

# Expression of Stress-Response Protein 60 in Iritis Associated With Experimental Autoimmune Encephalomyelitis

Takeshi Kumagami,\* Shinsuke Kato,<sup>†</sup>  
Ryoko Ishikura,\* Masao Nagata,\* Akihiko Tamai\* and Eisaku Ohama<sup>†</sup>

*\*Department of Ophthalmology and <sup>†</sup>Division of Neuropathology,  
Institute of Neurological Sciences, Faculty of Medicine, Tottori University, Yonago, Japan*

**Purpose:** To study the expression of stress-response proteins in the inflamed iris of rats with experimental autoimmune encephalomyelitis (EAE).

**Methods:** EAE was induced in Lewis rats by immunization with homogenized spinal cord of the guinea pig emulsified in complete Freund's adjuvant (CFA) (group EAE). Control rats included those immunized with only CFA (group CFA) and those that were untreated (group Normal). Immunohistochemical study for the localization of stress-response protein (srp) 27, srp 60, srp 72, ubiquitin, and  $\alpha$ B-crystallin was performed.

**Results:** All rats in group EAE developed iritis, whereas none of the rats in group CFA and group Normal developed iritis. No expression of ubiquitin,  $\alpha$ B-crystallin, srp 27, srp 60, or srp 72 was seen in the epithelium of the iris in group CFA rats. In the eyes of rats in group EAE, srp 60 was expressed in the epithelium of the iris in 20 of 22 (90.9%), ubiquitin in 4 of 22 (18.2%), and  $\alpha$ B-crystallin in 3 of 22 (13.6%). In the group Normal rats, only ubiquitin was expressed in the epithelium of the iris in 1 of 6 (16.7%) eyes examined.

**Conclusions:** These results suggest that srp 60 may be a potential uveitogenic antigen in the iris in EAE. **Jpn J Ophthalmol 1999;43:458-465** © 1999 Japanese Ophthalmological Society

**Key Words:** Experimental autoimmune encephalomyelitis, immunochemistry, iritis, posterior iris epithelium, stress-response protein 60.

## Introduction

Experimental autoimmune encephalomyelitis (EAE) is an inflammatory, demyelinating disease of the central nervous system (CNS) induced by immunization with myelin antigens. It is generally considered to be a useful animal model for studying the immunopathogenesis of multiple sclerosis. The clinical association of multiple sclerosis and anterior uveitis, which is characterized by inflammation of the iris or iritis, is well known in humans.<sup>1-6</sup> It is also well recognized that iritis is associated with EAE in al-

bino rabbits,<sup>7,8</sup> rhesus monkeys,<sup>9</sup> and Lewis rats.<sup>10,11</sup> However, the reasons for such an association are still unknown.

Stress-response proteins (srps) are induced in prokaryotic and eukaryotic species under various conditions of stress.<sup>12-15</sup> These srps have been studied extensively for their physiological roles in the maintenance of self-integration at the cellular level. Srps are named and classified in different families according to their apparent molecular mass. The srp 60 kDa family (srp 60 kDa or srp 60), which have retained a uniquely high level of sequence conservation during evolution, is the focus of interest as a potential antigen in autoimmune diseases<sup>16,17</sup> such as juvenile arthritis,<sup>18</sup> multiple sclerosis,<sup>19,20</sup> Behçet's disease,<sup>21</sup> and autoimmune hepatitis.<sup>22</sup> In the present study, we examined the expression of srps in the iris, the most anterior component of the uvea, in EAE.

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Correspondence and reprint requests to: Takeshi KUMAGAMI, MD, Department of Ophthalmology, Faculty of Medicine, Tottori University, 36-1 Nishimachi, Yonago, Tottori 683-8504, Japan

To the best of our knowledge, such an investigation has not been reported previously.

## Materials and Methods

### Animals

Forty female Lewis rats, aged 8–9 weeks and weighing 155–170 g, were purchased from Seiwa Experimental Animal Farm (Fukuoka). Water and food pellets were given ad libitum. Treatment of the animals adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the Guidelines for Animal Experiments in the Faculty of Medicine, Tottori University.

### Induction of EAE

The induction of EAE was a modified version<sup>23</sup> of the one described by Feurer et al.<sup>24</sup> In brief, 1 g of guinea pig spinal cord and 1 mL of 0.01 mol/L phosphate-buffered saline (PBS, pH 7.4) were homogenized. The homogenate was then emulsified with 2 mL of Difco's Bacto complete Freund's adjuvant (CFA) supplemented with 40 mg of *Mycobacterium tuberculosis* H37 Ra (Difco Laboratories, Detroit, MI, USA). Twenty-seven rats were immunized with 0.2 mL of this encephalitogenic emulsion by intradermal injection into both hind footpads (group EAE). Thirteen rats were used as controls: 3 rats were uninjected (group Normal) and 10 rats were inoculated with an emulsion of CFA and PBS (1 mL/1 mL) using the same dosage and method as in group EAE (group CFA).

### Evaluation of EAE and Iritis

Rats were weighed and assessed daily for clinical signs of EAE and iritis. EAE was characterized clinically by scoring the severity of limb paralysis according to the scale of Kato and Nakamura<sup>25</sup>: 0 = normal; 1 = limp tail; 2 = weakness of hind legs or mild ataxia; 3 = hind limb paralysis and severe ataxia; 4 = severe hind limb paralysis accompanied by urinary incontinence; and 5 = severe quadriplegia or a morbid state.

Eyes were examined for ocular signs by slit-lamp biomicroscopy. Because iritis is characterized by white membranous infiltrates confined to the iris, inflammation was scored as follows: 0 = normal; 1 = iris hyperemia around the pupillary margin; 2 = focal infiltrates; 3 = infiltrates present in less than 25% of the anterior surface of the iris; 4 = involvement of an area less than 50%; and 5 = involvement of an area greater than 50%.

### Histopathological and Immunohistochemical Examinations

At various times, experimental animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight) and perfused with fixative consisting of 4% paraformaldehyde in 0.1 mol/L cacodylate buffer (pH 7.3). The enucleated eyes were embedded in paraffin and 6  $\mu$ m-thick sections were prepared for hematoxylin and eosin staining and immunohistochemical studies. The histopathological severity of the iritis was evaluated by grading the degree of infiltration of inflammatory cells, mostly mononuclear cells, into the iris (4 degrees, from – to +++) and fibrin exudate on the anterior surface of the iris (as – or +).

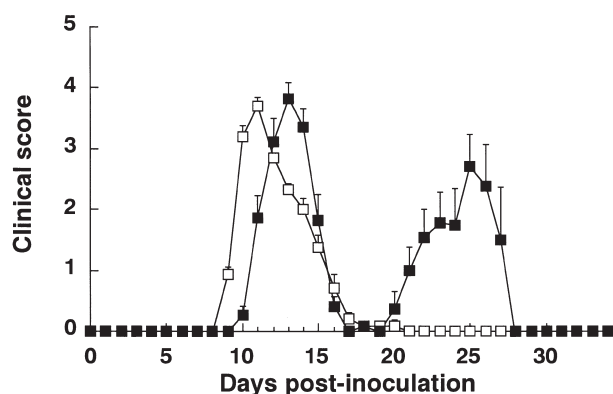
The sources of the primary antibodies and the dilution used are listed in Table 1. The primary polyclonal antibodies against ubiquitin and  $\alpha$ B-crystallin were supplied by Dr. S.-H. C. Yen (Albert Einstein College of Medicine, New York, NY, USA)<sup>26</sup> and Dr. J. E. Goldman (Columbia University College of Physicians and Surgeons, New York, NY, USA),<sup>27</sup> respectively.

Sections were deparaffinized, and the endogenous peroxidase activity was quenched by incubation for 30 minutes with 0.3% H<sub>2</sub>O<sub>2</sub>. After washing, normal sera homologous with the second antibody were used as blocking reagents. Sections were then incubated with each primary antibody overnight at 4°C, and control sections were exposed to PBS. Bound antibodies were visualized by the avidin-biotin-peroxidase complex (ABC, pH 7.4) system using

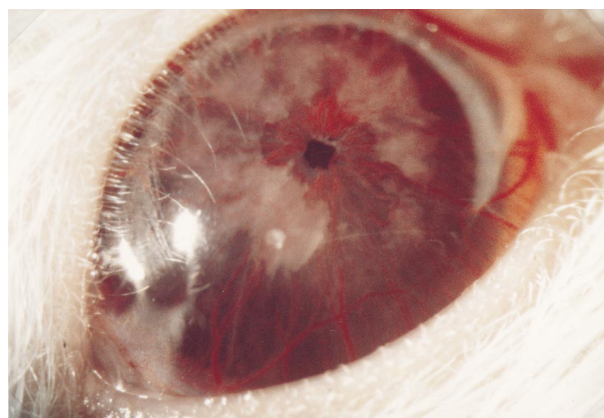
**Table 1.** Primary Antibodies Used in Present Study

Antibody Against	Clonality	Dilution	Source (Reference)
Srp 60	M	1: 800	Clone LK-2 (StressGen, Victoria, Canada)
Ubiquitin	P	1: 800	Dr. S.-H.C. Yen <sup>26</sup>
$\alpha$ B-crystallin	P	1: 500	Dr. J.E. Goldman <sup>27</sup>
Srp 27	M	Ready to use	Clone G3.1 (BioGenex, San Ramon, CA, USA)
Srp 72	M	1: 500	Code 3B6 (Amersham International, Little Chalfont, UK)

Srp: stress-response protein, M: monoclonal, P: polyclonal.



**Figure 1.** Clinical course of experimental autoimmune encephalomyelitis (■) and iritis (□) after immunization of Lewis rats with guinea pig spinal cord emulsified in complete Freund's adjuvant. ■: 27 to 2 (number of rats examined); □: 54 to 4 (number of eyes examined).



**Figure 2.** Anterior segment of eye of experimental autoimmune encephalomyelitis rat at peak of iritis. White membranous filtrates are observed on anterior surface of iris. In addition, iritis is characterized by engorged iris vessels, irregularity of pupillary margin, and miosis with posterior synechiae.

Vectastain ABC kits (Vector Laboratories, Burlingame, CA, USA), and 3, 3'-diaminobenzidine tetrahydrochloride (DAB; DAKO, Glostrup, Denmark) as the final chromogen.

### Ultrastructural Examination

Ultrastructural studies were performed at the peak of iritis in the EAE rats. Under general anesthesia by an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight), experimental animals were perfused with a fixative consisting of 2% paraformaldehyde and 5% glutaraldehyde supplemented with 0.1 mol/L cacodylate buffer (pH 7.4) containing 8%

sucrose. The eyes were enucleated and the anterior segment of the eye (the cornea, iris, and ciliary body) was taken for study. After postosmication and dehydration, the tissues were embedded in epoxy resin and ultrathin sections were cut with a diamond knife and an ultramicrotome (Type MT 6000-XT; RMC, Tucson, AZ, USA). The ultrathin sections were double-stained with uranyl acetate and lead citrate, and examined with a transmission electron microscope (Type H-300; Hitachi, Tokyo).

**Table 2.** Histopathological Findings in Iris of Rats in Group EAE

	Time Course of Disease Process				
	Peak of Iritis (n = 22)	1st Attack of EAE (n = 10)	1st Remission of EAE (n = 4)	2nd Attack of EAE (n = 10)	2nd Remission of EAE (n = 8)
Infiltration of mononuclear cells					
+++ <sup>†</sup>	22 (100)*	0 (0)	0 (0)	0 (0)	0 (0)
++	0 (0)	3 (30.0)	0 (0)	0 (0)	0 (0)
+	0 (0)	4 (40.0)	2 (50.0)	3 (30.0)	2 (25.0)
—	0 (0)	3 (30.0)	2 (50.0)	7 (70.0)	6 (75.0)
Exudation of fibrin					
+ <sup>‡</sup>	22 (100)	0 (0)	0 (0)	0 (0)	0 (0)
—	0 (0)	10 (100)	4 (100)	10 (100)	8 (100)

EAE: experimental autoimmune encephalomyelitis, n: number of eyes examined. Rats in Group EAE: Lewis rats immunized with homogenized spinal cord of guinea pig in Complete Freund's adjuvant.

\* Percentage of eyes examined.

<sup>†</sup> Degree of infiltration of mononuclear cells into iris graded from — to +++.

<sup>‡</sup> Exudation of fibrin on anterior surface of iris rated: +, none; ++, present.

## Results

### Clinical Findings

The rats in group EAE suffered two attacks of EAE (Figure 1), as previously reported.<sup>23</sup> The first peak of EAE appeared at 13 days postinoculation and the second peak appeared at 25 days postinoculation on the average. EAE developed with a score of  $3.8 \pm 0.3$  (mean  $\pm$  SE) in the first attack and  $2.7 \pm 0.5$  in the second attack.

However, iritis appeared only in one attack and the peak was observed at 11 days postinoculation on the average (Figure 1). The iritis score was  $3.7 \pm 0.1$  for this single peak. In group EAE, all rats developed bilateral iritis. The duration of the iritis varied considerably, the shortest episode being 5 days and the longest, 12 days. Iritis was characterized clinically by a white membranous infiltration on the iris surface, engorged iris vessels, irregularity of the pupillary margin, posterior synechiae, and miosis (Figure 2).

The time course of the disease process could be divided into five periods: the first peak of iritis, the first attack of EAE, the first remission of EAE, the second attack of EAE, and the second remission of EAE. We therefore made histopathological studies on the eyes of the EAE rats in the five periods.

In group CFA, none of the rats developed either EAE or iritis.

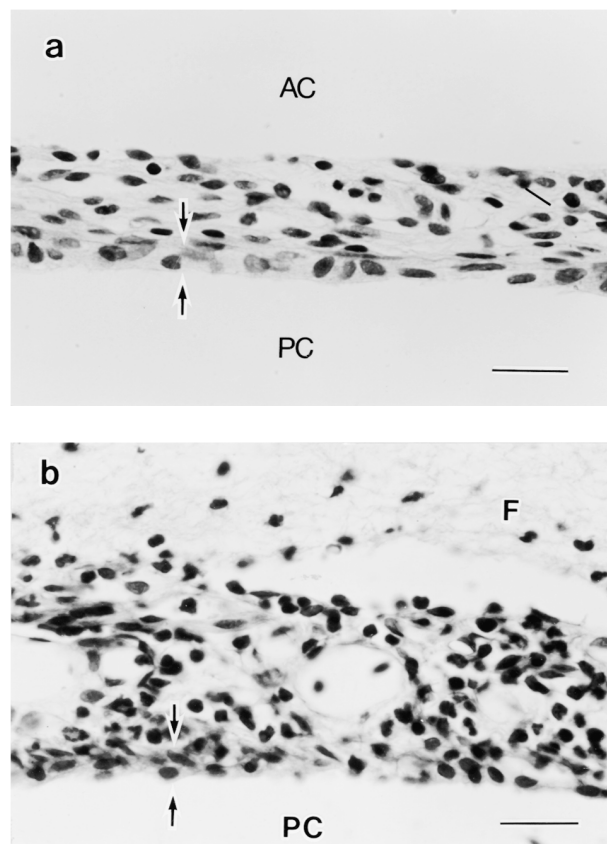
### Histopathological Findings

The iris of each of the rats in group EAE was examined because the major lesion existed in the iris. The histopathological findings during the course of the disease process are shown in Table 2. At the peak of iritis, 22 eyes of the 11 rats examined developed a high degree of iritis. Compared with the untreated rats in group Normal (Figure 3a), group EAE rats showed massive infiltration of inflammatory cells, mostly mononuclear cells, into the iris stroma, and exudation of a large mass of fibrin with mononuclear cells on the anterior surface of the iris (Figure 3b, Table 2). After the first attack of EAE, mild or slight infiltration of mononuclear cells was observed in the iris of 2–5 rats (4–10 eyes) but no fibrin exudate was detected (Table 2). There were no pathological changes in the choroid or retina.

The rats in group CFA had no histopathological evidence of iritis.

### Ultrastructural Findings

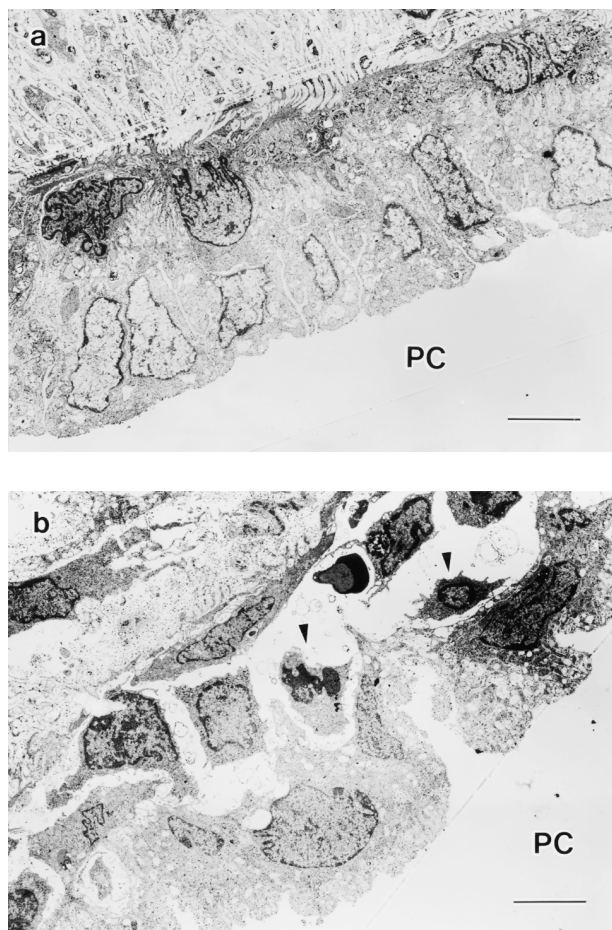
In the untreated rats, the deeply pigmented epithelium of the iris was arranged into a two-layered



**Figure 3.** Light microscopic findings of iritis. (a) Untreated rat. (b) Experimental autoimmune encephalomyelitis rat at peak of iritis. Massive invasion by inflammatory cells, mostly mononuclear cells, into iris. Large mass of fibrin with mononuclear cell exudates on anterior surface of iris. AC: anterior chamber, PC: posterior chamber, F: fibrin. Arrows indicate posterior epithelium of iris. Hematoxylin and eosin staining. Bars = 20  $\mu$ m.

epithelium. The layers of the iris epithelium consisted of the anterior iris epithelium, which gives rise to the pigmented myoepithelium, the dilator of the iris, and the posterior iris epithelium, which is made up of a single, deeply pigmented cuboidal cell layer (Figure 4a). The anterior layer of the iris, the stroma, consisted largely of delicate connective tissue and vessels admixed with dendritic melanocytes.

In the rats in group EAE, the iris pigment epithelium became disarranged, and mononuclear cells infiltrated the area between the anterior and posterior iris epithelium at the peak of the iritis (Figure 4b). The organelles of the iris pigment epithelium were not affected. Neither affected myelinated nerves nor high endothelial venules were observed in the inflamed iris of the rats with EAE.



**Figure 4.** Electron micrographs of epithelium of the iris. **(a)** Untreated rat, iris epithelium shows well arranged two-layered epithelium. **(b)** Experimental autoimmune encephalomyelitis rat at peak of iritis, iris epithelium is disarranged with infiltrating mononuclear cells (arrowheads). PC: posterior chamber. Bars = 5  $\mu$ m.

### Immunohistochemical Findings

No expression of srp 60 was seen in the epithelium of the iris of the rats in either group CFA or group Normal (Figure 5a). When control sections with EAE were incubated with PBS, no reaction products were seen (Table 3). Srp 60, however, was strongly expressed but limited to the posterior cuboidal epithelium of the iris at the peak of iritis (Figure 5b) in 20 of 22 (90.9%) eyes in the group EAE rats. There was a gradual decrease in the expression of srp 60 and iritis during the clinical course of EAE.

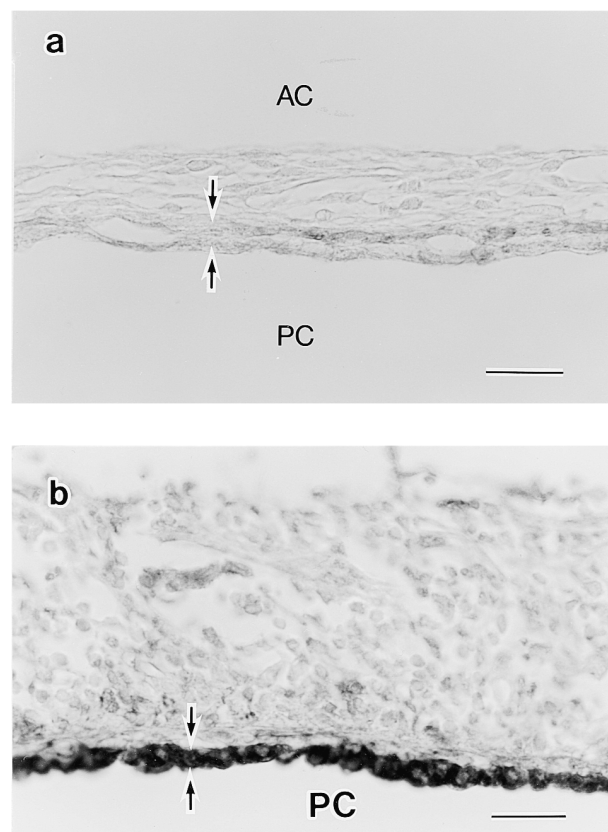
At the peak of iritis, ubiquitin and  $\alpha$ B-crystallin were also expressed in the posterior epithelium of the iris in 4 of 22 (18.2%) and 3 of 22 (13.6%) eyes of the rats in group EAE, respectively. Interestingly,

ubiquitin was expressed in the iris epithelium in 1 of 6 (16.7%) eyes examined from the untreated rats in group Normal. No expression of srp 27 or srp 72 was seen in the iris epithelium of the rats in groups EAE, CFA, or Normal.

No expression of the srps was seen in tissues of the rat other than the iris epithelium or the inflammatory cells of the eyes examined in the EAE, CFA, and Normal groups.

### Discussion

Experimental autoimmune encephalomyelitis is an organ-specific, cell-mediated inflammatory autoimmune disease of the CNS. It is elicited by the immunization of susceptible rat species with an emulsified suspension of CFA and either CNS tissue, myelin basic protein (MBP), proteolipid proteins, or



**Figure 5.** Expression of stress-response protein (srp) 60 in iris (light microscopy). **(a)** Untreated rat. No immunoreaction to antibody against srp 60 is observed in iris. **(b)** Experimental autoimmune encephalomyelitis rat at peak of iritis. Srp 60 is strongly expressed in epithelium of iris but limited to posterior cuboidal epithelium of iris. AC: anterior chamber, PC: posterior chamber. Arrows indicate posterior epithelium of iris. Srp 60 immunostaining. Bars = 20  $\mu$ m.

**Table 3.** Expression of Stress-Response Proteins (Srps) in Iris Epithelium of Rats in Groups EAE, CFA, and Normal

Srps	Time Course of Disease Process (Group EAE)*					Group <sup>†</sup> CFA (n = 20)	Group <sup>‡</sup> Normal (n = 6)
	Peak of Iritis (n = 22)	1st Attack of EAE (n = 10)	1st Remission of EAE (n = 4)	2nd Attack of EAE (n = 10)	2nd Remission of EAE (n = 8)		
Srp 60	20 (90.9)	6 (60.0)	6 (25.0)	2 (20.0)	1 (12.5)	0 (0)	0 (0)
Ubiquitin	4 (18.2)	1 (10.0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (16.7)
αB-crystallin	3 (13.6)	1 (10.0)	0 (0)	1 (10.0)	0 (0)	0 (0)	0 (0)
Srp 27	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Srp 72	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

EAE: experimental autoimmune encephalomyelitis, CFA: complete Freund's adjuvant, n: number of eyes examined.

Values in parentheses indicate percentage of eyes examined.

\*Rats in Group EAE: Lewis rats immunized with homogenized spinal cord of guinea pig in CFA.

†Rats in Group CFA: rats immunized only with CFA.

‡Rats in Group Normal: untreated rats (normal controls).

peptide fragments of the proteins. It is regarded to be a model of multiple sclerosis, although multiple sclerosis remains an extremely complex disease of unknown etiology. There is no spontaneous animal equivalent that includes all aspects of its pathogenesis.<sup>23</sup> EAE is a disease characterized histologically by a cellular infiltration of mononuclear cells into the CNS.<sup>28</sup>

In the present study, EAE was induced in Lewis rats by immunization with homogenized spinal cord of the guinea pig emulsified with CFA. All rats with EAE developed iritis, whereas no rats sensitized with only CFA developed iritis (group Normal). This indicates that the combination of spinal cord and CFA induced the iritis in the Lewis rats.

Verhagen et al<sup>11</sup> showed that MBP was uveitogenic in Lewis rats. They found that the iritis could be adoptively transferred by MBP-specific lines and clones. From this, they suggested that the myelinated nerves of the iris may possess CNS characteristics or at least some similarity to the MBP-specific T cells.

However, Shikishima et al<sup>10</sup> demonstrated ultra-structurally that the myelinated nerves remained intact in the inflamed iris associated with EAE in Lewis rats. High endothelial venules, which are responsible for the transcellular emigration of lymphocytes in various inflammatory diseases or in experimental models, perform a large part in the perivascular inflammatory process in the iris, retina, optic nerve, and CNS in EAE. In our study, the iritis at the peak of EAE was characterized histopathologically by massive infiltration of inflammatory cells, mostly mononuclear cells, into the iris stroma, and exudation of a large mass of fibrin with mononuclear cells on the anterior surface of the iris. As observed by

electron microscopy, the mononuclear cells invaded the area between the disarranged anterior and posterior iris epithelium. However, neither affected myelinated nerves in the iris nor high endothelial venules were observed in the inflamed iris in the rats with EAE. These findings present an enigma for the occurrence and development of iritis associated with EAE, but this discrepancy could be attributed to the difference in dose and content of the CFA supplemented with *M. tuberculosis* or *Bordetella pertussis*.

The immunohistochemical study revealed that ubiquitin, αB-crystallin, srp 27, srp 60, or srp 72 were not expressed in tissues other than the iris pigment epithelium or in the inflammatory cells in the rats in group EAE. This indicates that the iris epithelium is a target tissue and manifests the immunoreaction, ie, iritis.

No expression of the srps was seen in the epithelium of the iris in the rats in group CFA. In contrast, in the group EAE, srp 60 was highly expressed in the posterior cuboidal epithelium of the iris at the peak of iritis in 20 of the 22 (90.9%) of the eyes examined, ubiquitin in 4 of the 22 (18.2%), and αB-crystallin in 3 of the 22 (13.6%) eyes. In the untreated, group Normal rats, only ubiquitin was expressed in the epithelium of the iris in 1 of the 6 (16.7%) eyes examined.

In the group EAE rats, srp 60 in the iris epithelium also showed a gradual decrease in its expression during the clinical course of EAE and iritis, indicating the possibility that srp 60 may be a potential uveitogenic antigen in the iris in EAE. Our ultra-structural findings revealed that srp 60 became a target of the inflammatory cells; mononuclear cells infiltrated the area between the disarranged two-layered pigment epithelium of the iris.

In the lesion of the spinal cord of EAE rats, the inflammatory cells expressed srp 60 during the acute phase, and glial cells expressed srp 60 during the chronic phase.<sup>29</sup> This expression pattern of srp 60 is different from that in the iris of EAE rats. In the rats with EAE, srp 60 was expressed in a limited sense in the posterior cuboidal epithelium of the iris, which corresponds to the inner, retinal layer of the optic cup, regardless of the phase of EAE and iritis. This discrepancy in the pattern of srp expression may be attributed to the difference between bulbar lesion and spinal lesion.

In addition, small amounts of ubiquitin and  $\alpha$ B-crystallin were expressed in the epithelium of the iris at the peak of iritis in the rats with EAE. Small amounts of ubiquitin were also expressed in the epithelium of the iris in the untreated rats. It is known that ubiquitin plays a role in the management of degenerated proteins as the ubiquitin pathway for protein degeneration,<sup>30</sup> and that  $\alpha$ B-crystallin functions as a molecular chaperone.<sup>31</sup> Thus, it appears that these proteins were induced for natural environmental stress and/or protective reaction against inflammatory stress conditions, ie, an attack of the inflammatory cells.<sup>7</sup>

In conclusion, our results suggest that srp 60 may be a potential uveitogenic antigen to the iris in EAE. However, further immunohistochemical studies are needed to support a definite conclusion because we have not examined whether immunization of rats with srp 60 will induce EAE and iritis. Further studies are also needed to disclose how srp 60, although expressed in a limited sense in the posterior epithelium of the iris in the Lewis rats, can develop iritis.

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