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Suppression of Experimental Autoimmune Uveoretinitis by Dietary Calorie Restriction

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Purpose: To investigate the inhibitory effect of dietary calorie restriction on experimental autoimmune uveoretinitis (EAU) in rats, and its mechanism.

Methods: Lewis rats were maintained on a 50% calorie-restricted diet for 2 months or 6 months. The control group was maintained on a 90% ad libitum intake for the same length of time. Experimental autoimmune uveoretinitis was elicited in both groups by immunization with an inter-photoreceptor retinoid-binding protein or its peptide. Rats in both groups were examined clinically, histopathologically, and immunologically.

Results: The severity of EAU was milder in the restricted diet group than in the control group. In EAU rats, production of interferon-γ (IFN-γ) in eyes and of IFN-γ and tumor necrosis factor-α in draining lymph node cells was significantly lower in the restricted diet group than in the control group.

Conclusions: Our results indicate that a calorie-restricted diet suppresses the development of EAU. The suppressed Th1-dependent immunological response is one of the reasons for the mildness of EAU in the calorie-restricted diet group of rats. **Jpn J Ophthalmol 2001;45:46–52**

Key Words: Cytokine, dietary calorie restriction, experimental autoimmune uveoretinitis, immunoregulation.

Introduction

A calorie-restricted diet without malnutrition prolongs the life span and healthy period of a number of long-lived and autoimmune-disease-prone short-lived strains of rodents.[1–3] Recently, fasting and vegetarian diets have been reported to be useful adjuncts to the medical treatment of rheumatoid arthritis patients.[4]

Experimental autoimmune uveoretinitis (EAU) is a CD4 T-cell-mediated autoimmune disease that has been widely used as a model for human intraocular inflammatory disorders of unknown etiology, and it is easily induced by injecting retinal autoantigen.[5] In our previous study,[6] Lewis rats were maintained on 50% or 25% calorie-restricted diets (50% or 75% of the caloric level in the control rats) for 2 weeks or 4 weeks starting at 7 weeks of age. Experimental autoimmune uveoretinitis was then induced by immunization with inter-photoreceptor retinoid-binding protein (IRBP). Experimental autoimmune uveoretinitis was significantly suppressed when the level of calorie intake was decreased, and the duration of the restricted diet was prolonged. A 50% calorie restriction level for 4 weeks was sufficient to suppress EAU. Delayed type hypersensitivity against IRBP was also suppressed in the restricted diet group.

In the present study, the calorie-restricted diet was imposed earlier in life and continued for a longer period, and its suppressive effect on EAU was assessed. To investigate the mechanism of the suppression of EAU by calorie restriction, peripheral white blood cell (WBC) counts, antigen-specific antibody (Ab) IgG isotype assays, the proliferative response of lymph node (LN) cells in response to primary immunized antigen, and cytokine production were also studied.

To our knowledge, no previous reports other than our own[6] have examined the effects of calorie-

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restricted diet in an animal model of T-cell-mediated autoimmune disease. The results of this study are expected to provide new insights into the immunological effects of calorie restriction in animal models of autoimmune disease.

Materials and Methods

Diets

Male Lewis rats were fed semi-purified diets providing either 10.3 g/day (control group) or 5.6 g/day (restricted diet group), with the latter receiving 50% of the daily calorie intake of the control group (Table 1). Carbohydrates accounted for the nutritional difference between the two groups in total caloric intake. The control group was maintained on a 90% ad libitum intake. Age at the initiation of the control diet was 4 weeks. The restricted diet group of rats was maintained on a diet constituting 50% of the control group diet for 2 months or 6 months before immunization with IRBP or its uveitopathogenic peptide; then maintained in the same way for the remainder of the experimental period. All procedures conformed to the principles embodied in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Immunization

The IRBP was purified from bovine retina as reported previously.[7] Uveitopathogenic peptide derived from bovine IRBP, ie, R16,[8,9] located from 1177–1191, was synthesized as reported previously.[8,9] The IRBP or R16, emulsified (1:1) in complete Freund's adjuvant (Iatron, Tokyo), was injected into one hind footpad of rats at a dose of 20 μg (IRBP) or 10 μg (R16) in a volume of 0.1 mL. A suspension of *Bordetella pertussis* (Wako Pure Chemical, Osaka) was injected intravenously, 2×10^{10} organisms/rat, concomitantly with the antigen emulsion.

Clinical and Histopathological Findings

Rats in each group (the restricted diet group and the control group) were immunized with IRBP or R16 and euthanized on day 21 post-immunization. Disease progression was followed by daily slit-lamp biomicroscopy examination. The first day on which definite inflammation was observed in at least one eye was defined as the day of onset. The period from the day of onset to the day when inflammation had resolved in both anterior and posterior chambers was defined as the clinical disease duration.

The eyes were enucleated and fixed in 2.5% buffered glutaraldehyde for 4 hours, and then transferred into 10% buffered formaldehyde until processed. Tissue sections were prepared and stained with hematoxylin and eosin. Grading of clinical and histopathological severity on a scale of 0 to 5 as described previously[10] was used.

Stages of EAU

In this experiment, EAU stages were defined as follows: early-stage EAU (day 11 or day 12 post-immunization), established-stage EAU (day 14 post-immunization), and late-stage EAU (day 21 or day 23 post-immunization).

Peripheral WBCs

Peripheral WBCs were counted twice by standard procedures: before immunization with IRBP and in established-stage EAU in the 50% 6-month restricted diet group and the control group.

Assay for IRBP-Specific Ab IgG Isotypes

Serum was obtained from rats in the 50% 2-month restricted diet group and the control group in late-stage EAU after immunization with IRBP. Serum anti-IRBP IgG1 and IgG2b isotype levels were determined by enzyme-linked immunosorbent assay (ELISA), as previously described.[11,12]

Table 1. Composition of Diets

Ingredient	Control Group (%/Weight)	Restricted Diet Group (%/Weight)
Cornstarch	49.8	24.7
Casein	23.2	39.7
Dextrose	10.0	5.6
Corn oil	3.0	5.0
Cellulose	5.0	8.9
Amylopectin	1.0	1.9
Mineral mixture	7.0	12.3
Vitamin mixture	1.0	1.9
Kcal/100 g	360.0	327.6

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Table 2. Six-Month Restricted Diet Experiment

	Control Group (n = 8)	Restricted Diet Group (n = 8)
Body weight (g)		
Initial day	100 ± 7.2	100 ± 7.4
Day of immunization	280 ± 6.0	130 ± 11.2
White blood cells ($\times 10^2/mm^3$)		
Before immunization	37 ± 5.0	22 ± 1.6*
14 days post-immunization	85 ± 4.0	54 ± 24.1*
Clinical observation of EAU		
Incidence	8/8	5/8
Day of onset	11 ± 1.1	13 ± 1.0†
Disease duration (days)	5.0 ± 1.2	2.0 ± 1.1†
Clinical severity (score)	1.4 ± 0.5	0.6 ± 0.5†
Histological severity (score)	1.9 ± 0.4	0.6 ± 0.5*

EAU: Experimental autoimmune uveoretinitis. Restricted diet duration before immunization was 6 months; 20 μg of inter-photoreceptor retinoid-binding protein was administrated.
*$P < .01$, †$P < .05$.

Lymphocyte Proliferation Assay

Rats from each group (the 50% 2-month restricted diet group and the control group) were euthanized at established-stage EAU after immunization with R16, and their draining LN were removed. A single-cell suspension was obtained, and the cells were cultured in 96-well flat-bottomed plates as described previously.[10] The cells were stimulated with various concentrations of R16 or phytohemagglutinin A (PHA) (Sigma, St. Louis, MO, USA), and lymphocyte proliferation responses were determined as described previously.[10]

Cytokine Production

Rats in each group (the 50% 2-month restricted diet group and the control group) were immunized with R16 and euthanized in each EAU stage. The draining LN cells in each EAU stage were cultured with various concentrations of R16 or PHA for 48 hours. Enucleated eyes in early-stage EAU and late-stage EAU were homogenized separately in 0.5 mL of 0.01 M phosphate-buffered saline. The supernatants from cultured LN cells and homogenized eyes were collected and stored at −20° until assayed. interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) were determined by ELISA according to the manufacturer's recommendation (BioSource International, Camarillo, CA, USA). Cytokine values are expressed as pg/mL from duplicate samples using the standard curves.

Statistical Analysis

Data are reported as mean ± SE. Statistical analysis was performed by the Mann–Whitney *U*-test, and *P*-values <.05 were considered significant.

Results

Growth of Rats

Throughout the experimental period, all rats ate all the food provided each day. Changes in body weight are shown in Tables 2 and 3.

Table 3. Two-Month Restricted Diet Experiment

	Control Group (n = 10)	Restricted Diet Group (n = 10)
Body weight (g)		
Day of immunization	194 ± 6.0	111 ± 7.8
Clinical observation of EAU		
Incidence	10/10	9/10
Day of onset	9.0 ± 0	11.0 ± 2.5*
Histological severity (score)	3.5 ± 0.8	1.3 ± 1.2†

EAU: Experimental autoimmune uveoretinitis. Restricted diet duration before immunization was 2 months; 10 μg of R16 was administrated.
*$P < .05$, †$P < .01$.

EAU Incidence and Day of Onset

All rats in the control group developed EAU. In the 50% 6-month restricted diet group, 3 of the 8 rats did not develop EAU (Table 2), and in the 50% 2-month restricted diet group, one of the 10 rats did not (Table 3). Rats in both the 50% 6-month restricted diet group and the 50% 2-month restricted diet group showed a significantly later onset than any of the control group rats (Tables 2 and 3).

Clinical Duration and Severity of EAU

Rats in the restricted diet group had a significantly shorter period of clinically apparent disease than the control group (Table 2). Rats in the restricted diet group indicated a significantly milder clinical and histopathological grade than the control group (Figures 1 and 2, and Table 2).

WBC

Rats in the restricted diet group had significantly lower WBC counts than the control group before immunization and in established-stage EAU (Table 2).

Assay for IRBP-Specific Ab IgG Isotypes

In late-stage EAU, the anti-IRBP IgG1 level was significantly higher in the restricted diet group than in the controls (Figure 3). However, the anti-IRBP IgG2b level was significantly lower in the restricted diet group (Figure 3).

Lymphocyte Proliferation Assay

In the established EAU stage, draining LN cells from the restricted diet group proliferated in response to each concentration of R16 (Figure 4). The

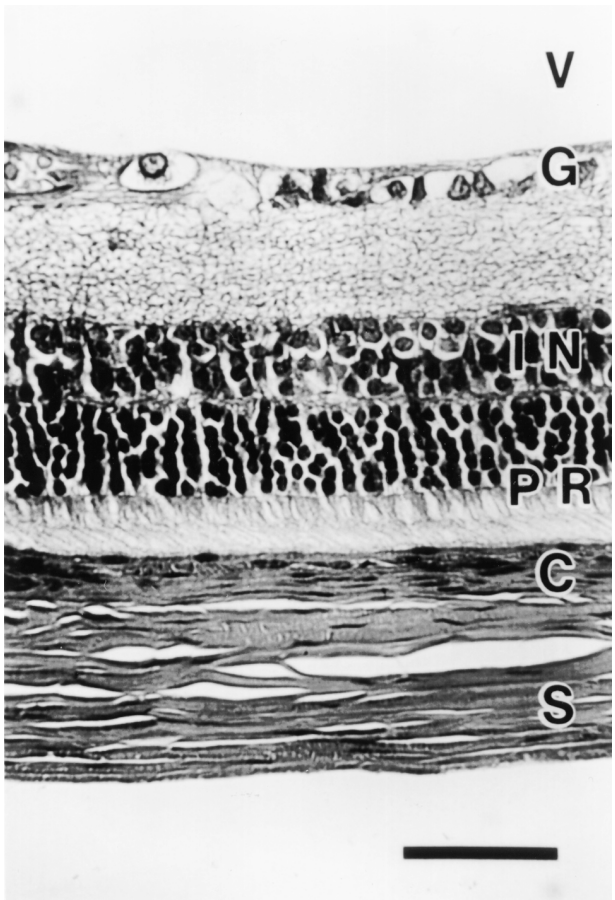

Figure 1. Histopathological changes in 50% 6-month calorie-restricted diet group 21 days after immunization with inter-photoreceptor retinoid-binding protein. Experimental autoimmune uveoretinitis is not observed. V: vitreous, G: ganglion cell layer, IN: inner nuclear layer, PR: photoreceptor layer, C: choroid, S: sclera. Bar = 5 μm.

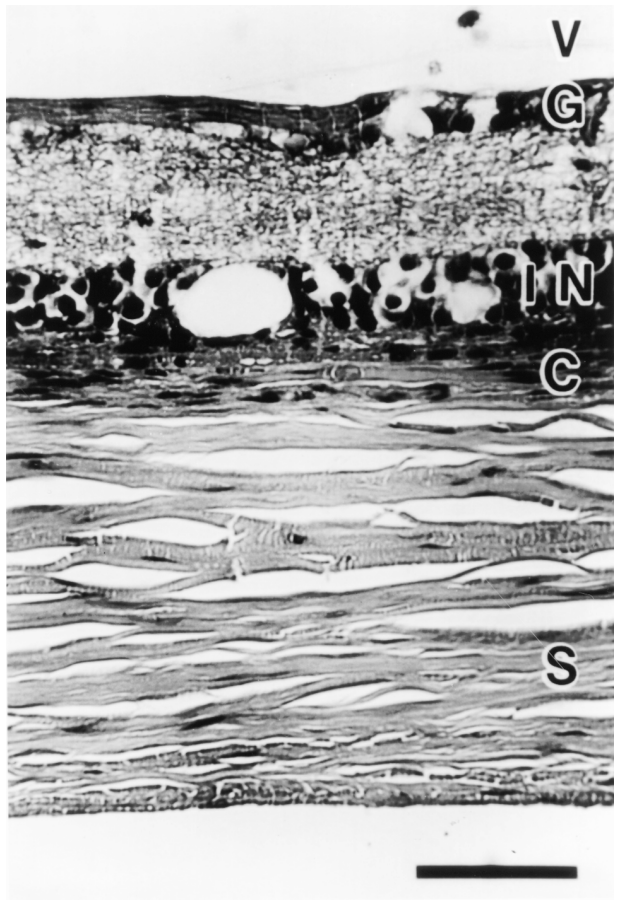

Figure 2. Histopathological changes in control group 21 days after immunization with inter-photoreceptor retinoid-binding protein. Photoreceptor layer is completely destroyed and inner nuclear cell layer is partially destroyed by inflammation. V: vitreous, G: ganglion cell layer, IN: inner nuclear layer, C: choroid, S: sclera. Bar = 5 μm.

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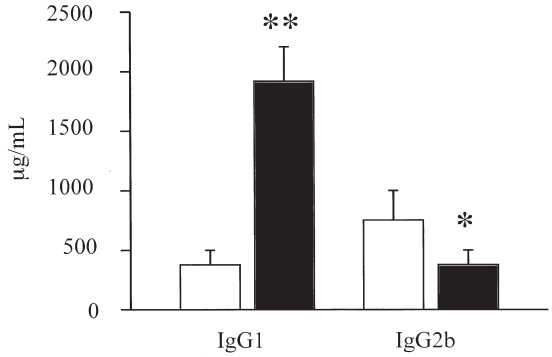

Figure 3. Serum levels of anti-inter-photoreceptor retinoid-binding protein Ig1 and IgG2b isotypes. Each bar represents SE. □: Control group (n = 7). ■: Restricted diet group (n = 7).**$P < .01$, *$P < .05$.

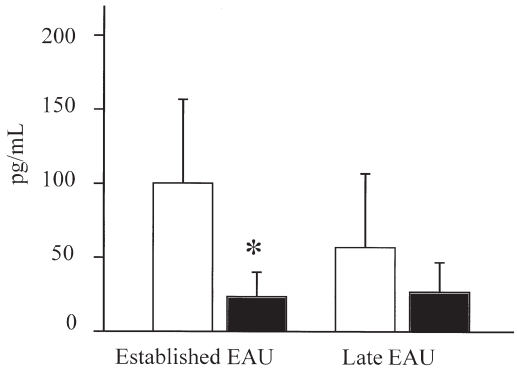

Figure 5. Production of interferon-γ in experimental autoimmune uveoretinitis (EAU) eyes. Each bar represents SE. □: Control group (n = 5). ■: Restricted diet group (n = 5). *$P < .05$.

proliferative responses to PHA were 8682 ± 1225 cpm in the control group and 9166 ± 2110 cpm in the restricted diet group without a significant difference between the two groups.

Cytokine Production in Eyes

Production of IFN-γ in both early- and late-stage EAU was lower in the restricted diet group than in the control group (Figure 5). There was no significant difference between the two groups in the production of TNF-α (Table 4).

Cytokine Production in LNs

Production of IFN-γ and TNF-α after stimulation with R16 was lower in the restricted diet group, at every EAU stage tested, than in the control group (Figures 6 and 7). IFN-γ and TNF-α increased after stimulation with PHA; however, no difference was observed between the two groups (Figures 6 and 7). Production of IFN-γ peaked at 1 mg/mL of R16, and was lower in the restricted diet group than in the control group at all concentrations of R16 tested (Figure 8).

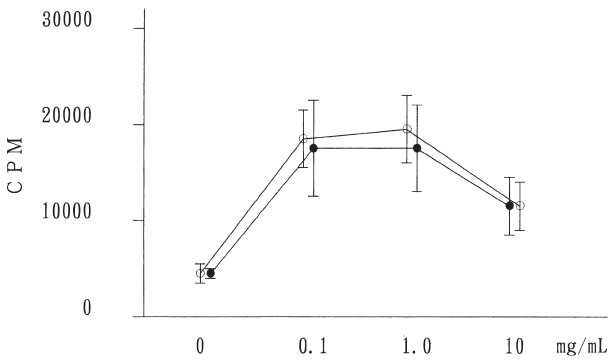

Figure 4. Lymphocyte proliferation assay of draining lymph node cells after stimulation with primary immunized antigen (R16). Each bar represents SE. ○: Control group (n = 5). ●: Restricted diet group (n = 5). CPM: counts per minute.

Discussion

In the present experiment, the rats in the control group were not maintained on ad libitum intake, but on 90% of ad libitum intake, in order to maintain the same caloric intake in all rats.

In our previous study,[6] a 50% 4-week restricted diet was adequate to suppress EAU in Lewis rats. We expected suppression of EAU to become more marked with the imposition of a restricted diet earlier in life and for a longer period, and a 50% 6-month restricted diet was therefore imposed at weaning, ie, at 4 weeks of age in the present study. Although the 50% 6-month restricted diet did not completely suppress EAU, 3 of the 8 rats did not develop clinical and histopathological EAU.

Table 4. Tumor Necrosis Factor-α (TNF-α) of Experimental Autoimmune Uveoretinitis (EAU)

	TNF-α (pg/mL)*
Early EAU	
Control group	63.2 ± 3.3
Restricted diet group	61.6 ± 4.3
Late EAU	
Control group	77.4 ± 42.3
Restricted diet group	62.8 ± 7.5

*n = 5.

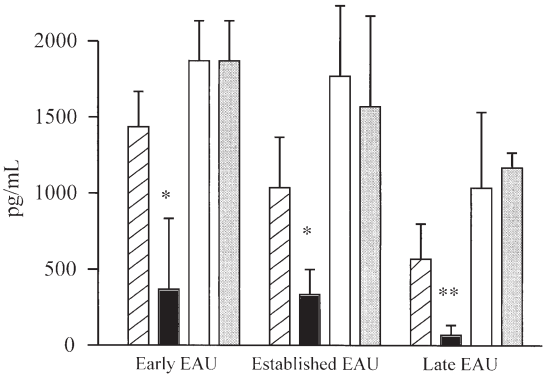

Figure 6. Production of interferon-γ by cells from lymph nodes of rats in each stage of experimental autoimmune uveoretinitis (EAU). Each bar represents SE. ▨: Control group stimulated with 10 mg/mL of R16 (n = 5). ■: Restricted diet group stimulated with 10 mg/mL of R16 (n = 5). □: Control group stimulated with phytohemagglutinin A (PHA) (n = 5). ▩: Restricted diet group stimulated with PHA (n = 5). $^{**}P < .01$, $^{*}P < .05$.

Peripheral WBC counts have been shown to be reduced by dietary calorie restrictions in rodents,[2,13] and our data showed a similar tendency. However, decreased WBCs alone did not seem to explain the suppression of EAU, because a restricted diet significantly ameliorated immuno-senescent phenomena.[14–18]

Previous reports have presented evidence implicating Th1 effector cells in the pathogenesis of EAU,[19,20] and the induction of the Th2-type response appears to be protective.[21] Production of

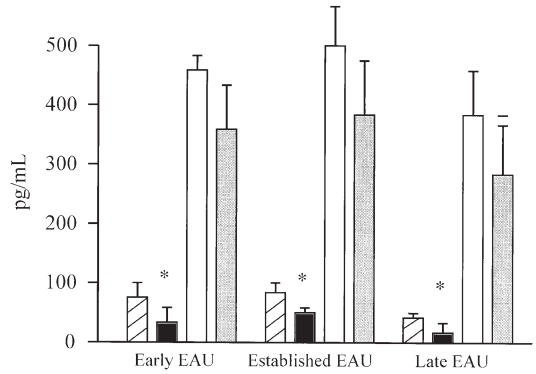

Figure 7. Production of tumor necrosis factor-α by cells from lymph nodes of rats in each stage of experimental autoimmune uveoretinitis (EAU). Each bar represents SE. ▨: Control group stimulated with 10 mg/mL of R16 (n = 5). ■: Restricted diet group stimulated with 10 mg/mL of R16 (n = 5). □: Control group stimulated with phytohemagglutinin A (PHA) (n = 5). ▩: Restricted diet group stimulated with PHA (n = 5). $^{*}P < .05$.

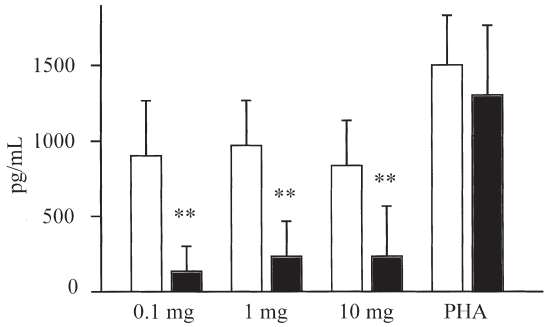

Figure 8. Production of interferon-γ by cells from lymph nodes of experimental autoimmune uveoretinitis (EAU) rats. Each bar represents SE. □: Control group stimulated with various concentration of R16 (n = 5). ■: Restricted diet group stimulated with various concentrations of R16 (n = 5). PHA: stimulated with phytohemagglutinin A (n = 5). $^{**}P < .01$.

IFN-γ and TNF-α and the delayed type hypersensitivity response are Th1-dependent.[22,23] Delayed type hypersensitivity to the immunized antigen (IRBP) was significantly suppressed in the restricted diet group.[6] In the present experiment, production of IFN-γ in the eyes and in the LN cells of EAU rats and of TNF-α in the LN cells of EAU rats were significantly lower in the restricted diet group. These results may reflect the mildness of the EAU in the restricted diet group. However, the levels of IFN-γ and TNF-α increased considerably after being stimulated with PHA. Thus, the IFN-γ or TNF-α production and secretion functions were not impaired in the restricted diet group, but the afferent pathway of antigen priming may have been suppressed. In the rat, the IgG2b isotype is Th1-dependent, and the IgG1 isotype is Th2-dependent.[21] The IRBP-specific IgG1/IgG2b ratio was higher in the restricted diet group. These results suggested that Th2 function was increased in the restricted diet rats. Probable elevation of the Th2 function may partially explain the result that LN cells from the restricted diet group proliferated significantly in response to R16.

Another possible mechanism of the suppression of EAU by dietary calorie restriction is elevated endogenous glucocorticoid levels. Serum glucocorticoid levels in the calorie-restricted diet animals were three times higher than in the control animals.[24] Ramierz et al[25] have reported that when lymphocytes from naive rats were stimulated with glucocorticoids, cytokines bearing Th1 were diminished, but expression of Th2-type cytokines was increased. In addition, proliferation of mitogen-stimulated lymphocytes increased after glucocorticoid treatment.[25] These results partially

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agreed with our own. The effect of glucocorticoids on the modulation of Th1- and Th2-cytokine production, however, has been controversial.[26–28] Moynihan et al[26] reported that glucocorticoids suppressed both IL-4 (Th2-cytokine[23]) and IFN-γ production in spleen cells. They[26] speculated that stress-induced alteration of the immune response was modulated not only by glucocorticoids but by other factors as well. Effros et al[17] reported that a restricted diet augmented the immune reaction against viral antigen (lymphocyte proliferation, Ab titer). Thus, elevated endogenous glucocorticoid levels cannot alone explain the suppression of EAU by calorie-restricted diet.

Further study is necessary to investigate the mechanism of the suppressive effect on EAU by dietary calorie restriction. As a calorie-restricted diet has few side effects and can be maintained simply, we anticipate that it will become an adjunctive therapy for human endogenous uveitis of unknown etiology.

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