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Rodent Models of Experimental Autoimmune Uveitis

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Summary

The model of experimental autoimmune uveitis (EAU) in mice and in rats is described. EAU targets immunologically privileged retinal antigens and serves as a model of autoimmune uveitis in humans as well as a model for autoimmunity in a more general sense. EAU is a well-characterized, robust, and reproducible model that is easily followed and quantitated. It is inducible with synthetic peptides derived from retinal autoantigens in commonly available strains of rats and mice. The ability to induce EAU in various gene-manipulated, including HLA-transgenic, mouse strains makes the EAU model suitable for the study of basic mechanisms as well as in clinically relevant interventions.

Key Words: Autoimmunity; EAU; IRBP; S-Ag; T cells; Th1; Th2; tolerance; uveitis; uveoretinitis.

1. Introduction

Experimental autoimmune uveitis (EAU) is an organ-specific, T-cell-mediated autoimmune disease that targets the neural retina and related tissues; it is induced by immunization with retinal antigens ***(1–3)***. The pathology of EAU closely resembles human uveitic diseases of a putative autoimmune nature in which patients display immunological responses to retinal antigens. Examples of such diseases, which have similar pathology to EAU and in which patients frequently have circulating lymphocytes that respond to retinal proteins, are sympathetic ophthalmia, birdshot retinochoroidopathy, Behcet's disease, and others ***(4,5)***. In the United States alone, there are approx 70,000 cases of uveitis per year, and autoimmune uveitis is estimated to account for approx 10% of severe vision loss.

Although none of the animal models mimics all the features of human disease, each has distinguishing characteristics reminiscent of different aspects of clinical uveitis. Even if the retinal antigens that might be involved in human

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uveitis have not been definitively identified, many uveitis patients respond to the retinal soluble antigen (S-Ag, arrestin) and to a lesser extent to other retinal antigens.

The EAU model has served as an invaluable tool to evaluate novel immunotherapeutic and conventional therapeutic strategies. The EAU model is also useful for study of basic mechanisms of tolerance and autoimmunity to organ-specific antigens in immunologically privileged sites ***(5)***. Thus, EAU is useful as a tool for clinical and for basic studies of ocular and organ-specific autoimmunity.

EAU can be induced in susceptible animals by peripheral immunization with a number of evolutionarily well-conserved uveitogens (purified protein antigens extracted from the retina or their peptides) in adjuvant or by adoptive transfer of lymphocytes specific to these antigens ***(2,5,6)***. In many cases, the sequence of these proteins is known, and the pathogenic fragments have been identified. The majority of the studies have been performed using heterologous bovine antigens because autologous rat or mouse retinal proteins cannot be obtained in sufficient quantities. Uveitogenic retinal proteins are molecules with homologues that can be found as far down the phylogenetic scale as the invertebrates ***(7–9)***. The uveitogenic retinal proteins identified so far are as follows:

1. S-Ag, arrestin. This 48-kDa intracellular photoreceptor protein is involved in the phototransduction cascade. It binds to photoactivated-phosphorylated rhodopsin, thereby apparently preventing the transducin-mediated activation of phosphodiesterase ***(10)***.
2. Interphotoreceptor retinoid-binding protein (IRBP). This 148-kDa protein is found in the interphotoreceptor matrix, which helps in transporting vitamin A derivatives between the photoreceptor and the retinal pigment epithelium. IRBP is composed of four evolutionary conserved homologous domains, which are thought to have arisen by gene duplication ***(8)***.
3. Rhodopsin and its illuminated form, opsin. This 40-kDa membrane protein is the rod visual pigment ***(7)***. Pathogenicity of this protein appears to be conformation dependent as rhodopsin is more pathogenic than opsin ***(11)***.
4. Recoverin, which is a 23-kDa calcium-binding protein.
5. Phosducin, which is 33-kDa soluble cytosolic photoreceptor protein.

Susceptibility to EAU is genetically controlled. It has been observed that different species, and strains within species, vary in their susceptibility. Thus, rats develop EAU after immunization with either S-Ag or IRBP. Guinea pigs are susceptible to S-Ag, but not to IRBP, and mice develop severe disease with IRBP, but not with S-Ag.

Within each species, there are susceptible and resistant strains. In mice and in rats, both major histocompatibility complex (MHC) and non-MHC gene

control have been implicated ***(12,13)***. MHC control is likely connected to the ability to bind and present uveitogenic epitopes. Non-MHC control is more complex and controlled by multiple genetic pathways that are not all defined.

One important factor is the type of effector response that a given strain is genetically programmed to mount. Strains that are dominant Th1 responders tend to be susceptible, whereas strains that are genetically low Th1 responders (e.g., AKR mouse, F344 rat) or overt Th2 responders (e.g., BALB/c mouse) tend to be resistant ***(14,15)***. Furthermore, skewing of the response toward a Th2-like phenotype, such as by treatment with the regulatory cytokines interleukin (IL)-4 and IL-10, can ameliorate EAU ***(16)***. Another factor is different levels of expression of retinal antigens in the thymus, provoking efficient central tolerance to antigens expressed in "adequate" amounts ***(17)***. Other factors, including the hypothalamus–pituitary–adrenal axis control and the number of mast cells in the eye, have also been implicated ***(18,19)***. Genetic control of clinical and experimental uveitis has been reviewed ***(20)***.

Although in many clinical diseases and in some autoimmunity models there is a gender bias of susceptibility, in EAU there does not seem to be an obvious difference in susceptibility between males and females. However, reduced susceptibility is seen in pregnant females ***(21)*** and in animals harboring an active infection (R. Caspi et al., unpublished). Both phenomena may be connected to the cytokine milieu elicited by the physiological state of the individual. Pregnant mice were found to have elevated transforming growth factor (TGF)-β levels ***(21)***, and infection elicits production of interferon (IFN)-γ, which has a protective role in tissue-specific autoimmunity, including EAU ***(22)***. There are no controlled studies of age dependency of susceptibility. We used animals between ages 6 wk and 8 mo without any noticeable differences in disease development (R. Caspi et al., unpublished observations).

EAU in many poorly susceptible strains can be enhanced by treatment with *Bordetella pertussis* in the form of heat-killed bacteria or, better yet, as purified pertussis toxin (PT), concurrent with immunization. This is similar to other autoimmune disease models, such as experimental autoimmune encephalomyelitis. Administration of PT concurrent with uveitogenic immunization permits expression of disease in resistant strains and enhances it in susceptible strains ***(14,23,24)***. The mechanism has for a long time been thought to involve the opening of the blood–organ barrier by PT ***(25,26)***; however, recently we showed that a dominant effect of PT in EAU is to enhance the Th1 response ***(14,23)***. This effect is at least in part because of maturation of dendritic cells by PT ***(15)*** and is mediated by the B subunit of PT ***(27)***. It is important to point out that PT can have strong inhibitory effects on disease as well if it is present at the time of cell migration to the target organ because of its inhibitory effects on chemokine receptor signaling through G protein inhibition ***(28)***. Therefore, not only the

timing of PT administration, but also the amount administered are important because excess PT administered at the time of immunization will persist into the effector stage of the disease and inhibit its expression ***(29)***.

In mice and in rats, EAU induced with the different uveitogenic proteins or their peptides appears to share essentially the same immunological mechanisms and histological features; however, there are species-specific and strain-specific differences in the course of disease ***(1,3,5,30,31)***. The type, number, and size of lesions serve as a basis for a semiquantitative grading system used to score disease severity. A degree of familiarity with ocular histology is needed to grade the disease in a specific fashion. In practice, disease scores assigned by different observers will not always be identical; however, when performed by the same person, grading should be consistent.

Although originally developed in the guinea pig, the two major EAU models in use today are the mouse and the rat. Each has its unique advantages. The mouse is immunologically well characterized, and many congenic and gene-manipulated strains are available that permit sophisticated studies of basic immunological mechanisms. The rat's larger size permits therapeutic and surgical manipulations that are more difficult to do in the mouse; traditionally, the rat is the preferred model for endocrine and physiological studies. More specific attributes of EAU in the rat and mouse models are described in **Subheading 3.** EAU models in the guinea pig, the rabbit, and the monkey have been reviewed elsewhere ***(1,5,32)***. The choice of model will therefore depend on the specific needs of the study.

The methods and descriptions in this chapter deal with the rat and the mouse models of EAU induced by defined synthetic peptides of retinal antigens. Synthetic peptides were chosen because they can be synthesized in every laboratory and do not require access to native retinal antigens from natural or recombinant sources. Last, **Subheading 4.** contains general information and troubleshooting information.

2. Materials

1. Mice (B10.RIII and C57BL/6) (Jackson Laboratories, Bar Harbor, ME).
2. Lewis rats (Charles River Laboratories, Wilmington, MA).
3. Pertussis toxin (cat. no. P-7208, Sigma, St. Louis, MO).
4. IRBP and S-Ag peptides.
5. Dulbecco's modified Eagle's medium (DMEM) and RPMI-1640 (Hyclone Laboratories, Logan, UT).
6. L-Glutamine, sodium pyruvate, nonessential amino acids, gentamicin (Invitrogen, Carlsbad, CA) as medium additives.
7. Fetal bovine serum (FBS; Gemini Bio-Products, Woodland, CA).
8. Freund's complete adjuvant (FCA) and *Mycobacterium tuberculosis* (Difco, Detroit, MI).

Table 1
Susceptibility to EAU of Some Inbred Rat Strains[a]

Strain	MHC	Susceptibility	Antigen	Reference
Lewis	$RT1^{l}$	High	S-Ag, IRBP	***(3,18)***
F344	$RT1^{lv1}$	Low	S-Ag, IRBP peptide R16	***(3,18,29)***
CAR	$RT1^{l}$	High	S-Ag	***(3,18)***
BN	$RT1^{n}$	Low[c]	S-Ag	***(5)***
PVG	$RT1^{c}$	High	S-Ag	***(37)***

[a]Please see other references for more detailed list ***(39,40)***.
[b]Resistance of this strain may vary in different colonies ***(18)***.
[c]Resistance is not overcome by PT treatment.

9. 10% glutaraldehyde (Fisher Scientific, Fair Lawn, NJ) solution was prepared in phosphate-buffered saline (PBS).
10. 10% phosphate-buffered formaldehyde (Fisher Scientific, Fair Lawn, NJ).
11. Ophthalmic dilating solutions: 1% tropicamide (Alcon Laboratories Inc., Fort Worth, TX) and phenylephrine (Akron Inc., Buffalo Grove, IL) for fundus examination procedure.
12. Sterile eye-irrigating physiological solution (Ciba Vision Ophthalmics, Atlanta, GA).

3. Methods

3.1. EAU in Rats

The model of EAU, originally established in the guinea pig using homologous uveal tissue ***(33–35)***, was adapted to the rat in 1973 by Wacker and Kalsow ***(36)*** using whole retinal extracts and was subsequently refined by de Kozak et al. using the retinal S-Ag ***(37)***. IRBP was shown to be uveitogenic in rats ***(38)***.

Susceptibility of EAU varies among different rat strains. The strain most commonly used for EAU studies is the Lewis rat. In the Lewis rat, the disease is monophasic, which develops characteristically severe uveitis and has served as a "standard" against which responses of other strains are compared. It appears that both MHC and non-MHC genes play a role ***(12)***; however, because of the limited availability of congenic and MHC-recombinant rat strains, their relative effects have not been well separated. **Table 1** summarizes the susceptibility of some common inbred rat strains to EAU induced with the native S-Ag or IRBP. A more detailed list has been published elsewhere ***(39,40)***.

The retinal uveitogenic proteins have historically been defined as such in the Lewis rat model, the most highly susceptible rat strain known. The normal

dose is 30–50 μg of S-Ag or IRBP or 50–100 μg of rhodopsin emulsified in FCA. Lewis rats do not require PT as part of the immunization protocol to develop disease, but if used, PT will cause earlier onset and enhanced disease scores. The immunizing protocol of 30 μg of peptide R16 of bovine IRBP (**Table 2**) in FCA normally results in disease onset on day 9 or 10. In contrast, immunization with 30 μg of S-Ag in FCA usually results in onset between days 12 and 14. Other strains may require PT as an additional adjuvant. Subcutaneous immunization in the thighs and base of the tail with an emulsion of S-Ag or IRBP in FCA was as good or better than the footpad route for induction of disease ***(41)***.

Table 2 shows the commonly used epitopes found consistently pathogenic in the Lewis strain. The peptides that are pathogenic at low doses are considered to contain a major pathogenic epitope.

3.1.1. Induction of EAU in the Lewis Rat by Active Immunization

The Lewis rat strain is highly susceptible to EAU. (In strains that are less susceptible, such as the F344, PT must be used as an additional adjuvant, and a higher dose of antigen is recommended.) Two peptides are recommended as strongly and consistently uveitogenic in the Lewis rat: peptide R16 of bovine IRBP (residues 1177–1191, sequence ADGSSWEGVGVVPDV) and peptide S35 of human S-Ag (residues 341–360, sequence GFLGELTSSEVATEVP FRLM). S35 tends to cause stronger disease, but onset is a day or two later than with R16.

1. Use 6- to 8-wk-old female Lewis rats (preferably housed in specific pathogen-free environment) with food and water *ad libitum*. If rats are procured from an outside vendor, it is best to acclimate them for a few days before immunization.
2. Prepare an emulsion of the chosen peptide: 30 μg of R16 or S35 in FCA (1:1 v/v) (**Support Protocol 3.3.3.**) by sonication to provide 30 μg of peptide in 200 μL per rat. Spin at 900*g* to remove any air bubbles embedded in the emulsion. A well-prepared emulsion should have the consistency of thick cream.
3. A 16-ga blunt-end needle is used to draw the emulsion into a 1-mL glass syringe, preferably with a Luer lock tip (rubber plungers in plastic syringes tend to soften and stick because of the oil in FCA). Carefully remove any residual air bubbles trapped in the syringe. Change to a 23-ga needle and subcutaneously inject 100 μL at the base of the tail and 50 μL in each thigh.
4. At 7–9 d after immunization, start inspecting the eyes with a flashlight for loss of red reflex (**Fig. 1**). Grade the disease on a scale of 0 (no disease) to 4 (severe disease) based on the scoring method in **Table 3**.
5. Approximately 16 d after immunization (or at least 7 d after onset of the disease), euthanize the rats. Remove the eyes and process them for histopathology (**Support Protocol 3.3.2.**).
6. Examine the hematoxylin- and eosin-stained sections under a microscope and grade the disease histopathologically following the guidelines listed in **Table 4**.

Table 2
Retinal Protein-Derived Peptides Pathogenic for Lewis Rats[a]

Source	Nickname (if any)	Position[b]	Amino acid sequence[c]	Minimal dose[d]	Reference
Bovine S-Ag	Peptide N	281–302 (287–297)	VPLLANNRERRGIALDGKIKHE	50 μg	***50***,***51***
	Peptide M	303–320 (303–317)	DTNLASSTIIKEGIDKTV	50 μg	***52***
Bovine IRBP	R23	1091–1115	PNNSVSELWTLSQLEGERYGSKKSM	100 n*M* (280 μg)	***53***
	R4	1158–1180	HVDDTDLYLTIPTARSVGAADGS	67 μg	***54***
	R14	1169–1191(1182–1190)	PTARSVGAADGSSWEGVGVVPDV	0.1 n*M* (0.2 μg)	***6***,***55***
	R16	1177–1191 (1182–1190)	ADGSSWEGVGVVPDV	0.1 n*M* (0.2 μg)	***6***
Human S-Ag	Peptide 19	181–200	VQHAPLEMGPQPRAEATWQF	25 μg	***56***
	Peptide 35	341–360 (343–356)	GFLGELTSSEVATEVPFRLM	5 μg	***56***,***57***
	Peptide 36	351–370 (356–366)	VATEVPFRLMHPQPEDPAKE	50 μg	***56***,***58***
Human IRBP	H-IRBP 715	521–540 (527–534)	YLLTSHRTATAAEEFAFLMQ[e]	0.1 μg	***59***

[a]More detailed list of other peptides pathogenic to Lewis rats can be found elsewhere ***(39***,***40)***.
[b]Parenthetical numbers indicate position of minimal sequence (if known).
[c]The minimal pathogenic sequence (if known) is underlined.
[d]Pathogenicity was tested in most cases using PT as an additional adjuvant ($1–2 \times 10^{10}$ heat-killed organisms per rat).
[e]An additional epitope may be encoded by the N-terminus (sequence YLLTSHRTATAA).

Table 3
Clinical Grading of EAU in the Rat

Grade[a]	Criteria
0	No disease; eye is translucent and reflects light (red reflex)
0.5 (trace)	Dilated blood vessels in the iris
1	Engorged blood vessels in iris; abnormal pupil contraction
2	Hazy anterior chamber; decreased red reflex
3	Moderately opaque anterior chamber, but pupil still visible; dull red reflex
4	Opaque anterior chamber and obscured pupil; red reflex absent; proptosis

[a]Each higher grade includes the criteria of the preceding one.

Table 4
Scoring EAU Histopathologically in the Rat

Grade	Area of retinal section affected	Criteria
0	None	No disease; normal retinal architecture
0.5 (trace)	<1/4	Mild inflammatory cell infiltration of the retina with or without photoreceptor damage
1	≥1/4	Mild inflammation and/or photoreceptor outer segment damage
2	≥1/4	Mild-to-moderate inflammation and/or lesion extending to the outer nuclear layer
3	≥1/4	Moderate-to-marked inflammation and/or lesion extending to the inner nuclear layer
4	≥1/4	Severe inflammation and/or full-thickness retinal damage

3.1.2. Induction of EAU in the Lewis Rat by Adoptive Transfer

EAU is a $CD4^+$ T-cell-mediated disease. The full histopathological picture can be obtained by adoptive transfer of immune lymph node or spleen cells or long-term $CD4^+$, MHC class II-restricted T-cell lines in the absence of detectable titers of serum antibodies ***(2,42)***. The cells must be activated with antigen or mitogen just prior to transfer to mediate disease efficiently, suggesting that

activation-dependent functions (lymphokine production, expression of adhesion molecules, etc.) are important. The minimal number of cells required to transfer the disease depends on their source and specificity ***(2,42,43)***. The following protocol describes induction of disease using primary cultures of lymph node cells and peptide antigen.

1. Rats to be used as cell donors are immunized as mentioned in **Subheading 3.1.1.** The donor rats are sacrificed 10–12 d postimmunization, and draining lymph nodes (inguinal and iliac) are harvested and cultured with the immunizing peptide as follows.
2. Keep isolated lymph nodes in RPMI-1640 media containing 1% FBS or rat serum. Prepare single-cell suspension by crushing the nodes using a plunger from a disposable plastic syringe on a sterile mesh in a Petri dish, with some sterile media to release the cells.
3. Transfer the cells to a 50-mL centrifuge tube and wash the Petri dish with additional media. Spin the cells at 300*g* and discard the supernatant. Resuspend the pellet in fresh media and repeat the washing procedure one or two times. Resuspend the final cell pellet in a small volume of RPMI-1640 media containing 1% FBS.
4. Count viable cells using an exclusion dye such as trypan blue.
5. Adjust the cell suspension to 5×10^6 cells/mL by adding complete RPMI media (**Support Protocol 3.3.4.**). Add the peptide used for immunization of the cell donors (R16 or S35) to a final concentration of 5 μg/mL.
6. Distribute 2-mL aliquots into 12-well tissue culture plates and incubate the culture at 37°C for 3 d in 5% CO_2 tissue incubator.
7. After 72 h, collect, wash, and count the cells (as described in **Subheading 3.1.2.**, **steps 3** and **4**). All procedures are preferably done using RPMI-1640 media with 1% serum for maximal cell viability.
8. Inject to $30–50 \times 10^6$ cells (in 0.3 to 0.5 mL) intraperitoneally into recipient Lewis rats.
9. Starting on day 3 after adoptive transfer, inspect the recipients for disease by examining the eyes with a flashlight for loss of red reflex (**Fig. 1**) and grade the disease as described in **Table 3**.
10. Between 11 and 14 d after adoptive transfer (around 7 d after disease onset), euthanize the rats, collect the eyes (**Support Protocol 3.3.2.**) and process them for histopathological grading of EAU according to **Table 4**.

3.1.3. Expected Course of Disease

The course of disease in the Lewis rat is typically acute and short. The time of onset will vary depending on severity of the developing disease, the antigen, and the antigen dose. S-Ag and its peptides tend to give more severe disease but a later onset than IRBP and its peptides. For instance, 30 μg of S35 peptide in FCA without PT results in onset around days 12–14, and a similar dose of R16 in FCA without PT results in onset around days 10–12. If pertussis is used

as additional adjuvant, the time of onset will be shortened by 2 d and will usually be more uniform than without pertussis. The amount of antigen used to elicit disease can be reduced severalfold if PT is used.

Onset of EAU induced by adoptive transfer is usually on days 4–7 (i.e., about a week earlier than for active immunization). The active EAU in the Lewis rats lasts 1–2 wk, and the disease does not relapse. The rapid onset and acute course of EAU in the Lewis rat makes it difficult to evaluate therapeutic intervention during active disease. Alternatively, to look at efferent stage disease, begin intervention 7 d after immunization, when immune lymphocytes are already present, or use an adoptive transfer system.

3.1.4. Quantitation

3.1.4.1. Clinical

Onset of disease in the albino Lewis rat can be recognized by inspecting the eyes with the aid of a good flashlight (**Fig. 1**). The normal eye appears translucent and reflects the light (red reflex). The first sign of uveitis is engorgement of blood vessels in the iris and an irregular pupil that cannot contract in response to light (caused by the iris adhering to the lens). Leukocyte infiltration and deposition of fibrin is first seen as dulling of the red reflex, progressing to complete opacification of the anterior chamber. The eye swells and can protrude from its socket (proptosis). In very severe cases, hemorrhages in the anterior chamber and even perforation of the cornea can occur. In the last case, the animal should be euthanized. We grade clinical EAU on a scale of 0 (no disease) to 4 (severe disease) using the criteria listed in **Table 3**.

3.1.4.2. Histopathology

EAU is defined primarily as a posterior segment disease because the target antigens reside in the retina. Lewis rats can develop very severe anterior chamber inflammation, which can lead to corneal perforation. Therefore, although clinical follow-up by anterior chamber inflammation is important and yields valuable information, the final readout should be recorded by histopathology. We grade EAU by histopathology based on number and extent of lesions and use an arbitrary scale of 0 (none) to 4 (maximum severity), in half-point increments, as shown in **Table 4** ***(40)***. Typical EAU vs normal histology is shown in **Fig. 3**. Although Fig. 3 shows EAU histopathology in the mouse, it is representative of that seen in the rat.

3.2. EAU in Mice

Mice are highly resistant to EAU induction with S-Ag, but some strains can develop severe EAU after immunization with IRBP ***(31)***. As in rats, age and

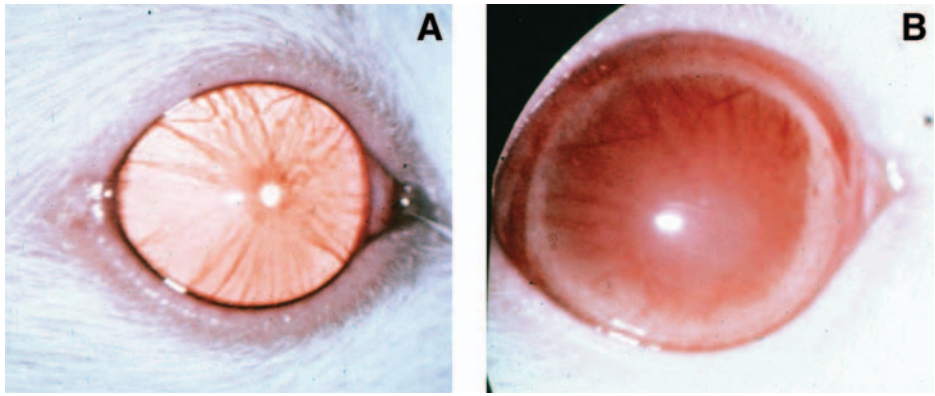

Fig. 1. Clinical appearance of EAU in the Lewis rat by anterior chamber examination. **(A)** Normal eye; translucent appearance; pupil and iris blood vessels are clearly visible, and the vessels are not congested. **(B)** Uveitic eye; the eye appears larger because of swelling and proptosis; red reflex is absent, and pupil is obscured.

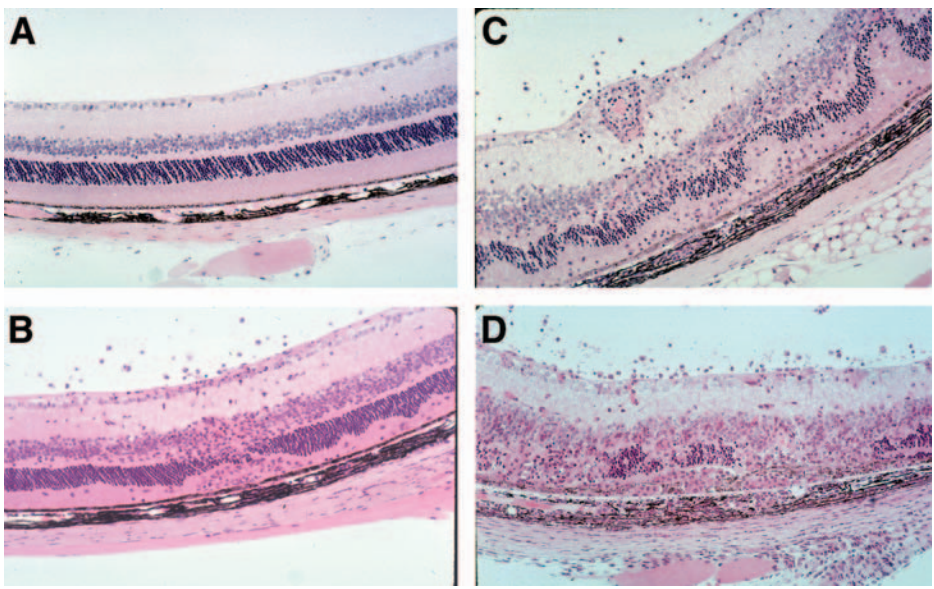

Fig. 3. Histopathology of EAU in the B10.RIII mouse. **(A)** No disease (score 0); note well-preserved photoreceptor cell layer. **(B)** Mild disease (score 1–2). **(C)** Moderate disease (score 2–3). **(D)** Severe disease (score 3–4). EAU in the rat shows essentially the same type of histopathology.

Table 5
Susceptibility of Different Mouse Strains to IRBP EAU[a]

Strain	H-2	Susceptibility[b]	Epitope, position, and reference
B10.RIII	r	Very high	SGIPYIISYLHPGNTILHVD (161–180) ***(46)***
B10.A	a (I-A^k)	High	ADKDVVVLTSSRTGGV (201–216) ***(45)***
B10.BR	k	High	Same as B10.A
A/J	a (I-A^k)	Medium	Same as B10.A
C57BL/6	b	Medium	GPTHLFQPSLVLDMAKVLLD (1–20) ***(44)***
C57BL/10	b	Medium	Same as C57BL/6

[a]More detailed list of other mouse strains can be found elsewhere ***(39)***.
[b]Mice were immunized by the split-dose method with 100 μg IRBP in FCA in the footpads and were given 1 μg of PT intraperitoneally. Eyes were harvested at 5 weeks. Adapted from Caspi et al. ***(13,22)***.

sex do not appear to have a major influence on susceptibility to disease. **Table 5** lists some common EAU-susceptible mouse strains and IRBP epitopes that have been found to induce disease in each.

The epitopes of the IRBP molecule pathogenic for the H-2^b, H-2^r, and H-2^k haplotypes have been identified ***(44–46)***. In the B10.A mouse (K^k, A^k, E^k, D^d), MHC control of susceptibility has been tentatively mapped to the I-A region, with modifying influences from the I-E region ***(13)***. In addition to a susceptible H-2 haplotype, which can bind and present the uveitogenic epitopes, the strain must also have a "permissive" background to express disease. Studies with MHC-congenic mice showed that a nonpermissive background can completely prevent expression of disease in mice with a "susceptible" H-2. Known susceptible H-2 haplotypes include $H2^r$, H-2^k, H-2^a (shares class II subregion with H-2^k), H-2^b, H-2^q, and H-2^d. The last two, initially thought to be resistant, can in fact be shown to be EAU susceptible when IFN-γ is neutralized or knocked out (***22*** and R. Grajewski and R. Caspi, unpublished, 2003).

The most susceptible mouse strain currently known is B10.RIII (H-2^r). Unlike other mouse strains, this strain does not require PT to develop disease by active immunization either with IRBP or with its major pathogenic epitope, residues 161–180 of human IRBP. B10.RIII does, however, require PT if the murine sequence of peptide 161–180 is used. This is likely because of thymic elimination of high-affinity T cells reacting with the endogenous version of peptide 161–180 (***46a***). In less-susceptible mouse strains, such as B10.A or C57BL/6, a higher dose of IRBP (50–150 μg) or of the peptide appropriate for the haplotype in FCA is injected subcutaneously concurrent with PT (0.5 μg) given intraperitoneally.

One of the strengths of the mouse model of EAU is the ready availability of many gene-manipulated mouse strains. Knockouts and transgenics for various immunologically relevant genes have been, and continue to be, instrumental in unraveling the basic mechanisms in uveitis. EAU can be induced with human IRBP residues 1–20 in mice of the H-2^b haplotype, which is expressed by the C57BL/6 and 129 strains, that typically serves for production of transgenics and knockouts ***(44)***.

It is also important to mention here one newly established model, the "humanized" EAU model in HLA class II transgenic mice. Uveitic diseases in humans show strong associations with specific HLA class I or class II alleles, which vary depending on the disease and the population studied. The genetic associations in uveitis have recently been reviewed ***(20)***. MHC association strongly supports a role for antigen presentation by HLA molecules in the etiology of uveitis. HLA class I and II transgenic mice afford a model to study these effects. Both HLA class I (A29) and class II transgenic (DR3, DR4, DR2, DQ6, DQ8) mice have been used to study uveitis. HLA A29 transgenics develop a spontaneous uveitis ***(47)***, whereas the various class II transgenic mice develop EAU after immunization with IRBP ***(48)***. Importantly, HLA-DR3 transgenic mice develop severe uveitis after immunization with S-Ag, which is thought to be involved in human uveitis but is not uveitogenic in wild-type mice. These humanized models support an etiological role for retinal antigens, which are uveitogenic in animals, in human uveitis and validate use of the EAU model for the study of human disease.

3.2.1. Induction of EAU in the B10.RIII Mouse by Immunization

The following protocol is given for B10.RIII mice, the most susceptible mouse strain currently known. Human IRBP peptide 161–180 is used. This peptide does not require PT as part of the immunization protocol, although PT will promote more severe disease and an earlier onset ***(23)***. A variation of the protocol is given for use in C57BL/6 mice.

1. Use 6- to 8-wk-old female B10.RIII mice ($H2^r$), preferably housed under specific pathogen-free conditions with food and water available *ad libitum*. If purchased from an outside vendor, let acclimate to animal facility for a few days before immunization.
2. Emulsify IRBP peptide 161–180 (*see* sequence in **Table 5**) in FCA (1:1 v/v) by sonication to provide 10–25 μg peptide in 0.2 mL emulsion per mouse. Severity of disease obtained will depend on the amount of peptide used ***(23)***. Spin at 900*g* to remove air bubbles trapped in the emulsion. A well-prepared emulsion has the consistency of thick cream.
3. Use a 16-ga blunt-end needle to draw the emulsion in a 1-mL glass syringe with a Luer lock tip (rubber plungers in plastic syringes tend to soften and stick

Table 6
Clinical Scoring of EAU in the Mouse

Grade	Criteria
0	No change
0.5 (trace)	Few (1–2) very small, peripheral focal lesions; minimal vasculitis/ vitritis
1	Mild vasculitis; <5 small focal lesions; ≤1 linear lesion
2	Multiple (>5) chorioretinal lesions and/or infiltrations; severe vasculitis (large size, thick wall, infiltrations); few linear lesions (<5)
3	Pattern of linear lesions; large confluent lesions; subretinal neovascularization; retinal hemorrhages; papilledema
4	Large retinal detachment; retinal atrophy

because of the oil in FCA). Carefully remove any air bubbles trapped in the syringe. Change to a 23-ga needle and inject each mouse subcutaneously with 0.2 mL emulsion, dividing the dose among the two thighs (50 μL each) and base of the tail (100 μL).

4. At 12 d postimmunization, monitor the eyes of these mice for disease induction by inspecting the fundus under a binocular microscope (**Support Protocol 3.3.1.**). Grade the animals on a scale of 0 (no disease) to 4 (severe disease) using the criteria described in **Table 6**.
5. Approximately 21 d after immunization (or 7 d after the disease onset), euthanize the mice and remove eyes for histopathology (**Support Protocol 3.3.2.**).
6. Examine the hematoxylin- and eosin-stained sections under a microscope and grade the disease histopathologically following the guidelines in **Table 7**.

3.2.2. Alternative Protocol: Induction of EAU in the C57BL/6 Mouse by Immunization

1. Use 6- to 8-wk-old female C57BL/6 mice ($H2^b$), preferably housed under specific pathogen-free conditions with food and water available *ad libitum*. If purchased from an outside vendor, let acclimate to animal facility for a few days before immunization.
2. Emulsify human IRBP peptide 1–20 (*see* sequence in **Table 5**) in FCA (1:1 v/v) by sonication to provide 200–300 μg peptide in 0.2 mL emulsion per mouse. Severity of disease obtained will depend on the amount of peptide used ***(44)***. Spin at 900*g* to remove air bubbles trapped in the emulsion. A well-prepared emulsion has the consistency of thick cream.
3. Prepare PT as adjuvant. Pertussis bacteria are not as potent as the purified toxin. If reconstituting a lyophilized preparation, bring up in PBS to 1 mg/mL or another convenient concentration with 1% mouse serum. The serum serves to prevent

Table 7
Grading EAU Histopathologically in the Mouse

Grade	Criteria
0	No change
0.5 (trace)	Mild inflammatory cell infiltration; no tissue damage
1	Infiltration; retinal folds and focal retinal detachments; few small granulomas in choroid and retina, perivasculitis
2	Moderate infiltration; retinal folds, detachments, and focal photo receptor cell damage; small-to-medium-size granulomas, perivas culitis, and vasculitis
3	Medium-to-heavy infiltration; extensive retinal folding with detachments, moderate photoreceptor cell damage; medium-size granulomatous lesions; subretinal neovascularization
4	Heavy infiltration; diffuse retinal detachment with serous exudate and subretinal bleeding; extensive photoreceptor cell damage; large granulomatous lesions; subretinal neovascularization

adsorption of the PT to plastic and glass tubes, pipets, and syringes. The stock solution can be kept at 4°C for up to 1 mo. For injection, prepare solution of 5 μg/mL just before use by diluting 1:20 in PBS with 1% syngeneic serum (0.1 mL will result in a dose of 0.5 μg).

4. Proceed as described in **Subheading 3.2.1.** for the B10.RIII mouse, starting from **step 3**.

3.2.3. Induction of EAU in B10.RIII or C57BL/6 Mice by Adoptive Transfer

Adoptive transfer of primed uveitogenic effector cells, just like in rats, can induce EAU in mice. Primary cultures from immunized donors, as described in the protocol in this section, can be used as a source of effector cells. Alternatively, long-term antigen-specific T-cell lines, which are typically $CD4^+$ cells of the Th1 phenotype, can be derived from draining lymph node cells of IRBP- or peptide-immunized mice ***(46)***.

1. Immunize donor mice as described in **Subheading 3.2.1.** and **3.2.2.** using peptide 161–180 of human IRBP for B10.RIII mice and peptide 1–20 of human IRBP for C57BL/6 mice, respectively.
2. After 10–14 d, harvest draining lymph nodes (inguinal and iliac) as well as spleens from donor mice.
3. Place isolated lymph nodes and spleens in DMEM media containing 1% FBS or mouse serum. Prepare a single-cell suspension (lymph nodes and spleen) by disrupting the spleens and the lymph nodes using a sterile rubber plunger from a

disposable plastic syringe on a sterile mesh in a Petri dish with some sterile media to release the cells.
4. Pool the suspensions of lymph node cells and of splenocytes and transfer to 50-mL centrifuge tubes. Wash the cells by centrifuging at 300*g*. Discard the supernatant and resuspend the pellet in fresh media. Repeat the washing one or two times. Resuspend the final cell pellet in a small volume of DMEM media containing 1% serum to maintain maximal viability of the cells.
5. Take a small aliquot and count the viable cells using a vital dye such as trypan blue.
6. Adjust the cell concentration to 10×10^6 live cells/mL in complete DMEM media (**Support Protocol 3.3.4.**) containing 20–25 μg/mL of the immunizing peptide. Optionally, including 5 ng/mL of IL-12 in the culture will result in a more highly Th1-polarized population that will transfer disease with fewer cells ***(44)***.
7. Distribute 150-mL aliquots into 175-cm^2 tissue culture flasks and incubate for 3 d in a humidified 37°C tissue incubator with 10% CO_2.
8. Important: After 24 h and again after 48 h of culture, bring the nonadherent cells into suspension by gently rocking the flasks and transfer the entire suspension to a new flask of the same size. This gets rid of excess (adherent) macrophages that produce inhibitory factors.
9. After 72 h of culture, collect, wash, and count the viable cells. There usually are many dead cells in the culture at this point. Purifying the cells by centrifugation over Ficoll (Lympholyte M, Accurate Biochemicals, Westbury, NY) can help obtain a more pure population of live cells and facilitate counting. Suspend the counted cells in DMEM media with 1% normal serum.
10. Inject cells intraperitoneally into syngeneic recipients in a volume of 0.5 mL or less. For B10.RIII mice, inject $30–50 \times 10^6$ cells. For recipient C57BL/6 mice, inject $50–100 \times 10^6$ cells. If IL-12 was used during culture, this number may be reduced.
11. Starting 4 d after adoptive transfer, inspect the recipients for disease induction by fundus examination (*see* **Support Protocol 3.3.1.**).
12. Between 12 and 14 d after adoptive transfer (at least 7 d after disease onset), euthanize the mice, collect the eyes, and process the eyes for histopathology. Score the eyes according to the criteria listed in **Table 7**.

3.2.4. Expected Course of Disease

The severity and clinical course of EAU induced with peptide 161–180 in B10.RIII mice is typically monophasic, resembling that for the Lewis rat, and lasts for 2–3 wk. High-intensity immunization results in an acute form of disease, with onset as early as day 8 or 9, and widespread photoreceptor damage, whereas a lower intensity immunization will result in milder disease with progressively later onset ***(23)***. In other strains (e.g., B10.A) it is possible to observe relapsing disease after a low-to-intermediate intensity protocol ***(49)***. As in the Lewis rat, the rapid onset and acute course of EAU induced with

peptide 161–180 in B10.RIII mice makes it difficult to evaluate therapeutic intervention during active disease. Alternatively, to look at efferent stage disease, begin intervention 7 d after immunization, when immune lymphocytes are already present, or use an adoptive transfer system.

3.2.5. Quantitation

3.2.5.1. Clinical

Unlike rats, in susceptible strains of mice (B10 background), black pigmentation of the eyes and an often mild involvement of the anterior chamber preclude detection of disease by anterior chamber examination. However, in pigmented strains, it is possible to observe changes in the fundus of the eye under a binocular microscope after dilating the pupil (**Support Protocol 3.3.1.**) (**Fig. 2**). Visualization of the fundus is possible if the anterior chamber does not become infiltrated with cells or after the infiltrate clears (usually within a few days after onset). The nature, number, and severity of the lesions are used as criteria for clinical scoring on a scale of 0 (no change) to 4 (severe disease) (**Table 6**).

3.2.5.2. Histopathology

Histological grading is the final readout of the disease and is performed on methacrylate-embedded tissue sections (**Support Protocol 3.3.2.**). Disease is scored on a scale of 0 (no disease) to 4 (maximum disease) in half-point increments according to a semiquantitative system described in **ref. *31***. Examples of various grades of pathology are shown in **Fig. 3**.

3.3. Support Protocols

3.3.1. Funduscopic Examination

Funduscopic examination can be used to detect and evaluate EAU in pigmented animals, provided that the anterior segment of the eye remains clear. In this procedure, the retina of the eye is examined through the dilated pupil under a microscope. Funduscopy is a good tool for determining the onset and clinical grading of disease in pigmented animals, but not in albinos. The following materials are used:

1. Ophthalmic dilating solutions: 1% tropicamide and phenylephrine.
2. Sterile physiological solution (either normal saline, PBS, or artificial tears).
3. Binocular microscope with coaxial illumination.
4. Microscope coverslip.

Anesthetize the animals and dilate the pupil with one to two drops of dilating solution. It takes several minutes for the medication to take effect. Place a

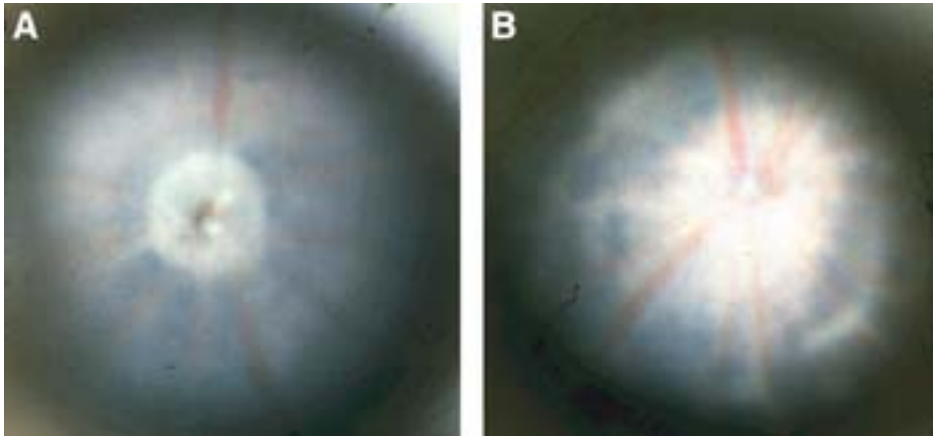

Fig. 2. Clinical appearance of EAU in the B10.A mouse by funduscopic exam. **(A)** Normal eye; note blood vessels radiating from the optic nerve head. **(B)** Uveitic eye; note retinal detachment obscuring the optic nerve head and numerous white linear lesions.

drop of sterile physiological solution and a microscope coverslip on the cornea to equalize refraction. Manipulate the head of the mouse under the microscope to inspect as far up the sides of the retina as possible. Look for engorged blood vessels, constricted blood vessels ("cuffing"), white linear lesions, subretinal hemorrhages, and retinal detachment. Typical appearance of a uveitic fundus is shown in **Fig. 2**. If you have difficulty in grading the disease, please *see* **Subheadings 4.1.** and **4.2.** for some possible reasons and solutions.

3.3.2. Handling of Eyes for Histopathology

Eyes should be collected within 15 min of euthanasia; otherwise, autolysis may preclude correct evaluation of the results. Enucleation in rats should be performed by carefully dissecting the globe from the periocular tissues and the optic nerve without applying pressure on the globe to avoid maceration of delicate ocular tissues, which become even more fragile when inflamed. In mice for enucleation, the eye should be made to protrude by applying pressure on the skull and plucked free of the tissue with a curved forceps.

Freshly enucleated eyes are fixed in 4% phosphate-buffered glutaraldehyde for 1 h and then transferred into 10% phosphate-buffered formaldehyde at least overnight or until processing. The brief fixation in 4% glutaraldehyde prevents artifactual detachment of the retina from the choroid. However, leaving the eyes in glutaraldehyde for too long will cause excessive hardening of the lens,

which will make sectioning difficult. The grading is conveniently done on methacrylate- or paraffin-embedded tissue sections cut up to 8 µ thick and stained with hematoxylin and eosin. To arrive at the final grading, several sections cut through the pupillary–optic nerve axis should be examined for each eye.

It should be remembered that, in this type of visual scoring, there is always an element of subjectivity. Therefore, it is important that the results be read in a masked fashion, preferably always by the same person. Whenever possible, both eyes should be evaluated for the disease as disease may be unilateral. If it becomes experimentally necessary to take only one eye, always collect the same eye to average out this random variation. Please *see* **Subheading 4.3.** for some potential problems and solutions.

3.3.3. Freund's Complete Adjuvant (FCA)

Using a porcelain mortar and pestle, 100 mg heat-killed *M. tuberculosis* (strain H37Ra) is crushed into a fine powder. Mix with 70 mL of FCA (1.0 mg/mL of *M. tuberculosis*). Store at 4°C until used. The suspension must be thoroughly mixed before each use as the mycobacterial particles settle quickly to the bottom.

3.3.4. Complete Medium for Cell Culture (RPMI-1640 or DMEM)

1. 500-mL bottle of RPMI-1640 or DMEM.
2. 50 mL FBS (10% final).
3. 5 mL 200 m*M* L-glutamine (2 m*M* final).
4. 5 mL 100 m*M* sodium pyruvate (1 m*M* final).
5. 5 mL 10 m*M* nonessential amino acids (0.1 m*M* final).
6. 0.5 mL 50 mg/mL gentamicin (50 µg/mL final).
7. 0.05 mL 0.5 *M* 2-mercaptoethanol (5 µ*M* final).

Filter though 0.2-µm filter and store up to 4 wk at 4°C.

4. Notes

1. Although the EAU model is very robust, problems can arise with the technique at a number of levels. If no disease is obtained, the following questions should be considered (some may seem trivial or obvious, but we have encountered all of them):
   a. Has the antigen been uveitogenic in previous experiment? If this is a new synthesis, a synthesis error might have changed the pathogenic epitope.
   b. Has a sufficient dose of antigen been used?
   c. Has the adjuvant been prepared correctly: use of enough mycobacteria, mixed before sampling (mycobacteria may have settled), use of a well-prepared (thick) emulsion?

d. Has the correct strain and substrain of mouse or rat been used? Substrains of the same strain, and even the same strain from a different vendor, may vary in their susceptibility.
e. Are the animals in poor health, stressed, or harboring an infection? Mice that are unhealthy, chronically stressed (have elevated corticosteroid levels), or actively infected (high levels of circulating interferon) will frequently fail to develop disease.
f. Have the mice developed abscesses at the site of immunization (reaction to the mycobacteria in FCA) and been promptly treated by the facility veterinarian with a nonsteroidal anti-inflammatory agent such as ibuprofen? These agents may inhibit induction of disease.

Susceptibility varies with strain. For some commonly used mouse strains immunized with IRBP, hierarchy of susceptibility is B10.RIII > B10.A = B10.BR > C57BL/10 > C57BL/6 ≥ 129.

Chronic exposure to strong light can damage photoreceptor cells and cause retinal degeneration. Albino animals are especially sensitive to this because of lack of pigment in their eyes. For this reason, it is important to protect animals that will be used in EAU experiments from strong light because the resulting retinal damage may confound correct EAU assessment. This includes frequent and prolonged funduscopies.

Some common laboratory mouse strains carry the rd (retinal degeneration) mutation and congenitally lack photoreceptor cells. These strains of course are not appropriate for EAU studies as they do not possess the target tissue. Strains carrying the rd gene include FVB/N, CBA, SLJ/J, PL/J, and most C3H substrains. However, F1 hybrids of these strains with a sighted strain such as B10.RIII or C57BL/6 will be sighted. As determined by crosses between sighted strains, a hybrid's susceptibility is usually intermediate between the two parental strains.

If a strain other than B10.RIII or a B10.RIII hybrid with a less-susceptible strain is used, PT may be needed. It is important to bring up lyophilized PT in a solution containing serum as carrier and to keep the reconstituted stock of PT for no longer than 1 mo. PT solution that has deteriorated will fail to support disease induction. Because different commercial lots of PT may vary in potency, each lot should be titrated for optimal disease-promoting activity. Depending on the lot and the manufacturer, this will usually be between 0.4 and 1 mg for mice and approximately double that for rats.

When anesthetizing animals for any reason, including funduscopy, it is important to keep in mind that anesthetized animals sleep with their eyes open and do not blink. Therefore, if animals are going to be asleep for more than just a few minutes, it is necessary to place an ointment on the eyes to prevent drying of the cornea. Drying of the eyes will inevitably result in exposure keratitis, which will cause corneal opacification and will make follow-up of clinical disease difficult or impossible.

2. Funduscopy is best done under general anesthesia. With some practice, funduscopy can be performed on nonanesthetized animals, but if disease is borderline or

severity scores are to be assigned, it advisable to anesthetize the mouse lightly prior to funduscopy to facilitate a more thorough inspection. Note that the dilating drops cause temporary opacification of the lens within 5–10 min after application, so it is important to complete the funduscopy within that time frame.

3. When cutting the embedded eye tissue, it is important to make sure that the cut is made through the pupillary–optic nerve plane. If the inflammation is mild, pathology is often most apparent around the optic nerve head. Therefore, if sections are cut more laterally, it can be missed. Especially in mild cases, specimens positive on funduscopy may appear to be negative on histology because of the fact that pathology is focal, and the sectioning may have missed it. Therefore, it is important to prepare and examine several nonconsecutive sections.

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