

Peripheral tolerance of T_H2 lymphocytes induced by continuous feeding of ovalbumin

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Abstract

We established conditions for inducing antigen-specific tolerance in T_H2 lymphocytes by means of oral tolerance. Mice were continuously exposed to ovalbumin in their drinking water for a minimal period of 20 days and then immunized against antigen in either complete Freund's adjuvant or Al(OH)₃. This feeding regimen tolerized both T_H2 and T_H1 responses as shown by diminished proliferation, cytokine secretion (IL-4, IL-2 and IFN- γ) and specific cytokine mRNA expression (IL-4, IL-2 and IFN- γ) *in vitro*, as well as by absence of specific antibody production (IgG1, IgG2a, IgG2b and IgE) *in vivo*. Conditions for generating T_H2 lymphocyte tolerance were different from those required to generate tolerance in T_H1 lymphocytes: these included extended, continuous exposure to high dosages of antigen, rather than a single or intermittent feeding regimen which was sufficient to induce tolerance in T_H1 lymphocytes. These findings suggest that continuous oral exposure to a tolerogen may be a biologically relevant strategy to tolerize both T_H1 - and T_H2 -dependent responses, and extend the potential clinical use of oral tolerance to ailments mediated by T_H2 lymphocytes.

Introduction

The gut associated lymphoid tissue is capable of generating both immunological tolerance (oral tolerance) and immune response (oral immunization) against antigens administered by the oral route. Conditions that distinguish between tolerance or immune response have not been clearly defined and might include sight of antigen entry (Peyer's patch or lamina propria) or the physical state of antigen (soluble or particulate) (1,2). Since both oral tolerance and oral immunization are relevant for treatment of human diseases (1–3), definition of these conditions is of major importance: immune responses should be avoided if tolerance is the objective and vice versa. Antigen feeding as a means to generate peripheral tolerance in CD4⁺ cells has been thus far successful in tolerization of T_H1 type responses, while those of T_H2 lymphocytes appeared to be intact (4–6). Similar difficulties in generating tolerance of T_H2 lymphocytes *in vitro* or of T_H2 -mediated antibody production *in vivo* have been encountered in other experimental systems in which tolerance was induced by i.v. or i.p. administration of soluble antigens (7–12). Thus, after tolerization by the oral or parenteral route, IL-2 and IFN- γ were not produced in cultures and a diminished IgG2a

antibody response was observed *in vivo* (tolerance of T_H1 responses). In contrast, IL-4 production *in vitro* and IgG1 responses *in vivo* were intact (intact T_H2 responses). The conclusion drawn from these studies was that T_H2 lymphocytes were resistant to tolerance induction in the periphery.

In contrast to these findings, earlier studies showed that feeding mice with large dosages of antigen led to tolerization of specific IgE-dependent cutaneous anaphylaxis. More recently, significant, though not complete, tolerization of T_H2 responses was achieved by the i.v. injection of deaggregated human γ -globulin. These studies indicated that oral, or peripheral, tolerization of T_H2 lymphocytes might require different conditions than those required to tolerize T_H1 lymphocytes and, thus, the present study was undertaken to determine conditions to orally tolerize T_H2 lymphocytes. Since T_H2 lymphocytes appeared to be more resistant to tolerance induction, we postulated that oral tolerization of T_H2 lymphocytes might require either larger antigen dosages or constant exposure to antigen in a manner that would prevent its rapid clearance from the blood. To directly evaluate these possibilities we modified a feeding regimen previously shown

to exclusively tolerize T_H1 lymphocytes and that delivered antibody-recognizable antigen into the blood, and demonstrate herein that both T_H1 - and T_H2 -dependent immune responses can be orally tolerized in an antigen-specific manner.

Methods

Experimental animals

Female BALB/C mice, 6–8 weeks of age, were used in all experiments. The mice were bred in our animal breeding unit (Rehovot) and were maintained in a temperature- and light-controlled environment with free access to feed and water. During experiments, mice continuously fed with ovalbumin (OVA, see below) had free access to OVA solution in water, instead of water alone. Each experimental group contained no less than five mice.

Antigens, feedings and immunizations

Antigens used were OVA (Sigma, St Louis, MO) and purified protein derivative of *Mycobacterium tuberculosis* (PPD; Statens, Denmark). Oral tolerance to OVA was induced by continuous oral exposure to a sterile solution of OVA in drinking water (1 mg/ml, unless otherwise stated) for a period of 20 days (unless otherwise stated). To reduce the effects of contamination by oral flora, the sterile OVA–water solution was replaced three times each day. Individually caged mice consumed 4 ± 0.25 ml solution/day (or 4 ± 0.25 OVA) and average consumption of grouped mice (five per cage) was 4.2 ± 0.35 ml solution/day; these values were similar to those previously published. In several experiments, mice were fed 10 intermittent boluses of OVA in water (8 mg OVA/feeding every second day) over the period of 20 days. Mice were immunized against OVA by injecting 20 μ g OVA/mouse, either emulsified 1:1 in complete Freund's adjuvant (CFA) or absorbed by 1 mg $Al(OH)_3$. Injections (100 μ l/mouse) were administered i.p.

Assay for anti-OVA antibodies

Presence of IgG1, IgG2a, IgG2b and IgE serum antibodies specific for OVA was tested by ELISA. Sera tested for presence of anti-OVA antibodies were placed on ELISA plates (Nunc, Denmark), previously coated with OVA (or BSA as negative control), followed by biotinylated rat anti-mouse IgG1, IgG2a, IgG2b or IgE detecting mAb (PharMingen, San Diego, CA) and finally by peroxidase–Streptavidin (Kirkegaard & Perry, Gaithersburg, MD). Bound antibodies were detected by TMB (Kirkegaard & Perry) and specific binding was determined by subtracting background binding to BSA. Standard curves were determined for each isotype in each assay by using unlabeled purified standards (all from PharMingen). Antibody concentrations are averages $\pm$ SEM/ml serum from no less than five individual mice and are expressed as mg/ml for IgG1, IgG2a, IgG2b and ng/ml for IgE.

Cell cultures

Spleen (erythrocyte depleted) cell cultures were used for proliferation, cytokine secretion and cytokine gene expression

assays. Cultures contained cells from individual mice and each experiment was performed with groups containing no less than five mice. The proliferation of T lymphocytes in response to OVA and PPD was assayed as described. Proliferation was measured by MTT oxidation (19) and results are averaged averages of quadruplicate cultures from individual mice expressed in absorbance units (at 570–630 nm) $\pm$ SEM. For cytokine secretion, 1×10^7 cells/well (in 1 ml) were cultured in 24-well plates (Nunc) with or without OVA (1 mg/ml). Cytokine secretion was determined temporally in supernatants collected from these cultures and was ascertained to be optimal after 9 h culture for IL-4 detection, 20 h for IL-2 detection and 48 h for IFN- γ detection. Collected supernatants were frozen and stored at -70°C till assayed (see below). For the assay of cytokine gene expression, 5×10^7 spleen cells/ml were cultured with or without OVA (1 mg/ml) for 6 h. After incubation, cells were collected and total RNA was isolated for RT-PCR (see below). DMEM was used for all cultures, and was supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids (all supplied by Biological Industries, Beit Haemek, Israel), 5×10^{-5} M 2-mercaptoethanol and 0.5% syngeneic serum.

Cytokine assays

Levels of IL-4, IL-2 and IFN- γ in supernatants were determined by capture ELISA as described. Briefly, supernatants were added to microtiter plates, previously coated with rat anti-mouse IL-4, IL-2 or IFN- γ mAb (capture antibodies, PharMingen) and blocked with BSA-diluent/blocking solution (Kirkegaard & Perry). Biotinylated rat anti-mouse IL-4, IL-2 or IFN- γ mAb (detecting antibodies, PharMingen) were added, and followed by peroxidase-labeled streptavidin. Bound cytokine was detected by the addition of ABTS (Kirkegaard & Perry). Cytokine levels were calculated from a log-log plot of absorbance versus concentration of recombinant cytokines (PharMingen) and results are expressed in pg/ml (for IL-4 and IL-2) or ng/ml (for IFN- γ). Threshold sensitivities of ELISA assays were 5 pg/ml, 10 pg/ml and 2.5 ng/ml for IL-4, IL-2 and IFN- γ respectively. IL-2 and IL-4 levels were also determined by bioassay using the CTLL-2 (IL-2-dependent) and CT.4S (IL-4-dependent; kindly provided by Dr W. E. Paul, NIH, Bethesda MD) cell lines as described (6).

Analysis of cytokine mRNA levels

Total RNA was isolated from cultured spleen cells using a TRI reagent (Molecular Research Center, Cincinnati, OH), according to a protocol provided by the manufacturer. mRNA was then reverse transcribed into cDNA, and the expression levels of IL-4, IL-2, IFN- γ and β -actin messages were determined by a quantitative PCR using cytokine specific primers. β -actin, IL-2 and IFN- γ primer sequences were those from Stratagene (La Jolla, CA). IL-4 sequences were as follows: IL-4 sense: 5'-CAGCTAGTTGTCATCCTGCTC-3' (76–97) and IL-4 antisense: 5'-CAGGAAGTCCTTTCAGTGA-TGTGAA-3' (445–421). All primers used spanned genomic introns such that any contaminating genomic DNA was detected by a higher molecular weight band. Quantitative PCR conditions were first established for all sets of primers using either cDNA from concanavalin A-activated spleen cells

or plasmid DNA. These were subjected to eight 2-fold serial dilutions and amplified for 25 cycles at 95°C for 25 s, 60°C for 60 s and 72°C for 60 s in a 9600 Geneamp PCR System (Perkin-Elmer Cetus, Branford, NJ). [^{32} P]dCTP was added directly into the PCR reaction as described (21) and the products were resolved on 5.5% acrylamide gel. mRNA was quantitated using a β -scope (Intelligent, Mountain View, CA). Tested cDNA samples were amplified undiluted and with two additional two-fold dilutions with cytokine specific primers, and at 1/100, 1/200 and 1/400 serial dilutions with β -actin primer sequences. The ratio of cytokine mRNA expression relative to β -actin was obtained for each dilution and expressed as the mean ratio $\pm$ SE.

Statistical analysis

The statistical significance of differences between experimental groups was determined using unpaired two-tailed Student's *t*-test, with differences considered significant at $P < 0.05$.

Results

Continuous exposure of mice to OVA induces specific T lymphocyte unresponsiveness *in vitro*

To determine if tolerance could be induced in both T_H1 and T_H2 lymphocyte subsets we exposed mice to OVA in their drinking water. Our rationale was to achieve conditions in which the experimental antigen was available to mice on a constant basis, rather than a pulse period characterized by a single dose feeding regimen (4). Exposure was continued for 20 days and then mice were immunized by OVA-CFA. Control mice were primed by OVA-CFA or CFA alone. Spleen cell cultures were prepared 10 days after immunization and T lymphocyte proliferation in response to OVA (Fig. 1A) or PPD (Fig. 1B) was determined. T lymphocytes did not proliferate in response to OVA but exhibited a dose dependent response to PPD which was similar to that of the OVA-primed, non-tolerant control group (Fig. 1; $P < 0.05$ for all antigen doses, representative of six experiments). Identical observations were made with lymph node (popliteal and mesenteric) cell cultures, with other protein antigens (human serum albumin and hen egg lysozyme) and with $Al(OH)_3$ as adjuvant (data not shown). Thus, continuous oral exposure to external antigens in solution is not an immunogenic stimulus, but induces antigen-specific T lymphocyte unresponsiveness as determined by absence of T lymphocyte proliferation.

Absence of T_H1 and T_H2 cytokine secretion in cultures derived from mice continuously exposed to OVA in drinking water

Absence of proliferation is only partially indicative of tolerance, since activated non-dividing cells may produce cytokines (22,23). Thus, to further establish an *in vitro* state of tolerance in T lymphocytes derived from mice continuously exposed to OVA in their drinking water, *in vitro* cytokine secretion was determined. Mice, continuously fed or non-fed controls, were immunized by OVA-CFA or OVA- $Al(OH)_3$; two additional non-fed groups were primed by either CFA or $Al(OH)_3$ alone. Spleen cell cultures were prepared 10 days after immunization, incubated with or without OVA, and supernatants were

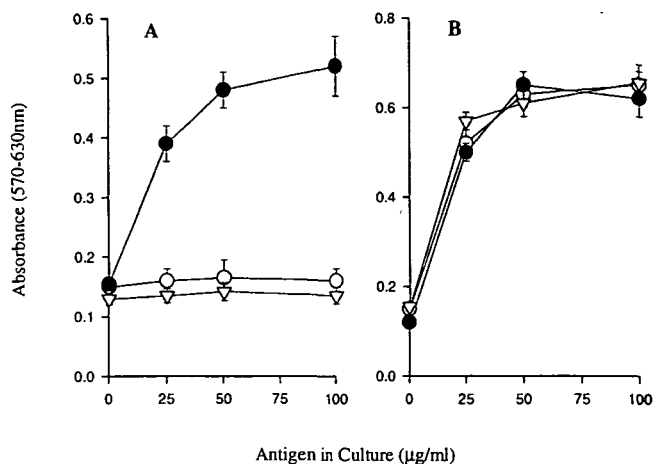


Fig. 1. Continuous oral exposure to OVA diminishes T lymphocyte proliferation response *in vitro*. Mice were continuously exposed to OVA in drinking water for 20 days and primed by OVA-CFA (open triangles). Control mice were primed by OVA-CFA (filled circles) or CFA alone (open circles). Erythrocyte depleted spleen cells from individual mice were prepared 10 days after immunization and stimulated by different concentrations of OVA (A) or PPD (B). Results are averaged averages $\pm$ SEM of quadruplicate cultures from individual mice ($n \geq 5$) and are expressed in absorbance units at 570–630 nm.

collected to determine secretion of IL-4, IL-2 and IFN- γ (Table 1, representative of five experiments). Mice continuously exposed to OVA did not secrete IL-4, IL-2 or IFN- γ in response to OVA (not at the designated time points and not at any other time point during a 48 h culture period), suggesting that both T_H2 (IL-4 secretors) and T_H1 (IL-2 and IFN- γ secretors) OVA-specific lymphocytes were tolerant. This conclusion is supported by the following observations. (i) The three cytokines were secreted in response to OVA in control cultures derived from OVA-CFA and OVA- $Al(OH)_3$ primed mice, but not in those derived from mice primed by CFA or $Al(OH)_3$ alone, thus indicating OVA specific activation of both T_H1 and T_H2 lymphocytes in two adjuvant systems ($P < 0.05$). (ii) Tolerance was OVA specific as evidenced by the capacity of cells from OVA-CFA exposed mice to secrete cytokines in response to PPD ($P < 0.05$). The possible presence of IL-4 and IL-2, undetected by ELISA, was further evaluated by bioassays using CT.4S and CTLL-2 cells respectively; IL-2 and IL-4 were not detected in cultures derived from mice continuously exposed to OVA, human serum albumin or hen egg lysozyme, and identical observations were obtained with lymph node cell cultures (data not shown). Hence, continuous oral exposure to antigen in solution induces specific tolerance of T_H2 and T_H1 lymphocytes as determined by *in vitro* proliferation and cytokine secretion.

Mice continuously exposed to OVA in drinking water do not express T_H1 and T_H2 cytokine genes

To further evaluate the level of tolerance induced by continuously exposing mice to OVA, we measured cytokine gene expression in response to OVA. Mice, continuously fed or

Table 1. Continuous exposure to OVA diminishes cytokine secretion *in vitro*

OVA fed ^a	Immunization	IL-4 (9 h, pg/ml) ^b		IL-2 (20 h, pg/ml) ^b		IFN- γ (48 h, ng/ml) ^b	
		OVA	PPD	OVA	PPD	OVA	PPD
—	CFA	<5	94 ± 7	<10	250 ± 13	<2.9	75 ± 4
—	OVA-CFA	41 ± 3	87 ± 5	133 ± 7	235 ± 17	37 ± 4	87 ± 9
Continuous	OVA-CFA	<5	98 ± 8	<10	262 ± 19	<2.8	75 ± 7
—	Al(OH) ₃	<5	ND	<10	ND	<2.6	ND
—	OVA-Al(OH) ₃	32 ± 4	ND	55 ± 2	ND	21 ± 2	ND
Continuous	OVA-Al(OH) ₃	<5	ND	<10	ND	<2.9	ND

^aMice were continuously exposed to OVA in drinking water for 20 days (1 mg/ml OVA in water), and then immunized i.p.

^bErythrocyte-depleted spleen cells (1×10^7 /ml) from individual mice were cultured 10 days after immunizations with or without OVA or PPD (1 mg/ml). Cytokine secretion was determined by ELISA. Cytokine levels were calculated from a log-log plot of absorbance versus concentration of recombinant cytokines. Results are averaged averages of quadruplicate cultures from individuals mice $\pm$ SEM ($n \geq 5$). Values in bold lettering indicate significant cytokine secretion above threshold levels (see Methods) ($P < 0.05$).

Table 2. Effect of continuous exposure to OVA on cytokine mRNA expression

OVA fed ^a	IL-4 mRNA ^b			IL-2 mRNA ^b			IFN- γ mRNA ^b		
	Medium	OVA	Ratio	Medium	OVA	Ratio	Medium	OVA	Ratio
—	0.7 ± 0.1	2.1 ± 0.1	3.0	2.9 ± 1.0	5.8 ± 1.0	2.0	0.9 ± 0.2	5.9 ± 0.5	6.6
Continuous	1.0 ± 0.0	1.0 ± 0.0	1.0	0.3 ± 0.0	0.2 ± 0.0	0.7	3.2 ± 0.6	2.4 ± 0.2	0.7

^aMice were continuously exposed to OVA in drinking water for 20 days (1 mg/ml OVA in water), and then immunized i.p. by OVA-CFA. Control non-fed mice were primed by OVA-CFA.

^bErythrocyte-depleted spleen cells (5×10^7 /ml) from individual mice were cultured 10 days after immunizations with or without OVA (1 mg/ml) for 6 (IL-2 and IL-4) or 12 (IFN- γ) h. Cells were collected and total RNA was isolated. mRNA was reverse transcribed into cDNA and the expression levels of IL-4, IL-2, IFN- γ and β -actin messages were determined by quantitative PCR using cytokine specific primers, as described in Methods. Visualized bands were quantitated using a β -scope and cytokine mRNA expression values are relative to those of β -actin mRNA used as an internal control $\pm$ SEM ($n \geq 5$). In addition, mRNA expression is presented as the ratio between mRNA expressed in response to OVA and that expressed in response to medium alone. Values in bold lettering indicate significant mRNA expression above levels expressed in response to medium alone ($P < 0.05$).

non-fed controls, were immunized by OVA-CFA; an additional non-fed group was primed by CFA alone. Spleen cell cultures were prepared 10 days after immunization, incubated with or without OVA, and cells were collected to determine IL-4, IL-2 and IFN- γ mRNA expression by quantitative RT-PCR (Table 2, representative of three experiments). The data show that IL-4, IL-2 and IFN- γ mRNA were not expressed in response to OVA by cells derived from mice continuously fed with OVA. In contrast, IL-4, IL-2 and IFN- γ mRNAs were specifically elevated in control cultures ($P < 0.05$). Hence, the state of tolerance in T_H2 and T_H1 lymphocyte subsets was confirmed by absence of cytokine gene expression following a specific antigen stimulus.

Mice continuously exposed to OVA do not secrete T_H2 - and T_H1 -dependent antibodies in vivo

The previous observations indicated that continuous feeding of OVA induced a state of tolerance in T_H2 and T_H1 lymphocyte subsets, as determined by *in vitro* analysis. To confirm that a similar state was present *in vivo* we studied profiles of antibodies produced in response to OVA stimulation in tolerant mice. Antibody isotype profiles serve as relative indicators of murine T_H subset activity *in vivo*: IgG1 and IgE production is regulated by IL-4 and represent a T_H2 -mediated response,

whereas IgG2a and IgG2b production is regulated by IFN- γ and represent a T_H1 -mediated response (24,25). Mice were continuously fed with OVA for 20 days and then primed by OVA-CFA. Control mice were primed by OVA-CFA or CFA alone. Serum samples were collected temporally after immunization (15–60 days) and analyzed for OVA-specific IgG1, IgE, IgG2a and IgG2b secretion (Fig. 2, representative of five experiments). Mice fed continuously with OVA produced very low, if any, detectable antibody response to OVA; thus anti-OVA IgG1 and IgE, as well as IgG2a and IgG2b, levels were completely diminished. In contrast, control mice primed by OVA-CFA developed significant responses that consisted of all antibody subclasses, indicating that CFA was capable of supporting both T_H2 - and T_H1 -mediated responses *in vivo* [Al(OH)₃ promoted selective T_H2 -mediated antibody production and could not be used to confirm T_H1 tolerance]. Antibody production was intact in tolerant mice as evidenced by presence of all four subclasses in response to PPD; levels were similar to those of control OVA-CFA immunized mice, and similar results were obtained 20, 30, 45 and 60 days after immunization (data not shown). Taken together the results suggest that both T_H2 and T_H1 responses are susceptible to tolerance induction by continuous oral exposure to antigen.

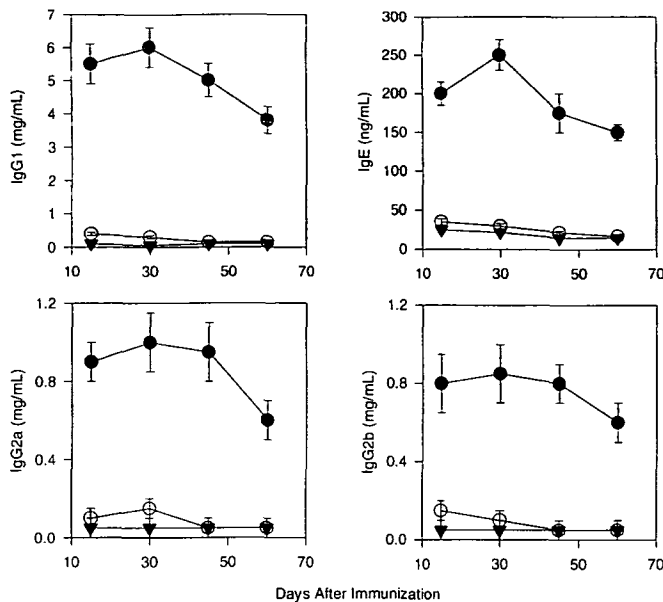


Fig. 2. Continuous oral exposure to OVA diminishes secretion of anti-OVA antibodies. Mice were continuously fed with OVA for 20 days (1 mg/ml) and primed by OVA-CFA (open circles). Control mice were primed by OVA-CFA (filled circles) or by CFA alone (filled triangles). Serum samples were collected temporally after immunization and individually assayed for IgG1, IgE, IgG2a and IgG2b by ELISA as described in Methods. Antibody levels are averages $\pm$ SEM/ml serum from individual mice (for all isotypes $n \geq 5$).

Characterization of the tolerogenic signal required for tolerization of T_H2 lymphocytes

Since selective tolerization of T_H1 lymphocytes was accomplished by means of a different feeding regimen (by a single or intermittent feeding regimen; 4), it was of interest to determine comparative requirements for inducing T_H2 lymphocyte tolerance. Three parameters were studied: (i) the rigidity of the feeding regimen, i.e. the necessity for continuous exposure compared with an intermittent feeding regimen, (ii) the minimal antigen dosage required for effective tolerization of T_H2 lymphocytes and (iii) the minimal period required for effective tolerization of T_H2 lymphocytes.

The importance of the feeding regimen for tolerization of

T_H2 lymphocytes was studied by comparing the degree of tolerance generated by continuous exposure to OVA in drinking water for 20 days to that generated by an intermittent feeding regimen in which mice received the same average dose of OVA (80 mg). Mice were then immunized by OVA-CFA and responses of both groups to OVA were compared 15 days after immunization (Table 3, representative of four experiments). T_H1 and T_H2 tolerance resulted from the continuous feeding regimen (both *in vitro* cytokine production and *in vivo* antibody secretion), whereas the intermittent feeding regimen caused selective T_H1 tolerance while T_H2 responses (cytokine and antibody) were unchanged, apart from a minor reduction in IL-4 secretion ($P > 0.05$). Hence, the condition of exposure to antigen was important for generation of T_H2 tolerance.

The minimal antigen dosage required for effective tolerization of T_H2 lymphocytes was determined by continuously exposing mice to different concentrations of OVA in drinking water (0–1 mg/ml) for 20 days. OVA-specific cytokine production *in vitro* and anti-OVA IgG1 and IgG2a production were assayed 15 days after immunizing mice with OVA-CFA (Fig. 3, representative of four experiments). Results show that tolerization of cytokine secretion *in vitro* and antibody responses *in vivo* required continuous exposure to 1 mg/ml OVA ($P < 0.05$). A dosage of 0.01 mg/ml had no effect on T lymphocyte functions ($P > 0.05$), and 0.1 mg/ml had only marginal effects on the measured functions ($P = 0.05$). Hence, oral tolerization of T_H1 - and T_H2 -controlled responses (cytokine and antibody production) required relatively high dosages of antigen.

The minimal period required for effective tolerization of T_H2 lymphocytes was determined by evaluating the temporal degree of tolerance generated *in vivo* by continuously exposing mice to OVA (1 mg/ml). Mice were immunized temporally after feeding was initiated, and serum samples were collected, in each case, 15 days after immunization, and then assayed by ELISA for IgG1 and IgG2a (Fig. 4, representative of five experiments). As shown, T_H1 -mediated responses were most sensitive to tolerance induction: a 1 day period of continuous exposure to OVA was sufficient to dramatically reduce specific IgG2a production, and which essentially ceased after 5 days of exposure. In comparison, T_H2 -controlled responses were highly resistant to tolerance induction and became so only

Table 3. Effect of feeding regimen on OVA specific tolerization of T_H1 and T_H2 lymphocytes

OVA fed ^a	Immunization	Cytokine secretion ^b			Antibody secretion ^c	
		IL-4	IL-2	IFN- γ	IgE	IgG2a
—	CFA	<5	<10	3.2 $\pm$ 0.3	20 $\pm$ 16	0.05 $\pm$ 0.02
—	OVA-CFA	35.4 $\pm$ 1.8	99.8 $\pm$ 7.4	43.5 $\pm$ 4.5	185 $\pm$ 19	0.81 $\pm$ 0.2
Intermittent	OVA-CFA	24.8 $\pm$ 0.9	<10	2.8 $\pm$ 0.3	164 $\pm$ 13	0.09 $\pm$ 0.05
Continuous	OVA-CFA	<5	<10	3.1 $\pm$ 0.2	28 $\pm$ 9	0.12 $\pm$ 0.04

^aMice were either continuously exposed to OVA (1 mg/ml) or received daily boluses containing 4 mg/ml for 20 days.

^bDetails of cytokine measurement are as in Table 1; concentrations are pg/ml for IL-2 and IL-4, and ng/ml for IFN- γ . OVA specific responses are averaged averages of quadruplicate cultures from individual mice $\pm$ SEM ($n \geq 5$) and values in bold lettering indicate significant cytokine secretion above background threshold levels (see Table 1) ($P < 0.05$).

^cSerum IgG2a (mg/ml) and IgE (ng/ml) levels were measured 15 days after immunization by isotype specific ELISA, and are averages of individual mice $\pm$ SEM/ml serum ($n \geq 5$). Values in bold indicate significant antibody secretion as compared to levels in naive serum ($P < 0.05$).

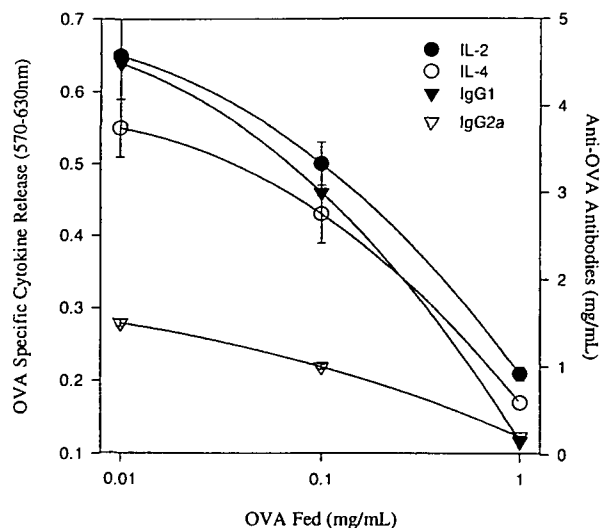


Fig. 3. Antigen dosage requirements for tolerization of T_H2 lymphocytes. Mice were continuously fed with different dosages of OVA for 20 days, and primed by OVA-CFA. Spleens and serum samples were collected 15 days after immunization to determine cytokine (circles) and antibody (triangles) secretion respectively. Erythrocyte depleted spleen cells were cultured as detailed in Table 1 and supernatants were added to cultures of CTLL-2 or CT.4S cells for detection of IL-2 (solid circles) and IL-4 (open circles) respectively. Results are averaged averages $\pm$ SEM of quadruplicate cultures from individual mice ($n \geq 5$) and are expressed in absorbance units at 570–630 nm. IgG1 (solid triangles) and IgG2a (open triangles) were determined by ELISA. Antibody levels are averages $\pm$ SEM/ml serum from individual mice ($n \geq 5$).

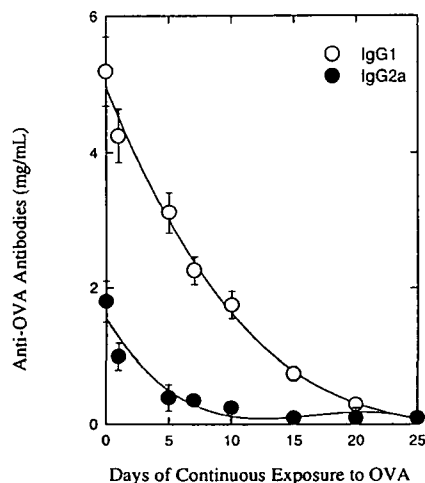


Fig. 4. Generation of T_H2 tolerance requires extended exposure period to antigen. Mice were continuously exposed to OVA for different periods (1–20 days). After each exposure period mice were immunized by OVA-CFA. Individual sera were prepared 15 days later and assayed by ELISA for anti-OVA IgG1 (open circles) and IgG2a (solid circles). Results are average antibody levels $\pm$ SEM/ml serum from individual mice ($n \geq 5$).

after 20 days of continuous exposure to antigen: anti-OVA IgG1 production gradually diminished with time of exposure to OVA and attained background values only after 15–20

days of exposure. To summarize, oral tolerization of T_H1 lymphocytes required relatively large dosages of antigen, and was achieved after a brief and intermittent exposure period to antigen. On the other hand, oral tolerization of T_H2 lymphocytes had the same requirements for antigen, but was achieved only after extended and continuous exposure periods.

Discussion

The administration of antigen via the oral route can lead to either tolerance or immunity (1–3). In cases where tolerization is the prime objective, feeding regimens and antigen structure appear to be of major importance to insure tolerance and to avoid responsiveness (1,2). The objective of the present study was to determine conditions for tolerizing T_H2 lymphocytes following oral administration of antigen. We show here that tolerization of T_H2 lymphocytes, as well as that of T_H1 lymphocytes, was successfully achieved by continuous oral exposure of conventional mice to OVA (and other proteins; data not shown) in water. Tolerance of lymphocyte responses was demonstrated at three levels: *in vivo* antibody secretion, *in vitro* proliferation and cytokine secretion, and *in vitro* cytokine gene expression. These results confirm and extend previous studies which demonstrated tolerization of IgE dependent cutaneous anaphylaxis after antigen feeding (13,14) and partial tolerization of T_H2 responses after i.v. administration of deaggregated human γ -globulin (15).

Oral tolerance was induced following continuous exposure to OVA in drinking water for 20 days, and was evaluated after immunization of mice by OVA in adjuvant. Tolerance of T_H1 and T_H2 lymphocytes was assessed by absence of proliferation and of IL-4, IL-2 and IFN- γ secretion or gene expression *in vitro*. These observations excluded a state of 'partial activation' in which cells are unable to divide but continued to produce cytokines (26,27). Absence of IL-4 secretion and expression *in vitro* was demonstrated by ELISA, CT.4S proliferation and RT-PCR; together these all indicate tolerization of T_H2 lymphocytes. Similar assays demonstrated T_H1 tolerance too. Support *in vivo* for these *in vitro* observations was demonstrated by absence of OVA-specific IgG1 and IgE (isotypes controlled by IL-4), as well as IgG2a and IgG2b (isotypes controlled by IFN- γ). Since IgE is considered to be exclusively T_H2 -dependent (24,25), its absence confirmed that T_H2 -mediated responses were tolerized. Thus, tolerant T_H2 and T_H1 lymphocytes did not secrete IL-4 and IFN- γ , and thus, did not provide cytokine signals necessary for isotype switching in B lymphocytes (28). The possible involvement of B lymphocyte tolerance is discussed below.

The induction of T_H2 tolerance required an extended period of continuous exposure to high dosages of antigen. These conditions were significantly different from those rendering T_H1 lymphocytes tolerant: T_H1 tolerance was achieved by a single or intermittent feeding regimen, did not require extended exposure periods to antigen, but was dependent upon continuous tolerogenic exposures to antigen (4,6,16). Thus, in spite of the fact that similar dosages of antigen were required for induction of tolerance in these T lymphocyte subsets, the distinctive time and exposure patterns indicate different sensitivities to tolerance induction (12). An interesting point raised by these studies relates to the feeding strategy

that induced tolerance in OVA-specific T_H2 lymphocytes. Administration of OVA by an intermittent feeding regimen for 20 days did not lead to T_H2 tolerance, while the identical antigen dosage administered *ad libitum* in drinking water did. A possible explanation for this observation is based on the role of intestinally absorbed antigen (or its fragments) in the induction of peripheral tolerance (6,29–31) and the physiology of renal clearance of proteins (or fragments) from the circulation (15,32). An intermittent exposure (every 2 days) to OVA was not sufficient to induce T_H2 lymphocyte tolerance, which are more resistant to tolerance induction than T_H1 lymphocytes (12), because OVA (or fragments) was rapidly being cleared from the circulation by glomerular filtration (32). On the other hand, an *ad libitum* feeding regimen provided OVA (or fragments) continuously, and when fed at an adequate dosage, provided the adequate signal for T_H2 tolerance induction. Thus, intra-circulatory OVA was replenished by drinking and possibly persisted for longer periods in body fluids to allow tolerance of all T lymphocyte populations (15).

Two additional issues are raised by this study: (i) does continuous exposure to antigen induce B lymphocyte tolerance and (ii) what is the mechanism for T_H2 tolerance? Since OVA-specific antibodies were absent in serum of mice continuously exposed to OVA, one could propose that tolerance resulted from both T and B lymphocyte inactivation. Most studies of oral tolerance have indicated T lymphocyte tolerance and intact B lymphocyte responsiveness (33–36); however, these studies were not programmed to achieve T_H2 tolerance. On the other hand, studies with transgenic mice have distinctly shown induction of peripheral B lymphocyte tolerance (37–40). Thus, although we clearly demonstrate tolerance at the T lymphocyte level, we cannot exclude at this time the additional possibility for B lymphocyte tolerance. This issue is currently being studied by using hapten-carrier tolerogens in an adoptive transfer model of tolerance we have developed (6).

The mechanism by which T_H2 lymphocytes, or their precursors, become tolerant *in vivo* has yet to be determined. Possibilities include clonal deletion, anergy and cytokine-mediated suppression (1–2,4,6,19,41–45). We have shown that a single feeding of antigen selectively induced anergy in T_H1 lymphocytes, and that *in vitro* treatment with IL-2 released the anergic cells for antigen-specific responses (6,19). This indicated that anergy could be achieved by exposing the immune system to antigen absorbed from the gut in the absence of co-stimulation (46). Whether or not this is the case for T_H2 lymphocytes too remains to be investigated, but it is tempting to assume a general mechanism for induction of peripheral tolerance, particularly if both lymphocyte populations require different co-stimulatory signals (23,47).

Abbreviations

CFA	complete Freund's adjuvant
OVA	ovalbumin
PPD	purified protein derivative of <i>M. tuberculosis</i>

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