

# Mucosal administration of IL-10 enhances oral tolerance in autoimmune encephalomyelitis and diabetes

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## Abstract

**IL-10 is an immunoregulatory cytokine that can modulate immune processes, inhibiting the expression of inflammatory T<sub>H</sub>1 type responses as well as affecting antigen-presenting cell function. In addition, IL-10 has been shown to be active at mucosal surfaces. In the present study, we examined the role of IL-10 on orally and nasally induced tolerance. Treatment of (PL/J × SJL)F<sub>1</sub> mice with low-dose oral myelin basic protein (MBP) (0.5 mg) and simultaneous oral IL-10 given 3 times reduced the severity and incidence of experimental autoimmune encephalomyelitis (EAE), whereas administration of oral IL-10 alone or MBP alone given in these doses had no effect. Lymphocytes from mice treated orally with MBP and IL-10 proliferated less, and produced decreased amounts of IFN- $\gamma$  and IL-2 and increased amounts of IL-10 and transforming growth factor- $\beta$  upon *in vitro* stimulation with MBP. Nasal administration of antigen and IL-10 reduced proliferative responses and IFN- $\gamma$  production, increased IL-10 production, and enhanced protection from EAE. In addition, oral IL-10 combined with oral myelin oligodendrocyte glycoprotein (MOG) 35–55 reduced relapses in MOG-induced EAE in the NOD mouse, as well as enhanced the protective effect of oral insulin in the NOD model of diabetes. These results demonstrate that IL-10 is biologically active at mucosal surfaces and can act synergistically to enhance the tolerogenic effects of mucosally administered antigen.**

## Introduction

Oral and nasal administration of antigen have been shown to be effective in inhibiting experimental models of autoimmune disease in animals and oral tolerance is also being tested for the treatment of human diseases (1). Oral tolerance is mediated by different mechanisms depending on the dose of antigen fed. High doses favor clonal deletion (2) and anergy (3,4), whereas low doses favor the induction of active suppression (5). Nasal administration of antigen also induces tolerance and suppresses experimental models of autoimmune disease in a number of systems (reviewed in 1). This route of administration has also been used to prime for local and systemic immune responses (6).

Cytokines play an important role in T cell differentiation and direct precursor T cells (T<sub>h</sub>p) toward T<sub>h</sub>1, T<sub>h</sub>2, T<sub>h</sub>3 or T<sub>r</sub>1 lineages. IL-4 directs precursor T cells to become IL-4-secreting T<sub>h</sub>2 cells (7), whereas IL-12 directs T<sub>h</sub>0 cells to

become IFN- $\gamma$ -secreting T<sub>h</sub>1 cells (8). Activation of CD4<sup>+</sup> T cells in the presence of IL-10 leads to the induction of cells which do not proliferate well and produce large amounts of IL-10 plus transforming growth factor (TGF)- $\beta$ , termed T<sub>r</sub>1 cells (9). Seder *et al.* demonstrated that TGF- $\beta$  promotes the differentiation of TGF- $\beta$ -producing T<sub>h</sub>3 cells (10) and we have shown that culture of MBP TCR transgenic spleen cells in the presence of IL-4 results in an increased frequency of TGF- $\beta$ -secreting cells as measured by limiting dilution analysis (11). IFN- $\gamma$  administration has been shown to inhibit (12), whereas anti-IL-12 treatment has been shown to enhance, oral tolerance and increase the secretion of TGF- $\beta$  (13) suggesting that *in vivo*, low-dose oral tolerance may be favored by factors which enhance the induction of regulatory cells or inhibit T<sub>h</sub>1 responses.

Cytokine-induced immune deviation has been investigated

as potential therapy for  $T_H1$ -type autoimmune diseases, as cytokine present at time of activation may alter the pathogenicity of effector T cells (14). However, there may be side effects of systemically administered cytokines, which may limit their use. Accumulating evidence suggests that oral administered cytokines are biologically active and may avoid the deleterious effects of systemic cytokine administration (15). Both oral and nasal routes of cytokine administration have been found to be biologically active including IL-4, IL-6, IL-10, IFN and TGF- $\beta$  (11,16–19).

Systemic administration of IL-10 has been tested in the experimental autoimmune encephalomyelitis (EAE) model with differing results. Administration of IL-10 during the inductive phase of disease was reported to suppress actively induced acute EAE in the Lewis rat (20), whereas IL-10 not only failed to abrogate chronic relapsing EAE in an adoptively transferred model of disease in SJL/J mice but exacerbated disease (21). Nasal administration of IL-10 was reported to suppress the clinical signs of acute EAE in Lewis rat and prevented relapses of protracted disease in DA rats (19). IL-10 is important for mucosal immune responses as IL-10-deficient animals develop chronic inflammatory bowel disease that shares histopathological features with human disease (22).

In the present study, we investigated the effect of mucosally administered IL-10 when low doses of antigen were orally or nasally administered. To determine whether IL-10 acts synergistically, we have used an antigen dose we have previously described (11) as a suboptimal tolerizing regimen. We found that IL-10 acts synergistically to enhance low-dose oral tolerance *in vivo* in the EAE model and enhances the production of IL-10 itself *in vitro*. In addition, oral IL-10 enhanced the protective effect of oral insulin in the NOD model of diabetes.

## Methods

### Animals

Female (PL/J $\times$ SJL) $F_1$  were purchased from the Jackson Laboratory (Bar Harbor, ME). These mice were 8–10 weeks of age and were housed in Harvard Medical School Animal Care Facilities according to institutional guidelines. NOD mice were purchased from Taconic Farms (Germantown, NY) at 3–4 weeks of age.

### Antigens, antibodies and recombinant cytokines

Mouse or bovine myelin basic protein (MBP) was prepared from brain tissue by a modified method of Deibler *et al.* (23); the purity of the MBP preparation was confirmed by gel electrophoresis and amino acid analysis. Ac1–11 peptide of MBP (Ac-ASQKRPSQRHG), myelin oligodendrocyte glycoprotein (MOG) peptide 33–55 and the insulin B chain peptide 10–24 were prepared using a peptide synthesizer and purified by HPLC (QCB, Hopkinton, MA). Ovalbumin (OVA) was purchased from Sigma (St Louis, MO). Polyclonal chicken anti-TGF- $\beta$ 1 antibody, human purified TGF- $\beta$ 1 and monoclonal mouse anti-TGF- $\beta$  antibody (Clone 1D11) were purchased from R & D Systems (Minneapolis, MN). The following reagents were purchased from PharMingen (San

Diego, CA): purified rat anti-mouse IL-2 (clone JES-1A12), IL-4 (clone BVD4-1D11), IL-10 (clone JESS-2A5) and IFN- $\gamma$  (clone R4-6A2) mAb; biotinylated rat anti-mouse IL-2 (clone JES6-5H4), IL-4 (clone BVD6-24G2), IL-10 (clone SXC-1) and IFN- $\gamma$  (clone XMG1.2); and recombinant mouse IL-2 (rIL-2), IL-4, IL-10 and IFN- $\gamma$ . For oral and nasal IL-10 experiments, recombinant murine IL-10 was a kind gift of the Schering Plough Research Institute (Kenilworth, NJ).

### Oral administration of autoantigens and of IL-10

(PL/J $\times$ SJL) $F_1$  mice were fed with 0.5 mg of OVA as a control protein, 0.5 mg of MBP and/or 0.1, 1 or 10  $\mu$ g of IL-10 dissolved in 0.25 ml PBS by gastric intubation with an 18-gauge stainless steel feeding needle (Thomas Scientific, Swedesboro, NJ). Animals were fed 3 times, every other day over a total of 5 days and immunized 2 days following the last feeding. NOD mice were fed with 250  $\mu$ g of insulin B chain peptide 4 times in the first week beginning at age 3–4 weeks and then once a week. Oral IL-10 (1  $\mu$ g) was given alone or at the time of B chain peptide administration. For MOG-induced EAE in the NOD mouse, 250  $\mu$ g MOG 35–55 peptide and/or 1  $\mu$ g IL-10 was given orally every day for 4 days prior to immunization.

### Nasal administration of MBP and of IL-10

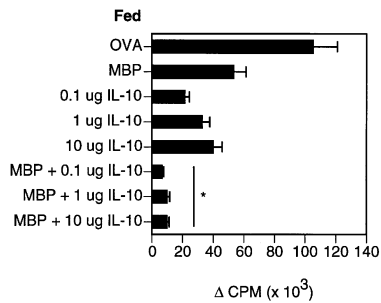
Mice were treated nasally 3 times with 0.5  $\mu$ g of MBP and/or 1  $\mu$ g IL-10 dissolved in 0.01 ml PBS (5  $\mu$ l/nostril).

### Induction and clinical evaluation of EAE and diabetes

(PL/J $\times$ SJL) $F_1$  mice received a s.c. injection in the flank of 100  $\mu$ g of mouse MBP in 0.1 ml of PBS emulsified in an equal volume of complete Freund's adjuvant (CFA) supplemented with 4 mg/ml of *Mycobacterium tuberculosis* H37 RA (Difco, Detroit, MI); mice also received an i.v. injection of 150 ng of pertussis toxin (PT) in 0.2 ml of PBS. A second injection of PT (150 ng/mouse) was given 48 h later. NOD mice were immunized in the hind footpads with 100  $\mu$ g MOG 35–55 peptide in CFA, as well as two injections of PT. Animals were monitored for symptoms of EAE and scored as follows: 0, no disease; 1, tail paralysis; 2, hind limb weakness; 3, hind limb paralysis; 4, hind limb plus forelimb paralysis; 5, moribund. NOD mice were monitored for diabetes development by urinary glucose testing and diabetes was confirmed by showing hyperglycemia (>250 mg/dl) for two consecutive testings. For all EAE experiments there were five mice per group and the experiment was repeated at least once. For studies of diabetes, 10 mice per group were used in each of two experiments.

### Cell culture of lymphocytes

For proliferation and cytokine assays, cells from similarly treated mice were pooled and were cultured in 96-well plates at  $5 \times 10^6$  and  $10 \times 10^6$  cells/ml respectively in serum-free medium, X-VIVO 20 (Biowhittaker, Walkersville, MD). To measure cytokines, culture supernatants were collected at 24 h for IL-2 and IL-4, 40 h for IL-10 and IFN- $\gamma$ , and at 72 h for TGF- $\beta$ . For proliferation, cells were pulsed with [ $^3$ H]thymidine at 72 h and radioactivity determined 16 h later.



**Fig. 1.** Proliferative responses of LNC from animals treated orally with MBP and/or IL-10. (PL/J×S/JL)<sub>F1</sub> mice were fed with OVA (0.5 mg), MBP (0.5 mg) or MBP (0.5 mg) and/or IL-10 (0.1, 1 or 10 µg). Two days after the last feeding mice were immunized with 100 µg MBP emulsified in CFA in the hind footpads. Ten days after immunization, popliteal LNC were cultured at  $0.5 \times 10^6$  cells/well with 50 µg/ml of MBP in 0.2 ml of medium. Proliferation was determined as in Methods. Data represent mean  $\pm$  SD of triplicate cultures, representative of three similar experiments. \*Proliferative responses of mice receiving MBP plus IL-10 at all doses were significantly lower than animals receiving IL-10 alone (all doses) ( $P < 0.05$ ) or MBP alone ( $P < 0.001$ ). The counts in all treated groups were significantly decreased compared to those in OVA-fed controls ( $P < 0.001$ ). Statistical significance was determined by ANOVA as described in Methods.

#### ELISA for cytokines

Quantitative ELISA for IL-2, IL-4, IL-10 and IFN- $\gamma$  were performed using paired mAb specific for corresponding cytokines per manufacturer's recommendations (PharMingen). TGF- $\beta$  was determined as previously described (11).

#### Statistical analysis

Maximum disease scores, day of disease onset, cytokine concentrations and thymidine incorporation were analyzed by Student's *t*-test, ANOVA,  $\chi^2$  or non-parametric analysis. Disease incidence was analyzed by Fisher's exact test.

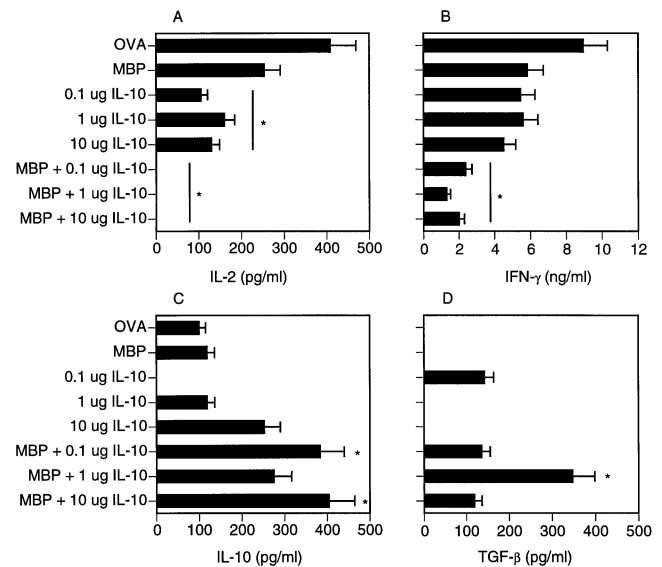
## Results

#### Proliferative responses of lymph node cells (LNC) are suppressed in animals treated with oral MBP plus oral IL-10

To investigate whether IL-10 was biologically active when administered orally and whether it could act synergistically with MBP in the induction of oral tolerance we measured the proliferative responses of LNC from mice fed with OVA, MBP, IL-10 or MBP plus IL-10. LNC were harvested 10 days after immunization with MBP and stimulated with MBP *in vitro*. As shown in Fig. 1, almost complete suppression of MBP-specific proliferation was observed in LNC from mice treated with MBP plus IL-10 at all doses compared to those fed OVA ( $P < 0.001$ ), IL-10 alone ( $P < 0.05$ ) or MBP alone ( $P < 0.001$ ). Mice that received IL-10 alone also showed significantly less proliferation compared to mice fed with OVA ( $P < 0.001$ ). Similar results were also observed in splenocytes and when cultures were stimulated *in vitro* with MBP Ac1-11.

#### Cytokine secretion pattern of LNC is altered in animals treated with MBP plus oral IL-10

We then tested the effect of oral IL-10 plus MBP on cytokine secretion. As shown in Fig. 2(A) oral administration of MBP



**Fig. 2.** Cytokine responses of LNC from animals treated orally with MBP and/or IL-10. (PL/J×S/JL)<sub>F1</sub> mice were fed with OVA, MBP (0.5 mg) or MBP (0.5 mg) and IL-10 (0.1, 1 or 10 µg) and immunized as described in Fig. 1. Ten days after immunization, popliteal LNC were cultured at  $1 \times 10^6$  cells/well with 50 µg/ml of MBP in 0.2 ml of medium. Cytokine production was determined as described in Methods. \*Significant differences as described in Results. Significance was determined by ANOVA as described in Methods.

or IL-10 alone suppressed IL-2 production compared to OVA-fed mice. However, the combination of MBP plus IL-10 led to a complete abrogation of IL-2 production ( $P < 0.0001$ ). For IFN- $\gamma$  production, enhanced suppression was also observed in animals treated with IL-10 plus MBP ( $P < 0.0001$ , Fig. 2B). Mice treated orally with either IL-10 or MBP alone also had decreased levels of IFN- $\gamma$  secretion, although not as marked as those treated with MBP plus IL-10 (Fig. 2B). An increase in IL-10 ( $P = 0.007$ ) and TGF- $\beta$  ( $P = 0.036$ ) production was found in animals treated with MBP plus IL-10 as compared to those receiving OVA, IL-10 or MBP alone (Fig. 2C and D).

#### Oral administration of IL-10 enhances suppression of EAE by oral MBP

Having observed that oral IL-10 acts synergistically with MBP *in vitro* we investigated whether oral MBP plus IL-10 could affect the disease model EAE. Animals were fed MBP 3 times at a dose that we have previously shown is not protective against EAE and 1 µg IL-10 was given orally at the time of each feeding. Three feedings of MBP at a dose of 0.5 mg per feeding or 1 µg IL-10 alone had no protective effect against onset or severity of EAE (Table 1). However, when MBP and IL-10 were given together, there was suppression of EAE severity ( $P = 0.019$ , IL-10 + MBP versus OVA;  $P = 0.036$ , IL-10 + MBP versus MBP alone) (Table 1). Disease incidence was also significantly decreased in mice fed with a subtolerogenic dose of MBP plus IL-10 compared to those receiving OVA alone ( $P = 0.04$ ) (Table 1). The course of disease was also altered with oral MBP plus IL-10 decreasing the disease burden in mice (Fig. 3). Although we observed

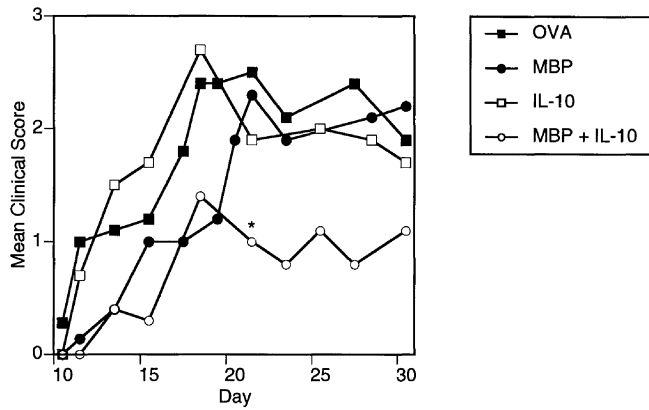
**Table 1.** Oral IL-10 enhances protection against EAE by oral MBP

Fed	Day of onset	Incidence	Mortality	Disease severity
OVA	14.3 ± 1.0	12/12	2/12	3.0 ± 0.3
MBP	16 ± 1.0	11/12	1/12	2.8 ± 0.3
IL-10 (0.1 µg)	18.2 ± 2.3	9/11	0/11	2.3 ± 0.4
IL-10 (0.1 µg) + MBP	17.3 ± 1.8	7/9	0/9	2.3 ± 0.5
IL-10 (1 µg)	15.2 ± 2.0	8/11	1/11	2.3 ± 0.5
IL-10 (1 µg) + MBP	20.9 ± 2.2	7/11 <sup>a</sup>	0/11	1.6 ± 0.4 <sup>b</sup>

(PL/J×S/JL)<sub>F1</sub> mice were fed with OVA, MBP (0.5 mg) or IL-10 (0.1 or 1 µg) every other day for a total of three feedings. One group was fed with both MBP (0.5 mg) and IL-10 (0.1 µg), and one group fed with both MBP (0.5 mg) and IL-10 (1 µg).

<sup>a</sup>Incidence of disease, MBP + IL-10- versus OVA-fed group ( $P = 0.04$ ) as determined by Fisher's exact  $t$ -test.

<sup>b</sup>Disease severity, MBP + IL-10- versus OVA-fed group ( $P < 0.019$ ), MBP alone ( $P = 0.036$ ) determined by Student's  $t$ -test.

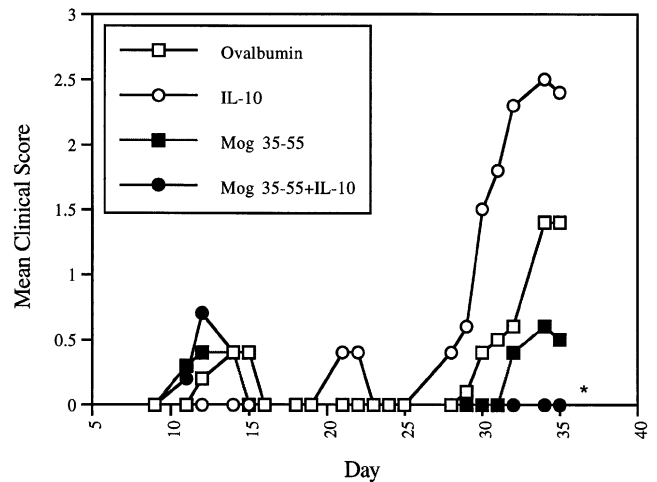


**Fig. 3.** Combined low-dose MBP and oral administration of IL-10 protects mice from EAE. (PL/J×S/JL)<sub>F1</sub> mice were fed with OVA (■), 0.5 µg MBP (□), 1 µg IL-10 (□) or 0.5 mg MBP plus 1 µg IL-10 (○) every other day for a total of three feedings. Two days after the last feeding, mice were immunized s.c. with 100 µg of mouse MBP in 0.1 ml of PBS emulsified in an equal volume of CFA supplemented with 4 mg/ml *M. tuberculosis* H37 RA. Immediately thereafter and again 48 h later mice received an i.v. injection of 150 ng of pertussis toxin in 0.2 ml of PBS. \* $P < 0.02$  by ANOVA.

a suppressive effect of 0.1 µg IL-10 *in vitro* on proliferation, IL-2 and IFN- $\gamma$  secretion (Figs 1 and 2) no effect of 0.1 µg IL-10 plus MBP treatment was seen upon disease (Table 1).

#### Oral IL-10 suppresses relapses in MOG-induced EAE

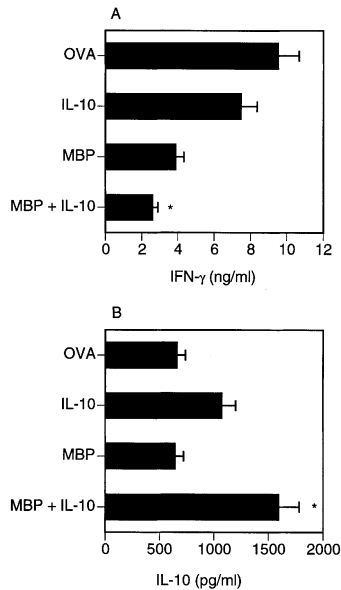
Having observed that oral IL-10 acts synergistically with MBP to suppress MBP-induced EAE, we tested the effect of oral IL-10 on relapses in MOG-induced EAE. NOD mice which develop relapsing EAE upon immunization with MOG 35–55 were fed 250 µg MOG peptide 4 times on consecutive days and 1 µg IL-10 was given orally at the time of each MOG feeding. Feeding MOG alone or MOG in combination with IL-10 had no protective effect on the first attack of EAE (day 12–14). However, at the time of the first relapse (day 28–30) animals fed MOG peptide or MOG peptide with IL-10 manifested a protective effect (Fig. 4). Whereas MOG feeding alone reduced the severity of the clinical score, the oral administration of MOG with IL-10 completely inhibited the second attack ( $P < 0.04$ ). Interestingly, IL-10 given orally alone increased disease severity as previously reported in an adoptive transfer model of relapsing EAE in SJL/J mice (21).



**Fig. 4.** Oral IL-10 in combination with oral MOG peptide 35–55 protects NOD mice from relapsing EAE. NOD mice were fed OVA (□), 250 µg MOG 35–55 (■), 1 µg IL-10 (○) or 250 µg MOG 35–55 plus 1 µg IL-10 (□) every day for a total of four feedings. Two days after the last feeding, mice were immunized in the hind footpads with 100 µg MOG peptide 35–55 emulsified in CFA supplemented with 4 mg/ml *M. tuberculosis* H37 RA. Immediately thereafter and again 48 h later mice were injected i.v. with 150 ng pertussis toxin. Results are representative of three similar experiments with five mice per group (determined by Student's  $t$ -test). \* $P < 0.04$  versus other groups.

#### Nasal administration of MBP and IL-10

A number of reports have suggested that nasal administration of antigen is effective in inducing tolerance (reviewed in 1). We therefore tested whether nasal administration of antigen plus IL-10 would enhance tolerance induced via this route. As shown in Fig. 5(A), nasal administration of MBP and IL-10 enhanced suppression of IFN- $\gamma$  production ( $P < 0.05$ ). Conversely, IL-10 production was significantly augmented by combined nasal treatment with MBP plus IL-10 ( $P < 0.05$ ) compared to OVA or MBP alone (Fig. 5B); however, no increase in TGF- $\beta$  was observed. This treatment was also moderately successful at suppressing EAE (Fig. 6). Interestingly, increasing doses of nasally administered MBP appeared to exacerbate disease; however, regardless of the dose of MBP, co-administration of IL-10 overcame the priming effect of MBP and suppressed disease (data not shown),



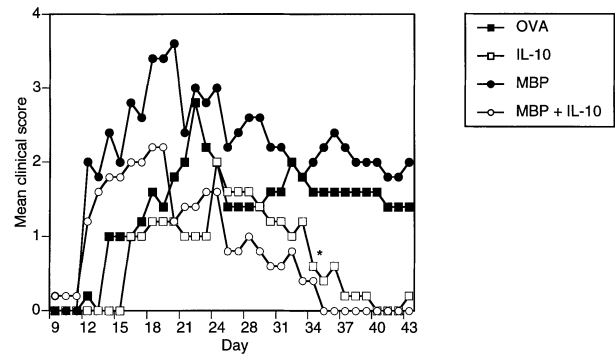
**Fig. 5.** Cytokine production of LNC from animals treated nasally with MBP and/or IL-10 (PL/J $\times$ SJL) $F_1$  mice were treated nasally with OVA, MBP (0.5  $\mu$ g) or MBP (0.5  $\mu$ g) and IL-10 (1  $\mu$ g) and immunized as described in Fig. 1. Ten days after immunization, LNC were cultured at  $1.0 \times 10^6$  cells/well with 50  $\mu$ g/ml of MBP in 0.2 ml of medium. Cytokines (A) IFN- $\gamma$  and (B) IL-10 were determined as described in Methods. \* $P < 0.05$  versus OVA control. Statistical significance was determined by ANOVA as described in Methods. Results are representative of three similar experiments.

suggesting IL-10 may be acting to suppress mucosal priming via the nasal route.

#### Oral IL-10 enhances suppression of diabetes by oral insulin in the NOD mouse

As we observed that oral IL-10 acts synergistically with MOG to enhance suppression of relapsing EAE in NOD mice, we investigated whether oral IL-10 could enhance the effect of oral insulin in inhibiting spontaneous diabetes development in the NOD mouse. Three-week-old female NOD mice were fed 4 times on consecutive days with either OVA, 250  $\mu$ g insulin B chain 10–24, 1  $\mu$ g IL-10 or B chain 10–24 with IL-10. For the duration of the experiment mice were fed once a week with IL-10, B chain 10–24 or both.

Three mice of each treatment group were immunized with insulin B chain 10–24, 2 days after the last of the four feedings in order to check the cytokine profile of lymphocytes from the immunized mice upon *in vitro* stimulation with the immunizing antigen. The data in Fig. 7 describe the marked decrease of IL-2 ( $P < 0.05$ ) and IFN- $\gamma$  ( $P < 0.02$ ) secretion in LNC harvested from mice fed with IL-10 in combination with insulin B chain 10–24. Mice fed with either IL-10 or B chain 10–24 alone showed a decreased  $T_H1$  response as well; however, IL-2 and IFN- $\gamma$  secretion was completely abrogated in lymphocytes from mice fed with the combination of IL-10 and insulin. Increase in TGF- $\beta$  secretion was observed only in the group fed with the combination of insulin and IL-10 ( $P < 0.004$ ) compared to all other treatment groups. IL-10 production was up-regulated in both the B



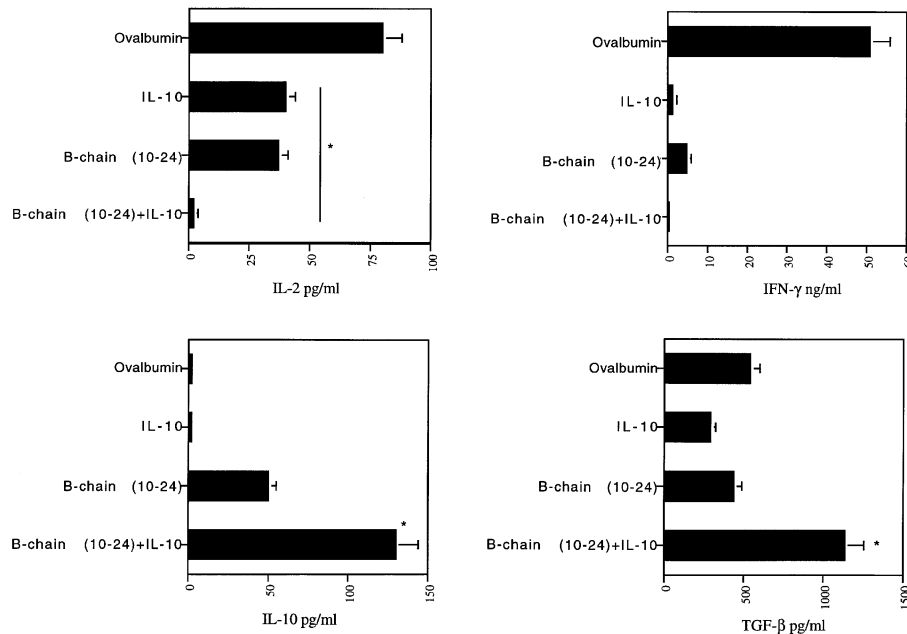
**Fig. 6.** Combined nasal MBP and nasal administration of IL-10 protect mice from EAE. (PL/J $\times$ SJL) $F_1$  mice were treated nasally with OVA (■), 0.5  $\mu$ g MBP (□), 1  $\mu$ g IL-10 (□) or 0.5  $\mu$ g MBP plus 1  $\mu$ g IL-10 (○) every other day for a total of three treatments. Two days after the last treatment, mice were immunized s.c. with 100  $\mu$ g of mouse MBP in 0.1 ml of PBS emulsified in an equal volume of CFA supplemented with 4 mg/ml *M. tuberculosis* H37 RA. Immediately thereafter and again 48 h later mice received an i.v. injection of 150 ng of pertussis toxin in 0.2 ml of PBS.

chain 10–24-fed group and the group fed with B chain 10–24 with IL-10 ( $P < 0.05$ ). The remaining mice in each group were monitored for diabetes development starting at 10 weeks of age. As can be seen in Fig. 8, by 32 weeks of age, 78% of the mice fed with OVA became diabetic. Fifty percent of mice fed with IL-10 and 40% of mice fed with B chain 10–24 were diabetic at 32 weeks of age. However, in the group fed with the combination of IL-10 and B chain 10–24, only 20% of the mice became diabetic ( $P < 0.02$ ). As in the EAE model IL-10 feeding further enhanced the protective effect of oral insulin in inhibiting diabetes development.

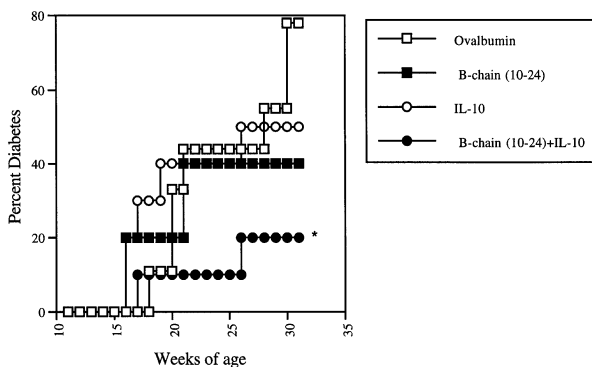
## Discussion

In the present study we demonstrate that oral administration of IL-10 enhances the production of IL-10 and TGF- $\beta$  generated by feeding MBP or MOG 35–55, and that this combination of oral IL-10 and oral autoantigens suppresses EAE. Nasal administration of MBP and IL-10 enhances tolerance by decreasing IFN- $\gamma$  and increasing IL-10 production as well as suppressing EAE. Furthermore, oral IL-10 enhances the suppression of diabetes in the NOD mouse treated with oral insulin.

One of the mechanisms by which oral tolerance is mediated involves the generation of regulatory cells including  $T_H2$ ,  $T_H3$  and  $T_H1$  cells. These cells are preferentially generated when low doses of antigen are fed (24). The factors which influence the generation of these cells, and the relationship of  $T_H3/T_H1$  cells to cells which secrete  $T_H1$  (IFN- $\gamma$ )- and  $T_H2$  (IL-4)-type cytokines has not yet been well defined. Given that low-dose oral tolerance involves the induction of cells that actively suppress  $T_H1$ -type responses, factors which favor the induction of regulatory  $T_H2/T_H3/T_H1$ -type cells would be expected to enhance low-dose oral tolerance. This is consistent with our previous findings that IL-4 enhanced the protection of oral MBP in the EAE model when suboptimal doses of MBP were fed (11), and with the findings of others that IFN- $\gamma$  inhibits



**Fig. 7.** Cytokine responses of LNC from NOD mice treated orally with insulin B chain peptide 10–24 and/or IL-10. NOD mice were fed with OVA, IL-10 (1  $\mu$ g), insulin B chain peptide 10–24 (250  $\mu$ g) or insulin B chain 10–24 in combination with IL-10. Two days after the last feeding, mice were immunized with 100  $\mu$ g of insulin B chain 10–24 emulsified in CFA in the hind footpads. Ten days after immunization, popliteal LNC were stimulated in culture at  $10 \times 10^6$  cells/ml for cytokine assays with 100  $\mu$ g/ml of insulin peptide in 0.2 ml medium. Cytokine production was determined as described in Methods. Data represent mean  $\pm$  SD of duplicate cultures. \*Significant differences as described in Results. Results are representative of three similar experiments.



**Fig. 8.** Effect of combined oral IL-10 and insulin B chain peptide 10–24 on diabetes development in the NOD mouse. NOD mice at 3–4 weeks of age were fed with OVA ( $\square$ ), 1  $\mu$ g IL-10 ( $\circ$ ), 250  $\mu$ g insulin B chain 10–24 ( $\blacksquare$ ) or 250  $\mu$ g insulin B chain 10–24 plus 1  $\mu$ g IL-10 ( $\square$ ) every day for a total of four feedings. The mice were then fed once a week for the duration of the experiment and monitored for diabetes development as described in Methods. \* $P < 0.02$  versus OVA (determined by  $\chi^2$  analysis). Results are representative of one of two experiments done with 10 mice per group.

(25) and anti-IL-12 enhances (13) oral tolerance. We and others (26–28) have also shown that i.p. or oral administration of the  $T_H2$ -inducing schistosomiasis soluble egg antigen in both EAE and diabetes suppresses  $T_H1$  and enhances  $T_H2$  (IL-10 and IL-4) cytokine responses to the autoantigens involved in disease as well as improves disease outcome. Factors which act as mucosal adjuvants and thus enhance the induction of gut immune responses have also been

reported to enhance oral tolerance, and include coupling of antigen to the B subunit of cholera toxin (29) or the use of multiple emulsions as an adjuvant (30). Different mechanisms of oral tolerance may be induced depending on the amount fed with high dose favoring deletion/anergy (2–4) and low dose favoring active suppression (5). In the present study we have shown the effect of oral IL-10 on low-dose oral tolerance. Aroeira *et al.* reported that anti-IL-10 antibodies did not affect high-dose oral tolerance (31), which is consistent with data we have obtained that oral IL-10 does not enhance oral tolerance when large doses of OVA (20 mg) are fed (A. J. Slavin, unpublished).

Although it is unexpected that an orally administered cytokine would be biologically active, oral cytokines have been shown to be active in other systems (15). It has also been shown that oral and nasal administration of cytokines may act as mucosal adjuvants and enhance down-regulatory immune responses to antigen given via the mucosal surfaces. For example, we have shown that oral administration of IL-4 increased oral tolerance to MBP decreasing local  $T_H1$  responses in the gut as measured by IFN- $\gamma$  message in the Peyer's patches and this protective effect was reversed by the administration of anti-TGF- $\beta$  antibody (11). Oral IFN- $\beta$  synergizes with the induction of oral tolerance in mice fed low-dose MBP (17). Oral IFN  $\alpha/\beta$  (type I) can suppress development of collagen-induced arthritis, and in a dose-dependent manner reduce delayed-type hypersensitivity, erythema and edema (32). Similarly Type I IFN can reduce the severity of EAE (33) as does orally administered IFN- $\gamma$  (16). By contrast, nasal administration of IFN- $\gamma$  can abrogate tolerance induced nasally with AChR in an experimental

model of myasthenia gravis (EAMG) (18). These combination therapies provide for the use of less tolerizing antigen, reducing exposure to autoantigen and decreasing the likelihood of developing autoantibodies or adverse immune responses to the tolerizing antigen.

Nasal administration of autoantigen to suppress autoimmune diseases is advantageous as relatively minute amounts are necessary. Intranasal administration of antigen has been used in several experimental models of autoimmune disease. Nasal application of retinal proteins can suppress EAU (34), collagen peptides suppress arthritis (35) and nasal insulin suppresses diabetes in NOD mice (36). Harrison *et al.* reported that inhaled insulin could increase IL-10 production (37). Intranasal GAD 65 has also been shown to induce immune deviation from T<sub>H</sub>1 to T<sub>H</sub>2 in NOD mice, increasing IL-5 and decreasing IFN- $\gamma$  with splenocytes from these mice able to transfer inhibition in NOD scid/scid mice (38). Inhalation of MBP or MBP peptide Ac1–11 can inhibit disease in rats and mice respectively (39,40). Intranasal administration of bacterial proteins and cholera toxin B subunit induce mucosal (especially salivary) IgA and non-mucosal antibody responses (41). It has also been reported that to induce tolerance via the nasal route required only 0.1% of AChR that was necessary for the induction of oral tolerance and protection from EAMG (42). In this model it appears that nasal tolerance to EAMG is mediated by TGF- $\beta$  (43) and nasal administration of IFN- $\gamma$  at the time of AChR administration abrogates the protective effect of nasal AChR, although IFN- $\gamma$  by itself does not alter the outcome of EAMG.

IL-10 has been used in the treatment of many disease models with varying results. IL-10 has been shown to augment humoral immune responses and inhibit certain aspects of cellular immunity (44). IL-10 mRNA was seen to be up-regulated during the remission phase of EAE (45) and IL-10-deficient mice fail to recover from MOG-induced EAE, while mice transgenic for IL-10 are completely resistant to the development of EAE (46). Nasal IL-10 prevented relapses in protracted disease in DA rats (19) and suppressed acute EAE in Lewis rat but s.c. administration did not (47). Systemic IL-10 can suppress acute EAE (20) but not relapsing EAE in an adoptive transfer model (21). Proteolipid protein-reactive T cells expressing transgenic IL-10 can suppress EAE after immunization with proteolipid protein (48) and the disease-promoting effects of IL-12 were inhibited by IL-10 (49). Development of diabetes is suppressed in mice treated s.c. with IL-10 (50) or a non-lytic IL-10/Fc fusion protein (51). The protective effect of NK T cells in an induced model of IDDM was neutralized by anti-IL-10 (52). By contrast expression of IL-10 under the insulin promoter in islets of transgenic mice results in macrophages, B cells, and CD4<sup>+</sup> and CD8<sup>+</sup> T cells infiltrating into the pancreas, and accelerated disease onset and increased prevalence when back-crossed onto NOD mice (53).

IL-10 may enhance oral tolerance by a number of mechanisms. It may act directly upon the epithelium, as intestinal epithelium has been shown to express IL-10 mRNA, protein and receptor (54,55). Indeed, jejunum and ileal tissue respond to serosal IL-10 by increasing Na<sup>+</sup> and Cl<sup>-</sup> absorption and increased bicarbonate production (56), and thereby IL-10 may increase time for antigen uptake by

preventing degradation and increasing pinocytosis by epithelium. Given the amount (1  $\mu$ g) we are feeding, if only 0.1% is not degraded the amount of IL-10 is in the nanogram range, which is physiologically relevant. Furthermore, exposure to digestive enzymes does not necessarily abrogate bioactivity as IL-6 retains bioactivity following treatment with trypsin chymotrypsin for 30 min (15). Another possibility is that by traversing the epithelium IL-10 influences local antigen presentation where regulatory cells are being generated. For example, the presence of dendritic cells in the airway epithelium may play an important role in either up- or down-regulating immunity, depending on their state of activation. Treatment of dendritic cells with IL-10 results in their decreased capacity to stimulate CD4<sup>+</sup> T cells in mixed lymphocyte reaction, and causes immature DC to become tolerogenic antigen-presenting cells (APC) (57) as well as preferentially primes naive T cells to generate T<sub>H</sub>2-type cells *in vitro* and *in vivo* (58,59). Other possible mechanisms include modulation of MHC class II on APC or effects upon costimulatory molecules. Although we suggest IL-10 may enhance antigen absorption or alter APC function, as yet we have no experimental data to support this hypothesis.

In summary, we have found that mucosally administered IL-10 can suppress T<sub>H</sub>1 responses and enhance production of TGF- $\beta$ /IL-10 when given with autoantigen, both in the EAE model and the NOD model of diabetes. Our results and those of others (15) suggest that *in vivo* administration of IL-10 enhances the effectiveness of oral tolerance for the treatment of autoimmune diseases and may ultimately be applied to human diseases in which oral tolerance is currently being tested as a therapeutic modality.

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### Abbreviations

APC	antigen-presenting cell
CFA	complete Freund's adjuvant
EAE	experimental allergic encephalomyelitis
EAMG	experimental autoimmune myasthenia gravis
LNC	lymph node cell
MBP	myelin basic protein
MOG	myelin oligodendrocyte glycoprotein
OVA	ovalbumin
PT	pertussis toxin
TGF	transforming growth factor

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