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Immunology

Induction of anergy or active suppression following oral tolerance is determined by antigen dosage

(peripheral tolerance/transforming growth factor β/interleukin 4)

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ABSTRACT **Oral tolerance was generated to hen egg white lysozyme in the mouse or to guinea pig myelin basic protein in the rat by a low-dose (1 mg) or a high-dose (5–20 mg) feeding regimen. High doses of antigen induced tolerance characterized by anergy with little or no active suppression and increased secretion of interleukin 4 (IL-4). Anergy was shown by an increase in frequency of IL-2-secreting cells following culture in recombinant IL-2. Low doses of antigen induced tolerance characterized by antigen-driven active suppression with increased secretion of transforming growth factor β (TGF-β) and IL-4 and minimal anergy. Without further immunization, spleen cells from animals orally tolerized by both regimens secreted increased levels of IL-4 and TGF-β in an antigen-specific manner. Animals fed high doses secreted more IL-4 and less TGF-β, whereas those fed low doses secreted more TGF-β and less IL-4. These results demonstrate that the two feeding regimens induced cell populations that differed in their cytokine secretion profile and their capacity to actively suppress *in vitro* and to induce anergy. Our results provide a basis for distinguishing different forms of antigen-driven peripheral tolerance and have important implications for orally induced antigen-specific modulation of human autoimmune diseases.**

The oral administration of soluble antigen is a long-recognized method to induce antigen-specific peripheral tolerance (1). Oral administration of autoantigens has also been shown to suppress experimental autoimmune diseases (reviewed in ref. 2) and is now being applied to treat human autoimmune diseases (3, 4), adding further relevance to understanding the mechanisms by which orally administered antigen induces peripheral tolerance. There is little evidence for clonal deletion following oral tolerance, as oral tolerance induced by a single gastric intubation or multiple feedings subsides after 45–60 days (5–7). Several studies have shown active suppression (1) as a mechanism for induction of oral tolerance. Our studies with myelin basic protein (MBP) in the Lewis rat model of experimental autoimmune encephalomyelitis (EAE) demonstrate that T cells induced after oral administration of antigen mediate active suppression. These T cells require specific antigen for activation and then suppress cellular immune responses *in vitro* and *in vivo* in an antigen-nonspecific fashion by the release of cytokines such as transforming growth factor β (TGF-β) (8–10). Other studies have shown that anergy may also be a mechanism for oral tolerance (11, 12). We postulated that these two forms of oral tolerance were related to the amount of antigen fed. Studies showing active suppression fed intermittent low antigen doses (8–10), whereas studies showing anergy fed high doses, either as a single bolus (12) or intermittently with soybean trypsin inhibitor (11). We report here that feeding dosage has profound effects on the mechanism of oral tolerance as defined by anergy, active suppression, and cytokine secretion profiles.

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MATERIALS AND METHODS

Animals. Female (SJL × PLJ)F_1 mice, 6–8 weeks of age (The Jackson Laboratory), and female Lewis rats, 8 weeks of age (Charles River Breeding Laboratories), were used.

Antigens, Feeding, and Immunization Regimens. Oral tolerance to hen egg lysozyme (HEL; Sigma) was induced in mice either by a single feeding of 20 mg or by five intermittent feedings of 1 mg given on alternate days over 10 days. HEL was administered in 0.25 ml of phosphate-buffered saline (PBS, pH 7.2) with a 24-gauge feeding needle (Thomas Scientific, Swedesboro, NJ). Oral tolerance to guinea pig MBP was induced in Lewis rats either by five every other day feedings of 1 mg over 10 days as described (9) or by four feedings of 5 mg, together with soybean trypsin inhibitor (Sigma), over a period of 8 days as described by Whitacre *et al.* (11). Mice were primed by hind footpad inoculation 7 days after feeding 20 mg and 2 days after the last 1 mg feeding with 20 μg of HEL or ovalbumin (OVA) absorbed by aluminum hydroxide (alum) (10 μg per footpad in 50 μl). Rats were immunized 2 days after the last feeding with 25 μg of MBP absorbed by alum (12.5 μg per footpad in 100 μl).

Cell Culture. Eight days after immunization, popliteal lymph node suspensions (LNCs) were prepared in Hanks' buffered salt solution containing 100 units of penicillin per ml and 100 μg of streptomycin per ml (BioWhittaker, Walkersville, MD). An erythrocyte-depleted spleen cell suspension was prepared from spleens of nonimmunized mice. T-cell proliferation and reversal of T-lymphocyte anergy were performed as described (12). In several experiments proliferation was measured in the presence of goat anti-murine interleukin 4 (IL-4) or chicken anti human TGF-β neutralizing antibodies (6–48 μg/ml) (R & D); an equivalent amount of normal goat IgG (Sigma) or chicken IgG (R & D Systems) was used in control cultures. Proliferation was measured after 4 days of culture by [^{3}H]thymidine incorporation [4-hr incorporation, 1 μCi per well; 2.00 Ci/mmol (1 Ci = 37 GBq), NEN]. Results, expressed in cpm, are averages of quadruplicate cultures of popliteal lymph node cells pooled from at least three mice or four rats, respectively. For cytokine production, primed LNCs or naive spleen cells were cultured in serum-free medium (X-vivo 20, Whittaker). Significance of differences between groups was determined by ANOVA or Student's *t* test. Limiting dilution analysis of cells secreting

Abbreviations: MBP, myelin basic protein; EAE, experimental autoimmune encephalomyelitis; TGF-β, transforming growth factor β; HEL, hen egg lysozyme; IL-4, interleukin 4; NS, not significant; r, recombinant; LNC, lymph node cell; OVA, ovalbumin; IFN-γ, interferon γ.

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IL-2 in response to HEL was performed according to standard procedures (11, 13).

Cytokines and ELISA. IL-2 and interferon γ (IFN-γ) were measured with murine cytokine ELISA kits (Genzyme) with listed threshold sensitivities at 15 and 125 pg/ml, respectively. For IL-4 and TGF-β, Maxisorp immunoplates (Nunc) were coated with either chicken anti-human TGF-β_1 or goat anti-murine IL-4 antibodies (5 μg/ml; both from R & D). After washing and blocking, samples and standards [murine recombinant IL-4 (rIL-4) and human rTGF-β_1; R & D] were added. Samples evaluated for TGF-β were activated by 10 mM HCl and then neutralized by 10 mM NaOH. Bound cytokine was detected by monoclonal mouse anti-human TGF-$\beta_{1,2,3}$ or monoclonal rat anti-murine IL-4 (1 μg/ml; both from Genzyme) followed by peroxidase-labeled goat anti-mouse IgG (H&L) or goat anti-rat IgG (H&L) (both from Kierkegaard & Perry Laboratories) and a one-component tetramethylbenzidine (TMB) reagent. Color development was stopped by TMB stop solution (Kierkegaard & Perry Laboratories) and absorbency was determined at 450 nm. Standard curves, plotting absorbance against log concentration, were linear between 500 and 7500 pg/ml and 500 and 5000 pg/ml for TGF-β and IL-4, respectively. Assay sensitivity was 250 pg/ml and 500 pg/ml for TGF-β and IL-4, respectively. IL-2, IL-4, and IFN-γ secretion were measured in 18-hr supernatants and TGF-β was measured in 72-hr supernatants.

RESULTS

Low-Dose Feeding Regimen Induces Active Suppression. HEL fed at either a low dose or a high dose diminished T-lymphocyte proliferation to HEL immunization (Fig. 1*A*). Tolerance was HEL specific, as HEL-tolerized mice responded normally when immunized with OVA (132,320 ± 3224 cpm and 118,252 ± 8261 compared to 125,655 ± 5639 cpm in nontolerant controls). Primed T lymphocytes from low- and high-dose tolerized mice were then tested for their capacity to actively suppress the *in vitro* proliferative responses of nontolerant, HEL-primed T lymphocytes in cell mixing experiments (Fig. 1*B*). When HEL-primed cells were mixed with those primed by alum alone, a dose-dependent reduction in response to HEL was observed, reflecting the proportional dilution of the responding cell population by that of the nonresponding alum-primed cell population (Fig. 1*B*, squares). When the same lymphocytes were mixed with cells derived from high-dose-tolerized mice, reduction of proliferation was not significantly different from that observed with alum-primed cells [Fig. 1*B*, triangles; not significant (NS)]. However, if nontolerant HEL-primed T lymphocytes were mixed with cells derived from low-dose-tolerized mice, significant suppression was observed at all cell ratios (Fig. 1*B*, circles; $P < 0.025$ for all points). Similar suppression was observed at multiple doses of HEL in culture (25–100 μg/ml). The induction of suppression was HEL specific and HEL driven, since LNCs from HEL low-dose-tolerized mice did not inhibit responses of OVA-primed T lymphocytes to OVA in the absence of HEL (Fig. 1*C*, squares vs. circles). However, if HEL was added to the culture, suppression was observed (Fig. 1*C*, triangles; $P < 0.02$ for all points).

High-Dose Feeding Regimen Induces Anergy. Preculture in rIL-2 led to complete restoration of the HEL-specific response in T lymphocytes derived from high-dose-fed mice (Fig. 2*B*; NS) but had little effect on the diminished response to HEL of T lymphocytes derived from low-dose-fed mice (Fig. 2*B*; high dose vs. saline, NS; low dose vs. saline, $P < 0.01$). Furthermore, LNCs and cells recovered from rIL-2 cultures were subjected to limiting dilution analysis (Fig. 2 *C* and *D*). The frequency of HEL-specific IL-2-secreting LNCs was significantly lower in cultures of mice fed high and low doses of HEL (Fig. 2*C*; f[saline] = 1/42,373 vs. f[low dose] = 1/1,250,000 and f[high dose] = 1/2,500,000, $P < 0.001$; f[low dose] vs. f[high dose], NS). Preculture of LNC in rIL-2 caused a significant increase in the frequency of HEL-specific IL-2-secreting cells in cultures derived from high-dose-fed mice (Fig. 2*D*; from $f = 1/2,500,000$ to $f = 1/58,823$, $P < 0.05$). A moderate increase in frequency of IL-2-secreting cells was also observed in cultures derived from low-dose-fed mice (from $f = 1/1,250,000$ to $f = 1/250,000$, NS), but this increase was significantly lower than that observed in cultures of high-dose-fed mice ($P < 0.05$). The <2-fold difference between the frequencies of IL-2-secreting cells in cultures from saline- and high-dose-fed mice was not statistically significant (saline, $f = 1/31,250$).

Active Suppression Is Mediated by TGF-β. Proliferation assays were performed in the presence of anti-IL-4 and anti-TGF-β neutralizing antibodies (Fig. 3 *A* and *B*, respectively). Anti-IL-4 had no effect on diminished HEL-specific responses in LNCs derived from mice fed low or high doses (compare to Fig. 2*A*). Anti-TGF-β antibodies, however, reversed the HEL-specific response in T lymphocytes from low-dose-fed mice (NS when compared to saline-fed con-

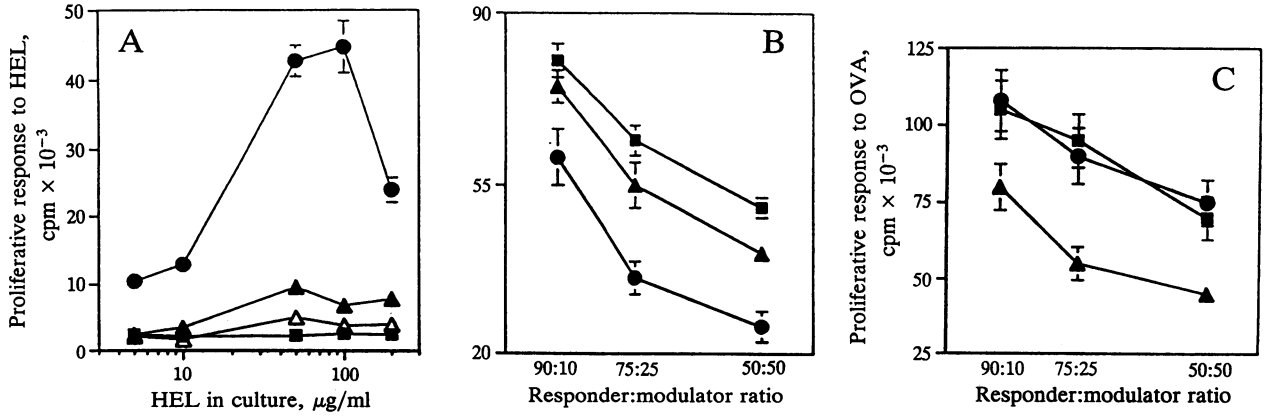

FIG. 1. Low-dose feeding regimen induces active suppression. (*A*) Mice were fed 20 mg of HEL (high dose, ▲) or five feedings of 1 mg (low dose, △). Controls were fed saline (●) or immunized with alum alone (■). Proliferative responses of LNCs to HEL were measured. (*B*) HEL-primed T lymphocytes, cultured in the presence of 50 μg of HEL per ml, were mixed with alum-primed LNCs (■) or with HEL-primed LNCs derived from high- (▲) or low-dose-fed (●) mice. (*C*) OVA-primed T lymphocytes, cultured in the presence of 50 μg of OVA per ml, were mixed with alum-primed LNCs (■) or with HEL-primed LNCs derived from low-dose-fed mice in the absence (●) or presence (▲) of 50 μg of HEL per ml. Results are averages of quadruplicate cultures ± SEM of cells pooled from at least three mice. Proliferation in response to medium alone was 2350–4600 cpm for all panels. Data are representative of 15 (*A*), 4 (*B*), and 2 (*C*) experiments.

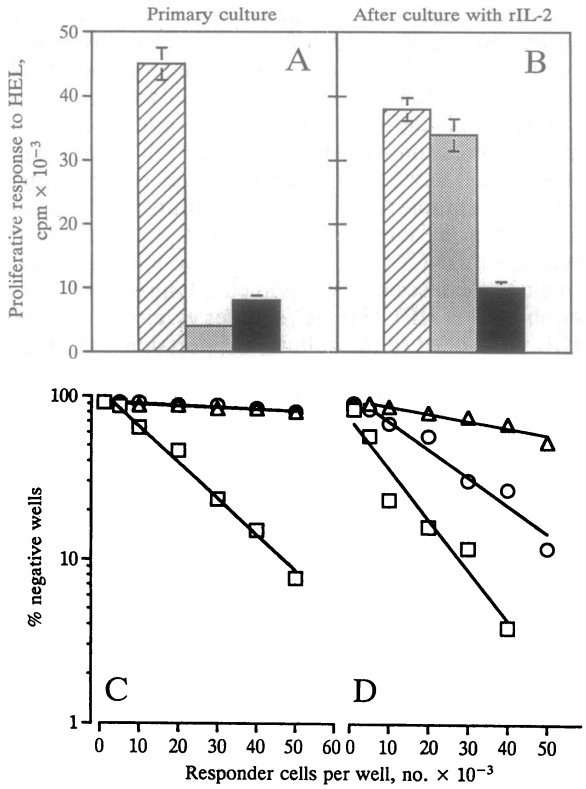

FIG. 2. High-dose feeding regimen induces anergy. Proliferative responses to HEL or limiting dilution analysis of HEL-specific IL-2-secreting cells were measured either directly (*A* and *C*) or after a 5-day culture in the presence of rIL-2 (50 units/ml, *B* and *D*). Proliferative responses shown are of nontolerant cells primed by HEL/alum (hatched bars and □) or of HEL-primed tolerant cells fed high (dotted bars or ○) or low (solid bars or △) doses. Results in *A* and *B*, expressed in cpm, are averages of quadruplicate cultures ± SEM (shown when larger than bar line thickness) of cells pooled from at least three mice. Results in *C* and *D* reflect IL-2 secretion in limiting dilution assays as determined by ELISA. Data are representatives of five (*A* and *B*) or two (*C* and *D*) experiments.

trols). There was only a minimal effect of anti-TGF-β antibodies on the response to HEL by T lymphocytes from high-dose-fed mice. The effect of anti-TGF-β was specific, since an equivalent dosage of normal chicken IgG had no effect on the diminished response of tolerized T lymphocytes to HEL (cpm of nontolerant controls = 49,831 ± 4533; cpm of high- and low-dose-fed mice = 5255 ± 877 and 7288 ± 693, respectively). Similar results were observed with several concentrations of neutralizing antibody (6–48 μg/ml; not shown). To confirm the role of TGF-β in suppression mediated by LNCs derived from low-dose-tolerized mice, cell mixing experiments were performed in the presence of anti-TGF-β neutralizing antibodies. As shown in Fig. 3*C*, anti-TGF-β totally abrogated the suppression by LNCs derived from low-dose-fed mice (NS when compared to saline-fed controls). Similar results were observed at several responder:modulator ratios (90:10, 75:25) and with several concentrations of neutralizing antibody (6–25 μg/ml).

High- and Low-Dose Feeding Regimens Increase IL-4 and TGF-β Secretion. IL-2 and IFN-γ secretion by tolerized LNCs was minimal in response to HEL (less than 15 and 125 pg/ml for IL-2 and IFN-γ, respectively, as compared to 250 and 1220 pg/ml in respective supernatants derived from primed nontolerant cell cultures). IL-4 secretion was observed in supernatants of tolerant cells cultured in the absence of HEL (Table 1, medium: $P < 0.025$ and $P < 0.01$ for 5 × 1 and 1 × 20 mg, respectively, vs. the saline group). When HEL was added to these cultures, secretion of IL-4 was reduced but was nonetheless higher than that produced by nontolerant controls (Table 1; $P < 0.05$ for both tolerant groups vs. saline control). LNCs derived from mice tolerized by the high dose produced more IL-4 than LNCs derived from mice tolerized by the low dose ($P < 0.05$), and both were markedly higher than that produced by nontolerant LNCs ($P < 0.05$ in presence or absence of HEL). TGF-β secretion was HEL dependent and occurred predominantly in cultures containing cells from the low-dose-fed group (Table 1; $P < 0.01$). Secretion of TGF-β in cultures containing cells from the high-dose-fed group was markedly lower than that secreted by low-dose-tolerized cells (1000 pg/ml compared to 3600 pg/ml; $P < 0.025$) but was significantly higher than that secreted by nontolerant cells (285 pg/ml; $P < 0.05$). IL-4 and TGF-β were secreted by tolerized unprimed spleen cells, but not by naive cells, in response to HEL (Table 1; $P < 0.025$ for all). Cells from high-dose-fed animals secreted more IL-4 than cells from low-dose-fed animals (2950 pg/ml compared to 1920 pg/ml; $P < 0.05$), and cells from low-dose-fed animals secreted higher levels of TGF-β than cells from high-dose-fed animals (3850 pg/ml compared to 2450 pg/ml; $P < 0.025$). No secretion of IL-2 or IFN-γ was observed (not shown).

High- and Low-Dose Feeding Regimens Induce Different Forms of Tolerance to MBP. MBP was fed to Lewis rats in low or high doses together with soybean trypsin inhibitor as described in *Materials and Methods*. Both feeding regimens suppressed proliferative responses to MBP in primary culture (Fig. 4). However, cells from low-dose-tolerized rats responded well to MBP in the presence of anti-TGF-β antibodies, but their diminished responses could not be reversed by preculture in rIL-2. In contrast, cells from high-dose-tolerized rats responded well to MBP after preculture in rIL-2 but did not respond to MBP in the presence of anti-TGF-β antibodies.

DISCUSSION

We have found that peripheral tolerance, induced by feeding HEL (an external antigen) or MBP (an autoantigen), is related to the nonmutually exclusive mechanisms of anergy and cytokine-mediated active suppression and that these mechanisms are dictated by antigen dosage. Furthermore, our findings confirm a proposed association between antigen, cytokine secretion, and level of peripheral tolerance (14, 15). Our observations with MBP reconcile the differences between previous studies of the mechanisms by which oral MBP suppresses EAE between our laboratory, in which cytokine-mediated suppression was demonstrated (9), and those of Whitacre *et al.* (11), in which anergy was demonstrated. Furthermore, since completion of our studies, a similar dose-related effect has also been reported for oral tolerance to S-antigen in the uveitis model, although cytokine patterns and IL-2 release were not measured (16).

In 1964, Mitchison (17) defined two zones of immunologic paralysis related to antigen dosage, and early studies of oral tolerances also suggested a relationship between dosage and the mechanism of tolerance. Mowat *et al.* (18) reported that high doses of OVA induced tolerance that was not abrogated by cyclophosphamide and that such tolerance affected antibody responses. Low doses of OVA induced a state of tolerance that could be reversed by cyclophosphamide and that primarily affected cell-mediated responses. Cyclophosphamide is believed to abrogate active suppression. It appears these studies were delineating components of active suppression vs. anergy depending on dose. In addition, Hanson and Miller (19) reported two components of oral tolerance following oral administration of OVA. They found tolerance was observed both in cyclophosphamide-treated and -untreated animals but that they were unable to transfer tolerance from cyclophosphamide treated animals.

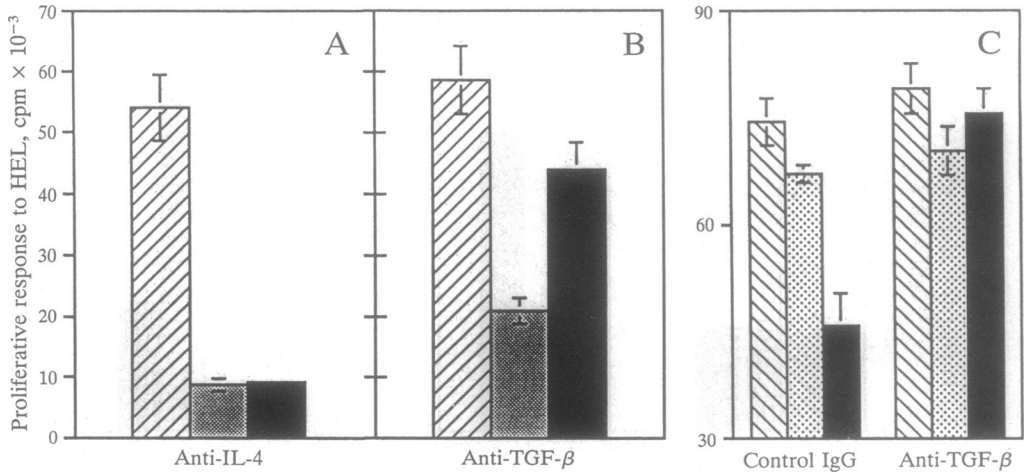

FIG. 3. Active suppression is mediated by TGF-β. Proliferative responses to HEL were measured in the presence of either anti-IL-4 neutralizing antibodies (12 μg/ml, *A*) or anti-TGF-β neutralizing antibodies (12 μg/ml, *B*). Proliferative responses shown are of nontolerant cells primed by HEL/alum (hatched bars) or of HEL-primed tolerant cells fed high (dotted bars) or low (solid bars) doses. (*C*) HEL-primed nontolerant T lymphocytes were mixed in a 1:1 ratio with cells derived from alum-primed nontolerant mice (hatched bars) or from HEL-primed high-dose (dotted bars) or low-dose (solid bars) tolerized mice. Proliferative responses to HEL (50 μg/ml) were measured in the presence of anti-TGF-β neutralizing antibodies or normal chicken IgG (12 μg/ml). Results, expressed in cpm, are averages of quadruplicate cultures ± SEM of cells pooled from at least three mice. Proliferation in response to medium alone ranged from 2400 to 3400 cpm for all groups. Data are representative of four (*A* and *B*) or three (*C*) experiments.

We postulate that the induction of active suppression following low-dose feeding occurs primarily in gut lymphoid tissue, whereas anergy associated with high-dose feeding is generated both systemically and locally. Suppression can be transferred by Peyer's patches from fed animals (20) and we have found antigen-driven TGF-β-secreting T cells in Peyer's patches of mice fed low doses of MBP within 24 hr after feeding (21). Other data in which high doses were fed indicate that peripheral tolerance generated after the oral administration of antigens is not limited solely to the gut since (*i*) antigen fragments are found in the bloodstream soon after feeding, (*ii*) serum has been shown to transfer tolerance, and (*iii*) antibodies directed against the fed antigen partially block the generation of tolerance (refs. 22 and 23; D. Melamed and A.F., unpublished data). These findings indicate that bloodborne antigen, in the absence of costimulatory signals, could induce tolerance via anergy, similar to that found by the direct i.v. or i.p. administration of aqueous antigen (24–26). It is unknown at this time why high doses of antigen do not induce equivalent amounts of active suppression as low doses. Possibilities include (*i*) anergizing regulatory cells or cells involved in their induction, (*ii*) differential stimulation via the T-cell receptor related to antigen concentration, and (*iii*) differential processing and presentation by antigen-presenting cells. The antigen itself may be a determinant of the dose required to induce active suppression as minute amounts of collagen (3–30 μg) induce active suppression whereas 300–1000 μg does not (27).

Table 1. Cells derived from mice fed high- or low-dose regimens differ in their IL-4 and TGF-β secretion profiles

Fed	*In vitro* stimulus	Cytokine production, pg/ml IL-4	TGF-β
	Primed LNCs		
Saline	Medium	950 ± 140	340 ± 110
	HEL	880 ± 120	285 ± 140
HEL			
1 mg × 5	Medium	**3120 ± 310**	830 ± 130
	HEL	**1960 ± 270**	**3600 ± 100**
20 mg × 1	Medium	**5600 ± 430**	540 ± 110
	HEL	**1790 ± 220**	**1000 ± 125**
	Nonprimed spleen cells		
Saline	Medium	550 ± 80	1120 ± 190
	HEL	610 ± 110	1150 ± 110
HEL			
1 mg × 5	Medium	720 ± 90	1500 ± 150
	HEL	**1920 ± 150**	**3850 ± 130**
	OVA	520 ± 60	1330 ± 220
20 mg × 1	Medium	580 ± 0.13	1220 ± 110
	HEL	**2950 ± 340**	**2450 ± 210**
	OVA	550 ± 90	1280 ± 90

HEL-primed LNCs or nonprimed spleen cells from saline-, low-dose-, or high-dose-fed mice were cultured in serum-free medium in the absence or presence of HEL or OVA (50 μg/ml). Supernatants were collected at 18 and 72 hr of culture, cleared by centrifugation, and measured for IL-4 and TGF-β content, respectively, by quantitative ELISA. Results, expressed in pg/ml, are averages of supernatants drawn from quadruplicate cultures ± SEM of cells pooled from at least three mice. Boldface values indicate significant secretion of cytokines above that of saline-fed controls (see *Results* for analyses). Data are representative of five experiments.

The oral administration of antigen, in the absence of additional immunization, primes for distinct cytokine patterns in spleen cell populations that reflect the feeding regimen. Thus, an orally administered antigen can stimulate cells to secrete IL-4 and TGF-β without proliferation or IL-2 production. These results provide a means for demonstrating immune responses and analyzing tolerance to orally administered antigen in the absence of immunization and proliferation. In primed LNCs from high- and low-dose-fed animals we observed constitutive secretion of IL-4 without antigen stimulation *in vitro* and reduction of IL-4 secretion when the fed antigen was added in culture. This was not seen for TGF-β. The mechanism for this observation is unexplained but was not related to the secretion of IFN-γ in these cultures. Other cytokines such as IL-12 may be involved. Note that animals fed high doses secreted more IL-4 and less TGF-β, whereas those fed low doses secreted more TGF-β and less IL-4. Increased IL-4 secretion by tolerized cells is indicative of the selective tolerization of Th1-type responses, while Th2-type responses appear to be more refractory to tolerization (24, 25). Thus, IL-4 secretion may not be indicative of an inhibitory function but, rather, the expression of a Th2-type response in a state of partial peripheral tolerance (28).

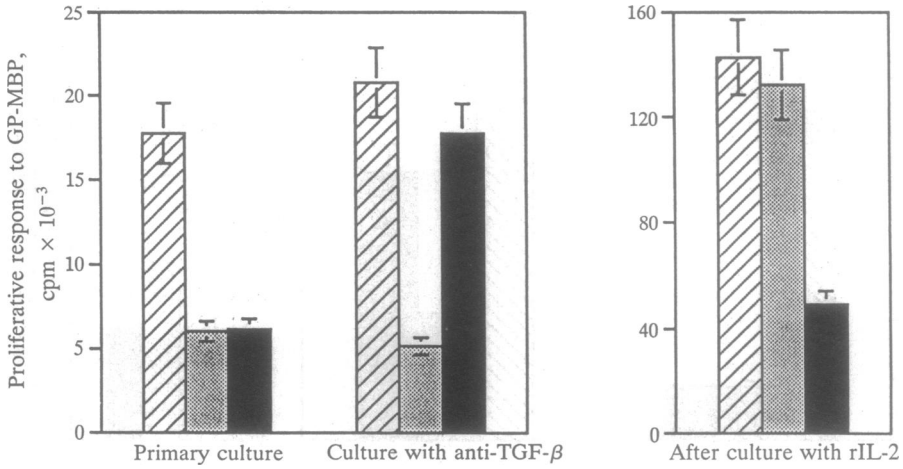

FIG. 4. High- and low-dose feeding regimens induce different forms of tolerance to guinea pig MBP (GP-MBP). Lewis rats were fed low (5 × 1 mg) (solid bars) or high doses (4 × 5 mg) (dotted bars) of MBP. Controls were fed saline (hatched bars). Rats were immunized and proliferation to MBP was measured in primary culture in the presence of anti-TGF-β neutralizing antibodies (12 μg/ml) or after a 5-day culture in the presence of rIL-2 (50 units/ml). Results, expressed in cpm, are averages of quadruplicate cultures ± SEM of cells pooled from at least four rats. Proliferation in response to medium alone ranged from 1500 to 4500 cpm for all groups. Data are representative of three experiments.

The present findings have important ramifications for the use of orally administered autoantigens as a therapeutic strategy to suppress autoimmunity (3, 4). Immunotherapeutic approaches based on anergy require knowledge of the disease inducing autoantigens and those antigens involved in the ongoing autoimmune process. Strategies based on cytokine-mediated active suppression, or bystander suppression (8), require only that the antigen be from the target organ and be capable of inducing regulatory T lymphocytes that secrete down-regulatory cytokines such as TGF-β (9, 29). Thus, oral but not i.v. MBP suppresses proteolipid protein-induced EAE (30). Because there is often reactivity to multiple autoantigens in an autoimmune disease and spreading autoimmunity (31–34), the generation of cytokine-mediated or bystander suppression provides an attractive immunotherapeutic approach.

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