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Research Article

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Interleukin-2 Treatment Potentiates Induction of Oral Tolerance in a Murine Model of Autoimmunity

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Abstract

The present study addresses the feasibility of potentiating oral tolerance by immunomanipulation, using the murine model of experimental autoimmune uveoretinitis (EAU) induced by immunization with the retinal antigen interphotoreceptor retinoid binding protein (IRBP). Three feedings of 0.2 mg IRBP every other day before immunization did not protect against EAU, whereas a similar regimen of five doses was protective. However, supplementing the nonprotective 3× regimen with as little as one injection of 1,000 U of human recombinant interleukin-2 (IL-2) resulted in disease suppression that was equal to that of the protective 5× regimen. The protective effect was maintained across a range of IL-2 doses and times of administration; none of the IL-2 regimens tested resulted in disease enhancement. Peyer's Patch cells of 3×-fed and IL-2–treated mice showed greatly increased production of TGF-β, IL-4, and IL-10 compared with animals given the nonprotective 3× regimen and to animals given the protective 5× regimen. We propose that IL-2 treatment enhances protection from EAU at least in part by stimulating production of antiinflammatory cytokines by regulatory cells in Payer's Patches. Moreover, the observed lymphokine production patterns suggest that whereas protection induced by the 3× + IL-2 regimen is likely to involve antiinflammatory cytokines, protection induced by the 5× regimen might involve anergy or deletion of the uveitogenic T cells. These results could have practical implications for use of IL-2 as a safe and effective way of potentiating oral tolerance. (*J. Clin. Invest.* 1994. 94:1668–1672.) Key words: autoimmune disease • uveitis • therapy • immunomodulation • cytokines

Introduction

The phenomenon of oral tolerance has been established in a number of experimental models of autoimmune disease where the ability to alter the immune response in an antigen-specific fashion has important implications for disease treatment. Although induction of tolerance to orally administered antigens has been recognized for a long time, its potential for treatment of autoimmunity and graft rejection has recently generated renewed interest (1–4). Oral administration of autoantigens suppresses a variety of experimental autoimmune diseases, including experimental autoimmune encephalomyelitis, (EAE)[1] collagen and adjuvant-induced arthritis, diabetes in the non-obese diabetic mouse and experimental autoimmune uveoretinitis (EAU) (4). Encouraging results have been reported in clinical trials that used oral administration of antigen to ameliorate symptoms of multiple sclerosis and arthritis (5, 6) and a clinical trial in uveitis is currently ongoing (Nussenblatt, R. B., unpublished data). The uveitis trial is a randomized, double-masked study, in which patients receive purified bovine Retinal Soluble Antigen (S-antigen, also known as arrestin), bovine retinal extract, or placebo. The data compiled from experimental models as well as from the clinical studies suggest that oral tolerance can control not only the induction stage of autoimmunity, but can also modulate an ongoing immune process (3, 4). Therefore, strategies that might augment the tolerogenic effect of antigen feeding have not only theoretical but also important practical implications.

Two major mechanisms that have been proposed to mediate oral tolerance are anergy and active suppression. Whereas anergy, manifested as functional silencing of effector T cells through a block in IL-2 production and proliferation in response to antigen, seems to predominate after administration of high doses of antigen, lower doses appear to favor induction of active suppression mediated by regulatory cells secreting antiinflammatory cytokines (7–11). Khoury et al. (7, 12) demonstrated in the model of EAE that secretion of antiinflammatory cytokines as well as protection from disease can be enhanced by feeding the bacterial adjuvant LPS together with the antigen. Because the regulatory cells involved in oral tolerance appear to be T cells, we decided to evaluate the effect of IL-2, the prototypic T cell growth factor, on induction of oral tolerance to retinal antigens in the B10.A mouse model of EAU. Our findings indicate that IL-2 can dramatically enhance protection from disease induced by the uveitogenic antigen interphotoreceptor retinoid-binding protein (IRBP). However, the resulting tolerance may be mediated by a different mechanism than the tolerance obtained after a protective feeding regimen alone.

Methods

Animals. Female B10.A mice, 5–8 wk old were obtained from the National Cancer Institute breeding facility in Frederick, Maryland, and were kept under specific pathogen-free conditions.

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1. *Abbreviations used in this paper:* EAE, experimental autoimmune encephalomyelitis; EAU, experimental autoimmune uveoretinitis; IRBP, interphotoreceptor retinoid binding protein; OVA, ovalbumin; PP, Peyer's Patch.

Table I. Five, but Not Three, Feedings of IRBP Protect Against IRBP-induced EAU

	EAU Score±SE (incidence)*		
	Control‡	IRBP 3×	IRBP 5×
Experiment 1	2.4±0.6 (5/5)	2.3±0.3 (5/5)	0.8±0.4 (3/5)
Experiment 2	1.5±0.4 (5/5)	1.9±1.0 (2/3)	0.6±0.5 (3/5)
Stats (vs. control)§		NS	$P < 0.02$

* Score is an average of both eyes of all mice in the group. In parentheses: incidence is shown as EAU-positive out of total animals in the group. ‡ Control animals in experiment 1 were fed PBS and controls in experiment 2 were fed OVA (0.2 mg/dose) 5×. § Snedcor and Cochran's test for linear trend in proportions. Experiments 1 and 2 were combined for analysis. *NS,* not significant.

Induction of oral tolerance and treatment with IL-2. Mice were fed 0.2 mg of bovine IRBP (13) either three or five times by gavage. Animals receiving three feeds were gavaged on days −7, −5, and −3 before immunization. Animals receiving five feeds were gavaged on days −11, −9, −7, −5, and −3 before immunization. Some groups of mice were treated with recombinant human IL-2 (Cetus, Emeryville, CA) injected intraperitoneally at the times and doses indicated.

Induction and scoring of EAU. Mice were immunized with 50 μg of IRBP emulsified in complete Freund's adjuvant given subcutaneously in the thighs and base of tail, and received 0.5 μg pertussis toxin intraperitoneally. Eyes were collected for histopathology 21 d after immunization (~ 7 d after disease onset), except where indicated otherwise. Serial sections (hematoxylin and eosin) were graded in a masked fashion by one of us, who is an ophthalmic pathologist (C. C. Chan). EAU severity was scored on a scale of 0 to 4 in half-point increments using a semiquantitative grading system based on the number, size and type of lesions present (14).

Measurement of cytokine production. Peyer's Patches (PP) of each animal were removed 21 d after immunization. The cells were cultured in 96-well plates (5×10^5 cells in each 0.2 ml well) with 20 μg/ml IRBP or with 2.5 μg/ml ConA in supplemented (15) DME (GIBCO BRL, Gaithersburg, MD) containing 0.5% fresh-frozen mouse serum. Supernatants were collected for cytokine assays after 24–36 h. TGF-β was assayed by inhibition of the mink lung carcinoma (CCL-64) cell line proliferation using [^{3}H]thymidine uptake. Total TGF-β was detected after heat activation of the supernatants at 80°C for 5 min and the active moiety was detected without pretreating the supernatants. IL-2 and IL-4 were assayed on HT2 cells (as [^{3}H]thymidine uptake) using the monoclonal antibodies S4B6 (anti-IL-2) and 11B11 (anti-IL-4) to neutralize the reciprocal cytokine. IFN-γ and IL-10 were measured by ELISA using the antibody pairs obtained from Pharmingen (La Jolla, CA). Biological activity in units or concentration in pg/ml was calculated from a standard curve established with the respective recombinant cytokine.

Statistical analysis. Statistical analysis of EAU scores was by Snedcor and Cochran's test for linear trend in proportions (nonparametric, frequency based) (16). Each mouse, not each eye, was treated as a statistical event. Statistical analysis of cytokine levels was by ANOVA, Fischer's exact test. Probability values of ≤ 0.05 were considered significant.

Results and Discussion

Five, but not three, feedings of IRBP protect mice against EAU. Protection from ocular disease by feeding of the uveitogenic S-antigen had been demonstrated in the rat EAU model (17), but a similar phenomenon has not previously been shown in mice. To test for induction of oral tolerance to the uveitogenic antigen IRBP groups of EAU-susceptible B10.A mice were fed 0.2 mg IRBP per dose either 3 or 5 times, approximately every other day. Control animals were given five doses of phosphate buffered saline (PBS) or 0.2 mg ovalbumin (OVA) on the same days. On the third day after the last feeding, all groups were challenged by immunization with a uveitogenic dose of IRBP. Eyes collected 21 d after the uveitogenic challenge showed significantly reduced EAU incidence and scores in animals fed IRBP five times before immunization, but three feedings of IRBP were not protective (Table I). The 5× feeding regimen appeared to afford long-term protection, rather than merely to change the kinetics of disease. Whereas onset of EAU did not appear substantially different in protected and unprotected groups (fundus examination on day 14 showed that onset had occurred in all cases, although symptoms were milder in 5×-fed mice), eyes harvested 28 and 35 d after immunization still showed substantially less pathology in the protected group. The absence of protection with the 3× feeding regimen did not appear to be due solely to the difference in the total amount of IRBP given, because another group of mice fed 3 × 0.5 mg IRBP was also not protected (data not shown). It is possible that not only the number, but also the shorter time interval over which the three feedings were administered may have constituted a contributing factor.

Parenteral administration of IL-2 potentiates oral tolerance. A single intraperitoneal injection of 1,000 U of IL-2, given at the time of immunization, converted the nonprotective 3× feeding regimen to a protective one. The reduction in disease scores was approximately equal to that induced by feeding IRBP five times (Fig. 1). IL-2 treatment given to mice that received the protective 5× feeding regimen did not appear to lower the disease scores any further; if anything, these mice were less well protected than the respective 5× group that did not receive IL-2. The same dose of IL-2 given at the time of IRBP challenge to control animals had no effect on subsequent disease. As seen

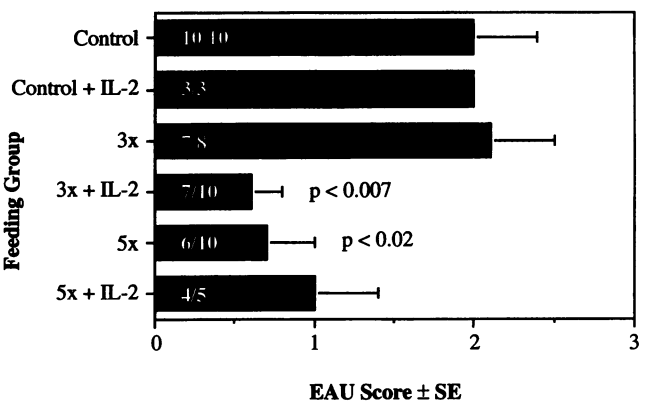

Figure 1. IL-2 treatment converts the nonprotective 3× feeding regimen into a protective one. Mice were fed with 0.2 mg of IRBP per dose either 3 or 5 times and the indicated groups received 1,000 U of IL-2 at the time of challenge. Control animals were fed 5× with OVA or PBS. On the third day after the last feeding all groups were immunized with a uveitogenic regimen of IRBP. The scores are calculated as average of all animals in the group. Incidence (as positive/total) is shown within the bars. Statistically significant *P* values (compared to control) are shown next to the bars. The results are a composite of two similar experiments.

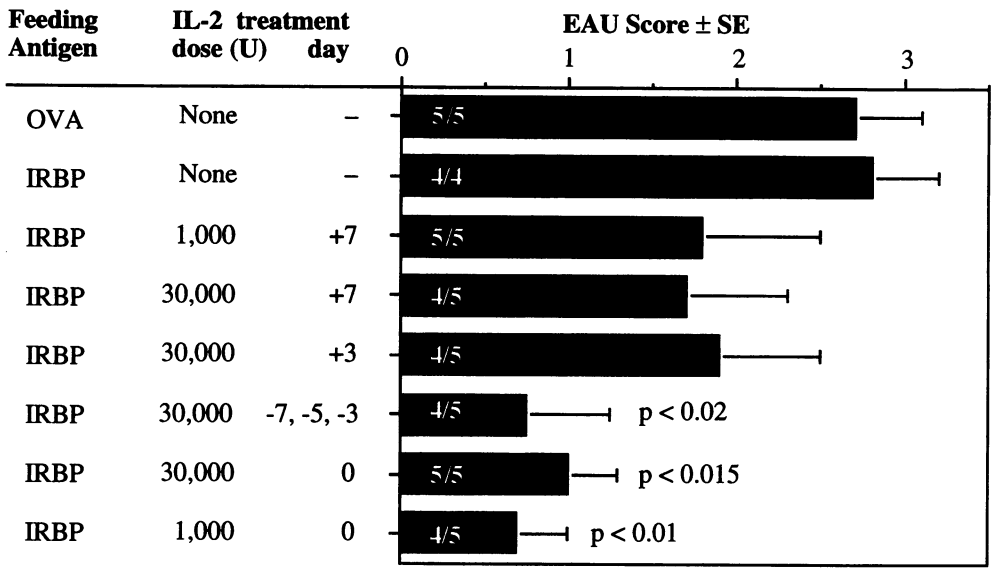

Figure 2. Effect of the dose and time of IL-2 administration on induction of protection from EAU. Mice were fed three times with 0.2 mg of IRBP per dose. Control animals were fed OVA. All groups were subsequently immunized with a uveitogenic regimen of IRBP (day 0). IL-2 treatment was given at the indicated doses and times. EAU scores are calculated as average of all animals in the group. Incidence (positive/total) is shown within the bars. Statistically significant *P* values are shown next to the bars.

previously with the 5× feeding, the protection achieved was prolonged: eyes harvested 42 d after immunization still showed considerably milder EAU in the 3× + IL-2 mice (score 0.6±0.5, incidence 3/3) compared to controls (score 3.8±0.3, incidence 5/5).

We studied the effect of dosage and timing of the IL-2 administration on induction of protection from EAU (Fig. 2). A single high dose of 30,000 U of IL-2 given at the time of uveitogenic challenge (day 0) resulted in a similar level of protection as the dose of 1,000 U. Three consecutive doses of 30,000 U each injected at the time of feeding (days −7, −5, and −3) had a protective effect equivalent to that of a single 30,000 U dose given at the time of challenge. In contrast, 30,000 U of IL-2 given 3 or 7 d after IRBP challenge, or 1,000 U given 7 d after challenge, failed to significantly protect from disease, although a distinct trend towards lower scores was apparent. Delayed hypersensitivity responses to IRBP, evaluated 21 d after immunization, closely followed the disease pattern in the various groups (data not shown).

These results indicate that, within the range of doses of IL-2 and IRBP tested here, (*a*) the best protection from EAU was achieved when the IL-2 treatment was given on, or before, day 0. It remains to be dissected whether the determining factor was temporal proximity to feeding or the timing relative to immunization; (*b*) the IL-2 treatment is effective across a wide range of doses, as 1,000 U in a single dose and 90,000 U divided into three consecutive doses were equally effective; and (*c*) the treatment has a wide margin of safety, since there was no enhancement of disease with any dose or timing of IL-2 administration. It is also worth noting, in view of the well known toxic effects of systemically administered IL-2, that the dose capable of potentiating oral tolerance is considerably below the toxicity range reported for mice, rats and humans (18–20).

The mechanism of IL-2–induced potentiation of tolerance appears to involve induction of anti-inflammatory cytokines. One of the postulated mechanisms of oral tolerance is by anti-inflammatory cytokines such as TGF-β and IL-4 (7, 8). Because it is likely that the first contact of immunocompetent cells with antigens taken up through the gut occurs in Peyer's Patches (PP), we measured production of IL-4, IL-10, and TGF-β by PP cells of mice fed the 3× regimen with or without IL-2 (Fig. 3). EAU scores developed by these mice are shown in Fig. 2. PP cells of protected groups produced significantly more ($P < 0.05$) TGF-β and IL-4 than did PP cells of nonprotected groups, and only protected animals produced IL-10. In the case of TGF-β the difference was particularly marked when total TGF-β was measured (after heat activation of the supernatant), although the active form by itself also tended to be higher in the protected, as well as the high-dose IL-2 treated, groups. It is uncertain what is the significance of the quantity of active vs. total forms of TGF-β measured directly after secretion, since the site of action of this cytokine could be far away from the Peyer's Patch, and the nonactivated form might become activated there. Mice that had been fed OVA before IRBP challenge had identical cytokine profiles to mice that received the nonprotective 3× feeding (Fig. 3 and unpublished data).

The finding that production of antiinflammatory cytokines was higher across the board in the protected groups, strongly suggests that it had a role in determining the clinical outcome. We interpret these results to mean that a population(s) of regulatory cells was induced by the 3× feeding regimen in the PP. An appropriately timed treatment with IL-2 was able to enhance this regulatory cell population(s) sufficiently to obtain a net protective effect from EAU.

The mechanism of tolerance induced by the 3×-feeding + IL-2 regimen may differ from tolerance induced by the 5× feeding regimen. The amount of IL-4 and (total) TGF-β were assayed in supernatants of PP cells from mice orally tolerized to IRBP using the different regimens. EAU scores of these mice are shown in Fig. 1. As before, PP cells of the protected 3× + IL-2 group produced high amounts of TGF-β and IL-4. In contrast, PP cells of mice protected by the 5× feeding regimen produced minimal amounts of these cytokines, which was not increased by treatment of the 5× fed mice with IL-2 (Fig. 4).

These findings can be interpreted as indicative of a difference in the mechanism of tolerance induced by these two feeding regimens. We propose that protection from EAU induced by the 3× + IL-2 regimen involves production of suppressive cytokines, whereas the 5× feeding regimen, which does not appear to result in suppressive cytokine production even after

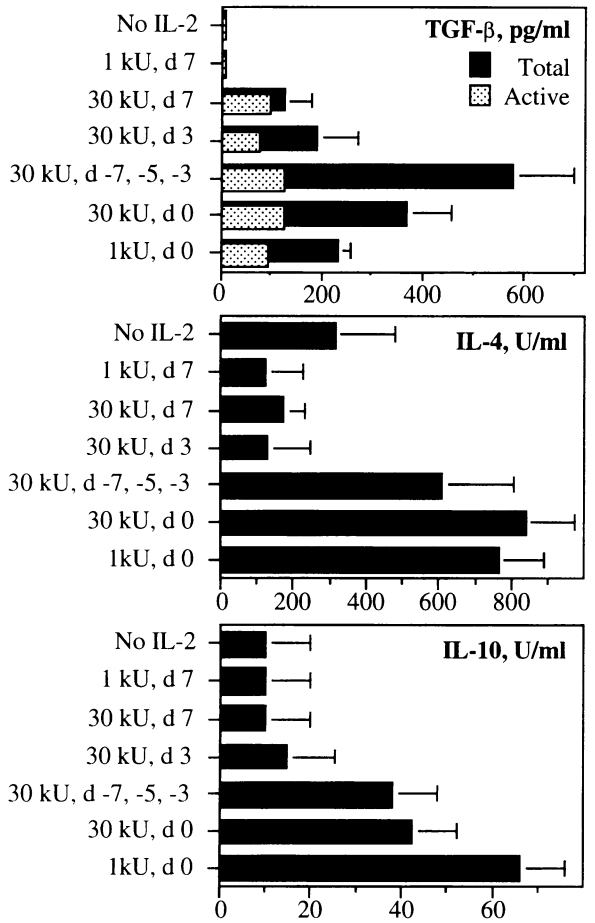

Figure 3. Peyer's Patch cells of protected mice produce elevated amounts of TGF-β, IL-4 and IL-10. Mice received three feedings of IRBP and a uveitogenic IRBP challenge. IL-2 treatment was given at the indicated doses and times. PP cells were collected 21 d after challenge and cultured with IRBP. Cytokines were assayed in culture supernatants as described in Methods.

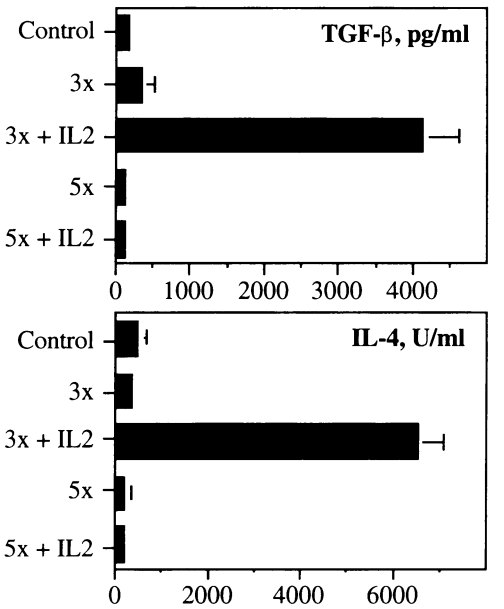

Figure 4. Mice protected by the 3× + IL-2 feeding regimen have a different cytokine profile than mice protected by the 5× regimen. Mice received 3 or 5 feedings of IRBP and a uveitogenic IRBP challenge. IL-2 treatment was 1,000 U on the day of challenge. PP cells collected 21 d after challenge were cultured with ConA. Cytokines were assayed in culture supernatants as described in Methods (IL-10 was not measured in this experiment).

IL-2 treatment, might conceivably involve silencing of the uveitogenic cells through a mechanism such as anergy or deletion, as demonstrated by others under certain experimental conditions (9–11). Although direct positive support for this hypothesis awaits further experimentation, such an interpretation is compatible with the observation that IL-2 treatment of the 5×-fed animals was unable to enhance the level of protection from EAU that was achieved by feeding alone, and may even have partly counteracted that protection. We infer from our findings that: (*a*) 5× fed mice had no regulatory cells that could have been stimulated by IL-2, and (*b*) a state of anergy may have been partially abrogated by the IL-2 treatment. Others have presented evidence that oral tolerance in the rat models of EAE and EAU can involve either anergy or active suppression, and the dominant mechanism may depend on the dose of the antigen fed, with high doses favoring anergy and low doses favoring suppression (7–10). We show evidence that oral suppression of EAU in mice might also be achieved by either of these two mechanisms, and that the mechanism preferentially evoked may be determined not solely by the dose and duration of feeding but, as in this case, by an appropriately timed dosing with the autocrine T cell growth factor IL-2. Which of the two tolerance mechanisms would be more beneficial in the long term from a therapeutic point of view, remains to be determined.

In conclusion, immunomodulation achieved by combining IL-2 treatment with oral administration of antigen could have practical clinical implications as a safe and effective way of potentiating oral tolerance. It is very encouraging that the therapeutic window with respect to both efficacy and safety is extremely broad, and the effective dose is well within the nontoxic range. Other routes of IL-2 administration, i.e., intravenous, subcutaneous, and oral, should be investigated. Future studies with clinical orientation should address the use of IL-2 for potentiation of tolerogenic oral regimens initiated after onset of disease.

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