

Fas Expression and Apoptosis in Rats with Experimental Autoimmune Uveoretinitis

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Purpose: To evaluate Fas expression on CD4 and CD8 T cells in each organ at each stage of experimental autoimmune uveoretinitis (EAU) and apoptotic cells within EAU eyes.

Methods: Rats were immunized with the uveitopathogenic peptide derived from interphotoreceptor retinoid-binding protein. Flow cytometry was performed in ocular cells, draining lymph nodes cells and splenic cells of EAU rats to investigate Fas expression by CD4 and CD8 lymphocytes. Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling staining of apoptotic nuclei was performed on sections of EAU eyes.

Results: Fas expression by both ocular and splenic CD4 and CD8 lymphocytes was significantly higher than in lymph nodes at each stage. In EAU eyes, there was a relatively large population of lymphocytes with Fas expression (19.6–25.6% of CD4 and 33.2–53.4% of CD8). Apoptotic cells were more prominent in the EAU eyes with established disease than in those with early or resolving disease.

Conclusions: These results suggest that the relatively large population of lymphocytes with Fas expression in EAU eyes reflects the activation of lymphocytes in these eyes, and that the increase in apoptotic inflammatory cells at the peak of established disease may participate in the spontaneous disappearance of EAU. **Jpn J Ophthalmol 2001;45:240–246** © 2001 Japanese Ophthalmological Society

Key Words: Apoptosis, CD4, CD8, experimental autoimmune uveoretinitis, Fas.

Introduction

Experimental autoimmune uveoretinitis (EAU) is a T cell-dependent disorder that can be induced by immunization with several retinal specific antigens such as retinal S-antigen,¹ interphotoreceptor retinoid-binding protein (IRBP),^{2,3} phosducin,⁴ recoverin,^{5,6} and the gamma subunit of cyclic guanosine monophosphate phosphodiesterase.⁷ Experimental autoimmune uveoretinitis in Lewis rats is characterized by an acute panuveitis 10–14 days after immunization with uveitopathogenic retinal antigens.¹ Most rats show disappearance of clinical signs of inflammation 7–10 days after disease initiation.¹ The mech-

anism of the spontaneous disappearance of EAU without recurrence is not well understood.

A possible mechanism of the spontaneous regression of EAU is immune privilege. Immune privilege is the mechanism by which vital organs, such as the eye, brain, testis, and ovary, are protected during immune and inflammatory reactions so that immune reactions either do not proceed, or proceed in a manner different from that in other areas.⁸ An antigenic encounter leads to the activation and expansion of reactive T cell populations. However, because the immune system remains relatively constant in size throughout life, there must be mechanisms that eliminate the expanded lymphoid populations that are no longer needed. One of these mechanisms involves the re-ligation of the T cell receptor on activated T cells, leading to apoptosis. This pathway requires interaction between Fas and FasL.⁹ Fas is a

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transmembrane protein, and transduces the apoptotic signal into susceptible target cells.¹⁰ FasL is also a cell-surface molecule, and binds to Fas on cells, inducing Fas-mediated apoptosis.¹¹ Recently, Fas and FasL have been considered to play an important role in the development of autoimmune diseases because mutations in either lead to immunologic disorders such as lymphadenopathy and proliferative abnormalities in mice and humans.^{12,13} Therefore, studies of the apoptotic process have focused on the pathophysiologic phenomena in several autoimmune disorders. Griffith et al¹⁴ have demonstrated high expression of Fas-FasL in normal mouse ocular tissues and the fact that Fas-FasL-mediated apoptosis is an important mechanism for the maintenance of immune privilege in the eye. To our knowledge, there are few reports of Fas expression in EAU animal models. In this study, Fas expression on CD4 and CD8 T cells in inflamed ocular, draining lymph node, and splenic tissues were investigated at each stage of EAU. In addition, apoptotic cells were detected in EAU eyes.

Materials and Methods

Uveitopathogenic Peptide

Uveitopathogenic peptide derived from bovine IRBP, R16,¹⁵⁻¹⁷ located from 1177 to 1191, was synthesized by a solid-phase procedure using a peptide synthesizer (Model 433A; Applied Biosystems, Foster, CA, USA).

Animals and Immunization Protocol

Twelve-week-old female Lewis rats were used as experimental animals. Rats were immunized with the peptide emulsified (1:1) in complete Freund's adjuvant injected into one hind footpad; 20 µg in a volume of 0.1 mL. A suspension of *Bordetella pertussis* (Wako Pure Chemical, Osaka) was injected intravenously; 2×10^{10} per rat at the time of immunization. Maintenance, control, experiments, and care of animals was performed in accordance with the Guidelines for Animals Experiments of ARVO.

EAU Evaluation

Rats were examined daily after day 7 post-immunization for clinical signs of EAU. Rats were divided into groups of 5 animals at each time of examination during the 5 stages of EAU. Preimmunization day: Lymph nodes and spleens were examined for flow cytometry, but eyes were not. Onset day of EAU (8–

9 days post-immunization, early disease stage): Initiation of EAU was confirmed by slit-lamp biomicroscope. Lymph nodes and spleens were examined for flow cytometry, but eyes were not. Three days after

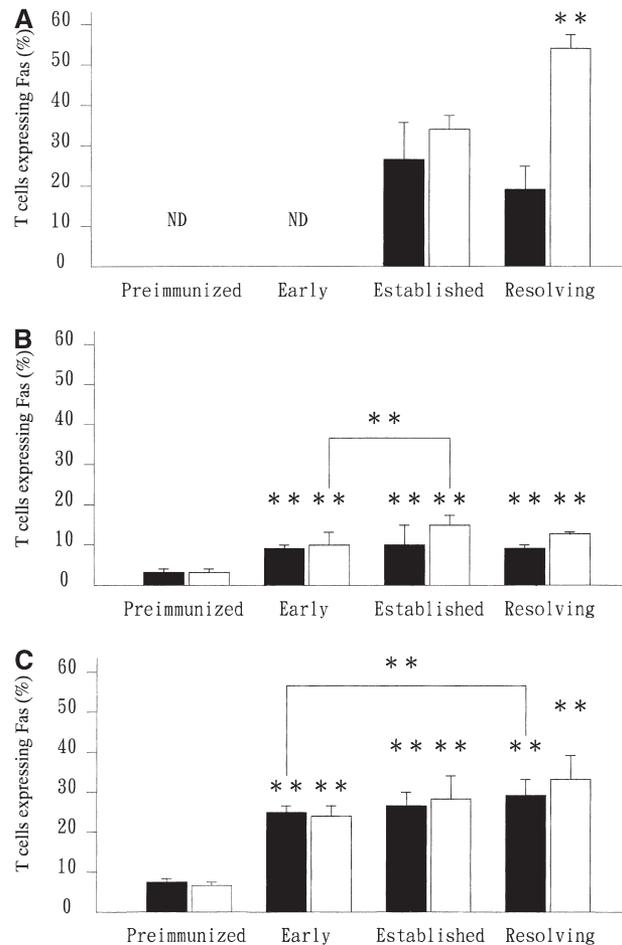
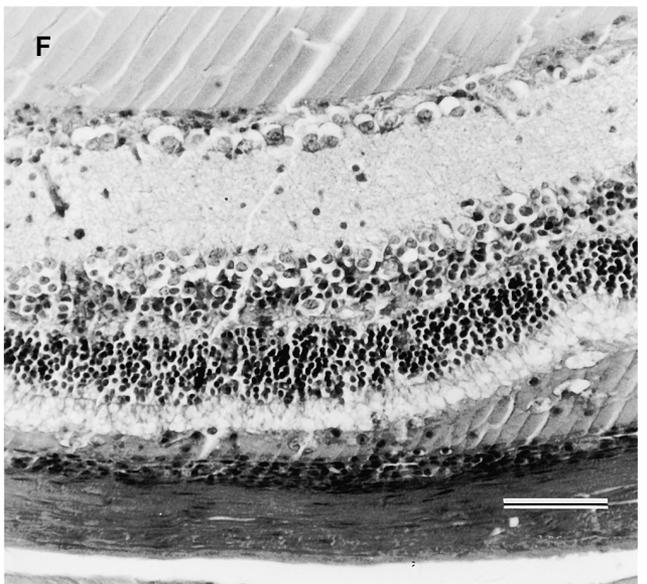
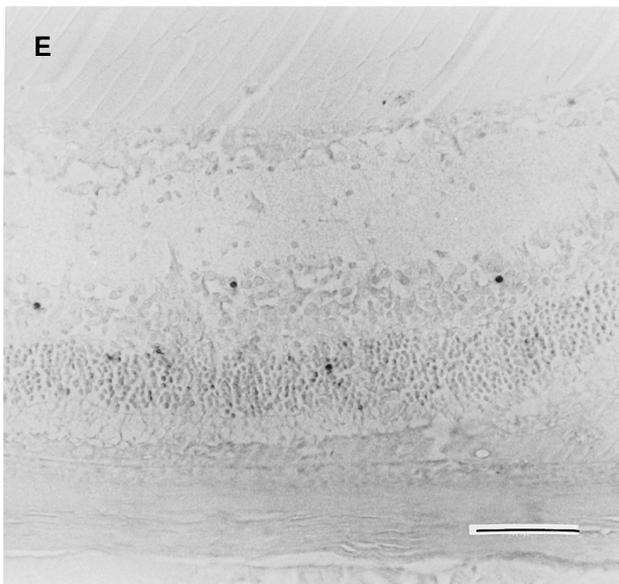
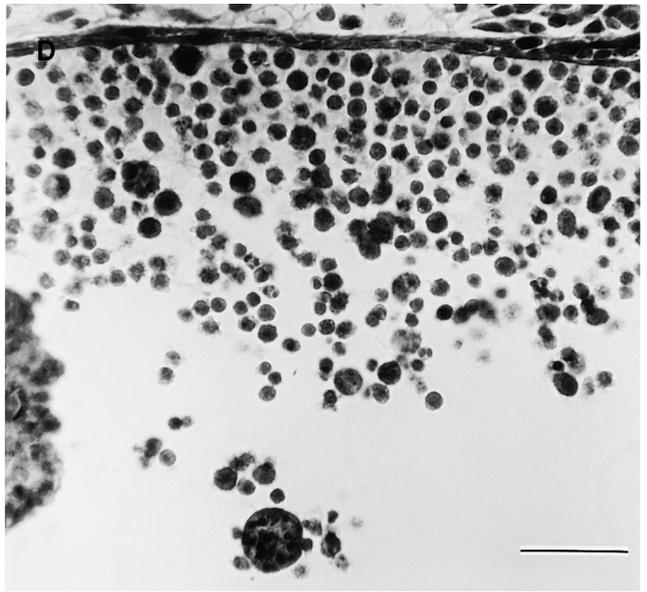
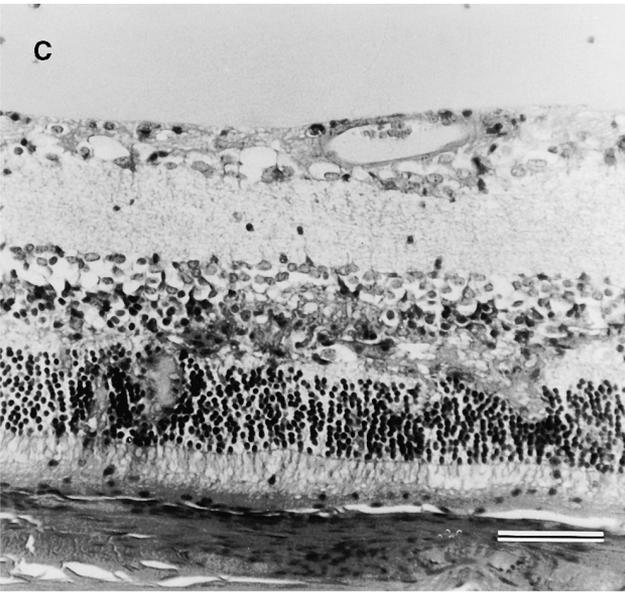
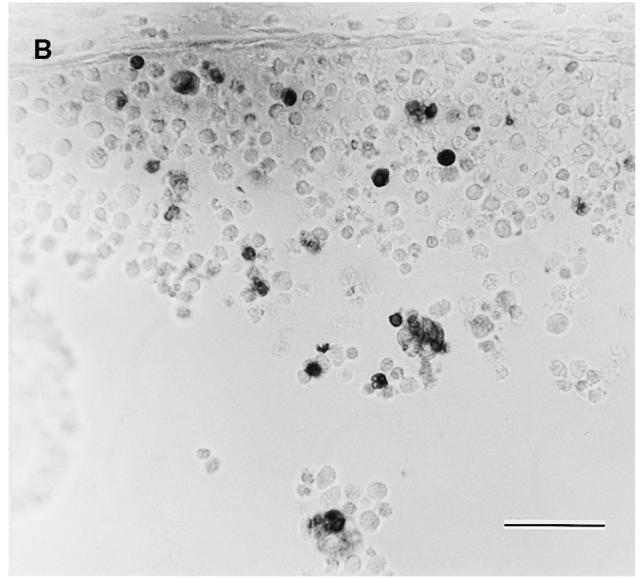
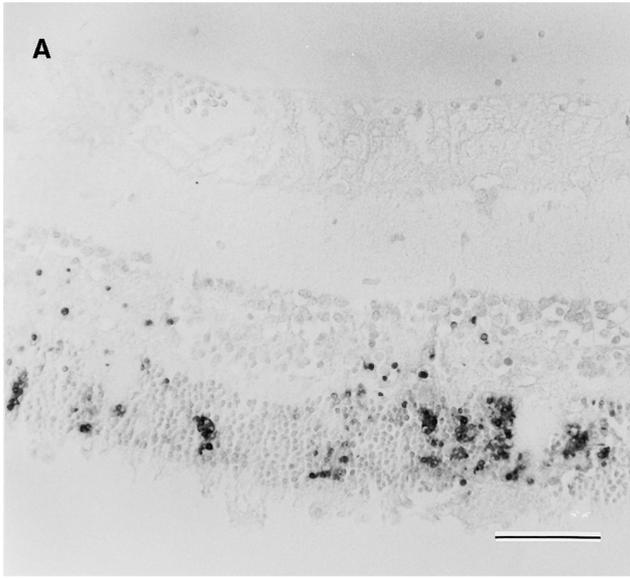


Figure 1. Fas expression by CD4 (■) and CD8 (□) T cells in (A) eye, (B) lymph node, and (C) spleen, at each disease stage. Early disease stage (8–9 days post-immunization, onset day of experimental autoimmune uveoretinitis [EAU]). Established disease stage (11–12 days post-immunization, 3 days after onset, disease peak of EAU). Resolving disease stage (14–15 days post-immunization, 6 days after onset, beginning of resolving inflammation of EAU). Late disease stage (21 days post-immunization, resolution of inflammation of EAU). In eye, $**P < .01$ compared to CD8 T cells in established disease stage. In lymph node, $**P < .01$ compared to preimmunized stage for both CD4 and CD8 T cells, and compared between early and established disease in terms of CD8 T cells (bar). In spleen, $**P < .01$ compared to preimmunized stage for both CD4 and CD8 T cells, and compared between early and resolving disease in terms of CD4 T cells (bar). ND: not done. (D) Representative flow cytometric analysis of cells in lymph node from EAU rat. Cells in lymph node were isolated at early disease and analyzed.



onset of EAU (11–12 days post-immunization, established disease stage): Degree of inflammation of EAU reached its peak. Eyes, lymph nodes, and spleens were examined by flow cytometry. Six days after onset of EAU (14–15 days post-immunization, resolving disease stage): Clinical signs of resolving inflammation of EAU were beginning. Eyes, lymph nodes, and spleens were examined for flow cytometry. Twenty-one days post-immunization (late disease stage): Clinical resolution of inflammation of EAU was confirmed. Eyes, lymph nodes, and spleens were examined for flow cytometry. The clinical grading of the ocular inflammation in EAU was from 0 to 5+ as described previously.¹⁸

Two-color Flow Cytometry

Two-color flow cytometry was performed according to Barton et al.¹⁹ In brief, the eyes, draining lymph nodes, and spleens were removed and placed in cold RPMI-1640 medium (Nissui, Tokyo) containing 10% fetal calf serum, and then gently pressed through a stainless steel mesh screen. Red blood cells were lysed with 0.9% ammonium chloride. The cells were washed and collected.

To quantify CD4 and CD8 T cell populations, respectively, cells were co-stained with monoclonal antibody (mAb) to rat CD4 or CD8 labeled with phycoerythrin (PE) (Serotec, Blackthorn, UK) and mAb to rat CD3 labeled with fluorescein isothiocyanate (FITC; DAKO, Copenhagen, Denmark). The antibody (Ab) concentrations used were those suggested by the manufacturers. After washing, the suspension was filtered through nylon mesh. Before analysis, cells were fixed with 2% paraformaldehyde and stored at 4°C in the dark. Within 24 hours, 10,000 cells from each sample were analyzed by flow cytometry (Epics Elite; Coulter Electronics, Hialeah, FL, USA).

To quantify populations expressing Fas, cells were incubated with mAb to rat CD4 or CD8 labeled with PE and non-labeled rabbit polyclonal antibody (Ab) to Fas (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing, the FITC-conjugated F(ab')₂

fragment of swine anti-rabbit immunoglobulins (DAKO) was added as a secondary Ab to anti-Fas Abs. The subsequent procedures were similar to those used in the CD4 or CD8/CD3 analysis described above.

Morphologic Examinations

For light microscopy, eyes were fixed in 2.5% buffered glutaraldehyde for 4 hours, and then transferred into 10% buffered formaldehyde for at least 24 hours. Fixed and dehydrated eyes were embedded in paraffin, and thin sections were cut. Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) staining of apoptotic nuclei²⁰ was performed on sections using a kit for in situ apoptosis detection (Intergen, Purchase, NY, USA), following the manufacturer's protocols. Some sections were also stained with hematoxylin and eosin.

Statistical Analysis

Values are expressed as means \pm standard deviation. The Mann-Whitney *U*-test was used to evaluate significance. $P < .05$ was regarded as statistically significant.

Results

Clinical Courses of EAU

The grading of EAU was 1.4 ± 0.5 in early disease, and 3.2 ± 0.8 in established disease. The EAU changes were bilateral and persisted for 6 to 8 days, ie, clinical signs resolved in the late stage of the disease (21 days after immunization).

Levels of CD4 and CD8 in CD3 Positive Cells in Organs

The relative proportion of ocular CD4 T cells was $79.5 \pm 4.5\%$ in established disease, decreasing to $60.7 \pm 11.3\%$ in late disease ($P < .01$). The relative proportion of CD4 T cells was 57.2% to 72.7% in lymph nodes, and 45.9% to 56.3% in the spleen. In both lymph nodes and the spleen, CD4 T cell popu-

Figure 2. Detection of apoptotic nuclei of cells in light microscopic sections by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL). In established experimental autoimmune uveoretinitis (EAU) (11 days post-immunization), (A) many TUNEL-positive cells are observed predominantly in outer nuclear layer ($\times 100$, bar = 20 μm) and (B) inflammatory cells in posterior chamber ($\times 400$, bar = 5 μm). (C, D) Hematoxylin and eosin staining of the same eye or serial sections as in (A) and (B), respectively. In resolving EAU (15 days post-immunization), (E) small populations of cells show signs of TUNEL positivity ($\times 100$, bar = 20 μm). (F) Hematoxylin and eosin staining of serial section designated (E).

lations in early disease ($57.2 \pm 8.3\%$ in lymph node and $45.9 \pm 1.7\%$ in spleen) were significantly smaller ($P < .05$) than those in other stages. The relative proportion of ocular CD8 T cells was $5.7 \pm 0.6\%$ in established disease, increasing to $9.3 \pm 2.3\%$ in late disease ($P < .01$). The relative proportion of CD8 T cells was 20.8% to 24.2% in lymph nodes, and 21.0% to 27.2% in the spleen without significant differences among disease stages.

Comparison of Fas Level in CD4 and CD8 T Cells Among Disease Stages

Eyes. Fas expression by CD8 T cells was lower in established disease ($33.2 \pm 3.6\%$, $P < .01$) than in resolving disease ($53.4 \pm 3.5\%$). Fas expression by CD4 T cells (19.6–25.6%) in eyes did not differ significantly between established (25.6%) and resolving (19.6%) disease stages (see Figure 1).

Lymph nodes. Fas expression by CD4 T cells was higher in all disease stages (early: $8.5 \pm 1.2\%$, established: $9.9 \pm 3.6\%$, resolving: $8.9 \pm 0.7\%$, $P < .01$) than in the preimmunization stage ($1.9 \pm 1.3\%$). There was, however, no statistically significant difference among disease stages. Fas expression by CD8 T cells was also higher in all disease stages (early: $9.9 \pm 1.9\%$, established: $14.0 \pm 3.2\%$, resolving: $10.9 \pm 0.9\%$, $P < .01$) than that in the preimmunization stage ($1.8 \pm 0.7\%$). Furthermore, the expression in established disease was higher ($P < .01$) than in early disease.

Spleens. Fas expression by both CD4 and CD8 T cells was higher in all disease stages (early: CD4, $23.6 \pm 2.0\%$, CD8, $23.0 \pm 3.1\%$, established: CD4, $26.4 \pm 4.2\%$, CD8, $28.4 \pm 5.2\%$, resolving: CD4, $29.4 \pm 3.0\%$, CD8, $32.0 \pm 7.4\%$) ($P < .05$) than in the preimmunization stage (CD4, $6.8 \pm 1.3\%$, CD8, $5.7 \pm 0.8\%$). Furthermore, the expression in resolving disease was higher (CD4, $P < .01$, CD8, $P < .05$) than in early disease.

Apoptotic Cells in EAU Eyes

In early disease, TUNEL-positive cells were seldom observed. In established disease, many positively stained cells were seen predominantly in the outer nuclear layer (Figure 2A), and infiltrating inflammatory cells in the posterior chamber were also stained (Figure 2B). There were also some positively stained cells from the outer plexiform layer to the ganglion cell layer, and infiltrating inflammatory cells in the vitreous cavity. In resolving disease, the

population of positively stained cells had decreased by approximately 10-fold as compared to established disease (Figure 2E).

Discussion

To induce EAU, retinal antigen (IRBP) was injected into one footpad of each rat. Activated and clonal expanded T cells in draining lymph nodes enter the general circulation. Activated T cells are able to home in on organs containing the specific antigen. Once enough of these activated, antigen-specific T cells enter a target organ such as the eye, they release cytokines, which then start an inflammatory cascade by breaking down the blood-ocular barrier and allowing additional leukocytes into the area.²¹

In our experiment, the relative proportion of CD4 T cells in the eye was 79.5% in established disease, decreasing to 60.7% in late disease. The relative proportion of ocular CD8 T cells was 5.7% in established disease, increasing to 9.3% in late disease. These profiles were essentially consistent with those of previous reports.¹⁹ In lymph nodes and the spleen, the relative proportions of CD4/CD3 T cells in early disease were significantly lower than those in the preimmunization stage and other disease stages. CD3 T cells include alpha beta T cells and gamma delta T cells.²² Alpha beta T cells expressed surface markers of CD4 and/or CD8, while gamma delta T cells did not.²² In another T cell-dependent autoimmune disease, experimental allergic encephalomyelitis (EAE), TCR gamma delta T cells were more frequently detected in the spleen than in the EAE lesions.²³ In EAE lesions,²⁴ a 60-kDa heat shock protein (hsp 60) colocalized with TCR gamma delta T cells, and hsp 60/CD3 was expressed more prominently in the early stage of acute EAE than in other disease stages.²⁴ There is a possibility that the relative proportion of gamma delta T cells increases in lymph nodes and in the spleen, in early EAU. However, for EAU, no data are yet available to support this speculation. Further study is necessary to answer the aforementioned questions.

Fas was expressed preferentially on memory or previously activated T cell populations, but not on naive T cells.²⁵ The evidence implicating Th1-like effector cells in the pathogenesis of EAU has been presented.²⁶ Barton et al¹⁹ reported that the population of memory CD4 T cells in the retina was larger than that in lymph nodes. In our study, Fas expression by both CD4 and CD8 T cells in eyes was also significantly higher than in lymph nodes at each stage. Considering our results together with those of others,¹⁹

Fas expression appears to reflect the degree of ocular lymphocyte activation. The Fas/CD4 profile in each organ was essentially the same as that of Fas/CD8, the exception being Fas/CD8 in the eyes. Fas expression by CD8 T cells was significantly higher in resolving disease (53.4%) than in established disease (33.2%); however, further study is necessary to reveal the significance of this discrepancy.

Fas expression by both CD4 and CD8 T cells was low in preimmunized lymph nodes (1.8–2.3%) and the spleen (5.7–10.5%). After immunization, in lymph nodes, Fas expression increased 4.8–7.9-fold, and in the spleen, Fas expression increased 3.5–5.6-fold. In a previous report, Fas was detected in <10% of normal splenic T cells derived from mice, and after activation, Fas expression increased 10-fold.²⁷

In passive transfer EAE,²⁸ at its clinical peak, Fas or FasL/T cells constituted a small proportion (1–3%) of cells, and a minority of the infiltrating cells were TUNEL-positive. Most of the TUNEL-positive cells were CD4+ or Mac-1+ (marker of resident microglia and infiltrating macrophages). At the beginning of the clinical recovery stage, the proportion of Fas or FasL/T cells increased to 20–30%, and about half of the infiltrating cells were TUNEL-positive. In our study, TUNEL-positive cells also constituted a small population in early disease, but increased at the peak of established disease. However, the types of TUNEL-positive cells have yet to be determined. At the beginning of the clinical resolution of EAU, TUNEL-positive cells were minimal as compared to the number in the established disease stage. In addition, in our study, in both early and established disease, the proportion of Fas/CD4 T cells was about 30%. The reason for these discrepancies between our results and those of Bonetti et al²⁸ is unknown. In our experimental model, EAU was induced by active immunization, rather than by passive transfer as in the study of Bonetti et al.²⁸ *Mycobacterium tuberculosis* and *Bordetella pertussis* were injected in our active immunization procedure, so that T cells were activated not only by IRBP peptide but also by their respective bacterial components. Passive EAU transfer studies are anticipated to provide further insights into the above-mentioned issues.

Our results suggest that a relatively large population of lymphocytes with Fas expression in EAU eyes reflect the activation of ocular lymphocytes. Fas-positive activated lymphocytes may interact with FasL, which is expressed constitutively in ocular tissues or activated T cells, and undergo apoptosis. Increased apoptotic inflammatory cells at the peak of established disease may participate in the spontane-

ous disappearance of EAU. FasL expression on CD4 and CD8 T cells in inflamed ocular, draining lymph node, and splenic tissues should be also investigated in the near future.

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