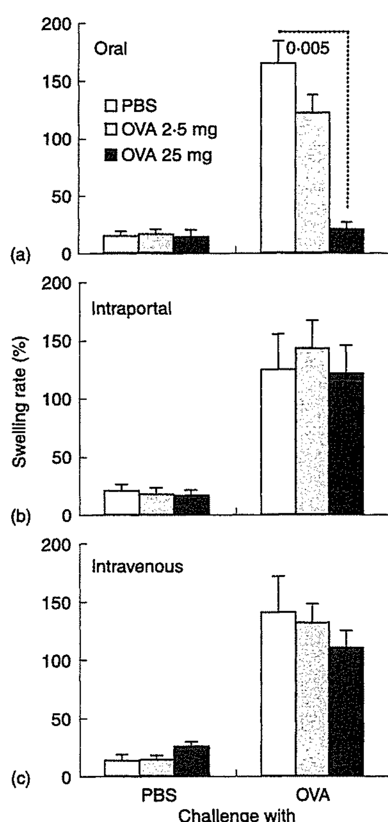


Figure 7. OVA-specific DTH response is suppressed by oral administration of intact OVA, but not by intraportal or intravenous injection of intact OVA. BDF_1 mice were orally administered (a) or injected into the portal (b) or peripheral vein (c) with 2·5 mg or 25 mg of OVA or PBS, and then i.p. immunized with OVA plus alum twice. DTH response was assessed 2 weeks after the second immunization of OVA plus alum. Ear thickness was measured 24 h after challenge of OVA or PBS into the ear. The data are expressed as the mean + SE of five to six mice. The value represents statistical significance ($P <$) compared with control mice treated with PBS in the same way.

at 1 week, IgG2a, IgG2b and IgM were significantly enhanced, although IgG1 at 4 weeks and IgE were suppressed (Fig. 8b, c).

The data clearly show that the injection of intact OVA into the portal or peripheral vein cannot induce immunological tolerance but rather enhances part of immune responses against OVA.

The injection of intact OVA into the lower intestinal tract is less effective in terms of OVA-specific tolerance induction

We therefore investigated whether the modification of intact OVA by gastrointestinal digestion was essential to induce oral tolerance. Mice were orally administered or injected into the guts, stomach, duodenum, ileum, or colon, with 25 mg of intact OVA. OVA-treated and untreated mice were i.p. immunized with OVA plus alum twice, and then OVA-specific plasma immunoglobulin and DTH response were assessed.

OVA-specific immunoglobulin production was remarkably suppressed by the oral administration of intact OVA, whereas immunoglobulin was sufficiently produced in untreated mice (Fig. 9a). The injection of intact OVA into the stomach, duodenum, ileum, or colon significantly enhanced OVA-specific immunoglobulin production ($P < 0{\cdot}05$), compared with the oral administration of intact OVA (Fig. 9a). DTH response against OVA was also significantly enhanced in mice injected with intact OVA into the ileum or colon ($P < 0{\cdot}05$ or 0·005, respectively), compared with the oral administration of OVA (Fig. 9b). Levels of OVA-specific immunoglobulins and DTH responses were higher in mice injected with intact OVA into the lower intestinal tract.

The results demonstrate that the appropriate digestion of intact OVA in the gastrointestinal tract is crucial for the induction of oral tolerance against OVA.

Discussion

In this study, we showed that after the oral administration of OVA, OVA antigens were absorbed via the small intestines, transferred into the liver via the portal vein, and circulated in the bloodstream. This suggests that digestive enzymes do not completely digest OVA to amino acids and small peptides that do not have antigenicity, and OVA antigens can cross the intestinal surface.

M cells that exist over PPs uptake soluble macromolecule proteins.[10–12] OVA antigens may be taken up by M cells at the intestinal surface and then they may be processed and presented by immature DCs in PPs, which are shown to be inductive sites for oral tolerance where T cells secreting regulatory cytokines, IL-10[21] and TGF-β,[22] are induced. DCs may capture OVA antigens, present antigen epitopes to naïve T cells, and induce regulatory T cells in PPs. M cells are shown to also exist in small intestinal villi,[37] suggesting that antigens may be taken up by M cells apart from PPs. Moreover, DCs may send dendrites between epithelial cells and acquire OVA antigens over epithelia. Thus, some of the OVA antigens that cross the intestinal surface are captured by DCs in these intestinal mucosal tissues.

A protein antigen is digested by digestive enzymes to amino acids and small peptides.[9] They are absorbed via epithelial cells, and enter the portal vein through capillary vessels in the small intestine. After the oral administration of OVA to mice, OVA antigens are detected in peripheral blood.[30,31] Furthermore, our results clearly showed that OVA antigens, 45 000 MW of proteins, enter the portal vein and then the bloodstream after the oral administration of OVA. OVA antigens taken up by M cells, or using other routes, may enter the portal vein via capillary

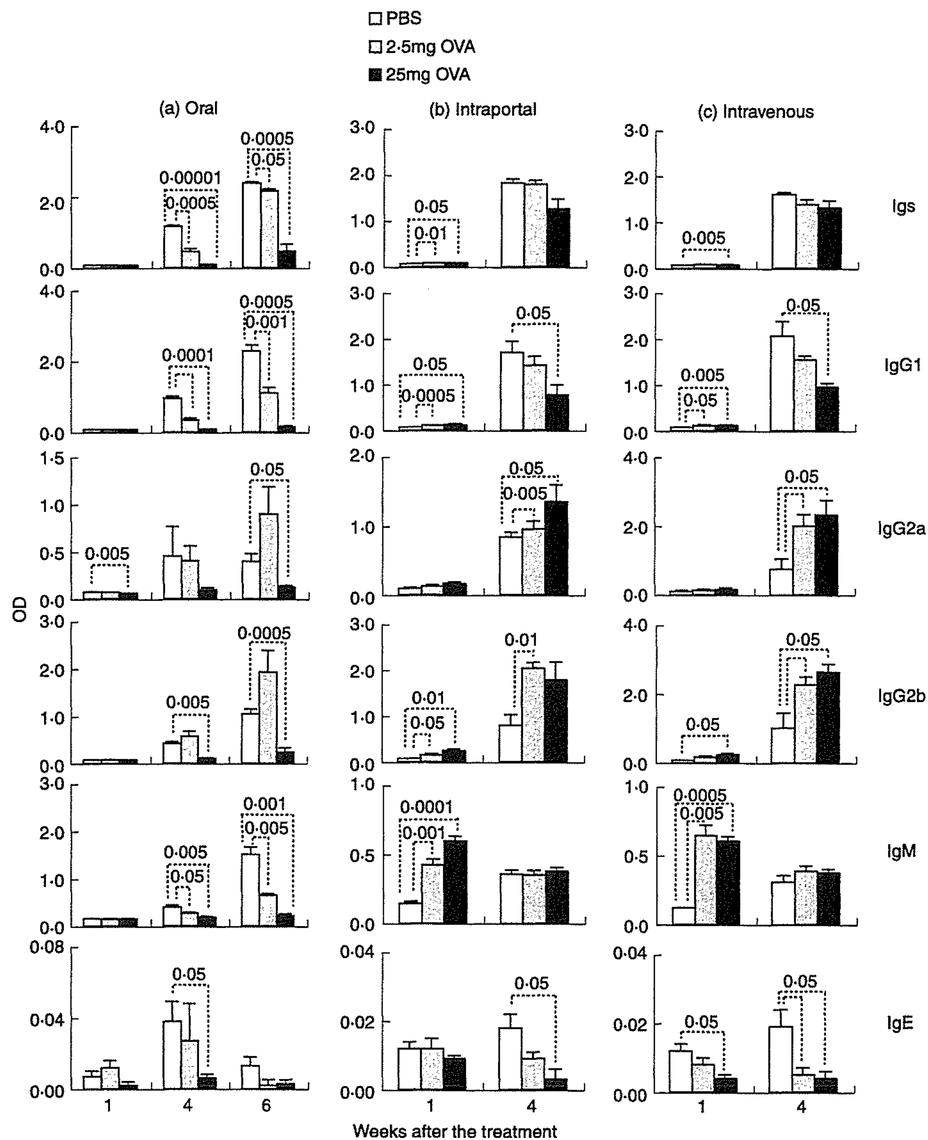


Figure 8. Production of anti-OVA immunoglobulins is suppressed by the oral administration of intact OVA, but not by intraportal or intravenous injection of intact OVA. BDF_1 mice were orally administered (a) or injected into the portal (b) or peripheral vein (c) with 2·5 mg or 25 mg of OVA or PBS. The mice were i.p. immunized with OVA plus alum 1 and 3 weeks after the first oral administration or injection of intact OVA. Orally treated mice were additionally i.p. immunized with OVA 5 weeks after the first oral administration. Peripheral blood was collected at various weeks. Anti-OVA immunoglobulins in plasma were assessed by ELISA. Data are expressed as the mean of OD (415 nm) + SE of five to six mice. Each value represents statistical significance ($P <$), compared with control mice treated with PBS in the same way.

vessels in the small intestine. Some allergens have been shown to cross the mucosal barrier by the disruption of tight junctions.[38,39] Notably, it has been shown that soluble protein antigens are rapidly pinocytosed by enterocytes and co-localized with major histocompatibility complex (MHC) class II in a vesicular compartment.[40] Bland *et al.* proposed that intestinal epithelial cells (IECs) presented antigens on their surface to local T cells[41] and induced suppressor T cells.[42] Recently, it has been reported that tolerosomes, which have an exosome-like structure and carry MHC class II with antigens, were released from IECs into serum.[43] It has also been shown that tolerosome from mouse serum 1 hr after the oral administration of OVA-induced oral tolerance and this induction was MHC class II dependent.[44] Hereafter, it is necessary to examine whether the 45 000 MW OVA antigens detected are free antigens or are included in exosomes in mouse serum.

© 2006 Blackwell Publishing Ltd, *Immunology*, **119**, 167–177

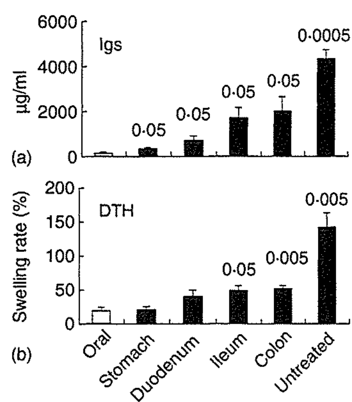


Figure 9. Less effective induction of immunological tolerance by injection of intact OVA into lower intestinal tract. BDF_1 mice were orally administrated or injected into the guts with 25 mg of OVA. OVA-treated and untreated mice were i.p. immunized with OVA plus alum 1 and 3 weeks after OVA treatment. Peripheral blood was collected 1 week after the second immunization of OVA plus alum. Plasma anti-OVA immunoglobulin was assessed by ELISA (a). DTH response against OVA was assessed 6 weeks after the second immunization (b). Data are expressed as the mean + SE of 3–9 mice. Each value represents statistical significance ($P <$), compared with mice orally administered with OVA.

It has been demonstrated that CD25-positive cells are crucial to immunological suppression by the transfer of serum after the oral administration of OVA.[32] The liver has been shown to contribute to tolerance induction because the intraportal injection of allogeneic cells[24,25] eggs of a parasite[26] or insoluble protein[27] induces immunological tolerance against the antigen. It is reported that liver endothelial cells endocytose OVA by a mannose receptor, CD206,[45,46] and antigen presentation by cells induces T-cell tolerance against OVA.[46] In this study, however, the injection of intact OVA into the portal or peripheral vein did not induce immunological tolerance but rather enhanced part of OVA-specific antibody production. As mannose receptors are also expressed on macrophages in red pulp in the spleen[47] intact OVA may be captured by macrophages in the spleen after intravenous injection. In our experimental system, these antigen-presenting cells (APCs) in the liver and spleen may not induce immunological tolerance when they endocytose intact OVA.

The uptake of intact antigens untreated with digestive enzymes may lead to immunological enhancement such as allergy. Ileal injection of BSA treated with pepsin induces immunological tolerance against BSA, whereas ileal injection of intact BSA enhances anti-BSA responses.[48] Correspondingly, in this study, the injection of intact OVA into the ileum or colon significantly enhanced both OVA-specific antibody production and DTH response. Induction of oral tolerance was more difficult when intact OVA was injected into the lower intestinal tract. It is reported that the impairment of gastric digestion of caviar extracts significantly enhanced caviar-specific IgG1, IgG2a, and IgE levels in mice.[49] In addition, cod proteins treated with pepsin show reduced IgE-binding capability and reduced histamine release from human basophils.[50] In the previous study, we demonstrated that oral tolerance against sheep red blood cells (SRBCs) was induced in young mice but rather SRBC-specific antibody response was enhanced in aged mice by the oral administration of SRBC.[51] Digestive and absorptive capacity is decreased in elderly people.[52] Reduced digestive capacity in aged mice might result in the failure of oral tolerance induction. In this report, it was shown that the absolute gastrointestinal ingestion of OVA via the upper gastrointestinal tract is crucial for oral tolerance induction. As macromolecular OVA antigens but not digested antigen fragments are detected in tolerant-mice serum, not only digestion but also some modification of macromolecular OVA in the gastrointestinal tract may be essential for oral tolerance induction.

The detection of OVA antigens has been shown in mouse serum 1 hr after the oral administration of OVA, and serum transfer induced significant suppression of OVA-specific immune responses.[31] Recently, it was shown that tolerosomes including MHC class II are produced by IEC at 1 hr after the oral administration of OVA, and tolerosomes induce oral tolerance.[44] Also in our results, OVA antigens were remarkably detected at 30 min and 1 hr after the oral administration of OVA.

In this study, it was clearly demonstrated that the absolute gastrointestinal ingestion of OVA via the upper gastrointestinal tract is crucial to the establishment of oral tolerance. Although macromolecular OVA antigens are detected after the oral administration of OVA in tolerant-mouse serum, the injection of intact OVA cannot induce tolerance. Therefore, some modification of macromolecular OVA in the gastrointestinal tract and ingestion may be essential for oral tolerance induction.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Young Scientists from the Japan Society for the Promotion of Sciences (JSPS). The authors are grateful to Dr Eiji Shinya, Ms Atsuko Owaki and Dr Megumi Takahashi for advice on the immunoprecipitation and immunoblotting.

References

1 Mowat AM, Strobel S, Drummond HE, Ferguson A. Immunological responses to fed protein antigens in mice. I. Reversal of oral tolerance to ovalbumin by cyclophosphamide. *Immunology* 1982; 45:105–13.

2 Lider O, Santos LM, Lee CS, Higgins PJ, Weiner HL. Suppression of experimental autoimmune encephalomyelitis by

oral administration of myelin basic protein. II. Suppression of disease and in vitro immune responses is mediated by antigen-specific CD8+ T lymphocytes. *J Immunol* 1989; **142**: 748–52.

3 Chehade M, Mayer L. Oral tolerance and its relation to food hypersensitivities. *J Allergy Clin Immunol* 2005; **115**: 3–12; quiz 3.

4 Breiteneder H, Ebner C. Molecular and biochemical classification of plant-derived food allergens. *J Allergy Clin Immunol* 2000; **106**(1 Part 1):27–36.

5 Sicherer SH, Sampson HA. 9. Food allergy. *J Allergy Clin Immunol* 2006; **117**(2 Suppl.):S470–5.

6 Khakoo A, Lack G. Preventing food allergy. *Curr Allergy Asthma Rep* 2004; **4**:36–42.

7 Weiner HL, Friedman A, Miller A *et al.* Oral tolerance: immunologic mechanisms and treatment of animal and human organ-specific autoimmune diseases by oral administration of autoantigens. *Annu Rev Immunol* 1994; **12**:809–37.

8 Ma S, Jevnikar AM. Autoantigens produced in plants for oral tolerance therapy of autoimmune diseases. *Adv Exp Med Biol* 1999; **464**:179–94.

9 Erickson RH, Kim YS. Digestion and absorption of dietary protein. *Annu Rev Med* 1990; **41**:133–9.

10 Bockman DE, Cooper MD. Pinocytosis by epithelium associated with lymphoid follicles in the bursa of Fabricius, appendix, and Peyer's patches. An electron microscopic study. *Am J Anat* 1973; **136**:455–77.

11 von Rosen L, Podjaski B, Bettmann I, Otto HF. Observations on the ultrastructure and function of the so-called 'microfold' or 'membraneous' cells (M cells) by means of peroxidase as a tracer. *Virchows Arch a Pathol Anat Histol* 1981; **390**:289–312.

12 Neutra MR, Phillips TL, Mayer EL, Fishkind DJ. Transport of membrane-bound macromolecules by M cells in follicle-associated epithelium of rabbit Peyer's patch. *Cell Tissue Res* 1987; **247**:537–46.

13 Wolf JL, Kauffman RS, Finberg R, Dambrauskas R, Fields BN, Trier JS. Determinants of reovirus interaction with the intestinal M cells and absorptive cells of murine intestine. *Gastroenterology* 1983; **85**:291–300.

14 Sicinski P, Rowinski J, Warchol JB, Jarzabek Z, Gut W, Szczygiel B, Bielecki K, Koch G. Poliovirus type 1 enters the human host through intestinal M cells. *Gastroenterology* 1990; **98**:56–8.

15 Amerongen HM, Weltzin R, Farnet CM, Michetti P, Haseltine WA, Neutra MR. Transepithelial transport of HIV-1 by intestinal M cells: a mechanism for transmission of AIDS. *J Acquir Immune Defic Syndr* 1991; **4**:760–5.

16 Owen RL, Pierce NF, Apple RT, Cray WC Jr. M cell transport of *Vibrio cholerae* from the intestinal lumen into Peyer's patches: a mechanism for antigen sampling and for microbial transepithelial migration. *J Infect Dis* 1986; **153**:1108–18.

17 Grutzkau A, Hanski C, Hahn H, Riecken EO. Involvement of M cells in the bacterial invasion of Peyer's patches: a common mechanism shared by *Yersinia enterocolitica* and other enteroinvasive bacteria. *Gut* 1990; **31**:1011–5.

18 Jones BD, Ghori N, Falkow S. *Salmonella typhimurium* initiates murine infection by penetrating and destroying the specialized epithelial M cells of the Peyer's patches. *J Exp Med* 1994; **180**:15–23.

19 Kelsall BL, Strober W. Distinct populations of dendritic cells are present in the subepithelial dome and T cell regions of the murine Peyer's patch. *J Exp Med* 1996; **183**:237–47.

20 Rescigno M, Urbano M, Valzasina B *et al.* Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nat Immunol* 2001; **2**:361–7.

21 Fujihashi K, Dohi T, Rennert PD, Yamamoto M, Koga T, Kiyono H, McGhee JR. Peyer's patches are required for oral tolerance to proteins. *Proc Natl Acad Sci USA* 2001; **98**: 3310–5.

22 Tsuji NM, Mizumachi K, Kurisaki J. Antigen-specific, $CD4^+$ $CD25^+$ regulatory T cell clones induced in Peyer's patches. *Int Immunol* 2003; **15**:525–34.

23 Spahn TW, Fontana A, Faria AM *et al.* Induction of oral tolerance to cellular immune responses in the absence of Peyer's patches. *Eur J Immunol* 2001; **31**:1278–87.

24 Nakano Y, Monden M, Valdivia LA, Gotoh M, Tono T, Mori T. Permanent acceptance of liver allografts by intraportal injection of donor spleen cells in rats. *Surgery* 1992; **111**:668–76.

25 Yu S, Nakafusa Y, Flye MW. Portal vein administration of donor cells promotes peripheral allospecific hyporesponsiveness and graft tolerance. *Surgery* 1994; **116**:229–34; discussion 34–5.

26 Cuison A, Tasaka K, Chuang CK, Minai M, Yoshikawa H, Nakajima Y. Schistosome eggs in the portal vein can induce tolerance. *Int J Parasitol* 1995; **25**:993–8.

27 Ogasawara H, Watari E, Shirai Y, Yokomuro K. [Inhibitory effects of portal venous injection of type II collagen in collagen-induced arthritis]. *Nippon Ika Daigaku Zasshi* 1997; **64**:220–4.

28 May CD, Remigio L, Feldman J, Bock SA, Carr RI. A study of serum antibodies to isolated milk proteins and ovalbumin in infants and children. *Clin Allergy* 1977; **7**:583–95.

29 Vance GH, Thornton CA, Bryant TN, Warner JA, Warner JO. Ovalbumin-specific immunoglobulin G and subclass responses through the first 5 years of life in relation to duration of egg sensitization and the development of asthma. *Clin Exp Allergy* 2004; **34**:1542–9.

30 Bruce MG, Ferguson A. The influence of intestinal processing on the immunogenicity and molecular size of absorbed, circulating ovalbumin in mice. *Immunology* 1986; **59**:295–300.

31 Peng HJ, Turner MW, Strobel S. The generation of a 'tolerogen' after the ingestion of ovalbumin is time-dependent and unrelated to serum levels of immunoreactive antigen. *Clin Exp Immunol* 1990; **81**:510–5.

32 Karlsson MR, Kahu H, Hanson LA, Telemo E, Dahlgren UI. Tolerance and bystander suppression, with involvement of CD25-positive cells, is induced in rats receiving serum from ovalbumin-fed donors. *Immunology* 2000; **100**:326–33.

33 Kato H, Fujihashi K, Kato R, Yuki Y, McGhee JR. Oral tolerance revisited: prior oral tolerization abrogates cholera toxin-induced mucosal IgA responses. *J Immunol* 2001; **166**:3114–21.

34 Wakabayashi A, Eishi Y, Nakamura K. Development of the immune system in severe combined immunodeficiency mice reconstituted with transferred fetal liver cells. *J Med Dent Sci* 1997; **44**:21–9.

35 Wakabayashi A, Eishi Y, Nakamura K. Regulation of experimental autoimmune orchitis by the presence or absence of testicular antigens during immunological development in SCID mice reconstituted with fetal liver cells. *Immunology* 1997; **92**:84–90.

36 Kweon MN, Fujihashi K, VanCott JL, Higuchi K, Yamamoto M, McGhee JR, Kiyono H. Lack of orally induced systemic unresponsiveness in IFN-gamma knockout mice. *J Immunol* 1998; **160**:1687–93.

© 2006 Blackwell Publishing Ltd, *Immunology*, **119**, 167–177

37 Jang MH, Kweon MN, Iwatani K *et al.* Intestinal villous M cells: an antigen entry site in the mucosal epithelium. *Proc Natl Acad Sci USA* 2004; **101**:6110–5.

38 Wan H, Winton HL, Soeller C *et al.* Der p 1 facilitates transepithelial allergen delivery by disruption of tight junctions. *J Clin Invest* 1999; **104**:123–33.

39 Mine Y, Zhang JW. Surfactants enhance the tight-junction permeability of food allergens in human intestinal epithelial Caco-2 cells. *Int Arch Allergy Immunol* 2003; **130**:135–42.

40 Gonnella PA, Wilmore DW. Co-localization of class II antigen and exogenous antigen in the rat enterocyte. *J Cell Sci* 1993; **106**:937–40.

41 Bland PW, Warren LG. Antigen presentation by epithelial cells of the rat small intestine. I. Kinetics, antigen specificity and blocking by anti-Ia antisera. *Immunology* 1986; **58**:1–7.

42 Bland PW, Warren LG. Antigen presentation by epithelial cells of the rat small intestine. II. Selective induction of suppressor T cells. *Immunology* 1986; **58**:9–14.

43 Karlsson M, Lundin S, Dahlgren U, Kahu H, Pettersson I, Telemo E. 'Tolerosomes' are produced by intestinal epithelial cells. *Eur J Immunol* 2001; **31**:2892–900.

44 Ostman S, Taube M, Telemo E. Tolerosome-induced oral tolerance is MHC dependent. *Immunology* 2005; **116**:464–76.

45 Kindberg GM, Magnusson S, Berg T, Smedsrod B. Receptor-mediated endocytosis of ovalbumin by two carbohydrate-specific receptors in rat liver cells. The intracellular transport of ovalbumin to lysosomes is faster in liver endothelial cells than in parenchymal cells. *Biochem J* 1990; **270**:197–203.

46 Limmer A, Ohl J, Kurts C *et al.* Efficient presentation of exogenous antigen by liver endothelial cells to $CD8^+$ T cells results in antigen-specific T-cell tolerance. *Nat Med* 2000; **6**:1348–54.

47 Linehan SA, Martinez-Pomares L, Stahl PD, Gordon S. Mannose receptor and its putative ligands in normal murine lymphoid and nonlymphoid organs: In situ expression of mannose receptor by selected macrophages, endothelial cells, perivascular microglia, and mesangial cells, but not dendritic cells. *J Exp Med* 1999; **189**:1961–72.

48 Michael JG. The role of digestive enzymes in orally induced immune tolerance. *Immunol Invest* 1989; **18**(9–10):1049–54.

49 Untersmayr E, Scholl I, Swoboda I *et al.* Antacid medication inhibits digestion of dietary proteins and causes food allergy: a fish allergy model in BALB/c mice. *J Allergy Clin Immunol* 2003; **112**:616–23.

50 Untersmayr E, Poulsen LK, Platzer MH, Pedersen MH, Boltz-Nitulescu G, Skov PS, Jensen-Jarolim E. The effects of gastric digestion on codfish allergenicity. *J Allergy Clin Immunol* 2005; **115**:377–82.

51 Wakabayashi A, Utsuyama M, Hosoda T, Sato K, Hirokawa K. Differential age effect of oral administration of an antigen on antibody response: an induction of tolerance in young mice but enhancement of immune response in old mice. *Mech Ageing Dev* 1999; **109**:191–201.

52 Masamune O. Digestive and absorptive capacity in the elderly. *Rinsho Byori* 1989; **37**:629–33.

AIDS RESEARCH AND HUMAN RETROVIRUSES
Volume 23, Number 2, 2007, pp. 000–000
© Mary Ann Liebert, Inc.
DOI: 10.1089/aid.2006.0098

Oral Attenuated *Salmonella enterica* Serovar *typhimurium* Vaccine Expressing Codon-Optimized HIV Type 1 *Gag* Enhanced Intestinal Immunity in Mice

YASUKO TSUNETSUGU-YOKOTA,[1] MASAYUKI ISHIGE,[2] and MASAHIRO MURAKAMI[2]

ABSTRACT

Oral immunization is a safe and easily applicable route to induce mucosal immunity to HIV infection. We examined the ability of oral attenuated *Salmonella typhimurium* (ST) vaccine expressing Gag for the efficiency of generating Gag-specific mucosal IgA and CD8$^+$ T cell responses in intestinal lymphoid tissues. By optimizing the codon of HIV-1 *gag* to the preferred codon bias of *Salmonella*, the expression of Gag in *Salmonella* was dramatically improved. The oral ST-Gag vaccine by itself was not so powerful and induces little Gag-specific CD8$^+$ T cell responses in the intestine. Nevertheless, we found that it potentiates otherwise weak intestinal CD8$^+$ T cell responses in nasally primed mice with Gag p24 and cholera toxin adjuvant. Thus, the oral delivery of *Salmonella* expressing Gag would be utilized in combination with other parenteral vaccine to direct and strengthen intestinal HIV-specific CTL responses.

INTRODUCTION

Transmission of human immunodeficiency virus (HIV) occurs mainly through mucosal surfaces during sexual contact. Recent studies revealed that the gastrointestinal and vaginal mucosa serve as sites for virus entry and are the initial and predominant sites where the virus replicates and amplifies itself; they are also the initial sites of CD4$^+$ T cell depletion.[1] Also in the macaque AIDS model, the quite recent reports that the massive destruction of resting memory CD4$^+$ T cells occurs early during simian immunodeficiency virus (SIV) infection further supports the importance of mucosal immunity.[2,3] Thus, it is currently believed that the new generation of candidate anti-HIV vaccines should elicit mucosal immune responses, including mucosal secretory IgA and, especially, CD8$^+$ cytotoxic T lymphocyte (CTL) responses, especially in intestinal and vaginal tissues. Furthermore, because of the economic concerns in many resource-poor countries, an ideal vaccine should be safe, have needle-free delivery, be cheap and easy to handle, and not need cold chain maintenance.

Mucosal immunization, especially by the oral route, has in recent years attracted great interest as a means of eliciting protective immunity against a variety of infectious diseases. The oral polio vaccine, which induces both systemic and mucosal humoral responses, is the most successful mucosal vaccine known to date.[4] Oral, live, attenuated *Salmonella* vaccine, Ty21a, is also known to induce strong and sustained humoral as well as cellular immune responses both in the mucosal and systemic compartments and now is licensed in 56 countries in Asia, Africa, Europe, and the Americas,[5] though a further improved vaccine is desired.[6] *Salmonella typhimurium* (ST) causes a self-limiting gastroenteritis in a wide range of mammals, including humans, and a systemic typhoid-like disease in mice. ST preferentially binds to the M cells in the follicular-associated epithelium (FAE) that is located above the Peyer's patch, and enters the submucosa either via the transcytosis of M cells or by disturbing the seal of the epithelial cell layer (which of these options mainly occurs is unclear at present).[7] ST is then phagocytosed by macrophages and dendritic cells (DCs) that are local to the area or recruited there by the infection. The bacteria taken up by these cells are known to persist in an intracellular membrane compartment and initiate T cell responses.[8,9] Thus, live *Salmonella* serves as an ideal carrier for the delivery of antigen to mucosa-associated lymphoid tissues in the intestine.

In animal models, attenuated ST has been used as a carrier to orally deliver the truncated HIV-1 env in mice[10,11] or SIV Gag in macaques.[12] However, these studies revealed that the

[1]Department of Immunology, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo 162-8640, Japan.
[2]Laboratory of Pharmaceutics, Faculty of Pharmacy, Osaka Ohtani University, Osaka 584-8540, Japan.