

Resting B Cells Are Not Antigen-Presenting Cells in the Induction of Oral Tolerance of Specific Th2 Immune Responses in Mice

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Key Words

B cells · Antigen-presenting cells · Oral tolerance · Ovalbumin · IgE · Th2 immune responses

Abstract

Background: It has been shown that antigen presentation by resting B cells can induce tolerance to intravenously administered protein antigens, but the role of resting B cells in the induction of oral tolerance is unclear. **Methods:** Mice continuously treated since birth with rabbit anti-mouse IgM serum for 5 weeks were depleted of B cells. When 4 weeks old, B cell-depleted mice drank 10% chicken egg white (EW) for 5 days. Ten weeks later, they were immunized with 10 µg of ovalbumin in alum and their T helper 2 (Th2) immune responses were tested. **Results:** Th2 cell-mediated IgE and IgG1 antibody responses and spleen cell production of IL-4 and IL-5 were suppressed by prior EW feeding during anti-IgM treatment. When anti-IgM-treated spleen cells collected 1 week after EW ingestion were transferred to naïve recipients, active suppression of Th2 immune responses was also demonstrated. **Conclusions:** Although resting small B cells aggregate in the mantle zone of follicles of intestinal Peyer's patches, the

present data suggest that they are not antigen-presenting cells in the induction of oral tolerance of Th2 immune responses to oral antigens.

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Introduction

Oral tolerance is the state of systemic immune hyporesponsiveness when animals and humans come into contact with protein antigens for the first time through the gut [1, 2]. Since the gut is continuously exposed to a great variety of dietary proteins, the induction of immune tolerance to oral antigens plays a crucial role in the maintenance of a homeostasis between food proteins and the body. Animal studies have further shown that ingestion of autoantigens can suppress experimental autoimmune disorders [3]. This natural route of tolerance induction has been applied in clinical trials investigating autoimmune diseases [4, 5]. However, which antigen-presenting cells (APCs) underlie the induction of oral tolerance is still unclear.

Several reports suggest that the tolerogenic moiety of gut-processed antigen may retain B cell epitopes [6–9], but the role of B cells in the induction of oral tolerance has

not been studied. As B cells can use their surface immunoglobulin receptors to catch specific antigens, activated B cells are very potent APCs for activated T cells [10]. Small resting B cells can present antigens to activated T cells [11, 12], but they fail to activate naïve T cells [11–14]. Recent studies have also shown that small resting B cells can present intravenously administered protein antigens to naïve T cells in a tolerogenic fashion and induce tolerance [14–17]. The tolerogenicity of resting B cells may be due to the absence of costimulatory factors such as B7, which are very rich in dendritic cells and activated B cells [17, 18]. Indeed, intestinal dendritic cells can present oral antigens and prime naïve T cells [19]. It has been shown that the B cell mitogen lipopolysaccharide can abrogate the induction of oral tolerance [20]. Resting B cells are very abundant in the mantle zones of follicles of Peyer's patches. As anti-IgM treatment from birth results in complete depletion of B cells [21–27], the role of resting B cells in the induction of oral tolerance can be examined in B cell-depleted mice. It has been shown that B cells are crucial for antigen presentation during systemic immunization [23–26]. Thus, in this study, anti-IgM-treated mice were sensitized 10 weeks after prior treatment, when sufficient regeneration of B cells was noted.

Materials and Methods

BALB/c Mice

Adult (6–8 weeks old) male and female BALB/c mice, purchased from the Animal Center, National Yang-Ming University, Taipei, Taiwan, were used as recipients for spleen cell transfer experiments and as breeding stock for a new colony in the Animal House, Veterans General Hospital – Taipei. Close to the expected date of delivery, pregnant mice were checked every day, and the offspring were treated within 24 h of birth. Mice were maintained on a standard laboratory diet (Lab diet, PMI Feeds, St. Louis, Mo., USA) without chicken egg white. All colony-bred mice were weaned onto the standard diet at 21 days of age.

Anti-IgM Treatment of Mice

Depletion of B cells was performed as described previously [27]. Briefly, rabbit anti-mouse IgM hyperimmune serum was prepared by monthly immunization with mouse myeloma IgM (MOPC 104E, Sigma, St. Louis, Mo., USA) in complete Freund's adjuvant (H37Ra, Sigma) once and then in incomplete Freund's adjuvant (Sigma). A serum pool containing 2.7 mg/ml rabbit IgG specific for mouse IgM was used undiluted for injections. BALB/c mice were injected intraperitoneally with antisera every day for the first week of life (0.1 ml/injection), on alternate days during the 2nd week (0.2 ml/injection), and 3 times per week thereafter for 3 weeks (0.3 ml/injection). The spleen cells collected from anti-IgM-treated mice showed no proliferation response to lipopolysaccharide and were analyzed on a cell sorter (FACScan, Becton Dickinson Electronics Lab., Calif., USA) using

1:20 diluted FITC-conjugated rat IgG2a anti-mouse LY-5 (B-220, Caltag Lab., Calif., USA) and rat IgG2c anti-mouse CD90 (Thy-1, Caltag), respectively for B and T cell markers. Using sandwich ELISA and immunodiffusion, it was seen that anti-IgM-treated mice had high titers of rabbit IgG, but not of mouse IgM.

Protein Antigens

Chicken eggs were purchased from the supermarket. Ovalbumin (OVA; grade VII), ovomucoid (grade III), conalbumin (type I) and lysozyme were obtained from Sigma.

Oral Administration of Antigens

Mice were fed 10% chicken egg white (EW; containing approximately 51% OVA, 12% conalbumin, 11% ovomucoid and 3.5% lysozyme) [13] or distilled water only for 5 consecutive days. The dried weight of 1 ml of EW was 124.5 mg. Each mouse consumed about 4 ml of 10% EW per day. This resulted in daily ingestion of approximately 25 mg of OVA, 6 mg of conalbumin, 5.5 mg of ovomucoid and 1.8 mg of lysozyme. B cell-depleted mice were fed when aged 28–32 days. Two days after the last feed, they received no more antiserum. To neutralize circulating anti-mouse IgM antibodies, they were then injected with 0.5 ml of normal mouse serum biweekly for 2 weeks. To permit sufficient regeneration of B cells, they were not treated for another 8 weeks. They were sensitized at the age of 15 weeks, when they had high titers of mouse IgM and IgG and regeneration of B cells was confirmed by a FACScan.

Preparation of Spleen Cells

Mouse spleens were resected and pressed through a sterile metal gauge. The spleen cells were collected and washed in RPMI-1640 with 10% fetal calf serum, 2 mM *L*-glutamine, 100 µ/ml penicillin and 100 µg/ml streptomycin. Red blood cells were depleted using EDTA-NH₄Cl lysing buffer. The suspension was filtered through a sterile gauge and washed three times with RPMI culture medium at 4°C.

Systemic Sensitization

Mice were sensitized with 10 µg (i.p.) of OVA, conalbumin, ovomucoid or lysozyme adsorbed onto 4 mg of alum and suspended in 0.5 ml of PBS. The day of sensitization is referred to as day 0 and the days of treatment are given relative to it. Mice were bled from the tail on days 14 and 21. The separated serum was stored frozen at –20°C until required.

Assessment of Specific IgG and IgG1 Antibody Responses

Serum-specific IgG and IgG1 antibody responses were assayed by an ELISA as previously described [17]. Briefly, 96-well vinyl plates were coated with 100 µg/ml OVA, conalbumin, ovomucoid and lysozyme in 0.05 M carbonate buffer (pH 9.6) overnight at 4°C. The plates were blocked with 3% skimmed milk in saline-Tween 20 for 1 h. Serum samples (1/300 or 1,000) and standards (pooled mouse sera collected 1 week after monthly immunization with OVA, conalbumin, ovomucoid or lysozyme in alum for 3 months) were added in duplicate and the plates incubated for 5 h at room temperature. After washing, horseradish peroxidase-conjugated goat anti-mouse IgG or IgG1 (1/5,000; SBA, Birmingham, Ala., USA) was added and the plates incubated overnight at 4°C. After washing, OPD (0.5 mg/ml) in citrate-carbonate buffer containing 0.015% H₂O₂ was added and the plates were incubated in the dark. Color development was stopped by 4 N H₂SO₄. Absorbance at 492 nm was read using a read-

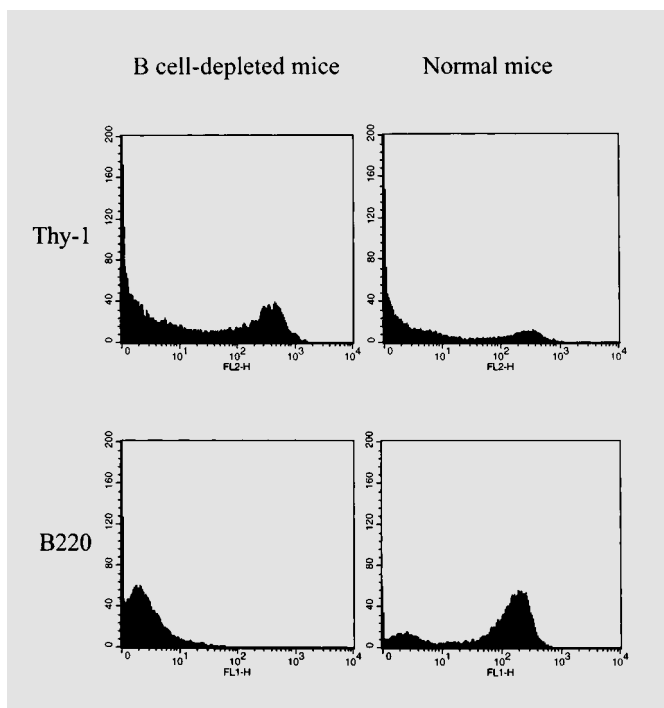


Fig. 1. Anti-IgM treatment from birth leads to complete elimination of splenic B cells. The spleen cells were collected and pooled from age-matched normal and anti-IgM-treated BALB/c mice. They were stained using FITC-conjugated rat monoclonal antibodies against T and B cell markers and then analyzed on a FACScan.

er (SPECTRAmax 250, Molecular Devices, Sunnyvale, Calif., USA) and unknowns were interpolated.

Passive Cutaneous Anaphylaxis

Serum IgE antibody responses to OVA, conalbumin, ovomucoid or lysozyme were assessed in triplicate by the passive cutaneous anaphylaxis test in male Sprague-Dawley rats purchased from the Animal Center, National Yang-Ming University, Taipei, Taiwan. Aliquots of 100 μ l of twofold diluted pooled mouse serum samples (1/10–1,280) were i.d. injected into the rats. All rats were challenged 48 h later by an intravenous injection of 1 ml of 0.9% saline solution containing 2 mg of protein antigens and 5 mg of Evans blue. The reaction was read 30 min after challenge. The passive cutaneous anaphylaxis titer was the highest dilution giving a positive reaction of at least 5 mm in diameter and was expressed as mean $\pm$ 1 SEM.

Spleen Cell Production of IL-4 and IL-5 in vitro

Spleen cells collected as above were cultured (4×10^6 cells/ml) with or without OVA (1 mg/ml) in 24-well flat-bottomed microtiter plates (Costar). Three days later, the supernatants were harvested, separated from the cells by centrifugation at 7,200 g for 5 min and stored at -20°C until required. The levels of IL-4 and IL-5 were assayed in duplicate using sandwich ELISA kits (Endogen, Cambridge, Mass., USA).

Statistical Analysis

Specific IgG and IgG1 antibody responses to OVA are presented as scatter plots with median lines indicated. Specific IgG and IgG1 antibody responses to conalbumin, ovomucoid and lysozyme are presented as means $\pm$ 1 SD. All group comparisons for specific IgG and IgG1 antibody responses were made using two-tailed Mann-Whitney U-tests.

Results

Effect of Anti-IgM Treatment on the Lymphocyte Population in Mice

Spleen cells were collected and pooled from 4-week-old normal and anti-IgM-treated mice. The population of splenic T and B cells was analyzed on a FACScan by direct staining using FITC-conjugated rat IgG2c monoclonal antibody anti-mouse CD90 (Thy-1) and FITC-conjugated rat IgG2a monoclonal antibody anti-mouse Ly-5 (B-220), respectively. The results are shown in figure 1. Consistent with a previous report [22], anti-IgM treatment from birth resulted in complete elimination of splenic B cells but did not deplete splenic T cells.

Oral Administration of EW to B Cell-Depleted Mice Leads to Oral Tolerance of Reaginic Antibody Responses to OVA

Groups of 4-week-old normal and anti-IgM-treated mice ($n = 6$) were fed 10% EW or distilled water only for 5 days. Anti-IgM treatment was stopped 2 days after the last feed. They were sensitized with 10 μ g of OVA in alum 10 weeks later. Their serum specific antibody responses were measured 14 days after sensitization (fig. 2). Compared with water-fed anti-IgM-treated mice, those anti-IgM-treated mice fed EW showed oral tolerance of IgG ($p = 0.005$), IgG1 ($p = 0.002$) and IgE antibody responses to OVA. Compared with normal controls, the EW-fed normal mice had profound tolerance of IgG, IgG1 ($p < 0.005$ for both) and IgE antibody responses to OVA. When ovomucoid, conalbumin or lysozyme was used instead of OVA to sensitize the mice in repeated studies, oral tolerance of reaginic antibody responses to ovomucoid, conalbumin or lysozyme was also found in those fed EW during anti-IgM treatment (data not shown).

Oral Administration of EW to B Cell-Depleted Mice Leads to Oral Tolerance of T Helper 2 Cytokine Responses to OVA

The effect of EW feeding on the spleen cell secretion of cytokines in vitro was further examined. Groups of 4-week-old normal and B cell-depleted mice ($n = 3$) were fed

distilled water or 10% EW for 5 days. They were sensitized with 10 µg of OVA in alum 2 or 10 weeks later, respectively. Their spleen cells were collected and pooled 12 days after sensitization. When spleen cells were cultured with OVA (1 mg/ml) in vitro, their production of IL-4 and IL-5 was suppressed in EW-fed mice with or without anti-IgM treatment, but not in the water-fed controls (table 1). When cultured without OVA, the levels of cytokines in the supernatants were very low in all groups.

Spleen Cell Transfer of Oral Tolerance of Reaginic Antibody Responses to OVA from EW-Fed B Cell-Depleted Mice to Naïve Recipients

Groups of 4-week-old normal and B cell-depleted BALB/c mice were fed distilled water or 10% EW for 5 days. One week after the last feed, their spleen cells were adoptively transferred into naïve BALB/c recipients (1 × 10⁸ cells per recipient). The recipients were sensitized with 10 µg of OVA in alum the next day. Their antibody responses were measured 14 days after sensitization (fig. 3). Compared with the recipients receiving spleen cells from B cell-depleted donors fed water only (n = 10), those receiving spleen cells from B cell-depleted donors fed EW (n = 7) showed oral tolerance of IgG, IgG1 (p < 0.005 for both) and IgE antibody responses to OVA. Compared with the mice receiving spleen cells from normal

Fig. 2. Oral tolerance of Th2 immune responses to OVA can be induced in B cell-depleted mice. Groups of normal and anti-IgM-treated BALB/c mice aged 28 days were fed 10% EW (n = 6) or distilled water only (n = 6 or 7) for 5 days. Anti-IgM treatment was stopped 2 days after the last feed of EW. Ten weeks later, they were sensitized with 10 µg of OVA in alum. Their serum reaginic antibody responses were measured 14 days after sensitization.

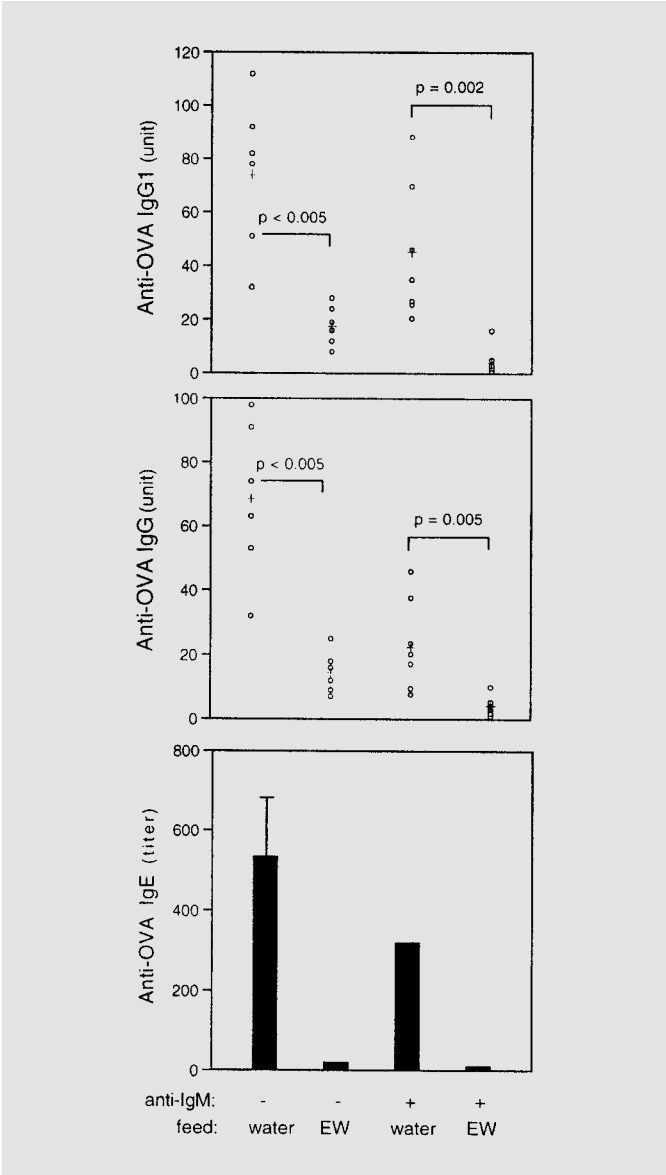


Table 1. Feeding 10% EW to B cell-depleted mice suppressed spleen cell production of Th2 cytokines IL-4 and IL-5 (pg/ml)

Mice	Feed	Secretion of IL-4		Secretion of IL-5	
		OVA	medium only	OVA	medium only
B cell-depleted mice	water	78.1	4.5	976.5	48.8
	EW	6.4	3.7	180.4	33.2
Normal mice	EW	8.9	5.6	232.7	57.2
	water	114.3	9.9	1,226.3	86.5

Groups of 3 normal and 3 B cell-depleted BALB/c mice were fed distilled water or 10% EW for 5 days. Anti-IgM treatment was stopped 2 days after the last feed. Ten weeks later, they were sensitized with 10 µg of OVA in alum. Their spleen cells were collected 12 days after sensitization and cultured with or without OVA in vitro for 3 days. Th2 cytokines IL-4 and IL-5 in the supernatant were assessed in duplicate by ELISA kits.

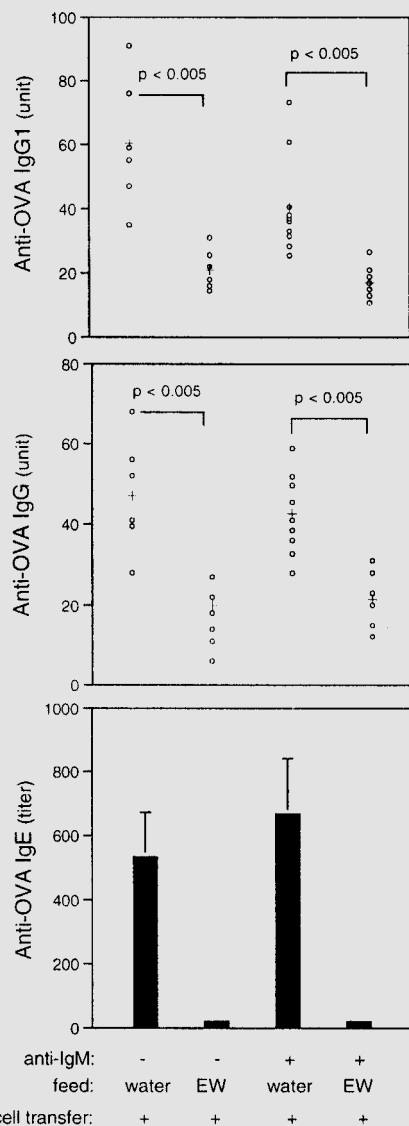


Fig. 3. Transfer of oral tolerance to OVA from B cell-depleted mice to syngeneic recipients. Groups of normal or anti-IgM-treated BALB/c mice aged 28 days were fed 10% EW or distilled water only for 5 days. Anti-IgM treatment was stopped 2 days after the last feed of EW. One week after the last feed, their spleen cells were collected and injected intraperitoneally into naïve BALB/c recipients (1×10^8 cells per recipient). Within 24 h, the recipients receiving normal spleen cells ($n = 6$) or anti-IgM treated spleen cells ($n = 7$ or 10) were sensitized with $10 \mu\text{g}$ of OVA in alum. Their serum reagenic antibody responses were measured 14 days after sensitization.

donors fed water only ($n = 6$), those receiving spleen cells from normal donors fed EW ($n = 6$) showed profound suppression of specific IgG, IgG1 ($p < 0.005$ for both) and IgE antibody responses.

Spleen Cell Transfer of Oral Tolerance to Ovomucoid, Conalbumin and Lysozyme from EW-Fed B Cell-Depleted Mice to Naïve Recipients

Groups of 4-week-old normal and B cell-depleted mice were fed distilled water or 10% EW for 5 days. One week after the last feed, their spleen cells were transferred into naïve recipients (1×10^8 cells per recipient). The next day, the recipients ($n = 6$) were sensitized with $10 \mu\text{g}$ of ovomucoid, conalbumin or lysozyme in alum. Their serum antibody responses were measured 14 days after sensitization (table 2). Compared with the mice receiving spleen cells from B cell-depleted mice fed water only, those receiving spleen cells from B cell-depleted donors fed EW showed suppression of IgG, IgG1 and IgE antibody responses to ovomucoid, conalbumin and lysozyme ($p \leq 0.005$). Similarly, those receiving spleen cells from EW-fed normal donors showed oral tolerance to ovomucoid, conalbumin and lysozyme ($p < 0.005$).

Discussion

We have demonstrated here that oral tolerance of T helper 2 (Th2) cell-regulated immune responses can be readily induced in B cell-depleted mice. It has been shown that resting B cells lack costimulatory factors and are defective APCs for primary immune responses to protein antigens [17, 18]. In contrast, activated B cells have increased expression of B7 and are more efficient in antigen presentation in mixed lymphocyte reaction and in antigen- or mitogen-triggered T cell proliferation [28, 29]. Although antigen-specific resting B cells are relatively uncommon, they may be the unique cells able to take up and present antigens to the unsensitized immune system [15]. It has been proposed that the defective antigen presentation of parenterally administered F(ab')_2 goat anti-mouse IgD in vivo by resting B cells to naïve T cells leads to low-zone tolerance [15]. Intestinal absorption of minute amounts of fed proteins has been repeatedly described [6, 30]. However, the present data suggest that the induction of oral tolerance may not be due to small B cell-mediated low-zone tolerance.

The correlation between suppressor T cells and immunoglobulin heavy chain-linked genes has been repeatedly shown by anti-idiotypic antibodies [21, 22]. Since B cells

Table 2. Feeding 10% EW to B cell-depleted mice induces oral tolerance of reagenic antibody responses to conalbumin, ovomucoid and lysozyme

	Feed	IgG	IgG1	IgE
<i>Conalbumin sensitization</i>				
B cell-depleted mice	water	76.7 ± 24.5	68.2 ± 35.9	533 ± 142
	EW	22.1 ± 14.4*	31.6 ± 16.7 ^a	27 ± 9
Normal mice	EW	32.2 ± 17.0*	27.5 ± 18.9*	4 ± 1
	water	97.2 ± 28.2	90.5 ± 26.9 ^a	640 ± 0
<i>Ovomucoid sensitization</i>				
B cell-depleted mice	water	216.3 ± 92.7	268.8 ± 135.6	427 ± 142
	EW	74.7 ± 29.1*	87.6 ± 24.5*	17 ± 4
Normal mice	EW	45.1 ± 16.4*	50.5 ± 19.7*	10 ± 3
	water	349.7 ± 75.2	434.4 ± 106.1	533 ± 142
<i>Lysozyme sensitization</i>				
B cell-depleted mice	water	422.8 ± 114.0	307.5 ± 102.3	533 ± 142
	EW	133.7 ± 55.9*	112.4 ± 50.3*	8 ± 2
Normal mice	EW	78.2 ± 27.3*	94.1 ± 38.5*	4 ± 1
	water	510.1 ± 96.8	426.6 ± 106.1	533 ± 142

Groups of normal and B cell-depleted mice were fed 10% EW or distilled water for 5 days. One week after the last feed, their spleen cells were transferred into naïve recipients (n = 6). The recipients were further sensitized with 10 µg of conalbumin, ovomucoid or lysozyme in alum. Two weeks after sensitization, their serum reagenic antibody responses were assessed and expressed as mean titers ± 1 SD. ^a p = 0.005; * p < 0.005.

and their products play a crucial role in the expression of the idiotype composition of suppressor T cell factors and repertoire of suppressor T cells, B cell depletion may interfere with the development of certain suppressor T cells [21, 22]. If the maintenance of oral tolerance is mainly regulated by idiotypic-specific suppressor T cells, it might be abrogated by B cell depletion. However, oral tolerance was readily induced in B cell-depleted mice in our study. Therefore, our result suggests that the induction of oral tolerance may not be due to the idiotypic-specific suppressor pathway. As oral tolerance has been shown here after transfer of spleen cells from B cell-depleted mice to naïve recipients, our results further suggest that oral tolerance to oral antigens may be due to other suppressor cells, but not idiotypic-specific suppressor cells.

Both intestinal epithelial cells and cells of macrophage and dendritic cell lineages in the lamina propria and Peyer's patches express class II MHC glycoproteins [31–33] and have the potential to present oral antigens. As the M cells overlying Peyer's patches play a critical role in oral antigen uptake [34], many studies have focused on Peyer's patches, but have been discouraged by the report that oral tolerance can be induced in rats without Peyer's patches [35]. Most dendritic cells isolated from Peyer's patches are immature [33], but dendritic cells from both lamina propria and Peyer's patches can present antigens and

induce T cell proliferation [32, 33]. Thus, it has been suggested that the presentation of antigens by dendritic cells in the gut is very similar to what occurs in other tissues [32, 33]. However, the possible APCs from different intestinal tissues revealed differences when cultured with naïve T cells. Cells from Peyer's patches activate mainly CD4+ T cells, while cultures with cells from lamina propria contained primarily of CD8+ cells [32]. Therefore, it is also possible that the lamina propria offers a unique microenvironment favoring the induction of CD8+ T cells.

It has been shown that intestinal epithelial cells play a crucial role in the induction of oral tolerance to haptens [36]. This is supported by early studies which found that intestinal epithelial cells could present OVA in vitro and selectively primed suppressor T cells [37], but is questioned by other reports that scid mice failed to generate the gut-processed tolerogenic moiety of OVA [6, 9]. Although scid mice lack functional T and B cells, they have normal somatic cells, including intact intestinal epithelium. The role of scid intestinal epithelial cells in the induction of oral tolerance may be further explored. On the other hand, many studies have shown that portal vein administration of foreign antigens induces portal vein tolerance [38, 39]. It has been shown that Kupffer cells are the major APCs in the induction of portal vein tolerance

[38, 39]. Most gut-absorbed oral antigens are transported through portal veins. It has been shown that oral tolerance can be abrogated by portacaval shunt [40]. Since Kupffer cell blockade by gadolinium can prevent the induction of portal vein tolerance [38, 39], it would be of interest to evaluate whether Kupffer cell blockade by gadolinium can abrogate the induction of oral tolerance or not.

The induction of oral tolerance to food proteins is a physiological defense mechanism of the gut [1]. However, peripheral tolerance to parenterally administered foreign antigens is an artificial phenomenon only demonstrated in experimental animals [12–18]. Whereas central tolerance is specific for self-antigens, peripheral tolerance is specific for parenterally or enterally administered foreign

antigens. In contrast to the reports that resting B cells can present parenterally administered antigens to naïve T cells and induce tolerance [14–17], our present data clearly reveal that resting B cells are not essential for the induction of oral tolerance of Th2 immune responses. Thus, the present results also point out that peripheral tolerance to parenterally and enterally administered protein antigens may be mediated by different mechanisms.

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