Serologic and Polymerase Chain Reaction Analysis of Intraocular Fluids in the Diagnosis of Infectious Uveitis

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• **PURPOSE:** Infectious uveitis entities are usually rapidly progressive blinding diseases that can be prevented by prompt administration of specific antimicrobial therapy. With the aim of improving early diagnosis in patients with infectious uveitis, intraocular fluid samples from patients with sight-threatening posterior uveitis were investigated to determine the causative agent.

• **METHODS:** Thirty-eight patients with acquired immunodeficiency syndrome (AIDS) and retinitis, eight immunosuppressed patients with retinitis, 16 immunocompetent patients with acute retinal necrosis, and 22 immunocompetent patients with toxoplasmic retinochoroiditis were analyzed

by polymerase chain reaction for the presence of herpesviruses and *Toxoplasma gondii* DNA and for local antibody production against these microorganisms.

• **RESULTS:** In patients with AIDS and retinitis, polymerase chain reaction was positive for cytomegalovirus DNA in 21 (91%) of the 23 ocular fluid samples obtained during active cytomegalovirus retinitis, whereas local antibody production analysis was negative in all cases. In acute retinal necrosis, varicella-zoster virus or herpes simplex virus could be established as the inciting agent in 81% of the cases, using the combination of both techniques. Polymerase chain reaction was positive in all samples obtained within two weeks after the onset of disease. *Toxoplasma gondii* DNA was detected in 4 of 13 samples (31%) from immunocompetent patients with active toxoplasmic retinochoroiditis; in each case, local antibody production was also detected. In contrast, no local antibody production was observed in two of three samples from transplant recipients that were positive for *T. gondii* DNA. All the control samples tested were negative for the above-mentioned tests.

• **CONCLUSIONS:** In patients with AIDS, polymerase chain reaction analysis is preferable above local antibody production in detecting the inciting agent of retinitis. In other cases, the combination of both techniques can make a valuable contribution to the diagnosis.

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HERPESVIRUSES AND *Toxoplasma gondii* are common inciting agents of ocular inflammation in immunosuppressed and immunocompetent individuals.¹⁻³ Cytomegalovirus is the major cause of retinitis in patients with acquired immunodeficiency syndrome (AIDS), and it is rarely seen in immunocompetent individuals.¹ Intraocular inflammation caused by other herpesviruses^{1,4} and even infections with multiple herpesviruses have also been reported in patients with AIDS.⁵ In immunocompetent patients, *T. gondii* is the most common infectious cause of posterior uveitis.³ Varicella-zoster virus and herpes simplex virus are the major causes of acute retinal necrosis, a severe inflammatory eye disease associated with a poor visual prognosis.⁶

Because retinitis can progress rapidly,^{4,6} early diagnosis and start with specific antimicrobial therapy is recommended.⁷ Until now, the diagnosis of infectious uveitis entities generally has been based on clinical characteristics combined with results of serologic laboratory examination. However, fundus examination can be complicated because of vitreous haze or opacities, and various syndromes share similar clinical features. Detection of local antibody production is beneficial in several infectious uveitis entities,⁸⁻¹⁰ but this indirect method is often negative early in the course of the disease.^{8,9} Polymerase chain reaction is a specific method directly detecting DNA of microorganisms, and its advantages include increased sensitivity and more rapidly available results than with viral culture.^{11,12} Polymerase chain reaction has already been used to detect various types of infectious uveitis.¹³⁻¹⁹ To improve early diagnosis, we analyzed intraocular fluid samples from immunosuppressed and immunocompetent patients who had posterior uveitis with a presumed infectious cause for both local antibody production against herpesviruses and *T. gondii*. We also tested for DNA of these microorganisms by polymerase chain reaction. The results show that polymerase chain reaction analysis is preferred to serologic techniques in patients with AIDS and retinitis, whereas in patients with other conditions, the combination of both techniques increases the detection of inciting agents in uveitis.

PATIENTS AND METHODS

A DIAGNOSIS OF UVEITIS WAS BASED ON CLINICAL CHARACTERISTICS according to the criteria of the International

Uveitis Study Group and the Research Committee of the American Uveitis Society (1994).^{20,21} Samples from thirty-eight patients with AIDS and retinitis, eight immunosuppressed patients without AIDS who had retinitis, 16 patients with acute retinal necrosis, and 22 patients with toxoplasmic retinochoroiditis were included.

Blood and aqueous humor or vitreous fluid samples were collected simultaneously. Aqueous humor samples were obtained by performing a paracentesis for diagnostic purposes, and vitreous fluid samples were collected during a therapeutic or diagnostic pars plana vitrectomy. In total, 58 aqueous humor samples and 26 vitreous fluid samples were collected and, depending on the amount of fluid, tested for the presence of different herpesviruses and *T. gondii*. In five patients with acute retinal necrosis, a second ocular fluid sample was obtained at a later stage during the disease.

Patients without intraocular inflammation included control subjects with 13 aqueous humor samples obtained during cataract or glaucoma surgery and vitreous fluid samples collected during a therapeutic vitrectomy in seven cases of proliferative vitreoretinopathy and six cases of diabetic retinopathy. The control patients were informed of these procedures and their consent was obtained. Twelve control vitreous samples were obtained from tissue donors. No information was available concerning seropositivity for herpesviruses or *T. gondii*.

Because it has been shown that aqueous humor and vitreous fluid contain polymerase chain reaction inhibitory factors,²² DNA was isolated from ocular fluid samples by using silica particles, according to the method of Boom and associates.²³ Fifty μ l of ocular fluid was used for DNA isolation, and afterward the template DNA was diluted in 50 μ l of distilled water. The amplifiability of the DNA was tested by adding 12.5 fg of plasmid-containing modified human β -globin DNA, and no polymerase chain reaction inhibitory activity was detected. Primers for cytomegalovirus, varicella-zoster virus, herpes simplex virus, and *T. gondii* were selected from published sequences (Table 1).^{11,12,24-26} We performed a single polymerase chain reaction and chose the outer primers from publications describing a nested polymerase chain reaction. All primers were tested for the optimal annealing temperature and magnesium²⁺ concentra-

TABLE 1

PRIMER SEQUENCES AND POLYMERASE CHAIN REACTION CONDITIONS
FOR HERPESVIRUSES AND *Toxoplasma gondii*

POLYMERASE CHAIN REACTION	PRIMER SEQUENCES (5'→3')	POSITION	MAGNESIUM ²⁺ (mmol)	ANNEALING TEMPERATURE (°C)	REFERENCE
Cytomegalovirus	AGCTGCATGATGTGAGCAAG GAAGGCTGAGTTCTTGGTAA	1767–1786 1894–1913	3.0	56	Cathomas and associates ¹²
Varicella zoster virus	TACGGGTCTTGCCGGAGCTGGTAT AATGCCGTGACCACCAAGTATAAT	51,066–51,090 51,314–51,338	2.0	60	Mahalingam and associates ²⁴
Herpes simplex virus 1	ATCACGGTAGCCCGGCCGTGTGACA CATACCGGAACGCACCACACAA	19–43 218–239	4.0	66	Aurelius and associates ²⁵
Herpes simplex virus 2	AACTCGGGRSCGTACTGYTT* CGGGAACGCGCCGGCCCAAC	1203 1600	5.0	70	Nahass and associates ¹¹
<i>Toxoplasma gondii</i>	GGCATTCTCTCGTTGAAGATT CCTTGGCCGATAGGTCTAGG	Ribosomal DNA	2.0	58	Cazenave, Cheyrou, and Begueret ²⁶

*R indicates A or G, S indicates G or C, Y indicates C or T.

tion (Table 1). Uracil DNA glycosylase was used to control carryover contamination.²⁷ The amplification mixture contained 0.4 pmol of 3' and 5' primer (Isogen Bioscience bv, Amsterdam, The Netherlands), 0.2 mmol of dUTP (Sphaero Q, Leiden, The Netherlands), dATP, dCTP, dGTP (Life Technologies, Breda, The Netherlands), 0.1 unit of *Taq* polymerase (Sphaero Q), 0.1 unit of uracil DNA glycosylase (Life Technologies), and 5 µl of isolated template DNA solution, diluted to a final volume of 50 µl with distilled water. The polymerase chain reaction was performed in a Biometra Trio-Thermoblock (Westburg, Leusden), as follows: the samples were incubated for ten minutes at 37 C and afterward at 95 C for five minutes, then 40 cycles were performed for one minute at 95 C denaturation, one minute at the optimal temperature for annealing, and one and a half minutes at 72 C for elongation. After the last cycle, the samples were incubated for seven minutes at 72 C for final elongation.

One fifth of the polymerase chain reaction product was subjected to electrophoresis on a 1.8% agarose gel, stained with ethidium bromide, and photographed. The gel was subjected to Southern blotting. The oligonucleotide probes (20 pmol) specific for the amplified fragments were labeled with 1.9 MBq of

adenosine 5'-[γ-³²P] triphosphate (ATP) by the method of Maniatis and associates.²⁸ After hybridization, the nylon membranes were washed and exposed to x-ray film at -80 C for 24 hours.²⁸ For varicella-zoster virus and cytomegalovirus, the oligonucleotide probes were designed with the following sequences: varicella-zoster virus: CTC ACT ACC AGT CAT TTC TAT CCA TC; and cytomegalovirus: GGC CTT AGC CTG CAG TGC AC. For herpes simplex virus 1, the probe was used as previously described, with the following sequence: TAC GAG GAG GAG GGG TAT AAC AAA GTC TGT.²⁵ For *T. gondii*, no Southern blotting was performed because of the short length of the polymerase chain reaction product, and for herpes simplex virus 2, no appropriate probe was available. In these cases, we identified the polymerase chain reaction product by ethidium bromide staining after electrophoresis on agarose gel and comparing the length of the product of experimental samples with a positive control.

Using known amounts of plasmid containing viral DNA, 20 target molecules were detected for cytomegalovirus DNA and varicella-zoster virus DNA, and 35 target molecules for herpes simplex virus 1 DNA. For herpes simplex virus 2 and *T. gondii* DNA, the detection limit of the polymerase chain reaction was

not determined because no plasmid containing the viral or parasitic DNA was available.

Toxoplasma gondii RH strain tachyzoites and human fibroblasts infected with the cytomegalovirus strain AD 169, varicella-zoster virus, or herpes simplex virus 1 and 2 strains isolated from patients were tested as positive controls. No cross-reactivity between each of the primer sets and positive control subjects for herpesviruses, *T. gondii*, or human DNA was observed.

Local antibody production analysis to herpesviruses and *T. gondii* was performed by indirect immunofluorescence, as described earlier.⁸ Local antibody production was determined by calculating the Goldmann-Witmer coefficient²⁹:

$$\frac{\text{antibody titer AH or VF}}{\text{antibody titer serum}} : \frac{\text{total IgG AH or VF}}{\text{total IgG serum}}$$

The ratio of anti-herpesviral antibody levels in serum and aqueous humor or vitreous fluid was compared to the ratio of total IgG in serum and aqueous humor or vitreous fluid. Theoretically, a Goldmann-Witmer coefficient greater than 1 should indicate intraocular antibody production. In view of the variability in the results of various measurements, local antibody production was defined as positive when the Goldmann-Witmer coefficient exceeded 3.^{8,30} Previously, we have shown that ten control samples from patients with cataracts and 14 vitreous fluid samples from patients with proliferative vitreoretinopathy and diabetic retinopathy were all negative for local antibody production against herpesviruses and 32 aqueous samples from subjects with cataracts were negative for local antibody production against *T. gondii*.^{8,30}

RESULTS

THE RESULTS OF POLYMERASE CHAIN REACTION ANALYSIS and local antibody production in the different diagnostic groups are provided in Table 2. None of the ocular fluid samples from the control subjects tested were positive for cytomegalovirus DNA (n = 38), varicella-zoster virus DNA (n = 35), herpes simplex virus DNA (n = 16), or *T. gondii* DNA (n = 25).

Thirty-one aqueous humor samples and seven vitreous fluid samples from 38 patients with AIDS

and retinitis were analyzed. The clinical diagnoses included active cytomegalovirus retinitis (n = 23), inactive cytomegalovirus retinitis (n = 5), toxoplasmic retinochoroiditis (n = 4), progressive outer retinal necrosis (n = 3), retinitis of unknown origin (n = 2), and AIDS-related retinopathy (n = 1) (Table 2). Cytomegalovirus DNA was detectable in ocular fluids from 21 (91%) of 23 patients with active cytomegalovirus retinitis, while no local antibody production against cytomegalovirus was observed in these samples (Table 2). One of the samples that was negative for cytomegalovirus DNA was positive for varicella-zoster virus DNA, which indicated that the clinical diagnosis may need to be reevaluated. In nine of ten cases of active cytomegalovirus retinitis tested, all positive for cytomegalovirus DNA in ocular fluid, cytomegalovirus DNA was also detected in peripheral blood leukocyte samples by polymerase chain reaction. In two cases of cytomegalovirus retinitis, local antibody production against varicella-zoster virus was observed, but the polymerase chain reaction was positive for cytomegalovirus DNA and negative for varicella-zoster virus DNA (Table 2). One of the four vitreous fluid samples and one aqueous humor sample obtained during inactive cytomegalovirus retinitis were still positive for cytomegalovirus DNA. However, the number of patients is too small to allow concluding whether polymerase chain reaction results for cytomegalovirus are influenced by disease activity.

Results were not influenced by treatment with antiviral medication because polymerase chain reaction for cytomegalovirus was positive in nine of the ten patients with active cytomegalovirus retinitis without treatment and all five cases during treatment with ganciclovir or foscarnet. For patients with inactive cytomegalovirus retinitis, polymerase chain reaction was positive in one of the three patients treated with antiviral medication and one of two patients not treated. In the other eight cases, no information about treatment with antiviral medication was available at the time the samples were collected.

Varicella-zoster virus DNA was detected in ocular fluid of all patients with the clinical diagnosis of progressive outer retinal necrosis (Table 2).^{4,31} Local antibody production against varicella-zoster virus in these three patients was negative. *Toxoplasma gondii* DNA was not detected in any of the patients with toxoplasmic retinochoroiditis (Table 2); two patients

TABLE 2

LOCAL ANTIBODY PRODUCTION AND POLYMERASE CHAIN REACTION ANALYSIS
OF OCULAR FLUIDS FOR HERPESVIRUSES AND *TOXOPLASMA GONDII*

NO. OF POSITIVE SAMPLES OF SAMPLES TESTED										
OCULAR DIAGNOSIS	NO. OF PATIENTS	CYTOMEGALOVIRUS		VARICELLA-ZOSTER VIRUS		HERPES SIMPLEX VIRUS			TOXOPLASMA GONDII	
		LOCAL ANTIBODY PRODUCTION	POLY- MERASE CHAIN REACTION	LOCAL ANTIBODY PRODUCTION	POLY- MERASE CHAIN REACTION	LOCAL ANTIBODY PRODUCTION	POLY- MERASE CHAIN REACTION HERPES SIMPLEX VIRUS 1	POLY- MERASE CHAIN REACTION HERPES SIMPLEX VIRUS 2	LOCAL ANTIBODY PRODUCTION	POLY- MERASE CHAIN REACTION
Patients with AIDS										
Active cytomeg- alovirus retinitis	23	0/23	21/23	2/20	1/22	0/19	0/22	0/17	0/11	0/18
Inactive cytomeg- alovirus retinitis	5	0/4	2/5	0/4	0/5	0/4	0/5	0/3	0/1	0/4
Progressive outer retinal necrosis	3	0/3	0/3	0/3	3/3	0/3	0/3	0/3	0/3	0/3
Toxoplasmic retino- chorioiditis	4	0/4	0/4	0/4	0/4	0/4	0/3	0/4	2/4	0/4
Retinitis of unknown origin	2	0/2	0/2	1/2	0/2	0/2	0/2	0/2	0/2	0/2
AIDS-related retinopathy	1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
Immunosuppressed patients (non-AIDS)										
Cytomeg- alovirus retinitis	2	2/2	1/2	0/2	0/2	0/2	0/2	—	0/1	0/2
Toxoplasmic retino- chorioiditis	3	0/3	0/3	0/3	0/3	1/3	0/3	—	1/3	3/3
Masquerade syndrome	3	0/3	0/3	0/3	0/3	0/3	0/3	—	0/2	0/3
Immunocompetent patients										
Acute retinal necrosis	16	0/12	0/13	10/16	7/16	4/15	1/15	0/10	0/13	0/12
Toxoplasmic retino- chorioiditis	22	0/8	0/21	0/12	0/20	0/11	0/20	—	15/22	4/22
Control subjects										
No uveitis	38	—	0/38	—	0/35	—	0/16	—	—	0/25

had local antibody production for *T. gondii*. In one patient with retinitis of unknown origin, there was local antibody production for varicella-zoster virus, but no varicella-zoster virus DNA was detected.

Five aqueous humor samples and three vitreous fluid samples from eight immunosuppressed patients with retinitis were investigated; the patients' diagnoses were non-Hodgkin's lymphoma ($n = 3$), heart transplant recipients ($n = 2$), renal transplant recipients ($n = 2$), and systemic lupus erythematosus ($n = 1$). *Toxoplasma gondii* DNA was detected in samples from three transplant recipients; in one of these samples, local antibody production against both *T. gondii* and herpes simplex virus was positive (Table 2). Local antibody production against cytomegalovirus was observed in samples from two patients suffering from non-Hodgkin's lymphoma and systemic lupus erythematosus, respectively, and cytomegalovirus DNA was detected in one of these cases.

Nine aqueous humor samples and seven vitreous fluid samples from 16 patients with acute retinal necrosis were analyzed. Local antibody production against varicella-zoster virus was positive in ten cases (63%). The polymerase chain reaction for varicella-zoster virus was positive in six (aqueous humor, $n = 4$; vitreous fluid, $n = 2$) of these ten samples positive for local antibody production against varicella-zoster virus (Table 2). Two of the samples negative for local antibody production were positive for varicella-zoster virus or herpes simplex virus 1 DNA, respectively. Samples from three patients were positive for local antibody production against both varicella-zoster virus and herpes simplex virus, and one sample solely for herpes simplex virus but without herpes simplex virus 1 or 2 DNA. The samples positive for varicella-zoster virus or herpes simplex virus 1 DNA were all obtained during the active stage of the disease, and the majority of these samples were collected within three weeks after the disease onset (Figure). In five cases, a second ocular fluid sample was obtained. One of these patients had a positive Goldmann-Witmer coefficient and polymerase chain reaction for varicella-zoster virus in the first sample, but the polymerase chain reaction became negative for varicella-zoster virus in the second sample four weeks later, whereas local antibody production against varicella-zoster virus was still readily detectable. In the other four cases, there were no changes in the

results of analysis of the first and second samples. Finally, in 12 cases (81%) of acute retinal necrosis, the inciting agent could be detected with both local antibody production and polymerase chain reaction (37.5%), local antibody production alone (31%), or polymerase chain reaction alone (12.5%).

In total, 22 samples (aqueous humor, $n = 13$; vitreous fluid, $n = 9$) from patients with toxoplasmic retinochoroiditis were analyzed. Local antibody production for *T. gondii* was observed in 15 of the 22 samples (68%) (Table 2). Local antibody production was positive in eight of the 13 samples (62%) obtained during active toxoplasmic retinochoroiditis and in seven of nine samples (77%) obtained during the convalescent stage of the disease. *Toxoplasma gondii* DNA could be detected by polymerase chain reaction in four of the 13 samples (31%) (aqueous humor, $n = 2$; vitreous fluid, $n = 2$) collected during active disease; these were all positive for local antibody production.

DISCUSSION

IN THIS STUDY, WE PERFORMED ANTIBODY AND POLYMERASE chain reaction analysis for herpesviruses and *T. gondii* on a relatively small amount (150 μ l) of ocular fluid. Local antibody production against cytomegalovirus was negative in all patients with AIDS and active cytomegalovirus retinitis, while in 21 cases (91%), cytomegalovirus DNA could be detected by polymerase chain reaction. Local antibody production against varicella-zoster virus was also negative in samples from patients with AIDS and progressive outer retinal necrosis⁴ in which varicella-zoster virus DNA could be detected with polymerase chain reaction. Currently, there is no explanation for the consistently negative Goldmann-Witmer coefficients in patients with AIDS and retinitis, but in some cases the relatively high serum antibody titers might have masked the local antibody production. However, in immunocompetent patients with acute retinal necrosis or toxoplasmic retinochoroiditis, local antibody production was more frequently positive than polymerase chain reaction. Furthermore, in two patients with AIDS and the clinical characteristics of cytomegalovirus retinitis, the results of the local antibody production, positive for varicella-zoster virus, were not

Figure (de Boer and associates). Relation between moment of sample collection after the onset of the acute retinal necrosis, and positive result for polymerase chain reaction or local antibody production. A total of 21 samples were obtained from 16 patients. The majority of the polymerase chain reaction-positive samples were obtained within three weeks after onset of the disease. GW indicates Goldmann-Witmer coefficient; PCR, polymerase chain reaction; M, months after the onset of symptoms; W, weeks after the onset of symptoms.

consistent with polymerase chain reaction results because in both cases cytomegalovirus DNA was detected. The interpretation of the positive Goldmann-Witmer coefficients in these cases is not clear because these patients had no signs of previous retinal lesions caused by a varicella-zoster virus infection. Therefore, polymerase chain reaction seems to be a preferable method for the diagnosis of retinitis in patients with AIDS.

In immunosuppressed patients without AIDS, local antibody production against cytomegalovirus was positive; therefore, the combination of both polymerase chain reaction and local antibody production might be beneficial in the diagnosis of retinitis in these patients. Furthermore, this study discloses that toxoplasmic retinochoroiditis appears to be an important differential diagnosis for transplant recipients with retinitis. The negative polymerase chain reaction in all four patients with AIDS and toxoplasmic

retinochoroiditis might indicate the lack of sensitivity of the polymerase chain reaction for *T. gondii*. More sensitive polymerase chain reaction techniques, for example, by the use of nested primer pairs, might improve the detection of *T. gondii* in ocular fluids in the future.

In both acute retinal necrosis and toxoplasmic retinochoroiditis, only samples obtained from patients during active ocular inflammation were positive for varicella-zoster virus, herpes simplex virus, or *T. gondii* DNA with polymerase chain reaction. However, local antibody production against these agents was still positive in 80% of samples from patients with acute retinal necrosis and 77% of the samples from patients with toxoplasmic retinochoroiditis collected during the convalescent stage of the disease. Previously, we and others observed negative results for local antibody production in patients with acute retinal necrosis and in those with herpes encephalitis

during the first weeks after the onset of disease.^{8,9,25} In the current study, all the samples collected within the first two weeks after the disease onset were positive for varicella-zoster virus or herpes simplex virus 1 DNA, including two cases that were negative for local antibody production against herpesviruses. The importance of the delay between sample collection and disease initiation was shown by a case where varicella-zoster virus DNA was detected in aqueous humor obtained one week after the onset of disease but not in vitreous fluid obtained three weeks later. Usui and associates¹³ reported positive results with polymerase chain reaction for varicella-zoster virus and herpes simplex virus DNA in samples from patients with acute retinal necrosis that had been obtained within one month after the onset of disease. Samples that had been obtained later were all negative. Therefore, in cases of acute retinal necrosis, the time of sample collection seems to be a crucial factor for serologic and polymerase chain reaction analysis of ocular fluids. In cases of acute retinal necrosis, polymerase chain reaction analysis can make a valuable contribution to early diagnosis in this sight-threatening disease.

In patients with posterior uveitis, positive polymerase chain reaction results for herpesviruses and *T. gondii* were obtained in aqueous humor as well as vitreous fluid. This is an important finding because aqueous humor is obtained by paracentesis, a safer and less invasive procedure than taking vitreous or chorioretinal biopsies. In immunocompetent patients, systemic treatment with corticosteroids is common in severe idiopathic uveitis but might be deleterious in cases of infectious origin. The ophthalmologist should consider performing a paracentesis before beginning treatment with systemic corticosteroids and while infectious cause is still being considered.

In this study, the diagnosis and therapy of several patients were changed because of laboratory analysis of intraocular fluids. In patients with AIDS, polymerase chain reaction was preferred to detection of local antibody production. In other cases, the combination of both serologic and polymerase chain reaction analysis of ocular fluids makes a valuable contribution to early diagnosis of infectious uveitis. However, polymerase chain reaction is a sensitive technique that needs to be used with strict precautions to avoid false-positive results by contamination of the samples.

Furthermore, the high sensitivity of this technique might result in the detection of latent viruses. Therefore, additional work in this area will be required before the true value of polymerase chain reaction analysis of ocular fluids in cases of infectious uveitis can be established.

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