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Differentiation of Th1 and Th2 Cells in Lymph Nodes and Spleens of Mice During Experimental Autoimmune Uveoretinitis

Masaru Takeuchi, Hidetoshi Yokoi,
Rintaroh Tsukahara, Jun-ichi Sakai and Masahiko Usui

Department of Ophthalmology, Tokyo Medical University, Tokyo, Japan

Purpose: Experimental autoimmune uveoretinitis (EAU) is a T-cell–mediated autoimmune disease that can be elicited in susceptible rodent strains by immunization with a retinal autoantigen, such as interphotoreceptor retinoid-binding protein (IRBP). In this study, we investigated whether there is a correlation between inflammation in the eye and T-helper (Th)1- and Th2-type responses in the lymph nodes and the spleen after immunization of B10.A mice with IRBP.

Methods: B10.A mice were immunized with IRBP emulsified with complete Freund's adjuvant (CFA), and eyes were then enucleated for histological examination of EAU at 1, 2, 4, 6, or 8 weeks after immunization. In addition, lymph node cells and spleen cells were collected, and cultured with IRBP to measure T-cell proliferation responses and Th1-type (interleukin [IL]-2, interferon [IFN]-γ), Th2-type (IL-4, IL-10) cytokine production.

Results: Pathologically, severe ocular inflammation occurred 2 weeks after IRBP immunization, persisted for 2 weeks, and then gradually resolved. Interleukin-2 and IFN-γ production were observed in draining lymph node cells at 1 and 2 weeks after IRBP immunization. Those responses then diminished, whereas IFN-γ production by spleen cells was observed from week 1, peaked at week 4, and gradually decreased. Alternatively, significant production of IL-4 or IL-10 by draining lymph node cells was not detected at any time point. Both IL-4 and IL-10 production by spleen cells was observed at week 6.

Conclusions: Th1-type responses were observed early in draining lymph nodes, then in the spleen after IRBP immunization. The levels of IFN-γ production by spleen cells reflected the severity of EAU, confirming their pathogenic role in this disease. Th2-type responses were generated in the spleen only as the disease receded, suggesting a role for Th2 cells in the spontaneous termination of EAU. **Jpn J Ophthalmol 2001;45:463–469** © 2001 Japanese Ophthalmological Society

Key Words: Autoimmune disease, experimental autoimmune uveoretinitis, immunoregulation, Th1 cells, Th2 cells.

Introduction

Experimental autoimmune uveoretinitis (EAU) is a T-cell–mediated, organ-specific autoimmune disease that is induced in susceptible animals by immunization with retinal antigen (Ag), such as interphotoreceptor retinoid-binding protein (IRBP), or by the adoptive transfer of $CD4^+$ T lymphocytes specific to the Ag.[1,2] The ensuing inflammatory process results in massive and irreversible destruction of the retinal photoreceptor cells and consequent loss of vision. Experimental autoimmune uveoretinitis is used to represent a number of human ocular inflammatory diseases of a presumed autoimmune nature that exhibit pathology similar to EAU and can be accompanied by the presence of immunologic responses to ocular Ag.[3]

It has been revealed that T helper (Th) cells comprise functionally distinct subsets that are characterized by the patterns of lymphokines they produce following ac-

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Correspondence and reprint requests to: Masaru TAKEUCHI, MD, PhD, Department of Ophthalmology, Tokyo Medical University, 6-7-1 Nishishinjuku, Shinjuku-ku, Tokyo 160-0023, Japan

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tivation.[4,5] In mice, Th1 cells secrete interleukin (IL)-2, interferon (IFN)-γ, and tumor necrosis factor (TNF)α, and support macrophage activation, delayed-type hypersensitivity (DTH) responses, and immunoglobulin (Ig) isotype switching to IgG2a, whereas Th2 cells secrete IL-4, IL-5, IL-6, IL-10, and IL-13, and provide efficient help for B-cell activation, for switching to the IgG1 and IgE isotypes, and for antibody production. Th0 cells are characterized by their production of cytokines of both the Th1 and Th2 types, and are thought to be obligatory precursors of Th1 and Th2 cells.

It has been found that the effector T cells in EAU possess a Th1-like phenotype (high IFN-γ, low IL-4), and this result is compatible with that found in other animal models of autoimmune diseases, such as experimental autoimmune encephalomyelitis, diabetes in NOD mice, and adjuvant-induced arthritis.[6,7] Moreover, genetic susceptibility to EAU is associated with a dominant Th1-type response to the uveitogenic retinal antigen.[8–10] By contrast, Th2 cells have been thought to play a protective role in EAU. In the absence of IL-12, which imposes Th cell differentiation into the Th1-phenotype, IRBP-specific Th2 cells develop, and in mice with this phenotype, EAU fails to occur.[11] Particularly, immunization with an IRBP-derived peptide that induces IRBP-specific Th2 cells prevents the development of EAU in rat-thymus grafted nude mice that otherwise develop EAU spontaneously. Recently, Th1- and Th2-type cytokine mRNA expression has been examined in the retina of Lewis rats and mice with EAU. It was reported that IL-2 and IFN-γ mRNA expression peaked during the active phase of the disease and declined in parallel with disease resolution. Interleukin-10 mRNA expression was found to increase during the late stage of EAU.[12,13] These results indicate that activation of retinal Ag-specific Th2-type cells may be involved in the subsidence of inflammation during the natural process of EAU. In this study, to investigate where and when Th1- and Th2-type cells specific for IRBP were generated postimmunization with IRBP, we monitored pathological change in the eye and Th1- and Th2-type cytokine production by lymph node cells draining the immunization site and spleen cells after immunization. Our results indicate that (1) IRBP-specific Th1-type cells are activated in draining lymph nodes in the induction phase of EAU and this cell activation moves to the spleen during the acute phase; (2) the levels of IFN-γ production by spleen cells is compatible with the severity of this disease in the eye; and (3) activation of Th2-type cells, which produce IL-4 and IL-10, occurred in the spleen as Th1 activity waned, and correlated with remission of the disease.

Materials and Methods

Animals

Pathogen-free female B10.A mice at 6–8 weeks of age were purchased from Charles River (Hino). All mice were housed under conventional conditions in the specific pathogen-free facility of Tokyo Medical University. Mice were treated according to the Association for Research in Vision and Ophthalmology (ARVO) Resolution on the Use of Animals in Research.

Reagents

Interphotoreceptor retinoid-binding protein was isolated from bovine retinas, as described previously,[14] by Con A-sepharose affinity chromatography and high performance liquid chromatography. The IRBP preparations were aliquoted and stored at −70°C. *Mycobacterium tuberculosis* strain H37RA and CFA were purchased from Difco (Detroit, MI, USA); *Bordetella pertussis* bacteria was obtained from Wako Pure Chemical Industries (Osaka).

Immunization

Mice were immunized by a hind footpad injection with 100 μg/mL of IRBP in 0.2 mL emulsion (1/1 v/v) with CFA that had been supplemented with *M. tuberculosis* to the final concentration of 2.5 mg/mL. An additional adjuvant, *B. pertussis* bacteria, was injected intraperitoneally, 10^{10} organisms/mouse, concurrently with the immunizations.

Lymphocyte Proliferation Assay

Single cell suspensions were prepared from the inguinal lymph nodes or the spleen. Red blood cells were lysed with Tris-NH_4CL. Triplicate 0.2 mL cultures were incubated in 96-well tissue culture plates (4×10^5 cells/well) with or without varying concentrations of IRBP in serum-free medium, which was composed of RPMI 1640 medium, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin (all from Gibco BRL, Rockville, MD, USA), 0.1% bovine serum albumin (Sigma Chemical, St. Louis, MO, USA), and ITS+ culture supplement with 1 μg/mL iron-free transferrin, 10 ng/mL linoleic acid, 0.3 ng/mL Na_2Se, and 0.2 μg/mL $Fe(NO_3)_3$ (Gibco BRL). Cells were cultured for 96 hours at 37°C in an atmosphere of 5% CO_2, then pulsed with 0.5 μCi ^{3}H-thymidine 16 hours prior to termination of culture and harvested onto glass filters using an automated cell harvester (Tomtec, Orange, CT, USA). Radioactivity was as-

sessed by liquid scintillation spectrometry, and the amount expressed as cpm.

Cytokine Production Assay

Cells (4×10^6 cells/well) were incubated in 24-well tissue culture plates with or without 10 μg/mL of IRBP in 2 mL of serum-free medium as described above. Supernatants in cultures were collected after 48 hours for IL-2 production assay and after 72 hours for IL-4, IL-10, and IFN-γ production assays, because IL-2 is produced and consumed earlier than the other cytokines tested here. The amounts of cytokines were measured by quantitative capture enzyme-linked immunosorbent assay according to the guidelines of the manufacturer (PharMingen, San Diego, CA, USA). Rat MAb to mouse cytokine IL-2 (JES-1A12), IL-4 (BVD4-1D11), IL-10 (JES5-2A5), or IFN-γ (R4-6A2) (PharMingen) was used for the coating antibody; Biotinylated rat MAb to mouse cytokines IL-2 (JES6-5H4), IL-4 (BVD6-24G2), IL-10 (SXC-1), or IFN-γ (XMG1.2) (PharMingen) was used as the detecting antibody.

Histopathology and EAU Grading

Eyes were enucleated 1, 2, 4, 6, or 8 weeks postimmunization with IRBP. Freshly enucleated eyes were fixed in Bouin's fixative for histopathological study. Fixed tissue was embedded in methacrylate, and 4- to 6-μm sections, cut through the pupillary-optic nerve plane, were stained by standard hematoxylin and eosin. Six sections cut at different levels were examined for each eye. Severity of EAU was scored on a scale of 0 to 3, according to a system described in Table 1.

Results

Inflammatory Changes in the Eye After IRBP Immunization

Figure 1 shows the EAU scores of individual mice as well as the average scores at 1, 2, 4, 6, and 8 weeks after IRBP immunization. Intensive inflammatory changes were observed from 2 weeks after IRBP immunization, and 4 of 10 mice displayed severe EAU considered to be grade 3. Although the outer nuclear cell layer and photoreceptor cell layer were gradually destroyed, intense infiltration of inflammatory cells was still observed at week 4. After week 6, the intensity of infiltrating inflammatory cells was reduced, and atrophic changes were observed in the outer nuclear cell layer and the photoreceptor cell layer. Most inflammatory cells disappeared from eyes with EAU at week 8 after IRBP immunization.

Table 1. Histopathological Grading Chart for Experimental Autoimmune Uveoretinitis

Grade 0: No inflammatory cell infiltration of vitreous, ciliary body, choroid, or retina
Grade 1: Mild inflammatory cell infiltration of vitreous, ciliary body, choroid, or retina
Grade 2: Moderate inflammatory cell infiltration into choroid and retina with retinal vasculitis
Grade 3: Intensive inflammatory cell infiltration into whole retina with retinal folding

Proliferation Responses of Lymph Node Cells and Spleen Cells to IRBP After IRBP Immunization

Draining lymph node cells and spleen cells collected from mice immunized with IRBP were pooled and assayed for proliferation responses to IRBP. The results of a representative experiment are shown in Figure 2. Lymph node cells collected from mice at 1 and 2 weeks after immunization exhibited high proliferation responses to IRBP. Surprisingly, these responses were markedly reduced from 4 weeks after IRBP immunization (Figure 2A). In contrast, spleen cells collected from mice 1 and 2 weeks after IRBP immunization showed no proliferation responses to IRBP. Instead, proliferation of spleen cells to IRBP first appeared 4 weeks after IRBP immunization (Figure 2B). These results suggest the possibility that IRBP-reactive T cells were proliferating in the drain-

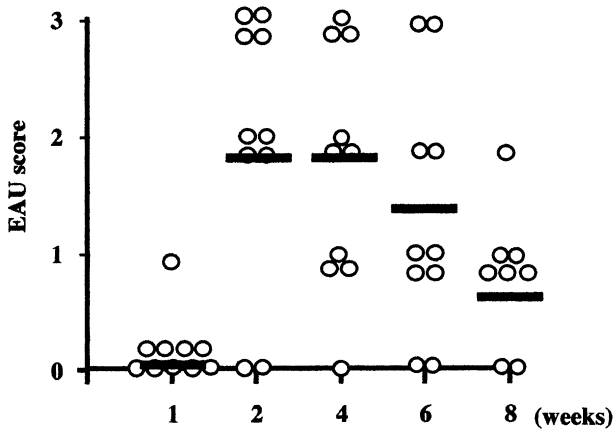

Figure 1. Experimental autoimmune uveoretinitis (EAU) scores and averages in interphotoreceptor retinoid-binding protein (IRBP) immunized B10.A mice. B10.A mice were immunized with IRBP and complete Freund's adjuvant (CFA), and whole eyes were enucleated and examined histologically at 1, 2, 4, 6, and 8 weeks after immunization. Scores were assigned on a scale of 0–3 according to our criterion described in Table 1. Each point represents an EAU score of one mouse and lines indicate averages of EAU scores at each examined week.

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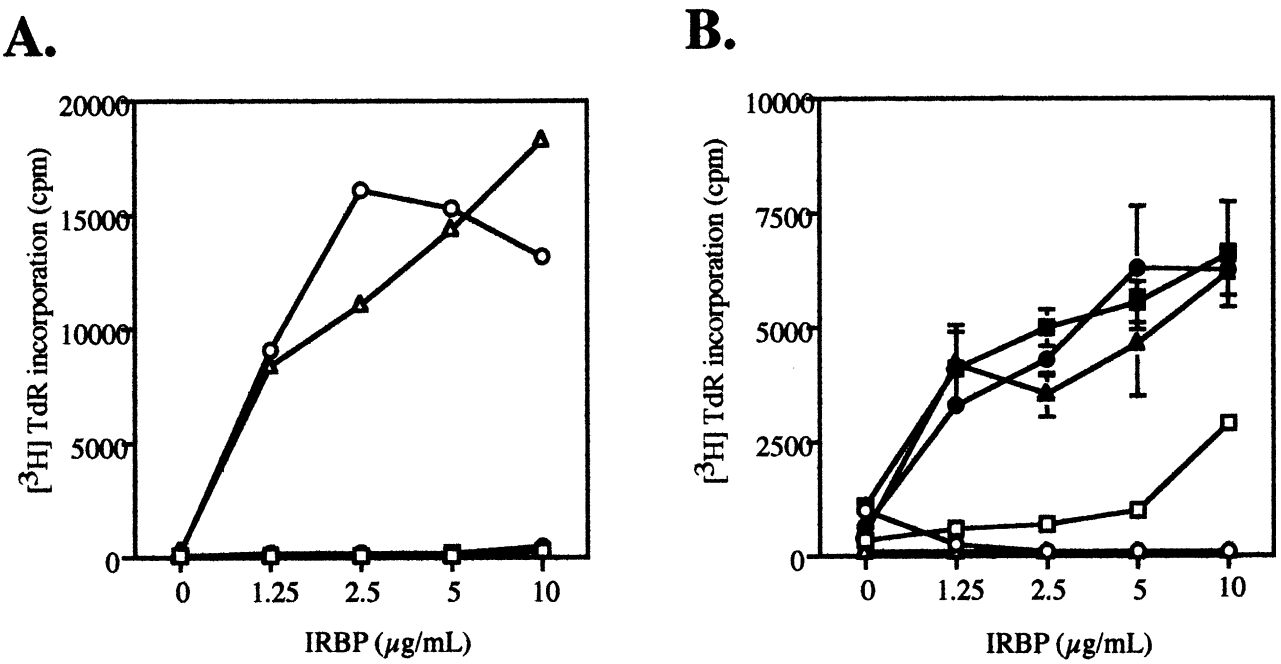

Figure 2. Kinetics of proliferation responses of draining lymph node cells and spleen cells collected from interphotoreceptor retinoid-binding protein (IRBP) immunized mice. Inguinal lymph node cells and spleen cells of intact mice, or those of mice immunized with IRBP at different time points were collected. Each group consisted of 5 mice, and their inguinal lymph nodes and spleens were pooled, respectively, and suspended into single cells. Proliferation responses of lymph node cells (**A**) and spleen cells (**B**) collected from intact mice (□), and mice with prior IRBP immunization are shown at 1 (○), 2 (■), 4 (■), 6 (●), and 8 (▲) weeks later.

ing lymph nodes during induction and early acute phase of EAU, and then migrated into the spleen prior to the remission phase of EAU.

Kinetics of Th1-Type Cytokine Secretion by Lymph Node Cells and Spleen Cells After IRBP Immunization

Subsequently, we examined the kinetics of Th1-type and Th2-type cytokine production by IRBP-reactive T cells in the draining lymph nodes and the spleen after IRBP immunization. Representative results of Th1-type cytokine production by lymph node cells and spleen cells following stimulation with IRBP are shown in Figure 3. Lymph node cells secreted large amounts of IL-2 at 1 and 2 weeks after IRBP-immunization, after which IL-2 production diminished. In contrast, IL-2 secretion by spleen cells began to increase from week 4. The results of IL-2 secretion by lymph node cells and spleen cells correlated with their proliferation responses. Alternatively, remarkable IFN-γ secretion by lymph node cells as well as their IL-2 production was observed only at 1 and 2 weeks after IRBP immunization. Spleen cells secreted large amounts of IFN-γ in a sustained fashion from 1-week post-IRBP immunization, peaking at week 2, and then decreasing gradually. The levels of IFN-γ production in the spleen corresponded with the severity of EAU in the eye.

Kinetics of Th2-Type Cytokine Secretion by Lymph Node Cells and Spleen Cells After IRBP Immunization

Figure 4 displays representative results of Th2-type cytokine secretion by lymph node cells and spleen cells stimulated with IRBP. As compared to naive controls, significant IL-4 or IL-10 secretion was not detected in cultures of lymph node cells collected in any week tested after IRBP immunization. Interleukin-4 secretion was observed in spleen cell cultures after week 6; their IL-10 secretion was also observed at weeks 1 and 2 after immunization. These results suggest that Th2-type cell activation is induced in the spleen in a late phase of EAU, which seems to be related mechanically to remission of EAU.

Discussion

Our results indicate that IRBP-specific Th1-type cells, which produce IL-2 and IFN-γ, are activated in

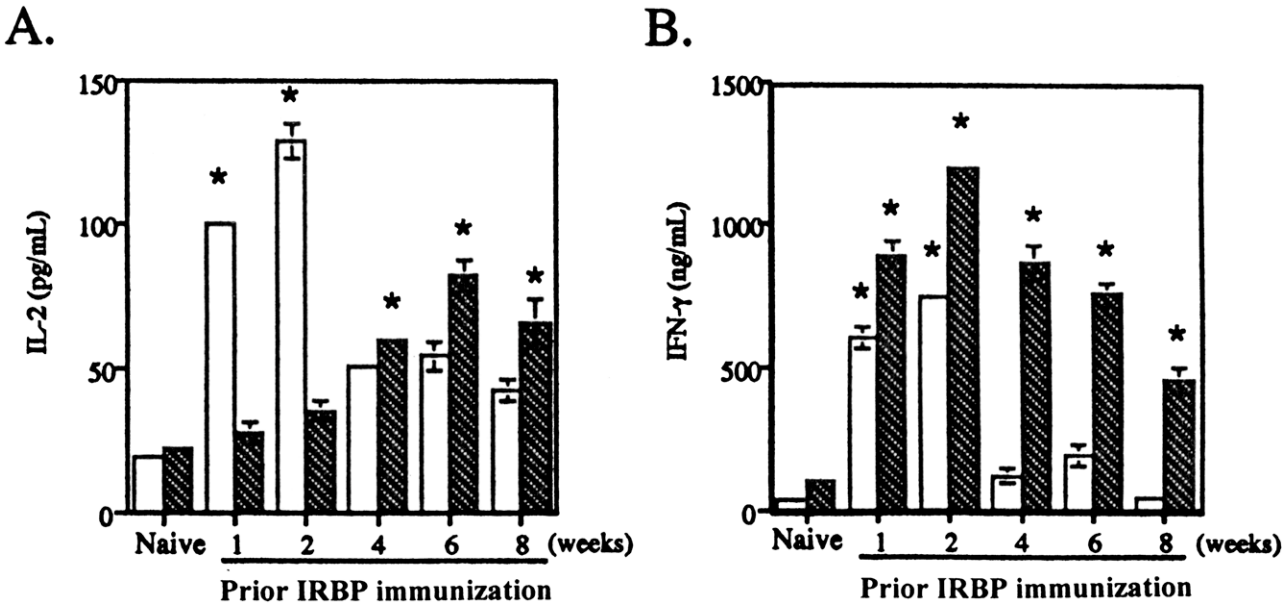

Figure 3. Kinetics of T-helper 1-type cytokine secretion by lymph node cells and spleen cells collected from interphotoreceptor retinoid-binding protein (IRBP) immunized mice. Inguinal lymph node cells and spleen cells of intact mice or those of mice immunized with IRBP at different time points were collected and incubated. Supernatants were collected after 48 hours for interleukin (IL)-2 assay, and after 72 hours for interferon (IFN)-γ assay. The amounts of cytokines were measured by quantitative capture enzyme-linked immunosorbent assay. IL-2 (**A**) and IFN-γ (**B**) production of lymph node cells (□), or those of spleen cells (■) are shown. Asterisks indicate mean values significantly greater than in naive mice ($P < .05$).

draining lymph nodes before the occurrence of EAU after IRBP immunization, while activation of IRBP-specific Th2-type cells, which produce IL-4 and IL-10, emerge in the spleen in the remission phase of EAU when Th1 cell activity recedes.

T-cell proliferation responses to IRBP were first observed in the draining lymph nodes, and then in the spleen after IRBP immunization. When proliferation responses to IRBP emerged in the spleen 4 weeks after IRBP immunization, proliferation of draining lymph node cells had already diminished. It is proposed that IRBP-specific T cells were first activated in the draining lymph nodes after IRBP immunization, and then circulated into the eye, spleen, and elsewhere during the acute phase of EAU. Based on the present cytokine profiles, IRBP-specific T cells, which are first activated in the draining lymph nodes and migrate into the spleen, would be Th1-type T cells. Interleukin-12 has been demonstrated to be a critical cytokine for induction of Th1 cell differentiation.[15,16] It has been shown that IFN-γ stimulates dendritic cells and macrophages to produce IL-12 and maintains IL-12 receptor β2 expression on Th1-type cells, which enhances the ability of these cells to be Th1 cells. Therefore, it is conceivable that Th1-type cells that migrate into the spleen might convert to Th2-type cells at the late phase of EAU, because their decreased IFN-γ production is insufficient to stimulate dendritic cells and macrophages to produce IL-12 or to maintain their IL-12 receptor β2 expression. Alternatively, because IFN-γ does not directly inhibit IL-4 production by T cells,[15,17] IRBP-specific Th2-type cells, which are generated in the spleen during the remission phase of EAU, might be a different population from that of Th1-type T cells.

In this study, predominant IL-10 production in the spleen was observed 1 and 2 weeks after IRBP immunization, even though IL-4 production was not. Interleukin-10 is known to be produced by Th2 cells, but it can also be produced by other cells, such as macrophages and B cells.[18,19] Because production of IL-4 was not significant in the spleen at 1 and 2 weeks after IRBP immunization, we speculate that the source of splenic IL-10 produced in the early induction phase of EAU might arise from non-T cells.

Although IRBP-specific Th1-type cells, which were detected in the draining lymph nodes and in the spleen before and during the acute phase of EAU in this study, are thought to act as effector cells for the occurrence of EAU,[7,8,20] it is not clear whether IRBP-reactive, Th2-type cells found in the

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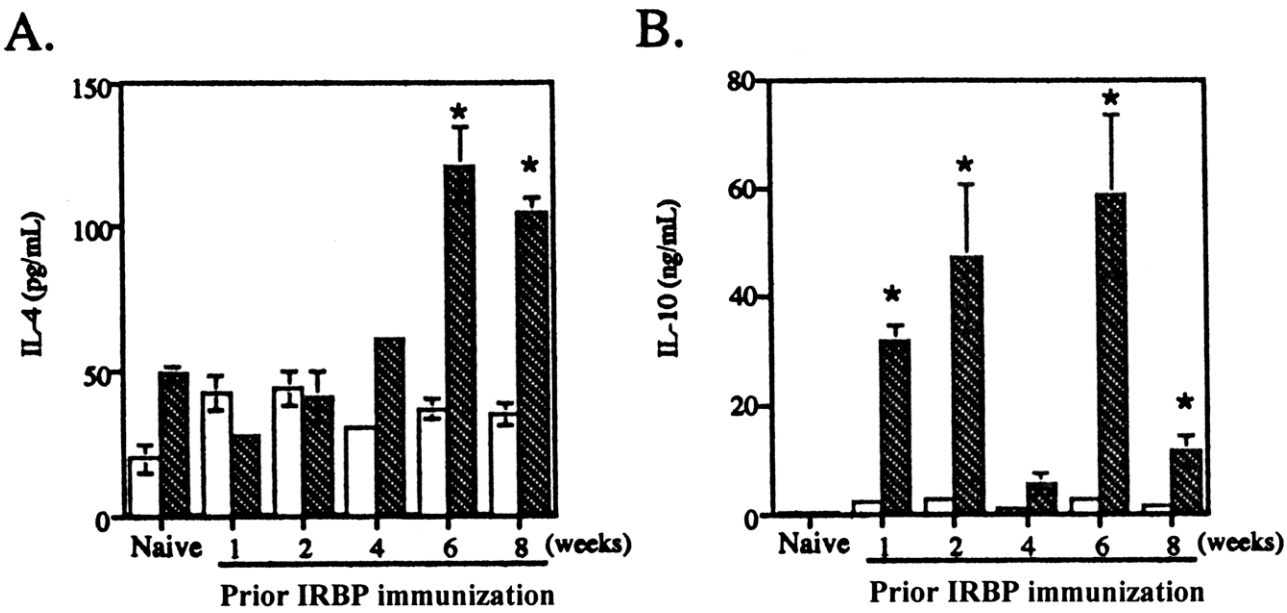

Figure 4. Kinetics of T-helper 2-type cytokine secretion by lymph node cells and spleen cells collected from from interphotoreceptor retinoid-binding protein (IRBP) immunized mice. Inguinal lymph node cells and spleen cells of intact mice, or those of mice immunized with IRBP at different time points were collected and incubated. After 72 hours, supernatants were collected for interleukin (IL)-4 and IL-10 assay. The amounts of cytokines were measured by quantitative capture enzyme-linked immunosorbent assay. IL-4 (**A**) and IL-10 (**B**) production of lymph node cells (□), or those of spleen cells (■) are shown. Asterisks indicate mean values significantly greater than in naive mice ($P < .05$).

spleen during the remission phase of EAU are responsible for the amelioration of EAU in the present study. Rizzo et al[13] have found that IL-4 and IL-10 mRNA expression in the eye coincided with down-regulation of Th1-cytokine mRNA expression and resolution of EAU. In addition, their results showed that IL-10-deficient mice were more susceptible to EAU and administration of IL-10 after uveitogenic immunization reduced the effects of the subsequent EAU.[13] It has been reported in most articles that Th2-type cells act as regulatory T cells to inhibit the occurrence of EAU by inactivating effector Th1-type cells.[1,20–22] However, it has not been shown directly that Th2-type cells play a critical role in the natural resolution of EAU. It is possible that Th2-type cell activation might result from down-regulation of Th1-type cell activation, which coincides with EAU remission caused by unknown factors.

In this study, because IRBP-reactive Th2-type cells were generated in the spleen at the remission phase of EAU, while IRBP-reactive Th1-type cells were in the draining lymph nodes, we hypothesized that the splenectomy of mice might prolong the ocular inflammation of EAU after IRBP immunization, and so we performed the following experiments. Under anesthesia, mice were gently splenectomized at 4 weeks of age, and immunized with IRBP 4 weeks after the splenectomy. However, EAU did not develop in any splenectomized mice (data not shown). This result indicates that IRBP-reactive Th1-type cells concerned with the induction of EAU require the involvement of the spleen also. Therefore, we are now planning to study the regulatory functions of Th2-type cells, which are generated in the spleen during the remission phase of EAU.

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