

The influence of initial exposure timing to β -lactoglobulin on oral tolerance induction

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Background: Although a number of studies have investigated the induction of oral tolerance to several proteins, relatively little is known about the induction of oral tolerance to β -lactoglobulin, one of the major antigenic proteins in milk.

Objective: We investigated the influence of the timing of the initial β -lactoglobulin exposure on oral tolerance induction and examined some characteristics of the tolerogenic immune response.

Methods: BALB/c mice were given β -lactoglobulin prenatally or from the third or fifth postnatal week, bred for 17 weeks, and compared with unexposed control mice. Specific plasma anti- β -lactoglobulin antibodies (total IgG, IgG subclasses, IgM, and IgE), antigen-specific splenocyte responses, frequencies of antibody-producing cells, and cytokine production by splenocytes, intestinal mucosal lymphocytes, and Peyer's patches were analyzed.

Results: Differences were observed among the 4 groups of mice in changes of plasma anti- β -lactoglobulin antibody titers, antigen-specific T-cell proliferation, and frequencies of antibody-producing splenocytes, intestinal mucosal lymphocytes, and Peyer's patch cells after the first exposure to β -lactoglobulin. The onset and duration of the immunologic responses were found to be dependent on the timing of antigen exposure. Prenatal exposure to antigen facilitated the induction of oral tolerance to β -lactoglobulin, whereas delayed antigen exposure retarded tolerance. The induction of oral tolerance was associated with increased IL-4 and/or IL-10 production and decreased IL-12 production.

Conclusion: Our results suggest that the timing of initial antigen exposure greatly influences the induction of oral tolerance to β -lactoglobulin and that altered secretion of regulatory cytokines may be responsible for the differences in antibody production and oral tolerance induction. (*J Allergy Clin Immunol* 1999;104:870-8.)

Key words: β -Lactoglobulin, oral tolerance, IgG, subclasses of IgG, IgE, intestinal mucosal lymphocytes, Peyer's patches, enzyme-linked immunospot assay, cytokine production

In recent years increasing interest has been shown in the phenomenon of oral tolerance,¹⁻¹⁰ which holds therapeutic potential in the treatment of certain autoimmune diseases and allergies. Adverse reactions to milk and

Abbreviations used

GALT:	Gut-associated lymphoid tissues
IML:	Intestinal mucosal lymphocyte
MTT:	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide thiazolyl
PP:	Peyer's patches
P/S:	Penicillin/streptomycin

eggs are most common during infancy and early childhood.¹¹ Although all children display humoral immune responses to basic food antigens, including the development of specific antibodies, these responses are suppressed by the induction of oral tolerance before leading to clinical manifestations in healthy individuals. A number of studies have investigated the induction of oral tolerance to ovalbumin^{4,12-17} and casein^{18,19}; however, there have been only a small number of reports concerning oral tolerance to β -lactoglobulin,^{20,21} one of the major antigenic proteins in milk.

The aim of this study was to investigate systemic and intestinal immune responses, including aspects of cytokine production, in response to oral β -lactoglobulin administration and the influence of the timing of initial antigen exposure on the induction of oral tolerance.

METHODS

Reagents

β -Lactoglobulin, *o*-phenylenediamine dihydrochloride, BSA, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, thiazolyl (MTT) blue, and 3-amino-9-ethyl-carbazole were obtained from Sigma Chemical Company (St Louis, Mo). Biotinylated rabbit anti-mouse IgG, biotinylated rabbit anti-mouse IgG1, biotinylated rabbit anti-mouse IgG2a, biotinylated rabbit anti-mouse IgG2b, biotinylated rabbit anti-mouse IgG3, biotinylated goat anti-mouse IgM, and biotinylated goat anti-mouse IgE were purchased from Zymed Laboratories, Inc (San Francisco, Calif). Peroxidase-conjugated streptavidin was supplied by Dakopatts (A/S, Glostrup, Denmark). RPMI-1640 and HBSS were from Nissui pharmaceutical Co, Ltd, Japan. L-glutamine, penicillin/streptomycin (P/S), and gentamicin reagent solution (gentamicin) were obtained from Gibco BRL Ltd (Gaithersburg, Md). FCS (6J4013) was supplied by Nichirei (Tokyo, Japan). Percoll was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). PHA and LPS were obtained from DIFCO Laboratories (Detroit, Mich).

Animals

BALB/c mice were divided into 4 dietary groups (p-0, p-3, p-5, and control) and bred for 6 to 17 weeks. Prenatal group mice (p-0, n = 20) were fed an experimental diet containing powdered milk from the beginning of the prenatal period (administered through mother mice) to the end of experiment. The postnatal to 3 weeks group (p-3, n = 20) and postnatal to 5 weeks group (p-5, n = 20)

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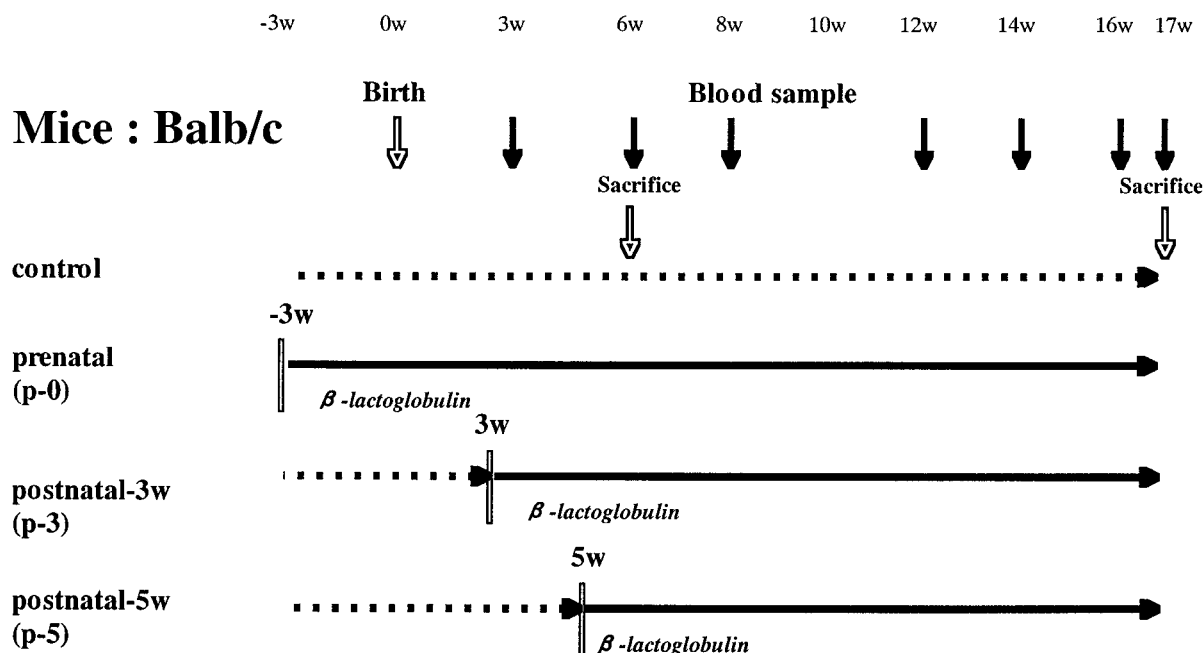


FIG 1. Experimental protocol. BALB/c mice were divided into 4 dietary groups (control, prenatal [p-0], postnatal 3 weeks [p-3], and postnatal 5 weeks [p-5]) and bred for 6 to 17 weeks. Blood samples were obtained from all mice at the end of weeks 3 and 6 and thereafter every 2 weeks until the end of experimentation.

were fed an experimental diet containing powdered milk from the third and fifth postnatal week, respectively, after feeding on standard solid fodder for 2 and 4 weeks, respectively. Control group mice ($n = 22$) were given standard solid fodder without milk throughout the entire experimental period (Fig 1). This study was performed in accordance with the Prime Minister's Office of Notification, and all animal protocols were approved by the Institutional Animal Care and Use Committee of Japan Women's University.

Preparation of blood samples

Blood samples were obtained from all mice at the end of weeks 3 and 6 and thereafter every 2 weeks until the end of the experimental period. One hundred microliters of blood taken from the orbit of each mouse was suspended in 400 μ L of iced saline and centrifuged (13,000g) for 5 minutes at 4°C to separate plasma. The samples were then stored at -80°C until analysis of β -lactoglobulin antibody titers.

Determination of antibody responses to β -lactoglobulin

Antibody responses of IgG, subclasses of IgG, IgM, and IgE to β -lactoglobulin were determined by ELISA. β -Lactoglobulin at 1 μ g/mL in 0.1 mol/L carbonate buffer (pH 9.6) was used to coat 96-multiwell plates (Sumilon MS-8496F, Sumitomo Bakelite Co, Ltd) overnight at 4°C. Plates were then blocked with 3% BSA-PBS for 4 hours at room temperature.

Plasma samples serially 2-fold diluted with 0.05% Tween-PBS (1:50 to 1:400) were added to wells in duplicate and incubated with coated antigen for 2 hours at room temperature. Detection antibodies, consisting of 1:4000 dilutions of biotinylated rabbit anti-mouse IgG, anti-mouse IgG1, anti-mouse IgG2a, anti-mouse IgG2b, anti-mouse IgG3, or biotinylated goat anti-mouse IgM in Tween-PBS, were then added to the plates and incubated overnight at 4°C. After adding a 1:4000 dilution of peroxidase-conjugated streptavidin (0.8 g/L, DAKO A/S Co, Ltd), plates were incubated at room tempera-

ture for 30 minutes. Reactions were developed by the addition of substrate solution consisting of 2 mg/mL *o*-phenylenediamine in 0.05 mol/L citrate-phosphate buffer, pH 5.0, containing 0.04% H_2O_2 for 15 minutes. Reactions were terminated by the addition of 25 μ L per well of 0.05 mol/L HCl. Optical densities were recorded by means of a microplate reader (MTP-22, Corona Electric Co, Ltd) at λ_1 :492 nm and λ_2 :405 nm. Standard curves performed by using hyperimmune anti- β -lactoglobulin sera as standard were run for each assay. Calculation of sample titers was made on the basis of those dilutions resulting in absorbances equal to 1:5000 diluted standard sera.

IgE antibody titers were determined by using a modified method, as described previously. Briefly, plates coated with β -lactoglobulin were blocked with 2% gelatin-PBS for 2 hours at 37°C. Undiluted plasma samples were added to the wells and incubated for 2 hours at 37°C. Plates were then incubated at 37°C for 45 minutes after the addition of detection antibody consisting of a 1:1000 dilution of biotinylated rabbit anti-mouse IgE. Reactions developed by a substrate solution were terminated after 30 minutes. A 1:8 diluted standard serum pool was used for calculating sample titers.

Isolation of intestinal mucosal lymphocytes and Peyer's patches

Intestinal mucosal lymphocytes (IMLs) were isolated by using the method of Davies and Parrott,²² with some modifications. Briefly, the small intestines were removed from mice and thoroughly washed with cold PBS by using a 20-mL syringe fitted with a short plastic tube. The mesentery, adherent connective tissue, and fat were dissected from the intestines, and Peyer's patches (PP) were carefully removed. A tube was run through the small intestine from the stomach side and tied to the large intestine with thread. The intestine was turned inside out and cut into 2- to 3-cm segments. Segments were placed into 20 mL of HBSS containing 1 U/mL P/S, 40 μ L/mL gentamicin, and 5% FCS (incomplete medium) in a 50-mL centrifuge tube and incubated for 45 minutes at

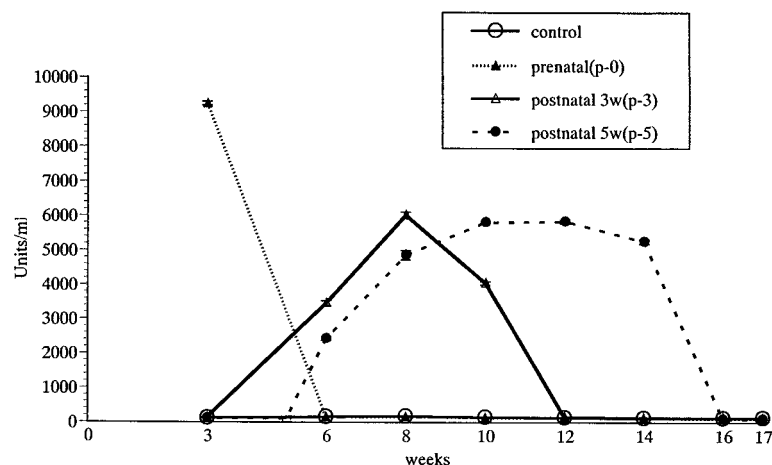


FIG 2. Plasma anti- β -lactoglobulin antibody (IgG) level. Induction of oral tolerance to β -lactoglobulin was shown to occur in all 3 test groups; however, the time required for the development of tolerance after the initial oral ingestion of β -lactoglobulin was estimated as 6, 9, and 11 weeks in the p-0, p-3, and p-5 groups, respectively. Results are expressed as units per milliliter, and each point represents means $\pm$ SEM from 7 mice per group at each time point.

37°C with continuous stirring. The tube was then centrifuged at 1500 rpm at room temperature for 10 minutes. Separated cells were passed through a 10-mL syringe column containing one layer of cotton and loosely packed glass wool to remove dead cells and tissue debris. Cells were then placed on a discontinuous Percoll density gradient (including 100% and 70%) to obtain the lymphocyte population.

Preparation and culture of spleen and PP cells

Spleens were aseptically removed from mice, and PP were carefully separated from intestines. Single-cell suspensions of spleen and PP lymphocytes were prepared as follows. Spleens and PP were squashed through a fine wire mesh and passed through an 80- μ m nylon filter. Cells were then suspended and washed in RPMI-1640. Cell suspensions were washed a further 3 times with RPMI-1640 before resuspension in complete culture medium containing 8% FCS, 2 mmol/L L-glutamine, 1 U/mL P/S, 5×10^{-5} mol/L 2-mercaptoethanol, and 2 mg/mL Na_2HCO_3 . Cell viability was determined by trypan blue exclusion.

Colorimetric MTT proliferation assay

The blastogenic response of spleen lymphocytes was determined by the MTT proliferation assay. Splenocytes (0.1 mL at 4×10^5 cells/well) in complete RPMI-1640 medium were incubated with dilutions of PHA (2 μg /well) in U-bottom, 96-well, tissue-culture plates (Falcon 3077, Becton Dickinson). Triplicate cultures were incubated at 37°C in a humidified 5% CO_2 atmosphere for 48 hours. Splenocytes (0.1 mL at 4×10^5 cells/well) were also incubated with LPS (1 μg /well) or β -lactoglobulin (0.4 mg/well) in flat-bottom, 96-well, tissue-culture plates (Falcon 3072 Becton Dickinson). Triplicate cultures were incubated at 37°C in a humidified 5% CO_2 atmosphere for 72 hours. Lymphocytes cultured without mitogens served as negative controls. Ten microliters of MTT (2.5 mg/mL) was then added to each well. After 3 hours' incubation at 37°C, 100 μL of extraction buffer (10% SDS, 0.01 normal HCl) was added. After overnight incubation, absorbances were measured at $\lambda_1:570 \text{ nm}$ and $\lambda_2:630 \text{ nm}$ by using a microplate reader (MTP-22 Corona Electric Co, Ltd). The proliferative response for mitogen-containing cultures was expressed as follows: proliferative response = $(\lambda_1:570 \text{ nm}, \lambda_2:630 \text{ nm with mitogens}) - (\lambda_1:570 \text{ nm}, \lambda_2:630 \text{ nm without mitogens})$.

Enumeration of β -lactoglobulin-specific IgG-, IgG1-, IgG2a-, IgG2b-, IgG3-, or IgM-producing cells by enzyme-linked immunospot assay

For the quantitation of IgG-, IgG1-, IgG2a-, IgG2b-, IgG3-, or IgM-producing cells in spleen cells, PP cells, and IMLs, a β -lactoglobulin-specific enzyme-linked immunospot assay was used.²³ In this assay 96-well plates with nitrocellulose bases (Millipore Corp, Bedford, Mass) were coated with carbonate buffer containing 0.05 mg/mL β -lactoglobulin overnight at 4°C. After blocking with RPMI-1640 medium containing 100 μL of 5% BSA at 37°C for 3 hours, spleen cells (4×10^6 /mL), IMLs (8×10^5 /mL), or PP cells (4×10^5 /mL) in culture medium (8% FCS, 2 mmol/L L-glutamine, 1 U/mL P/S, 5×10^{-5} mol/L 2-mercaptoethanol, and 2 mg/mL Na_2HCO_3) were added to individual wells. After incubation for 5 hours, the plates were washed and incubated with 1:200 diluted biotinylated rabbit anti-mouse IgG; 1:100 diluted biotinylated rabbit anti-mouse IgG1, biotinylated rabbit anti-mouse IgG2a, biotinylated rabbit anti-mouse IgG2b, or biotinylated rabbit anti-mouse IgG3; or 1:200 diluted biotinylated goat anti-mouse IgM in Tween-PBS overnight at 4°C. After adding 1:100 diluted peroxidase-conjugated streptavidin (0.8 g/L), plates were incubated at 37°C for 45 minutes. Spots representing single IgG-, IgG1-, IgG2a-, IgG2b-, IgG3-, or IgM-secreting cells were developed by the substrate, 3 amino-9-ethylcarbazole. The spots were then counted by using a dissecting microscope.

Measurement of in vitro cytokine production

Spleen cells, IMLs (5×10^5 /mL), or PP cells (2×10^5 /mL) were cultured in RPMI-1640 (containing 5% FCS, 2 mmol/L L-glutamine, 1 U/mL P/S, 5×10^{-5} mol/L 2-mercaptoethanol, and 2 mg/mL Na_2HCO_3) in flat-bottom, 24-well, tissue-culture plates (Falcon 3047, Becton Dickinson) with either PHA (20 μg /mL), LPS (10 μg /mL), or β -lactoglobulin (4 mg/mL). Triplicate cultures were incubated at 37°C in a humidified 5% CO_2 atmosphere for 72 hours. Culture supernatants were harvested, separated from contaminating cells by centrifugation at 13,000g for 5 minutes, and stored at -80°C until assay. IL-4, IL-10, IL-12, and TNF- α levels were determined by using a commercial ELISA kit (BioSource International Laboratories). IL-2 levels were measured by using a sandwich

TABLE I. Frequencies of splenocytes, IMLs, and PP cells producing IgG and IgG subclasses in response to β -lactoglobulin

			No. of spots forming cells/10 ⁶			
			Control group	p-0	p-3	p-5
Splenocytes	IgG	6 wk	0.6 $\pm$ 0.2	4.6 $\pm$ 0.3‡	3.3 $\pm$ 0.2‡	3.9 $\pm$ 0.2‡
		17 wk	0.7 $\pm$ 0.0	0.7 $\pm$ 0.0	3.6 $\pm$ 0.3‡	3.2 $\pm$ 0.2‡
	IgG1	6 wk	0.5 $\pm$ 0.1	3.5 $\pm$ 0.2‡	4.4 $\pm$ 0.3‡	1.9 $\pm$ 0.1‡
		17 wk	0.7 $\pm$ 0.1	0.8 $\pm$ 0.1	1.4 $\pm$ 0.1‡	1.0 $\pm$ 0.1*
	IgG2a	6 wk	0.1 $\pm$ 0.0	1.9 $\pm$ 0.1‡	3.3 $\pm$ 0.4‡	1.2 $\pm$ 0.1‡
		17 wk	0.3 $\pm$ 0.2	0.2 $\pm$ 0.1	0.9 $\pm$ 0.0†	0.9 $\pm$ 0.0‡
IMLs	IgG	6 wk	1.3 $\pm$ 0.4	8.4 $\pm$ 0.5‡	115.5 $\pm$ 4.2‡	5.8 $\pm$ 0.5‡
		17 wk	1.8 $\pm$ 0.5	7.5 $\pm$ 0.8‡	7.5 $\pm$ 0.8‡	11.1 $\pm$ 0.3‡
	IgG1	6 wk	1.4 $\pm$ 0.5	9.9 $\pm$ 1.4‡	44.6 $\pm$ 1.5‡	14.3 $\pm$ 0.9‡
		17 wk	2.1 $\pm$ 0.8	11.8 $\pm$ 1.0‡	12.0 $\pm$ 1.2‡	12.4 $\pm$ 1.2‡
	IgG2a	6 wk	1.1 $\pm$ 0.3	3.3 $\pm$ 0.5†	105.5 $\pm$ 2.9‡	13.9 $\pm$ 1.3‡
		17 wk	1.3 $\pm$ 0.3	2.1 $\pm$ 0.5	6.4 $\pm$ 1.4*	12.0 $\pm$ 1.9†
PP cells	IgG	6 wk	3.5 $\pm$ 1.2	20.8 $\pm$ 1.3‡	254.3 $\pm$ 9.2‡	53.3 $\pm$ 6.4‡
		17 wk	5.0 $\pm$ 1.4	7.5 $\pm$ 1.4	8.5 $\pm$ 0.9	16.0 $\pm$ 2.8†
	IgG1	6 wk	5.3 $\pm$ 1.7	71.8 $\pm$ 2.2‡	178.5 $\pm$ 9.4‡	39.3 $\pm$ 2.2‡
		17 wk	2.5 $\pm$ 1.2	6.0 $\pm$ 0.8*	6.8 $\pm$ 0.5*	9.3 $\pm$ 1.1†
	IgG2a	6 wk	2.3 $\pm$ 1.1	52.3 $\pm$ 2.6‡	204.3 $\pm$ 9.8‡	27.8 $\pm$ 1.2‡
		17 wk	1.5 $\pm$ 0.9	4.8 $\pm$ 1.9	8.3 $\pm$ 1.9*	6.8 $\pm$ 0.5‡

Data are presented as means $\pm$ SEM of 7 mice per group. Symbols indicate statistically significant differences determined by the Student *t* test: **P* < .05, †*P* < .01, and ‡*P* < .001 in comparison with the control group.

ELISA technique. Ninety-six–multiwell plates (Sumilon MS-8496F) were coated with 100 μ L of 5 μ g/mL anti-IL-2 antibody (JES6-5H4, Becton Dickinson) diluted in PBS and incubated overnight at 4°C. Wells were blocked with PBS and 0.05% Tween containing 1% BSA at room temperature for 2 hours. Serial 2-fold dilutions of the culture supernatants were added to duplicate wells and incubated overnight at 4°C. Wells were washed with Tween-PBS and incubated with 3 μ g/mL biotinylated rat anti-IL-2 mAb (JES6-1A12, Becton Dickinson) diluted in Tween-PBS with 1% BSA at room temperature for 3 hours. Wells were then washed with Tween-PBS and incubated with 0.5 μ g/mL biotinylated mouse anti-rat IgG2a (10092D, Pharmingen) diluted in Tween-PBS with 1% BSA at room temperature for 2 hours, washed with Tween-PBS, and incubated with 0.2 μ g/mL peroxidase-conjugated streptavidin diluted in Tween-PBS with 1% BSA at 37°C for 30 minutes. After 3 washing steps, reactions were developed by substrate solution consisting of 0.002% *o*-phenylenediamine in 0.05 mol/L citrate-phosphate buffer, pH 5.0, with 0.04% H₂O₂ for 15 minutes. Reactions were terminated by the addition of 50 μ L of 2 mol/L H₂SO₄. Optical densities were recorded by microplate-reader (MTP-22 Corona Electric) at 492 nm. A standard curve was generated by using murine recombinant IL-2 (Pharmingen).

Effect of intraperitoneal immunization with β -lactoglobulin on lymphocyte proliferation

Four groups of BALB/c mice (*n* = 10 each), treated as described above, were immunized intraperitoneally with 400 μ g of β -lactoglobulin in PBS 10 days before sacrifice at week 6, and proliferation of splenocytes in response to β -lactoglobulin was assessed.

Statistical analysis

Results were given as means $\pm$ SEM. After confirmation, the data were normally distributed by the Shapiro-Wilk test, and intra-group comparisons were made by using the 2-tailed paired Student *t* test. The 2-tailed unpaired Student *t* test was used for intergroup comparisons throughout.

RESULTS

Body weight

All groups gained weight rapidly from week 3 through week 8 and thereafter gained weight gradually. At week 6, the mean weights were 17.7 $\pm$ 0.9 g, 17.0 $\pm$ 0.4 g, 18.7 $\pm$ 0.7 g, and 17.8 $\pm$ 0.8 g in the control, p-0, p-3, and p-5 groups, respectively, and 25.8 $\pm$ 0.9 g, 26.3 $\pm$ 0.6 g, 26.0 $\pm$ 1.0 g, and 25.9 $\pm$ 0.5 g at week 17, respectively. There were no significant differences in body weight among the 4 groups at weeks 6 and 17.

Oral tolerance induction as assessed by antibody levels to β -lactoglobulin

As shown in Fig 2, plasma IgG titers to β -lactoglobulin remained low throughout the study period in control mice range (range 118 $\pm$ 3 U to 171 $\pm$ 5 U). In contrast, mice receiving β -lactoglobulin from the prenatal period (p-0 group) had extremely high IgG titers at week 3 (9245 $\pm$ 57 U). The titer then decreased rapidly to control levels by week 6 (161 $\pm$ 2 U), suggesting early development of tolerance to β -lactoglobulin after oral ingestion. Likewise, in the p-3 group mice fed the experimental diet from week 3, IgG antibody titers increased from week 6, reaching a peak at week 8 (6026 $\pm$ 85 U), and then decreased to control levels at week 12 despite continuous feeding of β -lactoglobulin, probably as the result of oral tolerance induction. P-5 group mice, given β -lactoglobulin from week 5, the time of usual weaning, had a similar pattern as p-3 group mice except for a far prolonged increase in IgG antibody titers lasting for more than 10 weeks, which might suggest delayed induction of oral tolerance. Thus the time required for the development of

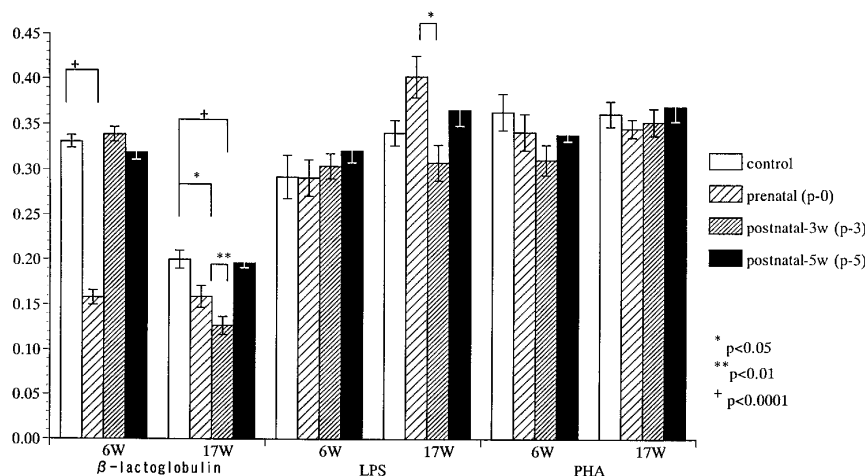


FIG 3. Proliferation of spleen cells stimulated with β -lactoglobulin, LPS, and PHA. At week 6, proliferation was significantly suppressed in the p-0 group with established oral tolerance compared with that found in the control group. In contrast, responses of splenocytes from the p-3 and p-5 mice were not significantly different from those of control mice at week 6. There were no significant differences in proliferative responses of splenocytes to LPS or PHA among the 3 test groups and control mice. At week 17, proliferation of splenocytes in response to β -lactoglobulin was suppressed in both the p-0 and p-3 groups compared with the control group. Responses to LPS were slightly greater in the p-0 group than in the control group. Data are presented as means $\pm$ SEM from at least 10 mice per group.

tolerance after the initial oral ingestion of β -lactoglobulin was estimated at 6, 9, and 11 weeks in the p-0, p-3, and p-5 groups, respectively.

IgG1 titers exhibited a similar pattern of changes to that of total IgG in all test groups. Titers of IgG2a were also increased similarly to total IgG in the p-3 and p-5 groups. However, IgG2a levels subsided earlier than IgG1 in both groups, and IgG2a levels in the p-0 group at week 3 were much lower compared with IgG1 levels. All 3 test groups showed similar patterns of IgG2b and IgG3 titers to total IgG; however, the increases were too small to be compared with those of IgG1 and IgG2a.

Antibody titers of IgM and IgE to β -lactoglobulin remained at low levels throughout the entire study period in all groups, and there were no significant differences among the 3 test groups and the control group at any time point.

Proliferative response of splenocytes to β -lactoglobulin

Fig 3 shows the proliferative responses of spleen lymphocytes to β -lactoglobulin, LPS, and PHA at week 6. Proliferation of splenocytes stimulated with β -lactoglobulin was significantly suppressed in p-0 mice compared with the control group ($P < .0001$). In contrast, proliferative responses of splenocytes from p-3 and p-5 mice were not significantly different from control mice at week 6. There were no significant differences in proliferative responses of splenocytes to LPS or PHA among the 3 test groups and the control mice (Fig 3).

At week 17, the proliferation of splenocytes in response to β -lactoglobulin was suppressed in both p-0 and p-3 mice compared with the control mice ($P < .05$ and $P < .0001$, respectively; Fig 3). P-3 mice showed a

larger significant decrease than p-0 mice ($P < .001$). Responses in p-5 mice were not significantly different from control mice at week 17, when increased IgG and IgG subclasses to β -lactoglobulin had already returned to control levels.

Responses to LPS were slightly greater in p-0 mice than in control mice ($P < .05$), although there were no significant differences between the p-3, p-5, and control mice (Fig 3). Proliferative responses to PHA in the 3 test groups did not differ from that found in control mice.

Enumeration of splenocytes producing β -lactoglobulin-specific antibodies

β -Lactoglobulin-specific IgG-producing splenocytes (B cells) were more frequent in the p-0 group compared with the control group at week 6 but not at week 17 (Table I). Likewise, the frequencies of splenocytes producing the 4 IgG subclasses were all higher in the p-0 group compared with the control group at week 6, although not different from the control group at week 17, with the exception of IgG3.

In contrast, significantly higher frequencies of splenocytes producing IgG and the 4 IgG subclasses were observed not only at week 6 but at week 17 in both the p-3 and p-5 groups. Although the frequencies were all reduced at week 17 compared with week 6, splenocytes producing β -lactoglobulin-specific antibodies were still present at week 17.

Unlike splenocytes producing IgG, the frequencies of splenocytes producing IgM to β -lactoglobulin did not change in any of the 3 test groups compared with the control group at weeks 6 or 17.

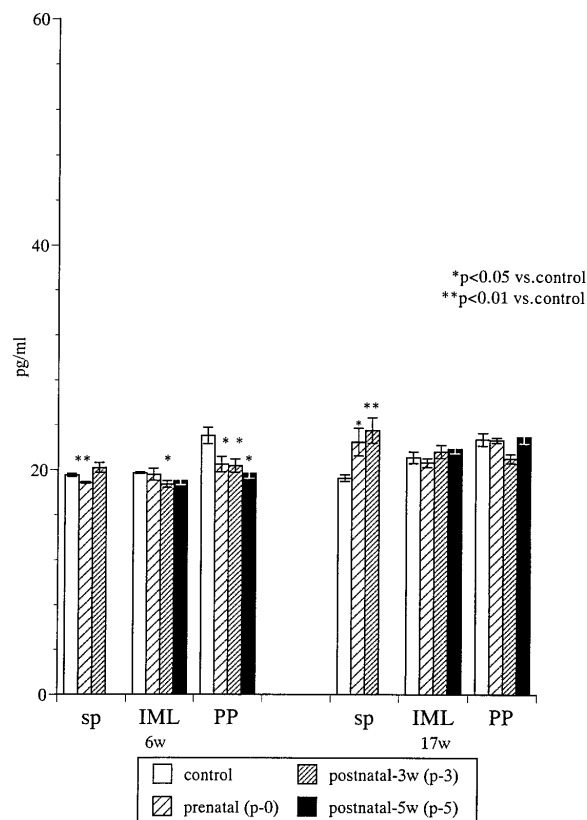


FIG 4. Levels of IL-12 from splenocytes, IMLs, and PP cells stimulated with β -lactoglobulin. Production of IL-12 by splenocytes was lower in the p-0 group than in the control group at week 6, production by IMLs was lower in the p-3 group, and production by PP cells was decreased in all 3 test groups at week 6. Data are presented as means $\pm$ SEM of 7 mice per group. Asterisks indicate statistically significant differences determined by means of the Student *t* test, as shown in the figure.

Enumeration of IMLs and PP cells producing β -lactoglobulin-specific antibodies

As shown in Table I, frequencies of IMLs producing IgG or IgG subclasses were higher than those of splenocytes in both the test and control groups at weeks 6 and 17. The frequencies of IMLs producing all the IgG subclasses except IgG2b were increased at week 6 in the p-0 group, and IMLs producing IgG, IgG1, and IgG3 were still increased at week 17 compared with the control group. In p-3 mice very large increases in the frequencies of IMLs producing IgG and each subclass, as much as 30-fold more than that found in the control mice, was observed at week 6. The increases were much decreased at week 17, although they remained significant compared with control mice. The p-5 group also showed significant increases in β -lactoglobulin-specific antibody producing IML frequencies at both weeks 6 and 17. However, the increases observed at week 6 were far smaller than those found in the p-3 group.

The frequencies of PP cells producing IgG and its subclasses were higher than IMLs in the control group at

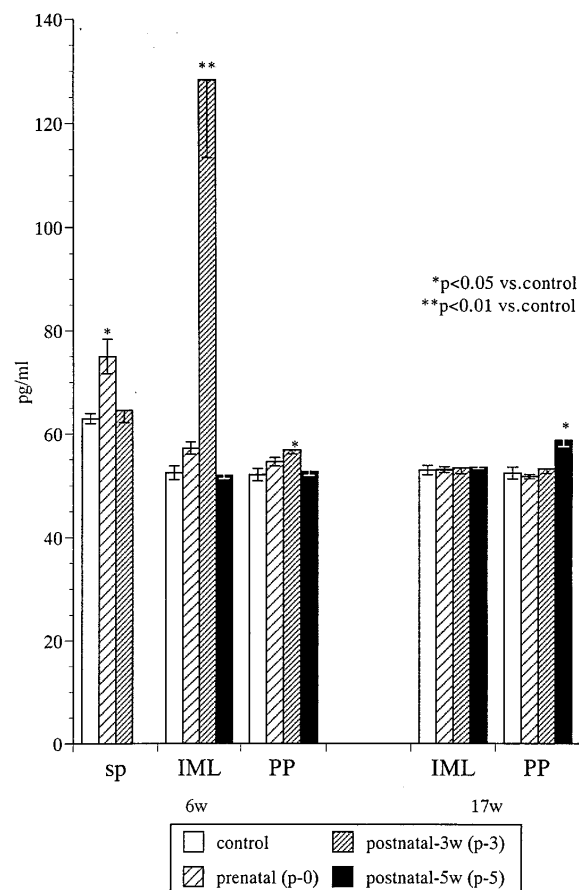


FIG 5. Levels of IL-4 from splenocytes, IMLs, and PP cells stimulated with β -lactoglobulin. Production of IL-4 by splenocytes was higher in the p-0 group, and production of IL-4 by IMLs and PP cells in the p-3 group was higher at week 6; production by IMLs in the p-3 group was markedly higher. At week 17, production by PP cells in the p-5 group was higher. Data are presented as means $\pm$ SEM of 7 mice per group. Asterisks indicate statistically significant differences determined by means of the Student *t* test, as shown in the figure.

both weeks 6 and 17. Frequencies were increased in the p-0 group mice at week 6, whereas only PP cells producing IgG1 differed from control levels at week 17 (Table I). The p-3 group mice showed highly significant (more than 30-fold of control group) increases in the frequencies of PP cells producing IgG and IgG subclasses at week 6. These frequencies were also significantly higher than those found in the control group at week 17, although the extent of the increase was lower than at week 6. Likewise, increased frequencies of PP cells producing IgG and IgG subclasses were observed in the p-5 group mice at both weeks 6 and 17; however, the increase at week 6 was much smaller compared with the p-3 group mice.

The frequencies of IMLs and PP cells producing antibodies to β -lactoglobulin were increased in all test groups, especially in the p-3 group. Furthermore, the increased frequencies remained significant even after the return of plasma immunoglobulin to control levels in the test groups. In

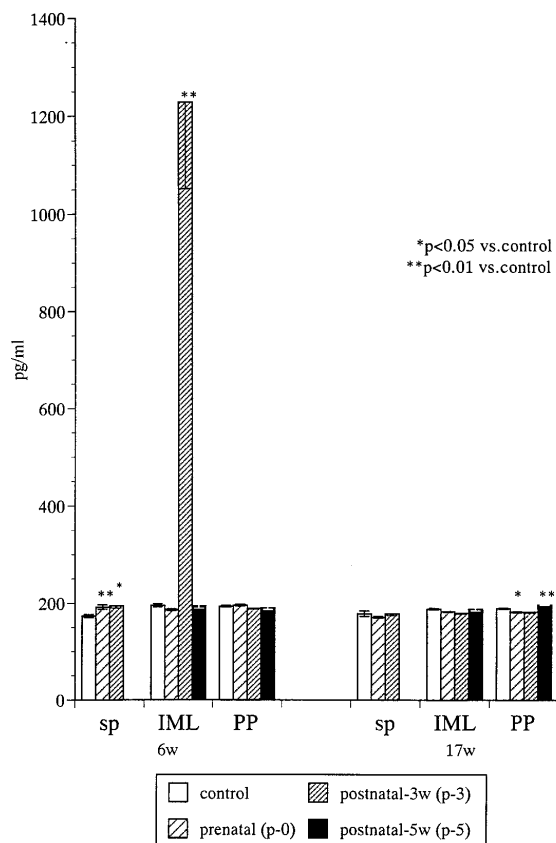


FIG 6. Levels of IL-10 from splenocytes, IMLs, and PP cells stimulated with β -lactoglobulin. At week 6, splenocytes of the p-0 and p-3 group produced higher amounts of IL-10. IL-10 produced by IMLs was markedly higher in the p-3 group mice compared with the control group. At week 17, decreased production of IL-10 by p-3 group IMLs and p-0 group PP cells were observed. In contrast, production of IL-10 by PP cells from p-5 group mice was significantly increased compared with the control group. Data are presented as means $\pm$ SEM of 7 mice per group. Asterisks indicate statistically significant differences determined by means of the Student *t* test, as shown in the figure.

addition, at week 17 in the p-0 group, frequencies of IMLs and PP cells that produced IgG2a and IgG2b were not different from those found in the control group, whereas the cells producing IgG1 remained higher.

The background frequencies of IMLs and PP cells producing IgM were significantly higher than those of cells producing IgG and IgG subclasses. There were no differences of frequencies of IgM-producing cells among the 3 test groups and control mice at both weeks 6 and 17.

Cytokine production by splenocytes, IMLs, and PP cells in response to PHA and β -lactoglobulin

None of the splenocytes from the 3 test groups fed an experimental diet produced different amounts of IL-2 in response to PHA compared with that produced by control mice at weeks 6 and 17. IL-4 production by spleno-

cytes stimulated with PHA was also not different between the experimental groups.

Accordingly, levels of cytokines from splenocytes, IMLs, and PP cells stimulated with β -lactoglobulin were investigated. Fig 4 illustrates production of IL-12 by these cells. The amounts of IL-12 produced by splenocytes were lower in the p-0 group than in the control group at week 6. IL-12 produced by IMLs was lower in the p-3 group. The production of IL-12 by PP cells was decreased in all 3 test groups at week 6. In addition, the amount of TNF- α produced by splenocytes was lower than that found in the control group at week 6. On the other hand, IL-12 production by splenocytes was higher in the p-0 and p-3 groups at week 17. The amount of IL-2 produced by splenocytes from the p-0, p-3, and p-5 groups of mice did not differ from that found in the control group at week 6, and neither IMLs nor PP cells produced different amounts of IL-2 from that found in the control group in the p-3 or p-5 groups. At week 17, no differences were observed in amounts of IL-2 produced by the splenocytes, IMLs, or PP cells from the 3 test groups and the control group.

As shown in Figs 5 and 6, p-0 group splenocytes produced higher amounts of IL-4 (Fig 5) and IL-10 (Fig 6) at week 6, whereas p-3 group splenocytes showed increased production of IL-10. Levels of IL-4 and IL-10 produced by IMLs were markedly higher in p-3 group mice compared with control mice but not in p-0 and p-5 group mice. At week 17, PP cells from p-5 group mice showed significantly increased production of both IL-4 and IL-10 at week 17.

Effects of intraperitoneal injection of β -lactoglobulin on lymphocyte proliferation

The effects of intraperitoneal injection of β -lactoglobulin, performed 10 days before sacrifice, on the proliferation of splenocytes stimulated with mitogens or β -lactoglobulin at week 6 were assessed. There were no differences in the proliferative responses of splenocytes in response to LPS or PHA among the 3 test groups. In contrast, the proliferation of splenocytes to β -lactoglobulin was significantly decreased in p-0, p-3, and p-5 group mice at week 6 ($P < .001$, $P < .01$, and $P < .05$, respectively; Fig 7). This indicates that some mechanism related to tolerance induction may have already started at week 6, even in the p-3 and p-5 groups in which antibody titers were just beginning to elevate.

DISCUSSION

Although some studies on oral tolerance to β -lactoglobulin have been reported,^{20,21} no systematic studies have yet been performed. The results of this study indicate that the timing of initial exposure to β -lactoglobulin influences the immunologic responses to β -lactoglobulin, including the induction of oral tolerance. In mice exposed to β -lactoglobulin during the prenatal period (p-0 group), plasma IgG titers to β -lactoglobulin were high at week 3, probably because of antigen exposure through

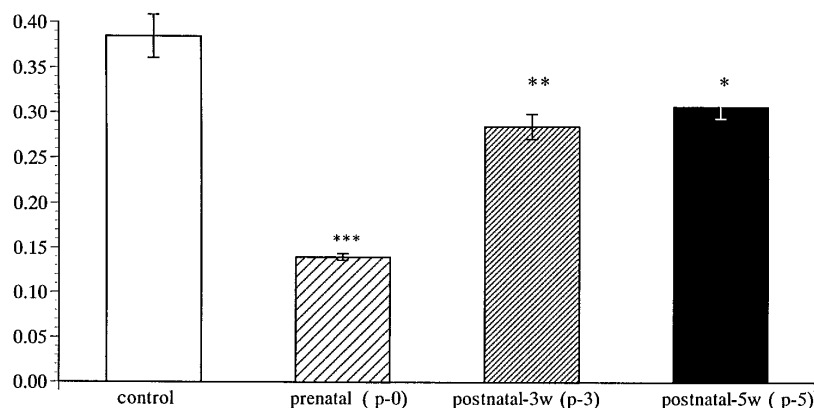


FIG 7. Effect of intraperitoneal injection by β -lactoglobulin on proliferation of splenocytes stimulated with β -lactoglobulin at week 6. Proliferation of splenocytes was significantly decreased in the p-0, p-3, and p-5 group mice at week 6 (*** P < .001, ** P < .01, * P < 0.05 in comparison with the control group). Data are presented as means $\pm$ SEM of 10 mice per group.

the placenta from the maternal system, but reduced rapidly to control levels at week 6. This abrupt change suggests the induction of oral tolerance against β -lactoglobulin was fully developed after 6 weeks. Likewise, oral tolerance developed in mice receiving β -lactoglobulin orally from week 3 after birth (p-3 group), as assessed by the decline in plasma IgG to β -lactoglobulin despite continuous feeding of the antigen at week 12. Mice given β -lactoglobulin from week 5 (p-5 group) after normal weaning showed the fall of specific IgG to β -lactoglobulin at week 16. The time intervals from the initial exposure to β -lactoglobulin to full development of tolerance as judged by antibody titers was estimated to be around 9 weeks in both the p-0 and p-3 groups if prenatal time was included. However, the period between the beginning of oral ingestion of β -lactoglobulin from mother's milk and induction of tolerance to β -lactoglobulin was 6 weeks in the p-0 group, which was much shorter than the 9 weeks in the p-3 group. Accordingly, the induction of oral tolerance to β -lactoglobulin, as assessed by specific antibody titers, appears to develop earlier in the p-0 group than in the p-3 group after the onset of oral ingestion of the antigen. These results indicate that the exposure to β -lactoglobulin from the prenatal period through the mother mice may have substantial effects on the accelerated development of oral tolerance. In addition, the longer time required for induction of oral tolerance in the p-5 group compared with the p-3 group indicates that later initial oral ingestion of β -lactoglobulin may be associated with delayed induction of oral tolerance.

Changes in IgG subclass titers, especially IgG1 and IgG2a, provided additional evidence for the influence of initial exposure time on the induction of oral tolerance. However, unlike IgG1, IgG2a was already low at week 3 in the prenatal group. Also, initial increases in IgG2a titers were reduced earlier than IgG1 titers in all test groups. It has been reported that helper T cells can be classified into 2 subpopulations (T_{H1} and T_{H2}) on the basis of patterns of cytokine secretion.²⁴ T_{H1} cells provide efficient help for IgG2a production, whereas T_{H2}

cells provide help for IgG1 and IgE production. Thus, the earlier inhibition of IgG2a antibody production compared with IgG1 in our results may indicate that T_{H1} -type responses could be preferentially inhibited in this oral tolerance to β -lactoglobulin.

Establishment of oral tolerance was also indicated by the decline of specific T-cell responses to the fed antigen. Suppressed proliferative responses of splenocytes (T cells) to β -lactoglobulin were observed at week 6 in the p-0 group, and at 17 weeks in the p-0 and p-3 groups. These results support the induction of T-cell hyporesponsiveness in the 2 groups. Moreover, the fact that the p-0 group showed significant suppression of splenocyte proliferation at both weeks 6 and 17 suggests that prenatal exposure to β -lactoglobulin had led to early induced and prolonged oral tolerance. In contrast, the p-5 group showed no change in the proliferative responses of splenocytes to β -lactoglobulin even at week 17, when plasma IgG titers to the antigen had already decreased to control levels. Complete suppression of IgG production presumably occurred before the suppression of splenic T-cell responses. However, there may be other explanations for the inhibition of antibody production to fed antigen in the p-5 group. Decline of antibody titers in a tolerant state could be achieved through many steps.

Anti- β -lactoglobulin antibody-producing cells were evaluated by enzyme-linked immunospot assay. Intestinal immune cells, namely IMLs and PP cells, were studied in addition to splenic lymphocytes. The frequencies of specific antibody-producing splenocytes significantly increased soon after the start of β -lactoglobulin ingestion in all test groups. This may indicate a systemic immune response to β -lactoglobulin in accordance with the increased plasma antibody titers. Increases in frequencies of IMLs and PP cells producing antibodies to β -lactoglobulin were more marked, and consistent with increased local immune responses to β -lactoglobulin. Interestingly, IMLs and PP cells in the p-3 group showed far greater numbers of antibody-producing cells to β -lactoglobulin at week 6 compared with the other 2 test groups. It is likely that imma-

ture gut-associated lymphoid tissues (GALT) have enhanced immune responses to β -lactoglobulin ingestion in the p-3 group mice that started ingesting β -lactoglobulin at week 3. In contrast, much smaller, although significant, responses developed in p-0 mice that received β -lactoglobulin through lactation before starting ingestion at week 3. The p-5 group mice, which had mature GALT on first ingesting β -lactoglobulin at week 5, had much smaller numbers of antibody-producing cells than the p-3 group. However, the frequencies of splenocytes, IMLs, and PP cells producing specific β -lactoglobulin antibodies were markedly reduced at week 17 in all of the test groups. This result also indicates the progression of oral tolerance in all test groups.

In addition, the results of this in vitro study show that the increased frequencies of antibody-producing cells in spleen cells, IMLs, and PP cells were found even after the titers of plasma antibodies decreased to the control levels in every test group. This may suggest that other additional regulatory mechanisms affect tolerance in vivo. Altered secretion of regulatory cytokines in the systemic or local immune systems may substantially influence the production of antibodies.

In this study the production of IL-2, a T_{H1} -type cytokine, by splenocytes, IMLs, and PP cells was not decreased in any test group at weeks 6 or 17. However, the production of IL-12 and TNF- α , relevant to T cell help, by splenocytes and IL-12 by PP cells decreased, whereas production of IL-4 and IL-10, T_{H2} -type cytokines, increased in the p-0 group at week 6. Likewise, the p-3 group showed decreased production of IL-12 by IMLs and PP cells along with increased production of IL-4 and/or IL-10 by splenocytes, IMLs, and PP cells. Furthermore, p-5 group mice showed decreased production of IL-12 by PP cells at week 6 and increased production of IL-4 and IL-10 by PP cells at week 17. These changes in cytokine production by splenocytes, IMLs, and PP cells are consistent with the suppression of T cell-proliferative responses and changes in the plasma IgG titers, implying that T_{H1} responses may be preferentially inhibited. This may result in T_{H2} dominance in the T_{H1}/T_{H2} balance. Moreover, increased IL-4 and IL-10 production by IMLs at week 6 was much greater in the p-3 group than in the p-0 group, probably because of heightened immunological activity of immature GALT around the time of weaning, as suggested in human subjects by Cummins et al.²⁵

In summary, results suggested that the timing of initial antigen exposure greatly influenced the induction of oral tolerance to β -lactoglobulin and that altered secretion of regulatory cytokines may be responsible for the differences in antibody production and oral tolerance induction. In any event, the precise mechanism of oral tolerance to β -lactoglobulin should be further investigated.

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