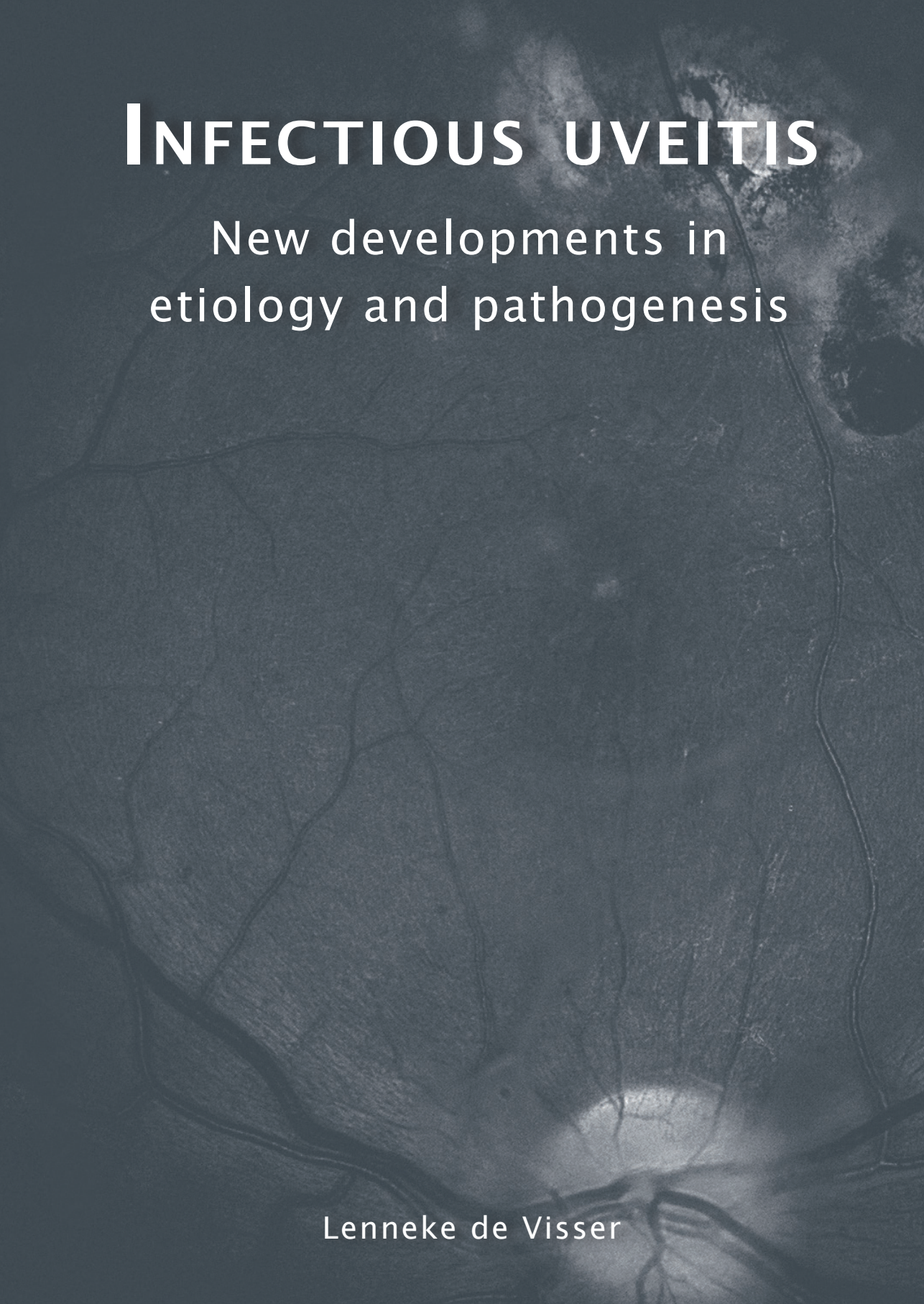

Infectious uveitis

New developments in etiology and pathogenesis

Lenneke de Visser

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Infectious uveitis

New developments in etiology and pathogenesis

Infectieuze uveïtis
Nieuwe ontwikkelingen in etiologie en pathogenese
(met een samenvatting in het Nederlands)

Proefschrift

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Lenneke de Visser

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Promotor: Prof.dr. A. Rothova

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Voor Joris en Lukas

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Contents

Chapter 1

Etiology and diagnosis of infectious uveitis

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Introduction

Uveitis is an inflammation of the uvea, which consists of the iris, the cilliary body, the choroid and of adjacent structures including the vitreous, retina and optic disc, and is initiated by various infectious and not infectious causes.[1]

The rapid identification of infectious uveitis entities is of crucial importance since treatment regimens and visual prognosis of intraocular infections are entirely different from noninfectious disorders.[2] The fast identification of specific infectious agents is particularly imperative in immunocompromised patients.[2,3] The prevalence of infectious causes depends on the geographic area; in Europe, approximately 20%-30% of uveitis entities are caused by an infectious agents. In posterior uveitis, however, this percentage increases to more than 50%. In the West, the most commonly involved pathogens are the parasite *Toxoplasma gondii*, Herpes simplex virus (HSV) and Varicella zoster virus (VZV).[4] In immunocompromised patients, CMV is the most common cause of uveitis, followed by *Toxoplasma* and *Treponema pallidum*.[3,5]

Research to ascertain novel causes of infectious uveitis is ongoing. In the clinical practice the presumed diagnosis of infectious uveitis is based on the specific clinical features, however laboratory data are mandatory for the confirmation of a suspected diagnosis, since similar clinical features might be caused by different microorganisms.

Laboratory tests based on the analysis of peripheral blood alone are of limited value, since these are not informative about what happens within the eye and positive results may be coincidental.[2,3,6-17] Negative peripheral blood results make a specific diagnosis unlikely but do not entirely rule out the possibility of infection.[10] The value of serologic results depends on the age of the patients and also depends on the prevalence of specific infections in a given population. At present, for the definitive diagnosis of intraocular infections, an analysis of intraocular fluids is required.

Intraocular fluid analysis in infectious uveitis

Existing diagnostic methods

The combined analysis by polymerase chain reaction (PCR) and determination of intraocular antibody production by calculation of the Goldmann-Witmer coefficient (GWC) has taken a prominent position within the laboratory diagnostic repertoire.[8,11,12,18,19] To obtain intraocular fluid for diagnostic purposes, a vitreous or aqueous tap can be performed.[6,20,21] A diagnostic vitrectomy can be simultaneously of therapeutic value since sight-impairing cloudy media are removed. This technique is more aggressive than an aqueous tap, but provides a larger amount of ocular fluid. A vitreous tap is mostly performed in the operating room using a surgical microscope. Around 0.5 to 0.7 mL of undiluted vitreous can be aspirated.[6] Possible complications of vitrectomy are endophthalmitis and retinal detachment, however, their incidence is low.[6] An aqueous tap can be performed in the outpatient setting, providing approximately 0.1 to 0.2 mL aqueous.[16] This procedure has been shown to be safe in the hands of an experienced ophthalmologist.[20,21] Various infrequent complications may occur, such as hyphema, occurring mostly in patients with a high intraocular pressure (IOP) at time of paracentesis and in patients with Fuchs Heterochromic Uveitis Syndrome (FHUS).[21] To date, no systematic studies have been done to determine whether vitreous or aqueous is superior in ocular fluid analyses, nor has been investigated whether the choice of aqueous or vitreous aspirate is dependent on the location of inflammation within the eye. However, it has been reported that aqueous tap and analysis provide a safe and useful first line diagnostic tool.[16,22]

Cultures

To establish the cause of infection microorganisms can be cultured from intraocular humors, however, not every pathogen has the ability to be cultured *in vitro*. Also, the pathogenic load in a sample has to be sufficient for culture.[23-29] Some viruses are unstable in a cell free environment and the infectious viral load may drop considerably in the period between sampling and application of the virus on to the cells. Moreover, as viruses are obligatory intracellular pathogens, they require susceptible host cells, which are not available for all viruses. Also some bacteria are difficult to culture, especially fastidious bacteria, which require

specialized environments due to complex nutritional requirements, such as *Bartonella henselae, Coxiella burnetii, Mycobacterium tuberculosis, Treponema pallidum, Rickettsia* species and *Borrelia burgdorferi*.[30] Culturing is often time consuming and renders results late in the disease process. However, it remains a prominent tool in the diagnosis of endophthalmitis.

Polymerase chain reaction analysis

PCR is a technique, whereby with the use of short complementary DNA fragments called primers, and DNA polymerase a single or few copies of a piece of DNA is amplified across several orders of magnitude, generating millions or more copies of a particular nucleic acid sequence.[31] The introduction of the PCR has greatly improved the detection of infectious agents and made the necessity to detect a pathogen by culture solely obsolete. PCR procedures are generally more sensitive than cultures and results are obtained faster.[32-34]

Next to basic PCR method, various more sensitive and specific techniques are available, like nested PCR and real time-PCR. Nested PCR is a modification of the PCR intended to reduce the risk of contamination due to binding of primers to incorrect regions of the DNA. This technique involves two sets of primers, used in two successive runs of PCR, the second set intended to amplify a target within the first run product, thereby increasing the specificity of the PCR.[31,35]

Real-time PCR is based on the basic PCR, and is used to amplify and simultaneously quantify a targeted DNA molecule by adding a fluorescent probe to the reaction. This not only increases the specificity, but also enables quantification of the nucleic acid load, and thus the pathogenic load of the original sample. Real-time PCR is applied to detect rapidly the presence of nucleic acid involved in infectious diseases, cancer and genetic abnormalities.[36] The introduction of real-time PCR assays to the clinical microbiology laboratory has led to significant improvements in the diagnosis of infectious disease, including infectious uveitis.[2,8,17,31,34,36-40]

Compared to nested PCR methods, real-time methods allow rapid DNA amplification, detection and quantitation of the pathogenic load. Moreover, as real-time PCR assays are performed in a closed-tube system, the risk of contamination is reduced.[41,42] However, real-time PCR assays may be less sensitive than nested PCR assays.[43,44]

PCR analyses, most notably real-time PCR, have proven to be valuable for the diagnosis of various intraocular infections, including cytomegalovirus (CMV) retinitis, ocular toxoplasmosis, acute retinal necrosis (ARN) and herpetic anterior uveitis.[14] PCR assays are also available for many bacteria, like *Bartonella henselae, Borrelia burgdorferi, Treponema pallidum, Mycobacterium Tuberculosis* and species, *Coxiella burnetii* and *Rickettsa* species and have been reported to be successful in the diagnosis of uveitis.[30,45-68] PCR directed to the 16S conserved gene sequences of bacteria is used to detect bacteria that cause endophthalmitis, but may also be used for the diagnosis of uveitis entities.[69-72] The 16S rRNA gene sequences contain hypervariable regions which can provide species-specific sequences which allow bacterial identification. As a result, 16S rRNA gene sequencing has become prevalent in medical microbiology as a rapid, accurate alternative to phenotypic methods of bacterial identification.[73] However, for intraocular fluid analysis, most notable in cases of endophthalmitis, one has to be aware of possible contamination, as the bacterium identified may have accidentally been introduced during surgical and/or laboratory procedures.

Positive PCR outcomes are directly related to the pathogenic load in the ocular fluid. It has therefore been suggested that the probability of detection of viruses by PCR is higher than in bacterial or parasitic infections, because viruses cause cell lysis and produce more offspring.[2,6,8] False-positive results may occur in PCR analysis due to contamination of samples, overflow of pathogens from the peripheral blood into the eye or the intraocular presence of infected cells not related to uveitis.[6,74] Therefore, positive PCR findings do not always prove causality. False-negative results might occur because of a low intraocular pathogenic load or due to the small volume of ocular fluid available for testing and might also depend on the time interval between the onset of infection and sampling. Therefore, negative PCR results do not entirely exclude the presumed diagnoses and other diagnostic tools may still be helpful.

Intraocular antibody analysis

Detection of specific intraocular antibody production is another indirect means to diagnose infectious uveitis. The mere presence of intraocular antibody is not indicative of local production as the blood-eye barrier may be compromised in uveitis and subsequently immunoglobulins may leak from the peripheral blood

into the ocular fluid. The Goldmann-Witmer coefficient corrects for this leakage by including total IgG as an indicator for leakage from the peripheral blood to the aqueous or vitreous fluid. The GWC compares the ratio of specific antibody in the eye and peripheral blood to the ratio of total IgG in the eye and peripheral blood ((specific IgG in aqueous/specific IgG in serum) / (total IgG in aqueous/total IgG in serum)). In case of leakage, division of the two ratios will approximate one.[2,11,12,19] Detection of antibodies in simultaneously collected ocular fluid and serum is most frequently performed by enzyme-linked immunosorbent assay (ELISA), by immunoblot or by indirect immunofluorescence assay (IFA).[7,8,10,12,13,16,17,75-81] GWC determination has been described for the most common causes of infectious uveitis; HSV, VZV, CMV, Rubella virus and *Toxoplasma gondii*, but also for Mumps virus, Measles virus, Parvovirus B19 and *Toxocara canis* (Chapter 8 and 9).[2,3,6-8,10-14,16,75,76,79,80,82,83]

False-negative results may occur in GWC analysis when high serum antibodies combined with extensive blood-aqueous barrier breakdown may mask a positive coefficient.[11,12] Occasionally, the GWC may become false-positive due to polyclonal B-cell activation. This can be explained by the tendency of the infecting organism to produce super-antigens that are capable of polyclonal activation of B-lymphocytes, and subsequent production of large amounts of antibodies of varying specificities. A patient may have multiple positive GWCs due to polyclonal B-cell activation. When analyzing for only one pathogen, polyclonal B-cell activation cannot be ruled out. In these situations, the C' coefficient can be calculated which compares the specific aqueous/serum antibody ratios from two pathogens. A C' value exceeding 4 is indicative of intraocular antibody production against the pathogen with the highest aqueous/serum ratio, whereas C' < 4 is considered indicative for polyclonal B-cell activation.[11,84] However, one should keep in mind that double infections with multiple positive GWC values may occasionally occur.

Contribution of polymerase chain reaction and Goldmann-Witmer coefficient

In general, both PCR and GWC contribute to the diagnosis of infectious uveitis. The extent of contribution of each test varies on the pathogen involved, immune status of the patient and the time of sampling.[8] In immunocompromised

patients with herpetic viral infections, PCR is positive predominantly early in the disease, whereas at later stages, GWC values are positive and PCR becomes negative.[2,8,85,86] This phenomenon might be explained by the fact that the pathogen is cleared in the late phase of the infection and the microbial load is reduced to below the detection limit, whereas intraocular antibody production is sustained for a longer period of time. Since aqueous sampling is performed most commonly in a chronic stage of the disease, viral DNA is often no longer present in the eye and GWC may contribute considerably to the diagnosis. One exception is ARN where patients, due to the progressive symptoms are tapped in the early stages of their disease. Indeed, in these patients PCR was found to be positive in over 90% of cases.[87] [JDF de Groot-Mijnes, personal observation] In patients with ocular toxoplasmosis GWC appears to be most important.[8] Negative PCR results observed in early stages of the disease might be explained by slow release of *T. gondii* tachyzoites from the cyst into the ocular fluid.[8]

In immunocompromised patients, PCR appears to be more informative, most notably in AIDS patients.[3] GWC does contribute to the diagnosis of uveitis in immunocompromised patients, but predominantly when *Toxoplasma* is involved. The contribution of the GWC depends also on the patient's degree of immunosuppression. The iatrogenic immunosuppression is more severe in stem cell and bone marrow transplants compared to solid-organ transplant recipients.[3]

In conclusion, both assays are helpful in the determination of infectious cause of uveitis. The contribution of the PCR and GWC may vary depending on the pathogen involved, the immune status of the patient and time of sampling. Although both PCR and GWC are required for the optimal diagnostic process of intraocular infections, we realize that both assays might not be available in a given clinical situation.[8] In these situations, one has to take into account the short-comings of the specific assay used.

Diagnosis of specific uveitis entities

Diagnosis of parasites

Ocular toxoplasmosis

Ocular toxoplasmosis (OT), caused by the parasite *Toxoplasma gondii*, is the most common identifiable cause of posterior uveitis in many parts of the

world and can be acquired either by congenital or postnatal route of infection.[88] Classically, OT presents as an unilateral focal retinochoroidal lesion, sometimes accompanied by one or more "satellite lesions" and typically by only one focus of active disease in immunocompetent patients.[17,38,77,89-92] In immunocompromised patients, OT may exhibit a variety of clinical lesions, including single foci of retinochoroiditis in one or both eyes, multifocal lesions, or diffuse areas of retinal necrosis, and occasionally as AU.[93-95] *Toxoplasma* infection may also mimic ARN and should be considered when diagnostic testing for HSV and VZV is negative.[96]

The presumed diagnosis is mostly based on the findings of focal chorioretinitis, usually in satellite formation. Clinical findings however may vary and be atypical.[76,97] Detection of anti-*T. gondii* IgG antibodies (and IgM in case of recently acquired infection) in peripheral blood is not sufficient for the diagnosis of OT as most adults (up to 60%) in continental Europe have been infected with *T. gondii*.[98,99] Moreover, focal retinal lesions are reported in other ocular infections, such as intraocular Rubella virus (Chapter 4) and *Toxocara canis* infection, and may also occur due to trauma or other damage in the retina. Peripheral retinal scars were also observed in the general population.[91,100-102]

To confirm the diagnosis of toxoplasmosis, intraocular fluid analysis can be performed to detect *T. gondii* DNA by PCR and/or to establish intraocular antibody production.[12,17,37,38,76,77,81,90-92,103-106] Local antibody production can be determined by ELISA or by IF assay followed by calculation of the GWC. Immunoblotting has also been described for the detection of serum and intraocular antibody, however, this method is elaborate and quantitation of specific bands is more complicated.[76] Several studies on PCR analysis of *Toxoplasma* in aqueous humor reported positive results ranging from 13 to 36%.[8,17,37,38] Analysis of intraocular antibody production reportedly yielded positive results up to 93%, and therefore, appears to play a more decisive role in the diagnosis of intraocular *Toxoplasma* infection.[3,8,16,17,38] In primary OT, both PCR and GWC analysis contribute equally to the diagnosis of ocular disease.[3,16,17,38,104] In immunocompromised patients, both assays appear to be valuable. Westeneng et al. reported that with PCR as the sole diagnostic approach, a diagnosis would have been missed in 60%, whereas GWC alone detected the parasite in 90% of cases.[3] However, PCR was reported to perform best results in atypical toxoplasmic chorioretinitis in immunocompromised patients.[38,104]

With regard to intraocular antibody production it is important to note that in patients with a *T. gondii* infection serum IgG titers may rise to such high levels that in the event of a severe blood-aqueous or blood-retina barrier breakdown, intraocular antibody production may be masked.[8,11,12,19] Still, by using the combination of GWC and PCR the diagnostic sensitivity can increase to 93%, as reported by Fekkar et al.. Various other studies also suggest the application of both diagnostic assays to establish the diagnosis of OT irrespective of the patient's immune status.[8,37,76]

Toxocara canis

Ocular toxocariasis or ocular larva migrans is a local complication of a *Toxocara canis* infection, which usually occurs in children, although it has been occasionally reported in adults (Chapter 6).[10,107-112] The clinical signs of ocular toxocariasis often include diminished vision, leukocoria and red eye. Focal chorioretinal granuloma is a typical lesion, which occurs mostly unilaterally and might be falsely diagnosed as retinoblastoma or endophthalmitis of bacterial origin.[10,107,109-113]

The presumed clinical diagnosis is usually based on the presence of chorioretinal granuloma, vitritis or focal lesions in the posterior eye segment in the presence of positive serology and after exclusion of other possible causes, such as *Toxoplasma gondii*, HSV and VZV.[107,110,111] *Toxocara* serology may confirm the suspected diagnosis, however low or undetectable *Toxocara* serum IgG titers have been reported in patients with ocular toxocariasis (Chapter 5).[10,114,115] De Visser et al. report on three children with positive GWCs despite negative or low serum titers.[10] Negative or low serum titers are probably due to waning antibodies, as demonstrated in a follow-up study of 20 patients with OT, where 85% showed a decrease in serum titers.[115] Thus, patients with a low or undetectable peripheral blood titer against *Toxocara* may have had higher titers in the past and ocular toxocariasis should not be excluded from the differential diagnosis. Conversely, the presence of serum IgG against *Toxocara canis* does not prove ocular involvement even in the presence of suspected clinical findings, since seroprevalence for *Toxocara canis* depends on the geographic area and may reach up to 46% in adults and 77.6% in children.[10,107-109,111,112,114,116-123]

To establish the diagnosis of intraocular *Toxocara* infection, intraocular

fluid analysis is warranted.[10] [Mayland Nielsen et al, unpublished data] Antibody detection in ocular fluid of patients suspected of ocular toxocariasis has been reported, but only two reports included GWC determination.[10,108,112,124,125] The sensitivity of GWC determination has not been investigated systematically, mainly due to the relative rareness of the disease. *Toxocara* larvae might induce a very strong local humoral immune response, which is exemplified by the fact that intraocular antibody titers often exceeded serum titers.[10,108,124,126,127] PCR assays have been described, but are mainly used for research purposes and their diagnostic value is not known.

Diagnosis of viruses

Ocular herpes virus infection

Herpetic uveitis is an ocular inflammation secondary to viral infection caused by Herpes simplex virus (HSV-1 and HSV-2), Varicella zoster virus (VZV) or CMV. Intraocular herpetic infections may either present as anterior (kerato)-uveitis or as characteristic types of posterior uveitis, such as ARN, Progressive Outer Retinal Necrosis (PORN) and CMV retinitis.[127, 128] Recently, non-ARN types of posterior ocular infections with herpes virus are being reported.[129] CMV retinitis and PORN occur predominantly in immunosuppressed patients whereas the other entities are prevalent mostly in patients with a competent immune system.

Herpes simplex virus and Varicella zoster virus anterior uveitis

HSV and VZV induced anterior uveitis (AU) usually presents as a unilateral AU, which is frequently recurrent and associated with high IOP during the episodes of active inflammation. Active or inactive keratitis, decreased corneal sensation, elevated IOP, keratic precipitates, posterior synechiae and (sector) iris atrophy may be observed.[131-134] Anterior chamber inflammation may be either mild or severe and may even produce a hypopyon or hyphema.[131-133,135,136]

Clinical distinction between HSV and VZV as the cause of AU is difficult, as both viruses can present with similar features.[133,137] Medical history and examination may suggest which virus is more likely.[137] HSV usually affects children and young adults, whereas VZV is more commonly seen in elderly and immunocompromised patients.[131,133,137] In VZV infections, ocular involvement

is preceded by skin involvement in the majority of cases, although there have been reports of ocular lesions preceding subsequent skin lesions, and even ocular lesions without any skin involvement.[138-143] VZV-associated uveitis can be accompanied by herpes zoster ophthalmicus, a systemic manifestation involving the ophthalmic division of the trigeminal nerve.[144] Ocular involvement occurs in 20% to 70% of cases, whereas anterior chamber inflammation occurs in up to 60% of immunocompetent patients with herpes zoster ophthalmicus (HZO).[131,144-148]
The presumed diagnosis of HSV or VZV AU is not difficult if typical ocular and non-ocular signs are present. However, in cases without preexisting HSV dermatitis or keratitis, the clinical diagnosis can be challenging.[131] Peripheral blood analyses for anti-HSV and anti-VZV antibodies are not useful, because the majority of adults are seropositive (up to 90% and up to 100% worldwide, respectively) even without a clear clinical history of disease.[131,149-151] A variety of laboratory techniques is available, including electron microscopy of vitreous, retinal biopsy, viral culture, local antibody production and PCR.[2,39,80,152-165] For the diagnosis of VZV-uveitis a Tzanck smear was often used to examine for the presence of multinucleated giant cells, however, this technique requires active surface disease and lacks specificity for differentiating VZV from HSV.[166,167] Culturing of corneal epithelial lesions can be performed, but this requires active epithelial disease, is time consuming and has a low sensitivity.[131,168] Therefore PCR and/or GWC analysis are most preferred laboratory techniques to diagnose herpetic AU.[2,3,6,8,80,131-133] PCR has proven to be a powerful tool for diagnosing herpetic uveitis anterior. Reportedly, PCR can provide a 80%-90% positive diagnosis rate by detecting the presence of HSV DNA in aqueous humor and vitreous.[2,78,169-173] In addition, GWC determination can aid in the diagnosis.[2,3,8,174]

Posterior segment manifestations of Herpes simplex virus and Varicella zoster virus

Posterior manifestations of HSV and VZV infection include progressive retinitis and choroiditis with vasculitis and papillitis, creating a specific clinical syndrome called ARN. ARN has a poor visual prognosis due to the frequent development of retinal detachment and optic disc atrophy. The American Uveitis Society has published diagnostic criteria for ARN. Clinical characteristics include one or more foci of retinal necrosis, with discrete borders in the peripheral retina,

a rapid progression of disease in the absence of treatment, circumferential spread, evidence of occlusive vasculopathy with arteriolar involvement, and a prominent inflammatory reaction in the vitreous and anterior chamber.[175]

In the immunocompromised a very aggressive variant named progressive outer retinal necrosis (PORN) may develop.[175,176] In neonates and infants, congenital posterior herpetic infections have long been recognized.[177]

VZV is the most frequent cause of ARN.[131] HSV-associated ARN often occurs in association with (meningo)encephalitis, although ocular disease may present years after resolution of the central nervous system disease.[131,160,178-181] Herpetic encephalitis preceding VZV-associated ARN has also been reported in immunocompromised patients, but less frequently.[182]

The diagnosis of ARN is generally based on clinical presentation, which is rather typical with peripheral retinal necrotic infiltrates and associated hemorrhages. Herpetic retinitis may be clinically confused with ocular toxoplasmosis, syphilis and CMV-retinitis.[183-186] The differentiation between the causative agents of retinal necrosis is mandatory for focused treatment and eventually prevention of infection in the contralateral eye.

Atypical presentations form a diagnostic challenge, and a delay in treatment can be harmful to vision. In such cases, quick laboratory testing of aqueous or vitreous specimens is beneficial. A variety of diagnostic techniques have been described, including antibody analysis of serum and/or intraocular fluid, pathologic examination of retinal biopsy specimens, viral culture from intraocular specimens, immunocytochemical studies, and a temporal relationship between ARN and herpetic dermatitis.[2,80,153,154,157,176,187-191] In addition, PCR analysis of ocular fluids has proven to be very useful.[2,160,192] With PCR–based assays it was demonstrated that most of the cases of ARN are caused by VZV or HSV and occasionally by CMV or *Toxoplasma*.[2,39,40,152,154,156,160-162,164,165,176,192-194] In addition to PCR, GWC determination can be applied.[80,87,154] In general, viral nucleic acid is readily detected in the early stages of the disease, whereas at later stage intraocular antibodies are produced and PCR tends to becomes negative.[2] When comparing the contribution of PCR and GWC, PCR appears to superior for the diagnosis of ARN, which might be explained by the fact that paracenthesis is usually performed early in the disease.[2,8,87,154] A delay in treatment of ARN patients can be detrimental to vision. Therefore, when ARN is suspected, therapy should

be initiated immediately without waiting for the laboratory confirmation of the diagnosis. If necessary, treatment can be changed when the results of ocular fluid analysis become available.

Cytomegalovirus anterior uveitis

In the past years, CMV-associated AU in immunocompetent patients has repeatedly been reported.[195-198] AU caused by CMV has a wide spectrum of clinical presentations.[196] It may present as recurrent episodic iritis with raised IOP resembling Posner-Schlossman syndrome (PSS) or manifest as a chronic AU with features suggesting FHUS, including small scattered keratic precipitaties and iris heterochromia in the absence of synechiae.[197-200] Other clinical features include endotheliitis and sector iris atrophy.[201-203] Anterior segment involvement of CMV infection has also been described in patients suffering from CMV retinitis in AIDS.[204] These patients presented with reticularly arranged, linear, or flecked corneal endothelial deposits.

CMV DNA and intraocular antibody production against CMV have been demonstrated in the ocular fluids of immunocompetent patients with unilateral recurrent hypertensive AU.[197,198,201] The clinical features of CMV-associated AU in 23 immunocompetent patients were assessed by Chee et al..[195] De Visser et al. found a positive GWC for CMV in 2 patients with mild recurrent unilateral AU with an elevated IOP and no posterior synechiae, which is in accordance with previous studies.[de Visser, unpublished data] Teoh et al. detected CMV DNA in a patient with PSS.[200] Chee et al. recently analyzed 104 patients with hypertensive AU and detected CMV DNA in 23 cases.[195] Seventy-five percent of the CMV DNA-positive patients had PSS, which strongly suggests involvement of CMV in the pathogenesis of PSS. Identification of CMV as a cause of AU in immunocompetent patients is important since this offers a potential for effective treatment.[195,197,198,205] So far, concurrent studies on GWC and PCR in CMV-associated AU have not been performed. Van Boxtel et al. performed both PCR and GWC on the ocular fluids of five patients, but the number of patients in this study is too small to draw any conclusions as to what is the best analysis. Like in other intraocular infections, cases with solely positive PCR and solely positive GWC have already been reported.[195,197,198,200,205] The exact contribution of either assay has to be determined. One would expect that GWC is more often positive in cases with long standing inflammation.

Cytomegalovirus posterior uveitis

CMV retinitis usually begins with small, white retinal infiltrates that may resemble a large cotton-wool spots if seen during the early phase of infection. Several clinical types of CMV retinitis have been reported including whitish necrotic lesions associated with hemorrhages (pizza pie retinopathy, cottage cheese and ketchup retinopathy) and a more indolent type with atrophic central lesions and granular whitish active borders.[82,101,206,207]

CMV retinitis usually affects immunocompromised patients, either those with HIV infection, or those with severe iatrogenic immunosuppression or neonates. It is the most frequent cause of infectious retinitis in patients with AIDS.[3,14,208] The introduction of HAART might influence the clinical presentation, which makes the clinical diagnosis more difficult. The clinical manifestations of congenital CMV infection resemble those in adults.

The diagnosis of CMV retinitis is usually based on the typical ophthalmoscopic picture in an immunosuppressed individual.[209,210] Serum antibodies can be detected in the majority of the normal population and thus do not have a significant diagnostic value.[209] Additional diagnostic tools usually consist of analysis of intraocular fluid, which can confirm the clinical diagnosis.[209] In AIDS patients, the clinical diagnosis of CMV retinitis can be thwarted by multiple agents co-infecting the retina, which underlines the importance of intraocular fluid analysis.[82]

Aqueous and vitreous analyses contribute to the diagnosis of CMV retinitis.[209,210] In AIDS patients with active, untreated CMV retinitis, PCR performed on vitreous has a sensitivity of 95%.[84,211] If the patient has already received treatment, the sensitivity declines to 47.5%. Determination of the GWC can support the diagnosis in difficult cases, but polyclonal stimulation and reduced antibody formation in immunosuppressed individuals may render interpretation of the result difficult.[84]

HIV-induced uveitis

Ocular infections in HIV-infected patients are mostly caused by opportunistic agents, such as CMV.[212] However, HIV was cultured from the ocular fluid of HIV-infected patients with anterior and/or posterior uveitis, in whom no other causative agents could be found.[213] Also, HIV RNA was detected by quantitative PCR in the ocular fluids of HIV seropositive patients with infectious retinitis.[214]

However, the HIV loads in the ocular fluids of these patients never exceeded those in the plasma and the presence of HIV in the eye was attributed to the entrance of circulating infected cells into the eye. Recently, Rothova et al. described a HAART-naïve patient with HIV-induced uveitis, whose HIV RNA loads were much higher than the plasma HIV loads, suggesting active intraocular HIV replication.[215] The patient presented with anterior uveitis and mild vitreous opacities and had no signs of any other identified cause of the intraocular infection. Following HAART treatment the ocular problems resolved.

HIV infection is initially diagnosed by detecting HIV antibodies by ELISA and by Western blot. Quantitative real time-PCR is subsequently used to determine the plasma HIV load and to monitor treatment efficacy and disease progress. Quantitative real time-PCR can also be applied to CSF and ocular fluid.[214,215] Rothova et al. suggested that quantitation of HIV RNA in intraocular fluids might be useful when evaluating HIV-infected patients with intraocular inflammation and without an identifiable opportunistic infection.[215] HIV GWC analysis on ocular fluids has not yet been reported.

Rubella virus-associated uveitis and Fuchs heterochromic uveitis syndrome

Clinical manifestations of 30 Rubella virus-associated uveitis patients included characteristics typical of FHUS and demonstrate that Rubella virus is involved in the pathogenesis of FHUS (Chapter 3).[216] FHUS is a chronic low-grade anterior chamber inflammation characterized by typical clinical signs such as fine keratic precipitates, diffuse iris atrophy and/or heterochromia, the development of cataract and the absence of posterior synechiae prior to surgery. The clinical diagnosis of FHUS is sometimes difficult, because not all symptoms are always present at the same time.[216] FHUS has been associated with multiple infections, including Rubella virus (Chapter 2), CMV, *Toxoplasma gondii* and *Toxocara canis*.[7,15,216-219] In Europe, almost 100% of FHUS cases are positive for intraocular antibody production against Rubella virus.[7,15] Birnbaum et al. found that FHUS is less common in patients born since the introduction of the US rubella vaccination program.[217] At the same time, an increase in the percentage of FHUS cases was observed among foreign-born individuals who did not have access to rubella vaccination and were naturally infected with Rubella virus. One may speculate that

with the introduction of the measles, mumps and rubella vaccine, the incidence of FHUS will decrease.[217]

Due to the high incidence of natural infection during the pre-vaccination era and recent vaccination programs, the seroprevalence for Rubella virus antibodies is very high (94%-96%).[220] Therefore, serology is not informative for the diagnosis of Rubella virus-associated uveitis and intraocular fluid analysis is essential. Several reports indicate that intraocular antibody production against Rubella virus is positive in 93%-100% of Rubella virus-associated uveitis cases, while PCR remains negative in the majority of cases.[7,15] This may be explained by a persistent low-grade infection yielding a low viral load in the aqueous humor.[15] However, FHUS representing a chronic auto-immune reaction triggered by the virus may also be a possibility.[218]

Parvovirus B19-associated uveitis

Systemic Parvovirus B19 infection causes erythema infectiosum in children, also known as the fifth disease. The virus has been associated with uveitis in several case reports.[221-223] De Boer et al. investigated intraocular antibody production against Parvovirus B19 in six patients with intermediate uveitis, but did not find a positive GWC.[75] The role of Parvovirus B19 was also investigated in 46 patients with *T. gondii*-negative focal chorioretinitis, intermediate uveitis and neuroretinitis, however, without positive results.[83] Recently, de Visser et al. demonstrated intraocular antibody production against Parvovirus B19 in 2 patients with idiopathic AU. [de Visser, unpublished data] Heinz et al. detected Parvovirus B19 antibodies in the ocular fluids of patients with uveitis, but did not determine whether this represented true intraocular antibody production or merely leakage from the peripheral blood.[224] The onset of uveitis after Parvovirus B19 infection might be explained by a persistent infection, which has been reported to occur in the peripheral blood, synovial fluid, cerebrospinal fluid and bone marrow.[225-227] Alternatively, the uveitis may be due to secondary autoimmunity, as chronic exposure to Parvovirus B19 has been shown to elicit the production of antiviral antibodies with auto-antigen binding properties.[228]

For the diagnosis of a current infection, especially in pregnant women, serology is commonly performed to detect anti-Parvovirus IgM and/or IgG by ELISA or IFA.[169,229-231] However, since Parvovirus B19 infection reaches a seroprevalence

in adults of 40-60%, serology alone is of limited value for diagnosis of ocular infections and intraocular fluid analysis should be performed.[232] In addition to GWC analysis, several techniques are available for molecular detection of Parvovirus B19. These include dot blot hybridization, and nucleic acid amplification, such as PCR, nested-PCR and real-time PCR.[233-236] Positive PCR results for Parvovirus B19 on ocular fluids have not yet been reported.

Human Parechovirus

Human Parechovirus is a Picornavirus of the genus Parechovirus and is known to cause gastroenteritis, encephalitis and flaccid paralysis in young children, but rarely causes disease in adults.[237] Recently, de Groot-Mijnes et al. found Human Parechovirus by PCR in the aqueous humor of four patients (Chapter 7), three of which were immunocompetent and had all similar clinical findings consisting of AU with corneal involvement. [De Groot-Mijnes, unpublished data] Certain types of Enteroviruses, a genus closely related to Parechovirus, were reported to cause uveitis in children in Russia, however, the detection of Human Parechovirus in ocular fluid is a novel finding and an association with ocular disease has not yet been reported. GWC for antibody detection against Human Parechovirus is not available and may not be feasible because seroprevalence for both Parechovirus and Enteroviruses is high and cross-reactivity is likely to occur.[238-242] Further investigation has to be performed to determine whether Human Parechovirus is a true cause of infectious uveitis.

Human herpes virus 6

Human herpes virus 6 (HHV6), a beta-herpes virus, has been associated with immunodeficiency disorders and neurologic diseases, and is the known causative agent of a childhood disease roseola infantum (or exanthema subitum).[243,244] Only few cases on the association of HHV6 and intraocular disease have been reported.[128,129,245,246] Majority of these cases exhibited the involvement of the posterior eye segment as panuveitis and optic neuritis.

Serum antibodies against HHV6 can be detected by IFA, however seroprevalence rates reach up to 80%, which renders serology of insufficient diagnostic value.[247-249] So far, HHV6 has been detected in ocular fluid by PCR

analysis only.[128,129,245,246] De Groot-Mijnes et al. detected HHV6 by PCR in the aqueous of a patient with AU, however, antibody analysis by immunofluorescence assay did not reveal the presence of IgG against HHV6 in the intraocular fluid.[De Groot-Mijnes, unpublished data; Chapter 7] GWC analysis should be feasible, but has not yet been reported. The role of HHV6 as a causative agent of uveitis is still uncertain and further studies are required.

Diagnosis of bacteria

Endophthalmitis

Infectious endophthalmitis is a progressive intraocular infection with subsequent inflammatory response, which initially affects the vitreous compartment and anterior chamber of the eye and quickly involves the whole intraocular space. The progression of infection might be extremely rapid and the risk of losing the useful vision is significant. Infectious endophthalmitis might either be exogenous (usually following intraocular surgery or perforating eye injury) or endogenous (preferentially occurring in immune deficient individuals with a potential infectious source as intravenous lines and catheters) and can be caused by a variety of bacteria and fungi. The most common cause of endophthalmitis is cataract surgery. Findings on examination include a classical combination of symptoms: redness, pain, and decreased vision. Typically, the eye lids and conjunctiva are injected and edematous, corneal haze or edema are present together with severe cellular reaction in anterior chamber sometimes combined with hypopyon. Posterior eye segment shows a varying degree of vitreous opacities and the view of the fundus is impaired. In the Endophthalmitis Vitrectomy Study, 94% of culture-confirmed cases involved Gram-positive bacteria; 70% of isolates were Gram-positive, coagulase-negative staphylococci, 10% were *Staphylococcus aureus*, 9.0% were *Streptococcus* species, and 2% were *Enterococcus* species. Various Gram-negative species made up 6% of the isolates.[250]

Detection of the causative microorganisms is essential for effective treatment of this progressive eye infection associated with a loss of vision and sometimes of the eye itself.[251] The diagnosis of endophthalmitis relies on isolation of the causative organisms, which is classically done by culture of an aqueous or vitreous sample.[29] To identify the causative agent, Gram stains, cultures and antibiotic sensitivities are usually performed. The culturing of ocular fluids can

bring up several difficulties, such as contamination. Also, many bacteria are slow-growing and fastidious. Anaerobic cultures should be kept for at least 14 days to recover slow-growing species.[29,252] Furthermore, experiments have shown that a delay in time between ocular fluid aspiration and application to the appropriate culture medium results in a significant decrease in yield of organisms.[29,252,253] In addition, the stains and cultures might be negative even in clinically evident cases, especially when the samples were collected in the late stages of the infection. The cultures from the vitreous are usually more informative than those from aqueous.[252] Molecular based diagnostic assays for bacterial endophthalmitis are currently being developed and show promising results in terms of sensitivity, most notably under therapy, and are characterized by a short time interval till laboratory diagnosis.[23,254,255] Endophthalmitis is a true ocular emergency and should be treated with broad-spectrum antibiotics immediately without awaiting the final results of ocular fluid laboratory analyses. Once the causative microorganism has been identified, the antimicrobial regimen can be adapted where necessary.[252]

Ocular tuberculosis

Mycobacterium tuberculosis primarily affects the lungs, although it may also involve other organs. Extrapulmonary involvement is seen in more than 50% of the patients who have AIDS.[256] The presence of a systemic tuberculosis infection may suggest but does not prove that tuberculosis is the cause of the ocular findings.[257] In contrast, the absence of active systemic tuberculosis does not exclude the presence of ocular tuberculosis. Recently, several cases of latent systemic tuberculosis were associated with active ocular infection.[52] Immunocompromised patients are at particular risk of reactivation of latent tuberculosis in the eye.[258] Intraocular tuberculosis is a great mimicker of various uveitis entities. The ability to mimic other infections is in part determined by the variable host response and to the fact that virtually all parts of the eye may be affected.[52] Ocular tuberculosis exhibits diverse manifestations including conjunctivitis, keratitis, scleritis, anterior granulomatous inflammation, retinal vasculitis, or chorioretinal lesions similar to serpiginous-like choroiditis.[52,258,259]

The large variations in clinical presentation make the diagnosis of intraocular tuberculosis difficult.[260] Clinical suspicion is an imperative first step toward the

correct diagnosis.[261-268] When patients are suspected of ocular tuberculosis, they generally undergo a complete physical examination, including a Mantoux tuberculin skin test (TST) and chest radiograph. However, the TST test results should be interpreted with care. Vaccination with BCG poses a potential source of cross-reactions and may yield false-positive results.[52,269] Recently, the interferon-gamma release assays (IGRAs), such as the QuantiFERON-TB Gold test and the T. Spot-TB® Elispot assay have been added to the diagnostic repertoire.[52,258,264,270] These are blood tests that measure the function of *M. tuberculosis*-specific $CD4^+$ T cells. The antigens used in these assays are specific for *M. tuberculosis* and are not shared by the Bacillus Calmette-Guérin vaccine strain nor by other *Mycobacterium* species.[52, 270] A positive TST or IGRA only indicates that a person has had tuberculosis or latent TB, not whether he has active disease. In fact, it has been reported that IGRAs may be unreliable in patients with active pulmonary tuberculosis.[271,272] In patients with extrapulmonary disease, the positive predictive value was 90.5%, suggesting that IGRAs may be useful for the diagnosis of ocular tuberculosis.[272] Several publications reported on the use of QuantiFERON-TB Gold test in patients with serpiginous-like choroiditis, chronic posterior uveitis and suspected tuberculous uveitis.[258,273,274] Overall, the IGRA performed equally or better than the TST, and was considered helpful in obtaining the diagnosis of ocular tuberculosis. However, negative QuantiFERON-TB results do not exclude ocular tuberculosis and should be interpreted with caution, as the test may be false-negative in AIDS patients with a low CD4 count.[273,275,276] *M. tuberculosis*-specific PCR assays are available and have been found useful for the early diagnosis of intraocular tuberculosis by using either aqueous or vitreous.[45-52,54,57-61] *M. tuberculosis*-specific antibodies are detectable during active and latent disease, however, sensitivity is rather low and serology is not commonly practiced for the diagnosis of tuberculosis.[278-281] The diagnosis of ocular tuberculosis is definitive when *M. tuberculosis* is cultured from the eye. However, this is rarely achieved, because mycobacterial culture facilities are not readily available.[271] Furthermore cultures may require several weeks for a positive result.[257] A rapid procedure for diagnosing tuberculosis is the examination of acid-fast (Ziehl-Neelsen) stained smears of infected ocular tissue or fluid. However, it has been estimated that at least 10^6 organisms/mL of sputum are required for detection on a smear.[52,277] Because the amount of organisms found intraocular fluids is low, direct

microscopy of the smears is usually not helpful.[52] The detection of intraocular antibody production against *M. tuberculosis* has not yet been investigated.

Ocular Borreliosis

Lyme borreliosis is a multisystem tick-borne disease caused by the spirochete *Borrelia burgdorferi*, although other *Borrelia* species can also cause Lyme disease. Ocular findings of Lyme borreliosis differ with the stage of the disease, but develop mainly in the late stages.[28,282] Early disease manifestations include mostly conjunctivitis and episcleritis.[28,282-286] During the disseminated stage, ocular disease may present with cranial neuropathy, optic nerve and papillary involvement, and orbital inflammation, whereas keratitis occurs in the persistent stage of the disease.[28] Intraocular inflammatory syndromes have been reported in both, early and late stages of infection.[28,282,287-290] Lyme borreliosis should be included in the differential diagnosis of retinal vasculitis, especially in endemic areas.[282]

The diagnosis of ocular borreliosis is generally based on clinical presentation supported by serological data.[6,28,291] Several serological assays are available to detect IgM and IgG antibodies, including ELISA and Western blot.[28] Lyme disease may be underdiagnosed because of borderline-seropositivity or seronegativity in ELISA assays.[6,55] False-negative results can occur when patients seroconvert late after infection or due to instant antibiotic treatment inhibiting or delaying the antibody response early in the course of the disease.[28] False-positive results may occur due to cross-reactivity with other spirochetes and even viruses.[292-295] Therefore, positive ELISA results should be confirmed by another assay, for instance immunoblot. Due to the different immune responses of each individual and the complex interpretation of the test results, serologic diagnosis of Lyme borreliosis remains equivocal and highly dependent on laboratory specialty.[6,296] The presence of a systemic infection is not proof of ocular disease, nor does seronegativity exclude ocular borreliosis.

PCR analysis of ocular fluid may become an additional tool to diagnose ocular Lyme disease, especially as positive PCR results have been reported in seronegative patients and were associated with a negative immunoblot.[30,53,55,56] Mikkilä et al. advised the combined application of ELISA and immunoblot on peripheral blood and PCR on ocular fluid for efficient diagnosis of ocular

borreliosis.[56] Intraocular antibody production against *Borrelia* has not been reported, but is occasionally performed in our laboratory. So far, positive results have not been obtained and the value of this assay for the diagnosis of ocular borreliosis remains to be established.

Ocular Bartonella infection

Cat scratch disease is the most frequently recognized form of systemic *Bartonella henselae* infection. Three to 10 days after inoculation, a small erythematous papule forms on the skin in 25% to 60% of infected patients. One to two weeks later constitutional symptoms might occur, including headache, anorexia, nausea, vomiting, and sore throat with regional lymphadenopathy.[297-299] Ocular involvement occurs in 5% to 10% of patients with cat scratch disease.[297] The presence of conjunctivitis accompanied by regional lymphadenopathy defines the clinical entity known as Parinaud oculoglandular syndrome and appears to be the most common ocular manifestation of cat scratch disease, affecting approximately 5% of symptomatic patients.[64,297] Conjunctival lesions may occur and necrosis with ulceration is common.[298] *B. henselae*–associated posterior segment complications have been well described and include neuroretinitis, focal retinitis, focal choroiditis, multifocal retinitis or choroiditis, vasculitis, intermediate uveitis, vascular occlusions, and bacillary angiomatosis.[68,300-308] Neuroretinitis appears to be most common intraocular manifestation and is usually unilateral.[64] The true prevalence of neuroretinitis in patients with systemic *B. henselae* infection is unknown, although it appears to be exceptional.[297,301,302] Among patients with neuroretinitis, nearly two thirds show serologic evidence of a past infection by *B. henselae* suggesting that cat scratch disease may be a common cause of this syndrome.[307]

The diagnosis of ocular *B. henselae* infection consists of clinical features supported by laboratory testing, which mainly relies on serology, and to a lesser extent on culture or PCR analysis of tissue and/or fluid samples.[64,68] Two serological tests are available for the detection of serum anti-*B. henselae* antibodies, an IFA and an ELISA. The sensitivities and specificities of the IFA are reported to be 90% or better for immunocompetent patients, but may fall to 70% or less in HIV-infected patients.[309] The ELISA is more variable in sensitivity and specificity, resulting in more false-negative results.[297] All serological tests for *B. henselae* have shown to cross-react with *B. quintana*, and cross-reactivity with other *Bartonella* species

can not be excluded.[68] It is extremely difficult to culture *Bartonella* species from biopsy specimens but can be accomplished using enriched agar incubated in 5% CO_2 at 35 to 37˚C. Growth of these fastidious colonies from tissue or blood can take up to 4 weeks.[68]

PCR-based techniques have been developed for the detection of *B. henselae*. Relman and associates developed the first primers for the detection of *Bartonella* DNA based on the *B. henselae* 16S ribosomal RNA gene.[64,67] These techniques are highly sensitive and are able to identify specific *Bartonella* species. Other PCR–based detection methods have since been developed, however, these are not yet commercially available and thus have so far mainly been used for research purposes.[62,63,68] GWC analysis for *B. henselae* has not been described.

Ocular Syphilis

The spirochete *Treponema pallidum* is the causative agent of syphilis, a sexually transmitted disease. Untreated syphilis manifests in several stages; primary, secondary, latent and tertiary syphilis, which are characterized by different clinical characteristics.[310-312] Ocular syphilis is usually a manifestation of secondary or tertiary syphilis. Uveitis is the most common ocular feature of syphilis and is often associated with neurosyphilis.[313] Signs of syphilitic uveitis include anterior segment inflammation, vitritis, papillitis and neuritis, macular edema, serous retinal detachment, retinitis and glaucoma.[313-317] No pathognomonic features exist for syphilitic uveitis and hence, the term “great imitator” applies not only to systemic syphilis, but also to the ocular disease. The incidence of syphilis among HIV-positive individuals has increased and all patients with syphilis should be tested for HIV as well.[3] Risk factors for acquiring the two infections are similar, and the presence of a genital chancre as seen in primary syphilis, increases the risk of acquiring or transmitting HIV.[310,318,319]

When ocular syphilis is suspected, initially standard syphilis screening assays are performed. It is common practice to include syphilis testing in the standard uveitis screening protocol. For syphilis screening, the treponemal tests (*Treponema pallidum* haemagglutination and particle agglutination assays (TPHA and TPPA, respectively)) are generally performed on peripheral blood. Enzyme immunoassays are also available and show promising results as screening assays in all stages of syphilis.[320,321] However, these tests do not discriminate between a previous or active infection. The non-treponemal veneral disease research

laboratory test (VDRL) is used to determine the activity of disease and can be useful for antibody quantitation during the course of treatment.[310] Neurosyphilis is confirmed by a positive VDRL in CSF or by the presence of intrathecal antibody production, using the TPHA or TPPA.[322-324] The applicability of VDRL and TPHA on ocular fluids remains to be investigated. Enzyme immunoassays have not proven to be useful in syphilitic uveitis yet.[325, 326]

Intraocular fluid analysis is not commonly used for the diagnosis of ocular syphilis. Direct pathogen or antigen detection, the treponemal and non-treponemal tests have been described in case reports or small studies in literature, but none yielded useful results.[327-332] Attempts for GWC determination have been reported, but without positive results.[3] Recently, positive PCR results on ocular fluids were reported in four cases, but large studies have not emerged so far.[65,66]

Diagnosis of fungi

Fungal endophthalmitis usually presents with creamy-white, well-circumscribed lesions of the choroid and retina, often accompanied by inflammatory infiltrates in the vitreous.[333] Endogenous fungal endophthalmitis is frequently an ocular manifestation of a systemic disease and mostly occurs in immunocompromised patients and intravenous drug addicts.[251,333-336] *Candida albicans* is the most common pathogen isolated in endogenous fungal endophthalmitis.[251,333,336] Other pathogens include *Aspergillus, Coccidioides, Cryptococcus, Blastomyces,* and *Sporothrix* species.[251,333,336] Exogenous infections usually are secondary to trauma with organic material or to surgery.[251,333,336]

Positive blood cultures might help in establishing the diagnosis of intraocular fungal infections while serology is not commonly practiced. For the diagnosis of fungal endopthalmitis direct smear and cultures are commonly used, but the intraocular samples of infected individuals might be culture-negative.[251,333,336,337] On the other hand, false positive results may occur due to contamination during sampling. In order to improve the value of microbiological diagnosis, PCR technology has successfully been applied to detect fungi in ocular samples.[337] Fungal endophthalmitis can be confirmed by PCR using panfungal primers complementary to 18S rDNA, and the primers targeting the internal transcribed spacer and 5.8S rDNA have also been reported.[6,338,339] PCR is considered a promising tool in patients with ocular *C. albicans*.[6,339]

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Chapter 2

Rubella virus is associated with Fuchs heterochromic iridocyclitis

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Abstract

Purpose: To determine whether Rubella virus (RV) is involved in the pathogenesis of Fuchs heterochromic iridocyclitis (FHI).

Design: Retrospective case-controlled study.

Methods: Intraocular immunoglobulin G production against RV, Herpes simplex virus (HSV), Varicella zoster virus (VZV) and *Toxoplasma gondii* was determined in the aqueous humor of 14 patients with FHI, 13 control subjects with herpetic anterior uveitis and 19 control subjects with ocular toxoplasmosis by calculation of the Goldmann-Witmer coefficient (GWC).

Results: All patients and control subjects were seropositive for RV. Intraocular antibody production (GWC > 3) against RV was found in 13 of 14 patients (93%) with FHI. Intraocular antibody production against HSV, VZV, or *T. gondii* was not detected. None of the control subjects with herpetic anterior uveitis or with toxoplasma chorioretinitis had a positive GWC for Rubella virus ($P < .0001$, Fisher exact test).

Conclusions: Rubella virus, but not HSV, VZV, or *T. gondii,* is associated with FHI.

Fuchs heterochromic iridocyclitis (FHI) is an intriguing ocular disease that occurs in approximately 2% of all patients with uveitis. The clinical criteria for the diagnosis of FHI include diffuse iris atrophy or heterochromia, cataract and stellate keratic precipitates, in principle in the absence of synechiae and acute signs of inflammation.[1] The pathogenic mechanism of FHI remains elusive. Fuchs[2] speculated that an unknown process might cause abnormal development of uveal pigment and chronic low-grade inflammation, eventually resulting in the secondary manifestations of iris atrophy and cataract.

Sympathetic nerve dysfunction, hereditary factors, intrauterine toxins, maternal illness, infections, and autoimmunity have all been considered in the cause of FHI.[1] Also, an association between FHI and ocular toxoplasmosis or Herpes simplex virus (HSV) infection has been suggested.[3,4] However, there is no convincing evidence for an involvement of either pathogen. Recently, chronic Rubella virus (RV) infection was implicated as a possible cause of FHI, based on the presence of RV-specific intraocular antibody production and intraocular persistence of the virus.[5] This unexpected and potentially very important finding requires confirmation.

We investigated the presence of RV, HSV, Varicella zoster virus (VZV) and *Toxoplasma gondii* in the aqueous humor (AH) of patients with clinically established FHI. Fourteen patients (nine men and five women) were included, 11 of whom fulfilled the aforementioned criteria for FHI. Two patients had synechiae in addition, one of whom also had acute signs of inflammation. The remaining patient had heterochromia and keratic precipitates but had no cataract. The patients' mean age at the time of sampling was 42 years (range, 23 to 73 years). None of the patients were immunocompromised. All patients were born before the implementation of childhood vaccination against RV at 14 months of age. Samples from age-matched patients with laboratory-confirmed herpetic anterior uveitis (10 patients with HSV and three patients with VZV) and with laboratory-confirmed toxoplasma chorioretinitis (n=19) served as controls. This study was performed according to the tenets of the Declaration of Helsinki and with earlier consent from all patients.

Paired AH and serum samples, which were taken for diagnostic purposes, were tested for intraocular antibody production against RV, HSV, VZV and *Toxoplasma gondii* by determination of the Goldmann-Witmer coefficient (GWC).[6,7]

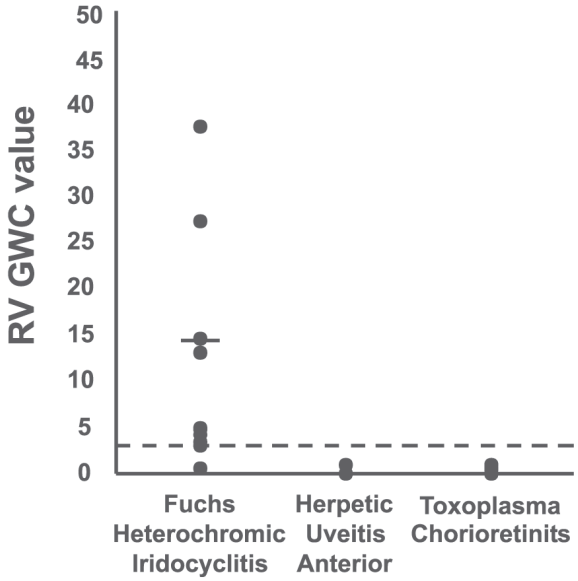

Figure. Analysis of the Rubella virus (RV) Goldmann-Witmer coefficient (GWC) values of 14 patients with Fuchs heterochromic iridocyclitis (FHI), 13 patients with herpetic anterior uveitis, and 19 patients with toxoplasma chorioretinitis. The threshold GWC value of 3 is indicated by the dashed line. The median value for the FHI patients with a positive RV GWC (14.46) is represented by a horizontal black line.

Specific antibody titers were determined by using the Enzygnost® anti-RV/IgG, anti-HSV/IgG, anti-VZV/IgG and toxoplasmosis/IgG enzyme-linked immunosorbent assay kits (Dade Behring, Marburg, Germany) essentially according to the instructions of the manufacturer. Total immunoglobulin G titers in serum and AH were determined by an in-house enzyme-linked immunosorbent assay with the use of commercially available reagents. Intraocular antibody production was considered positive when the GWC value exceeded 3.[7]

All patients and control subjects were seropositive for RV immunoglobulin G. Thirteen of 14 patients with FHI (93%) showed intraocular immunoglobulin G production (GWC > 3) against RV with a median GWC of 14.46 and a GWC range of 3.01 to 132.79 (Figure). These included the two patients with synechiae (GWC 27.16 and 123.47) and the one without cataract (GWC 26.48).

The one patient with FHI with a negative RV GWC value of 0.53 had a severe

blood-aqueous barrier breakdown, combined with a high RV immunoglobulin G serum titer, which may have obscured intraocular antibody production.[7] Thirteen of 14 patients with FHI (93%) were seropositive for both HSV and VZV and seven of 11 patients (64%) were seropositive for *T. gondii*, but none of the patients had a positive GWC for HSV, VZV or *T. gondii*. None of the control subjects had a RV GWC of > 1 (median, 0; Figure), whereas the GWC was positive for HSV or VZV in all patients for herpetic anterior uveitis (10 patients with HSV and three patients with VZV) and for *T. gondii* for all patients with toxoplasma chorioretinitis (n=19). The finding of intraocular RV immunoglobulin G production in 13 of 14 patients with FHI vs 0 of 32 control patients is statistically highly significant ($P < .0001$; Fisher exact test). Our data strongly support the conclusions of Quentin and Reiber[5] that RV, and not HSV, VZV or *T. gondii*, is associated with FHI.

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Chapter 3

Rubella virus-associated uveitis: clinical manifestations and visual prognosis

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Abstract

Purpose: To investigate the clinical profile of patients with chronic anterior uveitis and intraocular analyses positive for intraocular Rubella virus infection and assess eventual similarities to Fuchs heterochromic uveitis (FHU).

Design: Retrospective case-control study.

Methods: Clinical records of 30 patients with anterior uveitis positive for intraocular antibody production against Rubella virus by Goldmann-Witmer coefficient determination and/or polymerase chain reaction were reviewed and compared with clinical records of 13 patients with chronic anterior uveitis of undetermined origin. Multiple variables were assessed and patient records were evaluated at onset and at one year after their first visit to the University Medical Center Utrecht.

Results: Patients with Rubella virus-associated uveitis were younger at time of initial ophthalmologic presentation ($P = .014$). Rubella virus-positive patients presented more frequently with unilateral ocular disease ($P < .001$), keratic precipitates (KPs; $P = .014$), iris atrophy and/ or heterochromia ($P = .051$), associated vitreous opacities ($P = .024$), and cataract ($P = .004$). Also, the combination of KPs, absence of posterior synechiae, cataract and vitreous opacities occurred more often in the Rubella virus-positive group ($P = .026$) and the presence of three or four of these criteria occurred more frequently in the Rubella virus-positive group ($P = .004$).

Conclusions: Rubella virus causes a distinct clinical spectrum of ocular symptoms similar to the FHU syndrome which suggests that Rubella virus might be involved in the pathogenesis of FHU.

Introduction

The identification of infectious uveitis entities is of crucial importance since their treatment and visual prognosis differ entirely from noninfectious intraocular inflammations. Recent literature has suggested that Rubella virus may incite Fuchs heterochromic uveitis (FHU), but the clinical spectrum of uveitis associated with Rubella virus is not known.[1,2] Rubella virus represents a cause of congenital rubella syndrome, which is characterized by cataract and rubella retinopathy in the eye. Rubella virus was initially reported to cause uveitis in sporadic cases with postnatally acquired infections. Although the recent reports on the association of Rubella virus with uveitis have referred to FHU, the criteria of FHU were not specified in these studies.[1,2] In addition, FHU is often difficult to diagnose, because symptoms are not always present at the same time and sometimes only become obvious years later, when, for example, cataract causes visual deterioration.

In this study, we investigate the clinical profile and the course of the ocular disease in 30 patients with anterior uveitis associated with Rubella virus infection as judged by positive intraocular antibody production and/or by polymerase chain reaction (PCR) assays.

Methods

In this retrospective study, we reviewed the clinical records of 30 patients who presented with anterior uveitis and who had a positive outcome for intraocular antibody production against Rubella virus by determination of the Goldmann-Witmer coefficient (GWC) and/or PCR. All patients were seen at the Department of Ophthalmology at the University Medical Center in Utrecht, from November 1993 to June 2007. In addition to Rubella virus analysis, intraocular fluid samples from all patients were also assessed for Herpes simplex virus (HSV) and Varicella zoster virus (VZV) by PCR and GWC, yielding negative results.

For intraocular antibody production against Rubella virus, HSV and VZV, and GWC determination, paired aqueous humor and serum samples were tested at the laboratory of virology of the University Medical Center Utrecht. Aqueous humor samples were stored at -80°C in sterile screw-cap tubes within five hours of collection until subsequent laboratory analyses. The PCR and GWC assays for

Rubella virus, HSV and VZV were as described previously.[1,3] GWC values correct for leakage of serum antibodies into the ocular fluid attributable to blood-aqueous barrier breakdown, and values above three were considered indicative of intraocular antibody production.[4-7]

Thirteen patients with chronic anterior uveitis of unknown origin were included and served as controls. All control patients had negative results for PCR and for intraocular antibody production against HSV, VZV, and Rubella virus. Of the 30 patients positive for Rubella virus, two were found positive by both PCR and GWC determination, and one patient was positive only by PCR. All remaining patients had positive GWCs.

All patients and controls were tested for diagnostic purposes and had previously been subjected to extensive general screening, which included erythrocyte sedimentation rate, red and white blood cell counts, glucose levels, determination of serum angiotensin-converting enzyme levels, serologic tests for syphilis, HLA-B27 typing and chest radiography. In addition, the antinuclear factor was determined in all patients younger than 16 years. The results of this diagnostic examination were within the normal limits for all included patients. Based on the general screening and clinical presentation, none of the patients were considered immunocompromised, and there were no indications of systemic diseases.

Other pertinent patient information was recorded such as gender, age at time of the first consultation with ophthalmologist, the presence of systemic disease, ophthalmic history, unilaterality or bilaterality, various clinical manifestations, and visual acuity (VA). Findings on ocular examination, including abnormalities of the iris (specifically loss of anterior stromal details and crypts), presence of posterior synechiae and type of keratic precipitates, cells and flare in the anterior chamber and cells and opacities in the vitreous, retinal abnormalities and VA, were also registered. Treatment regimens and all eventual complications including cataract and glaucoma, were likewise assessed.[1,8-10]

Patient records were evaluated at time of the patients' initial visit at our institution and one year later. The one-year follow-up was available for 21 Rubella virus-positive and for five Rubella virus-negative patients.

The course of uveitis, as well as the classification of uveitis, grading of cells, and flare of the anterior chamber, were performed as previously recommended.[11]

In order to approximate criteria used in previous reports of the risk of glaucoma in patients with FHU, we evaluated the proportion of patients with an elevated ocular pressure above 21 mmHg in at least three measurements, which was not attributable to corticosteroid use, an optic disk ratio larger than 0.5, and/or demonstrated visual field (VF) loss.[11]

For statistical analysis of the data, the Pearson Chi-square test, the Fisher exact test, and the Mann-Whitney *U* test were used where appropriate. A probability (*P*) value of less than .05 was considered statistically significant. This study was approved by the institutional review board (Medical Ethics Review Committee) of the University Medical Center of Utrecht, The Netherlands.

Results

Clinical features of subject and control patients are given in Table 1. The male-to-female ratio was 2:1 in the Rubella virus-positive patients, and 1.2:1 in the Rubella virus-negative patients. The Rubella virus-positive patients were younger at the time of initial ophthalmologic presentation (32 years vs 44 years; $P = .014$).

Unilateral uveitis was more frequently present in Rubella virus-positive patients (28/30, 93%) compared to the Rubella virus-negative patients (six of 13, 46%; $P < .001$). Two Rubella virus-positive patients with bilateral uveitis were positive for intraocular antibody production against Rubella virus in both eyes. The previous ophthalmologic abnormalities, including amblyopia and cataract extraction, did not differ significantly between the Rubella virus-positive and Rubella virus-negative patients, nor did their presenting complaints (redness, pain and decreased VA). The recurrent and chronic courses of uveitis were similar for Rubella virus-positive and Rubella virus-negative patients.

The presence of keratic precipitates was more frequently observed in Rubella virus-positive patients (27/30, 90% vs seven of 13, 54%; $P = .014$). In six out of seven (86%) Rubella virus-negative patients with keratic precipitates, mutton fat keratic precipitates were observed. The presence of posterior synechiae at onset was observed in two out of 30 cases (7%); however, this was not statistically different from the controls (two of 13, 15%; $P = .366$). In addition, there was no development of posterior synechiae during the follow-up period in either group

Table 1. Clinical characteristics at presentation and at one-year follow-up of patients with Rubella virus-associated uveitis.

Follow-up time	Onset			1 year		
Clinical characteristics	RV-positive patients (%)	RV-negative patients (%)	*P*-value	RV-positive patients (%)	RV-negative patients (%)	*P*-value
	n = 30	n = 13		n = 21	n = 5	
Unilateral : bilateral involvement	28 : 2	6 : 7	<.001	19 : 2	3 : 2	n.s.
Anterior chamber findings						
Cells ≥ 1+	21 (70)	6 (46)	n.s.	12 (57)	4 (80)	n.s.
Keratic precipitates	27 (90)	7 (54)	.014	19 (90)	3 (60)	n.s.
Posterior synechiae	2 (7)	2 (15)	n.s.	2 (10)	0 (0)	n.s.
Iris atrophy and/or heterochromia	14 (46)	2 (15)	.051	9 (43)	2 (40)	n.s.
Iris nodules	2 (7)	2 (15)	n.s.	1 (5)	1 (20)	n.s.
Posterior segment involvement						
Cells in vitreous ≥ 1+	15 (54) (n = 28)	5 (38)	n.s.	11 (52)	1 (20)	n.s.
Vitreous opacities	21 (75) (n = 28)	5 (38)	024	17 (81)	2 (40)	n.s.
Chorioretinal scar	6 (20)	4 (31)	n.s.	4 (19)	2 (40)	n.s.
Cystoid macular edema	1 (3)	0 (0)	n.s.	1 (5)	0 (0)	n.s.
Complications of uveitis						
Cataract	23 (77)	4 (31)	.004	18 (86)	2 (40)	.029
Secondary glaucoma	7 (23)	4 (31)	n.s.	1 (5)	1 (20)	n.s.
Visual acuity ≤ 0,5	18 (60)	4 (31)	n.s.	6 (29)	3 (60)	n.s.
Visual acuity ≤ 0,1	7 (23)	3 (23)	n.s.	2 (10)	1 (20)	n.s.
Treatment of uveitis						
Topical corticosteroids	26 (87)	13 (100)	n.s.	20 (95)	5 (100)	n.s.
Topical non-steroid anti-inflammatory drugs	7 (23)	1 (8)	n.s.	5 (24)	1 (20)	n.s.
Periocular corticosteroids injections	21 (70)	3 (23)	.004	17 (81)	1 (20)	.008
Systemic corticosteroids	4 (13)	1 (8)	n.s.	3 (14)	0 (0)	n.s.
Surgical intervention	7 (23)	4 (31)	n.s.	19 (91)	2 (40)	.010
Cataract extraction	6 (20)	2 (15)	n.s.	19 (91)	1 (20)	<.001
Trabeculectomy	1 (3)	2 (15)	n.s.	0 (0)	1 (20)	n.s.

RV = Rubella virus; n.s. = not significant

(Table 1). Iris atrophy and/or heterochromia were more frequently observed in the Rubella virus-positive patients (14/30, 46% vs two of 13, 15%; *P* = .051). No additional cases with development of iris abnormalities were noted during the follow-up.

The presence of iris nodules did not differ between the groups. Also, the intensity of cellular reaction in the anterior chamber observed at time of initial presentation was similar for patients and controls.

At the time of the initial examination, cataract was more frequently present in the Rubella virus-positive patients (23/30, 77% vs four of 13, 31%; *P* = .004). This difference remained significant at the one-year follow-up (*P* = .029). The development of cataract increased with time in the Rubella virus-positive patients. By the one-year follow-up of Rubella virus-positive patients, cataract incidence had increased to 27/30 (90%), in contrast to the Rubella virus-negative patients, whose cataract frequency remained unchanged (four of 13; 31%).

No differences between Rubella virus-positive and Rubella virus-negative patients were noted in the presence of associated chorioretinal scars and cystoid macular edema. In addition, none of the 10 patients with chorioretinal scars (n = 6 of the Rubella virus-positive patients and n = 4 of the Rubella virus-negative patients) was positive in GWC and/or PCR for *Toxoplasma gondii*.

Vitreous opacities were more frequently observed in Rubella virus-positive patients (21/28, 75% vs five of 13, 38%; *P* = .024). Development of secondary glaucoma occurred in seven of 30 (23%) of the Rubella virus-positive and four of 13 (31%) of the Rubella virus-negative patients (*P* = .608).

At onset, a decrease in VA worse than 0.1 was observed for seven of 30 (23%) patients in the Rubella virus-positive group, which did not differ from the Rubella virus-negative controls (*P* = .985; Table 1). The main causes of decreased VA in the Rubella virus-positive group were the presence of cataract and vitreous opacities. The main causes for the Rubella virus-negative group were cataract and glaucoma. At the one-year follow up, a VA of more than 0.5 was found for 15/21 (71%) of the Rubella virus-positive patients and for two of five (40%) of Rubella virus-negative patients (*P* = .184).

The presence of ocular features regularly observed in FHU —1) keratic precipitates, 2) iris atrophy and/or heterochromia, 3) absence of posterior synechiae, 4) cataract — is indicated in Table 2 for the Rubella virus-positive

Table 2. Clinical criteria of Fuchs heterochromic uveitis in Rubella virus-positive patients with anterior uveitis.

Patients (n = 30)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1. Keratic precipitates	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■	■	■		■		■	■	■	■
2 .Iris atrophy and/or heterochromia	■	■	■	■	■	■	■	■	■	■										■		■	■	■						
3. Absence of posterior synechiae	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■	■		■	■	■	■	■
4. Cataract	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■			■	■				
Fulfillment of criteria of FHU	4 criteria										3 criteria													2 criteria						
Vitreous opacities	■	■	■	■	■	■	■				■	■	■	■	■	■	■	■	■		n.d.	n.d.	■	■	■	■	■			

FHU = Fuchs heterochromic uveitis; n.d. = not determined

Table 3. Clinical criteria of Fuchs heterochromic uveitis in Rubella virus-negative patients with chronic anterior uveitis.

Patients (n = 13)	1	2	3	4	5	6	7	8	9	10	11	12	13
1. Keratic precipitates	■	■	■	■		■	■	■					
2. Iris atrophy and/or heterochromia	■	■			■								
3. Absence of posterior synechiae	■	■	■	■	■	■	■		■	■	■	■	
4. Cataract	■	■	■	■									
Fulfillment of criteria of FHU	4 criteria		3 criteria		2 criteria			1 criterion				no criteria	
Vitreous opacities	■	■				■			■	■			

FHU = Fuchs heterochromic uveitis

patients and in Table 3 for the Rubella virus-negative patients.[8,9,12]

All four criteria were fulfilled at the time of the initial presentation by 10/30 (33%) of the Rubella virus-positive patients, compared to two of 13 (15%) of the Rubella virus-negative patients (P = .228). The presence of three or four of the criteria occurred more frequently in the Rubella virus-positive group (23/30, 77% vs four of 13, 31%; P = .004). In addition, the combination of keratic precipitates, the absence of posterior synechiae, cataract, and associated vitreous opacities was observed for nine of 30 (30%) of the Rubella virus-positive patients. In contrast, this combination was not found in any of the Rubella virus-negative patients (zero/13, 0%; P = .026).

Topical and systemic corticosteroid therapy did not differ between Rubella virus-positive and Rubella virus-negative uveitis patients. However, periocular corticosteroid injections were more frequently required in Rubella virus-positive patients (21/30, 70% vs three of 13, 23%; P = .004). This difference remained significant at the one-year follow-up (P = .008). The corticosteroid injections were often administered in conjunction with ocular surgery, accounting for 12/21 (57%) and two of three (67%) of the injections in the Rubella virus-positive and the Rubella virus-negative patients, respectively.

Discussion

In this study we observed a distinct clinical profile of patients with Rubella virus-associated anterior uveitis that differed from the clinical characteristics of patients with anterior uveitis of unknown etiology and without intraocular antibody production against Rubella virus.

Rubella virus-associated uveitis was characterized by a specific combination of clinical features, specifically keratic precipitates, iris abnormalities, and absence of posterior synechiae, and chronic character of predominantly unilateral intraocular inflammation. In addition, vitreous opacities were present in 75% of the Rubella virus-positive patients and 90% developed secondary cataract. This clinical profile of features was manifested preferentially in young adults. The ocular features observed in Rubella virus-positive patients were similar to those observed in the syndrome of FHU.

Rubella virus is transmitted by virus-laden droplets from the respiratory secretions of infected persons and can cause fever and rash.[13] While the postnatal

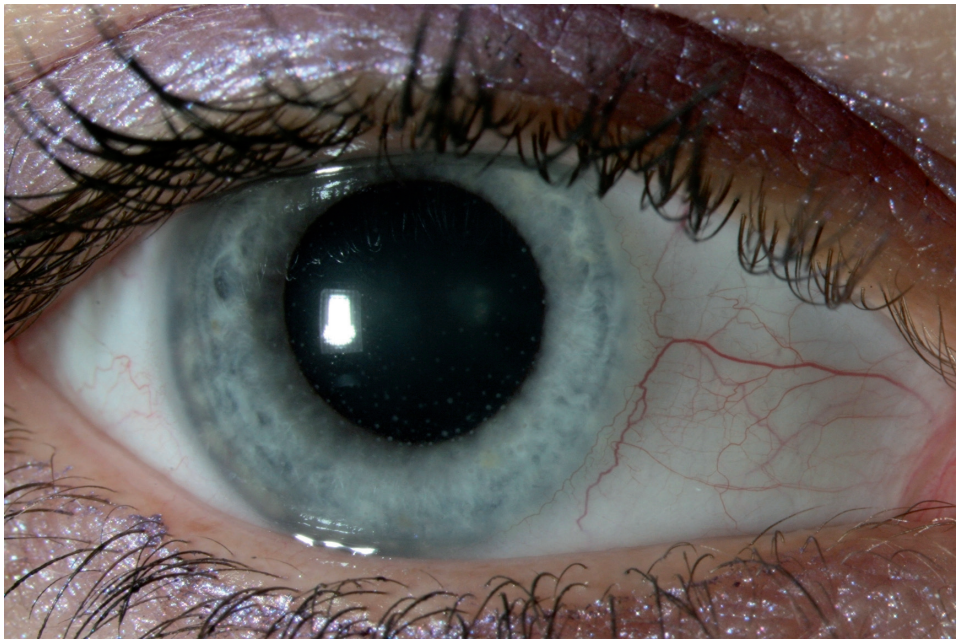

Figure. Characteristic keratic precipitates in a patient with Fuchs heterochromic uveitis.

infections are generally mild, congenital infection with Rubella virus is known to cause devastating manifestations giving rise to congenital Rubella syndrome, of which the classic triad consists of cataracts, cardiac abnormalities, and deafness.[13-15]

FHU is a chronic ocular disease of low-grade anterior chamber inflammation with typical clinical signs such as: 1) characteristic keratic precipitates (Figure), 2) diffuse iris atrophy and/or heterochromia, 3) absence of posterior synechiae, and 4) the development of cataract. Sometimes, vitritis and glaucoma also develop.[8,9,12,16] In the present study, 33% of the Rubella virus-positive patients with anterior uveitis fulfilled all four of the above criteria. Seventy-five percent of the Rubella virus-positive patients had a combination of two or more of the above criteria together with vitreous opacities.

It is interesting to note that the majority (16/30, 53%) of the Rubella virus-positive patients exhibited an absence of iris atrophy and/or heterochromia. The absence of heterochromia in FHU has commonly been reported, especially in dark

irides.[12,17,18] A possible explanation might also be that, in our retrospective study, the subtle hypochromia and iris atrophy were not always noted, especially in darker eyes. In addition, iris nodules have been described as a common symptom of FHU, occurring in more than 28% of cases.[12,19-21] We found only 7% of the cases in the Rubella virus-positive group, which did not differ from the Rubella virus-negative group. In this retrospective study, the subtle iris changes might have been overlooked.

The keratic precipitates typical of FHU are characteristic small translucent keratic precipitates scattered over the entire endothelium.[26] The appearance of keratic precipitates in Rubella virus-positive uveitis was not described in detail in most of the patients, whereas in the Rubella virus-negative group, most keratic precipitates were qualified as mutton fat. Vitreous opacities (present in 75% of Rubella virus-positive patients) have also been described as common symptoms in FHU.[12,21,22]

The presence of cataract is a common finding in all types of uveitis, but it is especially frequently noted in FHU, where it is one of the major signs, usually already present at the initial presentation. According to Mohamed and associates, FHU should be given serious consideration in any young person with unilateral cataract and no history of trauma or steroid use.[12] Our findings of cataract in Rubella virus-positive patients are consistent with the occurrence of cataract described in FHU. The favorable visual prognosis (after cataract extraction) described for FHU was also observed in our Rubella virus-positive patients.

The absence of posterior synechiae (before intraocular surgery) is considered to be one of the criteria of FHU.[12] In our study, we found that only two out of 30 Rubella virus-positive patients (of whom one was a child and the other had a history of ocular trauma) had posterior synechiae at time of presentation.

Although a standard definition for uveitic glaucoma has not been agreed upon, previous reports of the risk of glaucoma in FHU have ranged from 15% to 59%.[9,12,17,19,21,23] In our study, 25% of the Rubella virus-positive patients had intraocular pressure elevation, an optic disk ratio larger than 0.5 and/or VF defects, the occurrence of which did not substantially differ from the group with Rubella virus-negative patients. This implies that the development of glaucoma is a common complication of uveitis, rather than a development specific to Rubella virus-positive uveitis.

Peripheral chorioretinal scars in FHU have already been described by Fuchs, and subsequent studies have reported their occurrence at variable frequencies.[8,9,19,24,25] Because of the toxoplasmosis-like appearance of these scars, *Toxoplasma gondii* was thought to be associated with FHU. However, definitive laboratory proof of *Toxoplasma* infection has yet to be found in FHU patients with these chorioretinal scars.[1,2,24] In our study, the associated chorioretinal scars were also found in patients with Rubella virus-associated uveitis, but in none of them was a positive PCR and/or GWC for *Toxoplasma gondii* found. Therefore, the cause of chorioretinal scars in Rubella virus-associated uveitis remains unknown.

Other rare associations with FHU, such as sympathetic nerve dysfunction and hereditary factors, have been reported and considered in the pathogenesis of FHU,[8,12,26] but were not encountered in our series.

A shortcoming of this retrospective study is that the appearances of the iris and keratic precipitates were not systematically examined and concisely documented. Therefore, the exact determination of clinical features was not possible. A prospective study of Rubella virus-associated uveitis might be valuable to identify the exact clinical manifestations, especially the appearance of keratic precipitates and degree of iris atrophy in Rubella virus-positive patients. We think that we kept the possibility of bias toward FHU limited, because we report also on the clinical manifestations of patients who were Rubella virus-negative in ocular fluid testing. We tested all our patients with anterior uveitis for Rubella virus as well, and compared the two groups: negative and positive. If FHU features were not associated with Rubella virus, this would be apparent in our results.

In conclusion, Rubella virus-associated anterior uveitis appears to be characterized by a clinical spectrum of symptoms similar to the syndrome of FHU. Three or more criteria of FHU were observed in 77% of Rubella virus-positive patients, suggesting that Rubella virus might be the causative agent of a substantial number of cases of FHU. The observation that 15% of the Rubella virus-negative patients fulfilled the four criteria of FHU indicates that Rubella virus is probably not the only cause of FHU.

Our study underlines the similarities of Rubella virus-associated uveitis to FHU and might lead to a better understanding of the pathogenesis of this intriguing ocular disease.

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Chapter 4

Characteristics of focal retinal scars in Rubella virus-associated uveitis and ocular toxoplasmosis

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Abstract

Purpose: To assess clinical differences of focal (chorio-)retinal lesions between patients with intraocular Rubella virus infection and patients with ocular *T. gondii* infection.

Methods: Photographic and angiographic records of 28 patients with focal (chorio-)retinal scars, of which 11 patients with Rubella virus-associated uveitis and 17 patients with ocular toxoplasmosis (OT) were masked for identification and for infectious agent and evaluated by four specialists in the field of OT. Multiple characteristics of the lesions were assessed.

Results: No differences were observed between the chorioretinal lesions in Rubella virus-positive and *T. gondii*-positive patients. Chorioretinal lesions were considered consistent with the diagnosis of OT in 55% of Rubella virus-positive patients and in 88% of *T. gondii*-positive patients by at least three out of the four experts. Two experts considered the retinal lesions in *T. gondii*-positive patients more frequently "consistent with the diagnosis of ocular toxoplasmosis" ($P = .010$ and $P = .011$). There was a 'substantial agreement' between the four experts (Fleiss' Kappa = .623).

Conclusion: No single clinical factor differentiated focal chorioretinal lesions in patients with intraocular Rubella virus infection from those of patients with OT, which suggests that the etiological diagnosis of these lesions cannot be made solely on clinical grounds.

Introduction

Focal (chorio-)retinal scars can be observed in various ocular infections, the most representative being ocular toxoplasmosis (OT). Focal retinal scars have been reported in the ocular infections with Cytomegalovirus (CMV), Herpes simplex virus (HSV) and *Toxocara canis*, and may also occur due to trauma or other focal damage in the retina.[1-5] Peripheral retinal scars may also be observed in the general population.[6] The typical aspect of toxoplasmic scars consists of a focal, usually oval or round, white atrophic lesion with irregular pigmentations, frequently positioned next to each other (satellite formation).[1,7] Focal retinal scars described as "toxoplasmosis-like scars" were also observed in 7 to 65% of patients with Fuchs heterochromic uveitis (FHU).[8,9] The pathogenesis of retinal scars in FHU is not known. Such scars have usually been ascribed to presumed *T. gondii* infection; however, their toxoplasmic origin has not been proven.[10,11]

Recent literature has suggested that Rubella virus may incite uveitis with clinical features typical of FHU, including the associated focal retinal scars in 20% of cases.[8,12,13] However, the observations described above raise the questions about the origin of the focal retinal lesions in FHU and highlight the possibility that Rubella virus might also induce focal retinal lesions.

In this study, we assess clinical differences of the focal retinal scars in 11 patients with intraocular proof of Rubella virus and in 17 patients with intraocular proof of *T. gondii* infection.

Methods

In this retrospective study, we assessed 41 patients with Rubella virus-associated uveitis, who were positive in ocular fluid analysis by Goldmann-Witmer coefficient (GWC) determination and selected all 11 patients who exhibited anterior uveitis with associated retinal scars of which four were bilateral, resulting in 15 affected eyes (Figure 1A-F). In addition, we included 17 patients with unilateral retinal scars and ocular fluid analysis positive for *Toxoplasma gondii*. by calculation of the GWC (Figure 2A-F). All included patients were negative for other microorganisms tested in intraocular fluids by GWC and by PCR (HSV, Varicella zoster virus, CMV). Based on the general uveitis screening and clinical presentation, none of the patients was considered immunocompromised, and

there were no indications of associated systemic diseases.

Photographic and angiographic records of all patients were masked for the identification and for infectious agent causing the ocular disease and recorded on a CD-ROM in a random order and sent for the evaluation to four specialists in the field of ocular toxoplasmosis, working in Europe (the Netherlands and France), the United States and Brazil. To assess the characteristics of the retinal lesions, a questionnaire was developed to register the following characteristics per affected eye: number and location of retinal lesions, description of the lesion(s), extent of the retinal lesion(s), occurrence of satellite lesions, and other particular features, such as the hyperpigmentation, characteristics of the lesion borders and the presence of associated vasculitis and papillitis. Because the (chorio-) retinal lesions found in FHU patients are mostly inactive, we only included photographs and angiographs of *T. gondii*-positive patients with quiet, inactive retinal lesions. Finally, consistency with the diagnosis of ocular toxoplasmosis and presumed diagnosis were assessed (Table 1).

All patients were tested in serum and aqueous samples for Rubella virus and *T. gondii* IgG antibodies by enzyme-linked immunosorbent assay (ELISA) to calculate the Goldmann-Witmer coefficient (GWC) for intraocular antibody production as described previously (Table 2).[12,14] All patients with a positive GWC for Rubella virus infection tested negative for *T. gondii* in aqueous, but their serum samples tested positive for IgG against *T. gondii* in variable titers (6 – 283 IU/ml), except for one patient, who was entirely seronegative for *T. gondii*. One patient classified as Rubella virus-associated uveitis had a positive GWC for Rubella virus with a coefficient of 18. Two years previously, this patient tested positive for *T. gondii* with a value of 6. We assumed that this patient was Rubella virus-positive, since the Rubella virus GWC had the highest value.[15]

All patients with intraocular proof of OT tested negative for Rubella virus in aqueous. Their sera were positive for IgG against Rubella virus in 15 cases; the 2 remaining patients tested negative for IgG against Rubella virus (both were not vaccinated against Rubella virus). Aqueous samples in all patients were collected for diagnostic purposes by paracentesis or during cataract surgery. The Rubella virus analyses in OT patients were performed using the remainders of diagnostic samples according to institutional regulations.

For statistical analysis of the data, the Pearson Chi-square test, the

Fisher exact test and the Mann-Whitney *U* test were used where appropriate. A probability (*P*) value of less than 0.05 was considered statistically significant. The Fleiss' kappa was calculated to measure the degree of agreement between the four experts. The K (kappa) value ranges from < 0 to 1.00, where a K value of < 0 is considered to represent 'poor agreement', 0.0 to 0.20 'slight agreement', 0.21 to 0.40 'fair agreement', 0.41 to 0.60 'moderate agreement', 0.61 to 0.80 'substantial agreement' and a K value within 0.81 to 1.00 was considered to represent 'almost perfect agreement'.[16-18]

Results

The results of questionnaire analyses and the examples of the photographs are given in Table 1 and Figure 1 and 2. The male-to-female ratio was 4:7 in the Rubella virus-positive patients, and 15:2 in the *T. gondii*-positive patients (*P* = .004). The mean age at onset of uveitis was 34 (range 22 – 56) years for the Rubella virus-positive patients and 30 (range 21 – 39) years for the *T. gondii*-positive patients (*P* = .412). All Rubella virus-positive patients had low-grade anterior uveitis associated with small keratic precipitates scattered over the corneal endothelium and 9/11 had associated cataract, of which 6 had a cataract extraction and none had synechiae. Thereby, all had at least three signs typical of FHU. Also, none of the scars in these patients had ever been seen as a focus of active disease. In the toxoplasmosis group, 5/17 of the patients (29%) exhibited primary ocular toxoplasmosis and 12/17 (71%) of the patients had a recurrence. Congenital infection was assumed in at least 1/17 (6%) patients because retinal scars had been present in this patient since the age of 3 years. Regarding the other patients it was unclear whether OT was postnatally or congenitally acquired. One patient with OT had cataract and subsequent cataract extraction. None of the patients in both groups had nystagmus.

The mean number of retinal lesions per affected eye in the Rubella virus-positive group was 2.7 lesions per eye and 2.8 lesions per eye in the *T. gondii*-positive group. The occurrence of one single lesion per patient in the Rubella virus-positive patients was 6/11 (55%) and 5/17 (29%) in the *T. gondii*-positive patients (*P* = .184).

Both retinal and choroidal layers were considered to be involved in most patients. No differences in retinal and choroidal involvement were found between

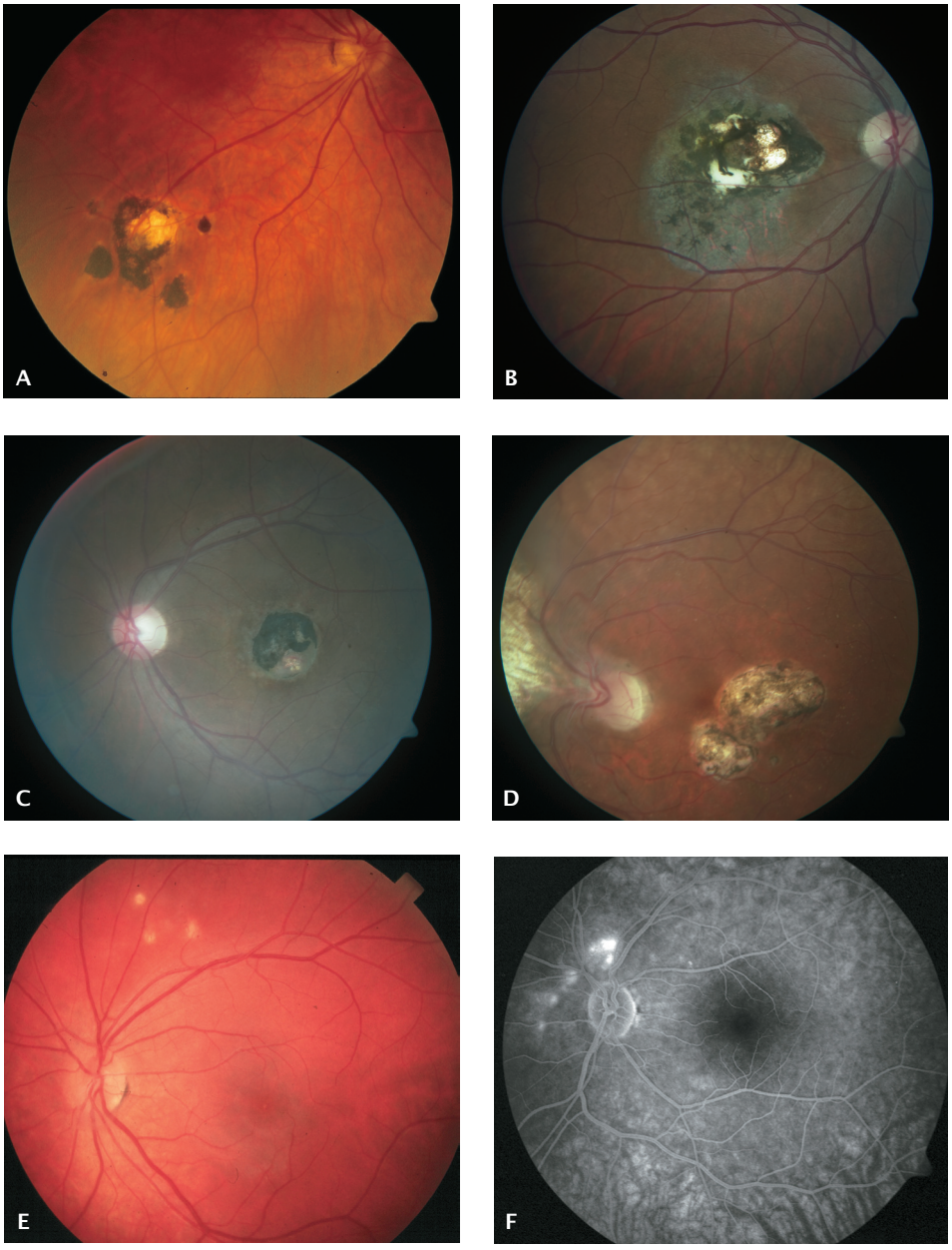

Figure 1A-E.Chorioretinal lesions of patients with Rubella virus-associated uveitis.

Figure 1F. Retinal lesion in a patient with Rubella virus-associated uveitis and seronegative for *T. gondii.*

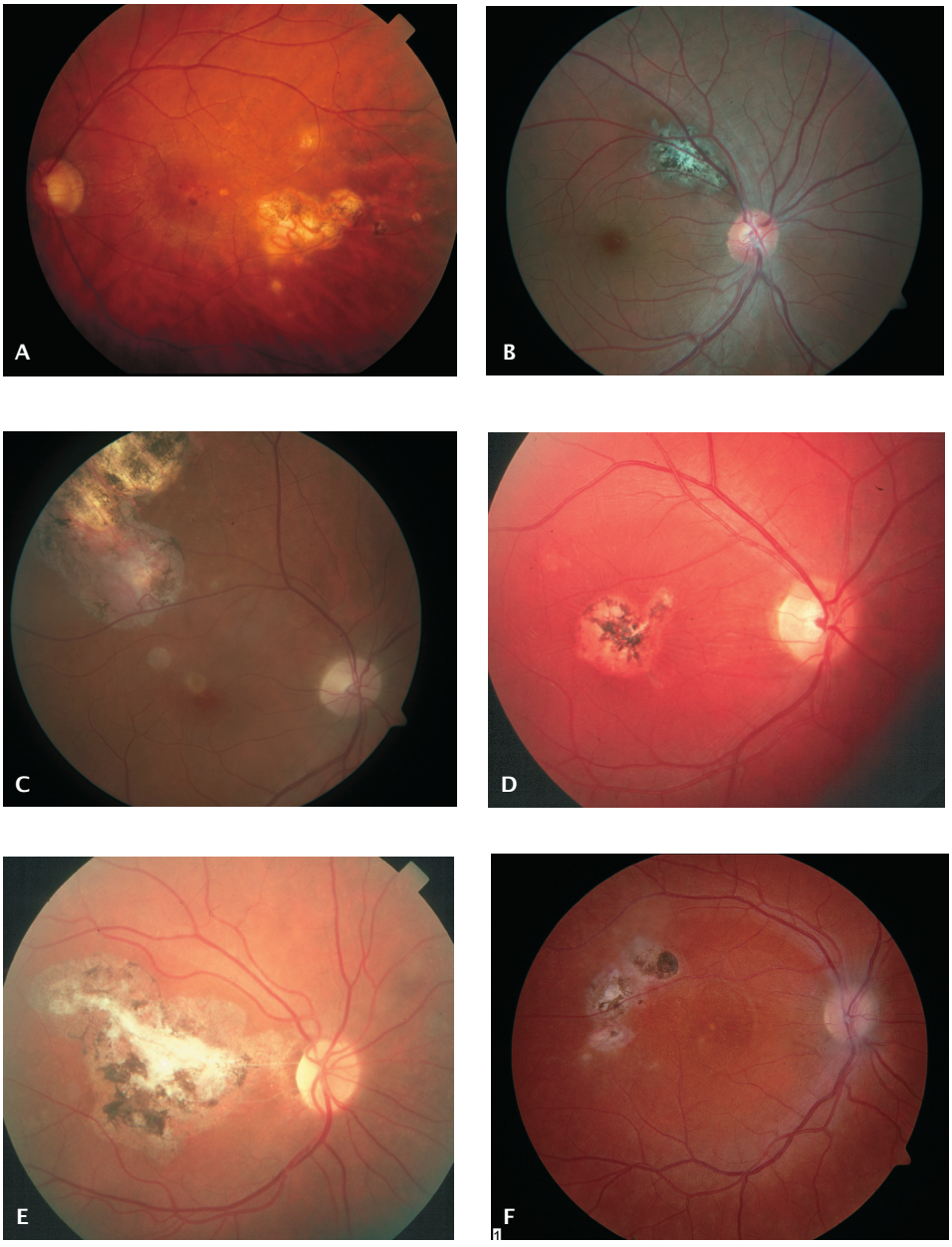

Figure 2A-F. Chorioretinal lesions of patients with intraocular proof of ocular toxoplasmosis.

Table 1. Characteristics of chorioretinal lesions on the photographs of patients with Rubella virus-associated uveitis and ocular toxoplasmosis per affected eye according to four evaluating experts.

Expert	Expert 1			Expert 2		
	RV positive (%) n = 15	Toxo positive (%) n = 17	*p*-value	RV positive (%) n = 15	Toxo positive (%) n = 17	*p*-value
Description of retinal lesion						
Retinal	2 (13)	2 (12)	n.s.	5 (33)	3 (18)	n.s.
Choroidal	13 (87)	15 (88)	n.s.	10 (67)	14 (82)	n.s.
Atrophic border	9 (60)	14 (88)*	n.s.	13 (93)*	14 (93)*	n.s.
Pigmented border	6 (40)	6 (38)*	n.s.	9 (64)*	13 (87)*	n.s.
Location of retinal lesion						
Central	6 (40)	8 (47)	n.s.	6 (40)	9 (53)	n.s.
* Macular	6 (100)	7 (88)	n.s.	6 (100)	8 (89)	n.s.
* Adjacent to optic disc	3 (50)	1 (6)	n.s.	3 (50)	1 (6)	n.s.
Peripheral	7 (47)	5 (29)	n.s.	5 (33)	3 (18)	n.s.
Central and peripheral	2 (13)	4 (24)	n.s.	4 (27)	5 (29)	n.s.
Associated papillitis	0 (0)	1 (6)	n.s.	0 (0)	1 (6)	n.s.
Associated vasculitis	0 (0)	4 (24)	0,045	1 (7)	2 (12)	n.s.
Satellite lesions	7 (47)	13 (76)	0,082	7 (47)	11 (65)	n.s.
Extent of largest retinal lesion †						
< 3 optic disc diameters	6 (55)	8 (47)	n.s.	9 (60)	7 (41)	n.s.
> 3 optic disc diameters	5 (45)	9 (53)	n.s.	6 (40)	10 (59)	n.s.
Consistent with the diagnosis of ocular toxoplasmosis						
yes	9 (60)	14 (82)	0,160	10 (67)	17 (100)	0,010
no	6 (40)	3 (18)	0,160	5 (33)	0 (0)	0,010

n.s = not significant; RV= Rubella virus
* not all patients were evaluated for all included specific aspects;
† per patient

Expert 3			Expert 4		
RV positive (%) n = 15	Toxo positive (%) n = 17	*p*-value	RV positive (%) n = 15	Toxo positive (%) n = 17	*p*-value
6 (40)	10 (59)	n.s.	15 (100)	13 (67)	0,045
12 (80)	14 (82)	n.s.	11 (73)	15 (88)	n.s.
10 (71)*	14 (82)	n.s.	12 (86)*	16 (94)	n.s.
8 (57)*	14 (82)	n.s.	9 (64)*	13 (76)	n.s.
4 (27)	5 (29)	n.s.	4 (27)	7 (41)	n.s.
4 (100)	5 (100)	n.s.	4 (100)	7 (100)	n.s.
0 (0)	0 (0)	n.s.	0 (0)	0 (0)	n.s.
8 (53)	4 (24)	n.s.	4 (27)	5 (29)	n.s.
3 (20)	8 (47)	n.s.	7 (47)	5 (29)	n.s.
0 (0)	1 (6)	n.s.	0 (0)	0 (0)	n.s.
0 (0)	2 (12)	n.s.	1 (7)	4 (24)	n.s.
11 (82)	12 (71)	n.s.	7 (47)	15 (88)	0,011
7 (64)	8 (47)	n.s.	6 (55)	7 (41)	n.s.
4 (36)	9 (53)	n.s.	5 (45)	10 (59)	n.s.
7 (47)	15 (88)	0,011	11 (73)	15 (88)	0,281
8 (53)	2 (12)	0,011	4 (27)	2 (12)	0,281

Table 2. Detection of *T. gondii* and Rubella virus IgG by enzyme-linked immunosorbent assay in both serum and aqueous of patients with Rubella virus-associated uveitis and retinal lesions.

Patient	Rubella virus GWC in aqueous	Rubella virus IgG in serum (IU/ml)	*T. gondii* IgG in serum (IU/ml)	*T. gondii* GWC in aqueous
1	4	316	14	Neg
2	21	137	73	Neg
3	11	135	254	Neg
4	206	75	283	Neg
5	4	117	6	Neg
6	14	180	49	Neg
7	10	542	57	Neg
8	28	674	Neg	Neg
9	49	74	91	Neg
10	18	530	164[a]	6[a]
11	121	23	10	Neg

[a] determined 2 years before the Rubella virus testing; the concurrent analyses were not possible due to a small volume of the samples.

GWC = Goldmann-Witmer Coefficient
Neg = negative

Rubella virus-positive and *T. gondii*-positive patients. In addition, no differences were found with respect to the location of the lesions (central, peripheral or both) and other lesions characteristics, including the aspect and (hyper-) pigmentations of the lesions and their borders. The sizes of the retinal lesions were also similar in the OT and Rubella virus-positive groups.

One of the experts (in contrast to the other three) considered associated vasculitis more frequent in *T. gondii*-positive eyes (0/15, 0% versus 4/17, 24%; $P = .045$). Satellite lesions were more frequently considered in OT by two experts, whereas the other two did not find this association (Table 1). The conclusion

that the photograph was "consistent with the diagnosis of ocular toxoplasmosis", was more frequently made in *T. gondii*-positive patients than in Rubella virus-positive patients by 2 of the 4 experts (10/15, 67% versus 17/17, 100%; $P = .010$ and 7/15, 47% versus 15/17, 88%; $P = .011$; Table 1). At least three of the four experts considered the retinal lesions consistent with the diagnosis of OT in 6 of 11 (55%) of Rubella virus-positive patients and in 15 of17 (88%) of *T. gondii*-positive patients. The degree of agreement, Fleiss' Kappa, over the consistency of the lesions with the diagnosis of ocular toxoplasmosis in all patients between the four experts was 0.623, which corresponds to a 'substantial agreement' between the four experts.

Discussion

In this study, four experts from different ophthalmologic centers found no obvious differences between the characteristics of chorioretinal lesions with laboratory proven Rubella virus-associated uveitis and ocular toxoplasmosis. There was a substantial degree of agreement found between the four experts, and the majority of Rubella virus-associated chorioretinal scars were considered to be consistent with the diagnosis of ocular toxoplasmosis. The only slight difference found by 2 out of the 4 experts was in the frequency of satellite formation of the lesions, which was considered to be more frequent in the OT group.

The chorioretinal scars in OT may vary from small focal to large destructive retinal lesions. The scars usually have an atrophic center and are surrounded by irregular hyperpigmentation. Classically, OT is associated with a unilateral focal chorioretinal scar, sometimes together with one or more "satellite lesions" and only one focus of active disease in immunocompetent patients.[7] Usually, the diagnosis of OT is based on clinical features only. However, the appearance of the focal lesions and subsequent atrophic scars is not unique to OT and may be mimicked by myopic scarring, by scars following focal retinal and choroidal vascular accidents and may also be related to other infections. For example, lymphocytic choriomeningitis virus can cause lesions that resemble toxoplasmic retinochoroiditis scars.[19,20] The fact, that patients exhibiting focal lesions are not always positive for *T. gondii* in serology and/or in intraocular fluid analysis is consistent with possible alternate causes of focal retinitis.

The occurrence of focal chorioretinal lesions in patients with FHU and patients with Rubella virus-associated uveitis has long been described.[6,8,10-13,21-24] Toxoplasmic etiology of (a part of) FHU has been examined and cases of proven OT followed by mild chronic uveitis suggesting FHU have been described.[6,10,11,22-27] A study assessing the ocular fluids of patients with FHU revealed negative results for *T. gondii*.[21] Although our Rubella virus-positive patients had active uveitis, their chorioretinal scars appeared quiet. There is a possibility that the chorioretinal lesions in Rubella virus-associated anterior uveitis represent old toxoplasmosis scars. If so, this would suggest a double infection, first toxoplasmic retinitis and second Rubella virus-associated anterior uveitis. The GWC values of patients with quiet toxoplasmic scars have not been systematically investigated and it is possible that these have become negative over time. Our patients positive for Rubella virus did not report (previous) symptoms compatible with ocular toxoplasmosis and active retinal lesions were never encountered.

Seropositivity for *T. gondii* in the general population in the Netherlands is about 40%, which does not correspond with the seroprevalence of 90% (10/11) found in our Rubella virus-positive group.[28] Our serologic tests were developed for diagnosis of ocular infections and use markedly lower cut-off points than commercial screening tests and therefore the results of these two studies cannot be compared.[28] The probability (*P*) of randomly sampling 10/11 patients with retinochoroidal scars who are seropositive for *T. gondii* is $P = .0008$, which suggests that the observed lesions might be linked to toxoplasmosis. Although the double infections are not probable, the high prevalence of serum toxoplasmic antibodies in Rubella virus-associated uveitis suggests that the involvement of *T. gondii* in the formation of chorioretinal scars in Rubella virus-associated uveitis cannot be entirely excluded.

Previous laboratory studies suggested that the chorioretinal scars in Rubella virus-associated uveitis might be induced by Rubella virus itself.[8] Support for this hypothesis is that congenital rubella infections can cause progressive pigmentary retinopathy with retinal scarring.[29] Another possibility might include immune-mediated processes, such as an autoimmune reaction against retinal antigens brought up by molecular mimicry, or exposure to autoantigens by retinal damage.

The high number of male patients in our OT group is striking (15 out of 17) and represents an accidental availability of remainders of diagnostic aqueous

samples solely. The male-to-female ratio of 121 patients with laboratory proven OT (GWC>3 and/or positive PCR) tested between 2001 and 2007 at our institution is 1.6:1.

Our results show that the distinction of chorioretinal scars in Rubella virus-associated uveitis and OT is not possible purely on clinical grounds. Despite our detailed analysis of retinal features, we were not able to identify specific characteristics that could discriminate OT from Rubella virus-associated scars. Associated ocular features other than the chorioretinal scars can, however, help with clinical diagnoses: low grade chronic uveitis and associated cataract without synechiae suggest Rubella virus-associated uveitis, and active chorioretinal lesions with overlying vitreous infiltrate suggest OT.[1,8] In addition, the presence of a "satellite lesion" an active lesion adjacent to old scar(s) makes the diagnosis of OT more probable.

In conclusion, no single clinical factor differentiated focal chorioretinal lesions associated with OT from those in patients with Rubella virus-associated uveitis. Our findings illustrate that the etiological diagnosis of focal chorioretinal scars cannot be made solely on clinical grounds. Further study is needed to determine the exact origin of the chorioretinal scars in Rubella virus-associated uveitis.

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Chapter 5

Diagnosis of ocular toxocariasis by establishing intraocular antibody production

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Abstract

Purpose: To investigate the role of *Toxocara canis* in posterior uveitis of undetermined origin.

Design: Retrospective case-study.

Methods: Paired ocular fluid (47 aqueous humor (AH) and two vitreous fluids) and serum samples of 37 adults and 12 children with undetermined posterior uveitis were retrospectively analyzed for intraocular IgG antibody production against *Toxocara canis* by enzyme-linked immunosorbent assay and Goldmann-Witmer coefficient (GWC) determination. Previous diagnostic investigation by polymerase chain reaction and GWC for Herpes simplex virus, Varicella zoster virus and *Toxoplasma gondii* had not provided a cause of the posterior uveitis.

Results: Three of 12 (25%) children showed intraocular IgG production against *Toxocara canis*. One child had vitritis, one presented with a low-grade uveitis and a peripheral retinal lesion and the third had posterior uveitis and a chorioretinal scar. All three children had AH IgG titers exceeding those of the corresponding serum. In fact, two children had low *Toxocara* serum IgG titers (<1:32) and would have been considered seronegative upon routine serology screening. Intraocular antibody production against *Toxocara canis* was absent in all 37 adults, including five seropositive patients.

Conclusions: Our results indicate that ocular toxocariasis is mainly a pediatric disease. Serological screening is not informative for the diagnosis of intraocular *Toxocara* infection. *Toxocara* GWC analysis, however, can be of value when diagnosing patients with posterior focal lesions or vitritis of unknown etiology.

Introduction

Toxocara canis is a roundworm with the dog as its natural host. Humans can become infected by ingestion of soil or contaminated meat containing *Toxocara* larvae. In particular, children eating dirt or in close contact to puppies are at risk of being infected. In humans the *Toxocara* larvae can invade several organs, such as the lungs, liver, brain and eye, where they are encysted by a granulomatous cellular reaction.[1-3]

Not much is known about the prevalence of human toxocariasis, but the disease occurs worldwide. Seroepidemological studies may vary widely depending on the population examined. Reported *Toxocara* seroprevalences range from 4 to 46% in adults and can be as high as 77.6% in schoolchildren.[2,4-12] High percentages are associated with low hygienic standards and high exposure to infected dogs.

Ocular toxocariasis or ocular larva migrans (OLM) is a local complication of a *Toxocara canis* infection and is usually suspected in children,[1-3,13] although it has been reported in adults.[14,15] The clinical signs of ocular toxocariasis often include diminished vision, leukocoria, red eye and strabismus. Lesions occur mostly unilaterally and might be falsely diagnosed as retinoblastoma or endophthalmitis of bacterial origin. The diagnosis is usually based on the presence of chorioretinal granuloma or focal lesions in the posterior eye segment in the presence of positive serology.[1,2,15] However, low or undetectable *Toxocara* serum immunoglobulin (Ig) G titers have been reported in patients with ocular toxocariasis.[16] Therefore, the diagnosis of ocular toxocariasis is difficult and in the majority of cases remains only presumptive.

In this study, we examined the possibility of intraocular infection with *Toxocara canis* in 49 patients (37 adults and 12 children) with posterior uveitis of undetermined origin by means of serum antibody and Goldmann-Witmer Coefficient (GWC) determination.

Methods

From 2001 to 2006, 49 patients with posterior and panuveitis of unknown etiology were examined at the Department of Ophthalmology (n=43) at the University Medical Center in Utrecht (UMCU), The Netherlands, or at ophthalmology clinics in other Dutch hospitals (n=6). From all patients the clinical

characteristics where recorded. All patients had been subjected to extensive general screening, which included erythrocyte sedimentation rate, red and white blood cell counts, glucose levels, determination of serum angiotensin-converting enzyme levels and serological tests for syphilis, and chest radiography. Based on the general screening and clinical presentation, none of the patients were considered immunocompromised. Moreover, intraocular fluid analysis was performed at the UMCU for Herpes simplex virus (HSV), Varicella zoster virus (VZV) and *Toxoplasma gondii* by polymerase chain reaction (PCR) and GWC determination with negative results.[17] All patients had clinical characteristics compatible with ocular toxocariasis (granuloma, focal chorioretinitis, or multiple focal chorioretinal lesions) and *Toxocara canis* serology had been previously requested, but did not provide conclusive evidence about the cause of uveitis. Of 49 patients, 12 (24%) were children (under 17 years of age) and 37 (76%) were adults. The children included seven (58%) boys and five (42%) girls, with a mean age of 9.6 years at the time of sampling (range, two to 16 years). The adults included 20 (54%) men and 17 (46%) women, with a mean age of 35.9 years at the time of sampling (range, 17 to 65 years) (Table 1).

The simultaneously taken serum and ocular fluid samples (46 aqueous humors (AH) and three vitreous fluids) previously used for examination for HSV, VZV and *T. gondii*, were retrospectively analyzed for intraocular antibody production against *Toxocara canis* by GWC determination ((specific IgG in AH/specific IgG in serum) / (total IgG in AH/total IgG in serum)).[18,19] *Toxocara canis*-specific serum and AH IgG were determined by using the *Toxocara canis* IgG enzyme-linked immunosorbent assay (ELISA) kit (DRG Instruments, Marburg, Germany), which contains micro test wells coated with an excretory/secretory antigen derived from second-stage larvae of *Toxocara canis*. The assays were performed according to the instructions of the manufacturer. However, instead of a single 1:64 dilution, which is the manufacturer's screening dilution for seropositivity, four two-fold dilutions ranging from 1:32 to 1:256 were used for both serum and AH. Serum and AH IgG titers were calculated using the Mikrowin software version 3.0 (Mikrotek Laborsysteme, Overath, Germany). In case of undetectable serum IgG (titer <32), the GWC value was calculated using a serum titer of 32 and referred to as larger than (>) the outcome of the GWC. Total IgG titers in serum and aqueous humor were determined by an in-house ELISA which

has been previously described.[17] Intraocular antibody production was considered positive when the GWC value exceeded three.[18,19] This study was performed in accordance with institutional regulation of the University Medical Center Utrecht, The Netherlands.

Results

General clinical characteristics are given in Table 1. Two of 37 adults were seropositive for *Toxocara canis* at the screening dilution of 1:64 and an additional three were positive at the 1:32 dilution (5/37; 14%). Of the children, one was seropositive at 1:64, one was just positive at the 1:32 dilution (2/12; 17%) and the remainder were negative. Intraocular antibody production against *Toxocara canis* (GWC >3) was absent in all 37 adults, including the five seropositive adult patients. Moreover, in none of the 37 adults *Toxocara canis* IgG was detected in the aqueous humor. In contrast, three of 12 (25%) children demonstrated intraocular IgG production against *Toxocara canis*. Two of these three children were negative at dilution 1:32. The third child was positive at dilution 1:64. All three children had an intraocular *Toxocara* IgG titer which exceeded that of the serum (Table 2). In the remaining nine children no *Toxocara canis* IgG was detected in the aqueous humor. The three children with a positive *Toxocara canis* GWC are described below.

Table 1. Clinical characteristics of patients with uveitis suspected for ocular toxocariasis.

	Gender	Mean age (y)	Uni-or bilateral	Focal chorioretinitis	Multiple focal lesions	Chorioretinal granuloma
Children n = 12	7 (58%) M 5 (42%) F	9.6	11 (92%) uni 1 (8%) bi	5 (42%)	none	none
Adults n = 37	20 (54%) M 17 (46%) F	35.9	31 (84%) uni 6 (16%) bi	29 (78%)	3 (8%)	6 (16%)
Total n = 49	27 (55%) M 22 (45%) F	29.4	42 (86%) uni 7 (14%) bi	34 (69%)	3 (6%)	6 (12%)

M = male; F = female; Uni = unilateral; Bi = bilateral

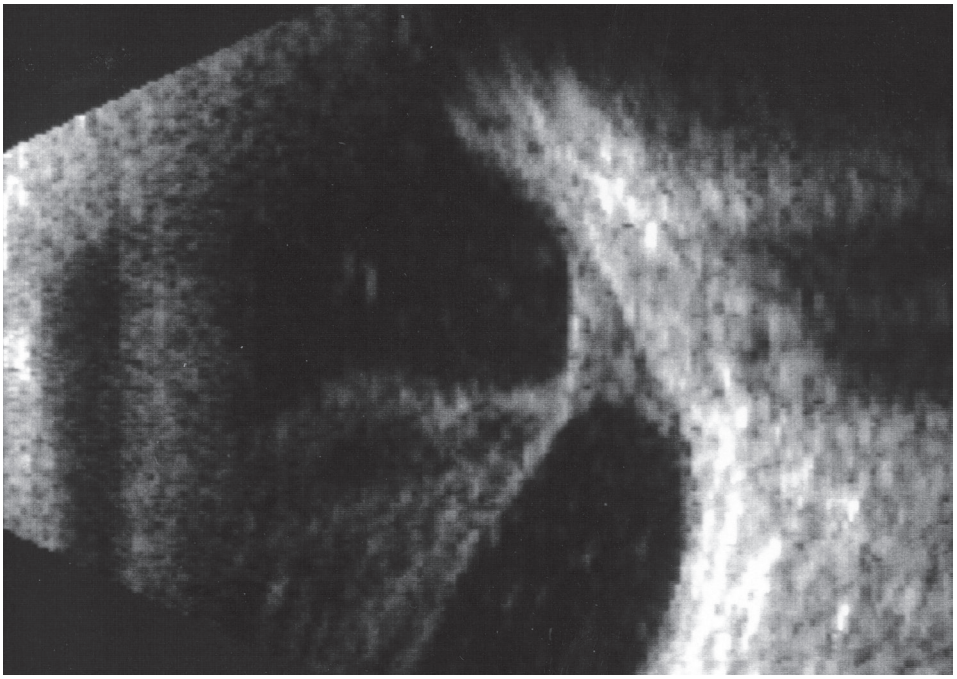

Figure 1. Ultrasonography revealing a funnel-shaped structure and adhesion to the optic disk in a child with ocular toxocariasis.

Case 1. A 7-year-old Turkish boy was referred to our clinic because of recently detected decrease in visual acuity of the left eye (LE). He had no ophthalmic history, except intermittent redness of the LE for several months. Ocular examination of this eye revealed the presence of keratic precipitates, cells in the anterior chamber, iris bombé with papillary seclusion, mature cataract, and dense vitreous membranes. Funduscopy was not possible attributable to mature cataract. Ultrasonography revealed vitreous opacities with a funnel-shaped structure in the vitreous, adhesion to the optic disk, and disk edema (Figure 1). The right eye (RE) was unremarkable. He was referred to a pediatrician, but there were no indications for tuberculosis, juvenile idiopathic arthritis, and sarcoidosis by Purified Protein Derivative, anti-nuclear antibodies, and radiological examination of the chest. *Ascaris lumbricoides* serology was negative, *Toxocara canis* serology was positive, aqueous analysis revealed negative GWC results for HSV, VZV, Rubella virus, *T. gondii* and *Borrelia Burgdorferi*, and negative

PCR results for HSV, VZV and *T. gondii*. At that time the diagnosis remained inconclusive. Cataract extraction was performed in combination with vitrectomy with silicone oil, because of retrolental vitreous membranes and tractional retinal detachment. After removing the silicone oil, the patient developed proliferative vitreoretinopathy. Subsequently, the eye became atrophic and because of severe psychological and cosmetic problems, enucleation followed and the eye was investigated at the pathology laboratory. Microscopic examination revealed a hyperplastic cornea and a round nuclear inflammatory infiltrate in the underlying fibrous tissue. In addition to fibrosis, neovascularization, and papillary seclusion, the retina was completely detached and prolapsed anteriorly with adhesion to the fibrous tissue. This piece of the retina showed reactive gliosis. The angles of the anterior chamber were completely obstructed, partly with reactive choroid proliferation. Locally, macrophages with multinuclear giant cells were observed on the retinal pigment epithelium. Eosinophils were not observed. Stainings to detect microorganisms were all negative, but this does not exclude an infectious cause. Based on histopathological analysis, no specific diagnosis could be made, other than evidence for recurrent uveitis.

Retrospectively, serum and AH were analyzed for *Toxocara canis* immunoglobulin, yielding a very high AH titer (1609), exceeding the serum titer (94). The resulting GWC was positive (144), and the diagnosis of ocular toxocariasis was made.

Case 2. An 8-year-old boy was referred to our clinic because of recently detected uveitis of the LE with vitreous cells and a peripheral retinal scar. The RE was normal. The initial visual acuity of the left eye was 1.0. The diagnosis ocular toxocariasis or toxoplasmosis was suspected. The general medical history was not remarkable, however the patient was born in Sri Lanka and visited it several times. On ocular examination, the visual acuity of the LE was 0.8, the anterior chamber revealed sporadic cells, the lens was clear, the vitreous exhibited cells and opacities, and in the inferior peripheral retina a white lesion was observed. The RE had full visual acuity and no abnormalities. Fluorescein angiography demonstrated a peripheral active lesion, possibly a granuloma with vitreous traction (Figure 2). Ultrasonography revealed a vitreous density inferiorly, however a prominent granuloma was not observed.

The patient was referred to a pediatrician for examination for systemic diseases. The erythrocyte sedimentation rate was 5 mm/hour and blood counts and angiotensin-converting enzyme were within normal range. Radiological chest examination was normal. *Ascaris* serology was negative and *Toxocara* titers were less than 1:32. *Toxoplasma* IgM was negatieve and IgG was positive. There was no evidence for an active infection with *Coxiella burnetii, Rickettsia conorii, Rickettsia typhi, Strongyloides stercoralis*, *Filaria*, Cytomegalovirus, HSV, and VZV. Aqueous analysis was negative for HSV, VZV, *T. gondii* and Rubella virus, both by PCR and by GWC. Despite undetectable serum IgG against *Toxocara*, the AH titer was clearly positive (109) and a GWC value of > 243 was determined, establishing intraocular antibody production against *Toxocara canis.* The patient was not treated for toxocariasis because the lesion became quiet and atrophic over time. The uveitis, however, persisted and was treated with topical corticosteroids.

Case 3. A 13-year-old boy was seen at the ophthalmology clinic because of a decrease in visual acuity of the LE existent for 6 months. He was in general good health and had no ophthalmic history. On ocular examination, the visual acuity of the LE was 0.1, the anterior chamber revealed no cells, and the vitreous exhibited

Table 2. Clinical and laboratory data of the three children with a positive Goldmann-Witmer Coefficient for *Toxocara canis*.

Patient	Gender	Age	Immune status	Location uveitis	Uni-or bilateral	Activity	Vitritis	Retinitis
1	male	7	normal	panuveitis	unilateral	yes	yes	nd
2	male	8	normal	posterior	unilateral	yes	no	focal
3	male	13	normal	posterior	unilateral	no	no	focal

IgG = immunoglobulin G; GWC = Goldmann-Witmer Coefficient; OT = ocular toxocariasis; Nd = could not be determined

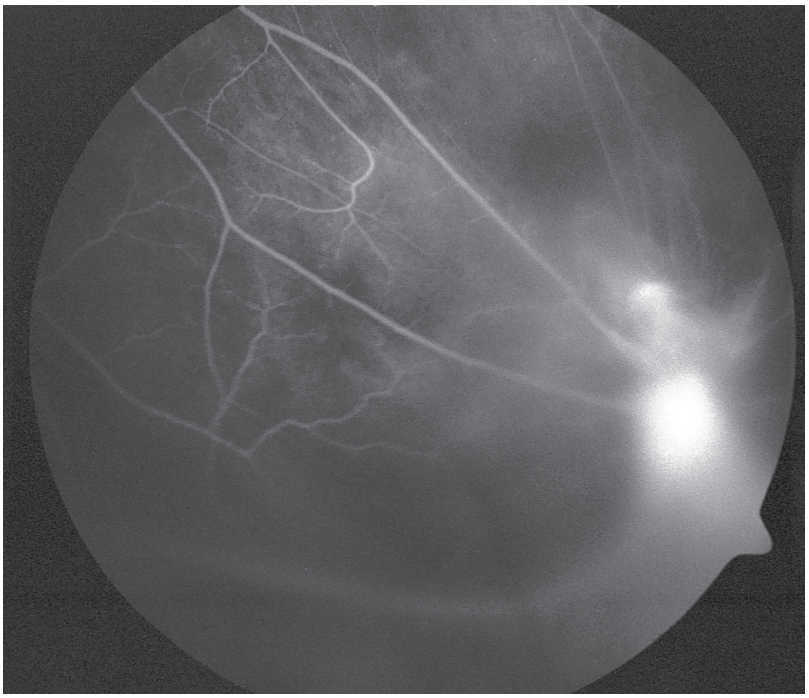

Figure 2. Fluorescein angiography demonstrating a peripheral active lesion, possibly a toxocaral granuloma.

Granuloma	Vasculitis	Papillitis	Anterior segment involvement	Presumed diagnosis	Serum IgG titer	Aqueous IgG titer	GWC
nd	nd	nd	nd	unknown	94	1609	144
no	yes	no	no	ocular toxoplasmosis or OT	<32	109	> 243
no	no	no	no	ocular toxoplasmosis or OT	<32	103	>1085

some pigment cells, vitreous strands with retinal traction, and a macular scar with a pucker. Ultrasonography revealed a posterior vitreous detachment with adhesion to the optic disk. On fluorescein angiography no vasculitis was seen. The RE was normal. Vitrectomy with removal of internal limiting membrane was performed. A vitreous sample was obtained and subsequent screening by *Toxocara canis*, *Ascaris lumbricoides* serology was negative. Serum IgG against *Toxocara* was undetectable, however the IgG titer in the vitreous was 103, resulting in a GWC value of at least 1085, establishing ocular toxocariasis. Visual acuity did not improve after vitrectomy.

Discussion

In this study we found three children with local antibody production against *Toxocara canis*. Antibody detection in serum and in ocular fluid of patients suspected of ocular toxocariasis has been reported,[13,14, 20] but only one report included GWC determination to correct for passive leakage of antibodies from the serum in the aqueous attributable to blood-aqueous barrier breakdown.[21]

The three children with a positive GWC had very low serum IgG titers. One child was positive at the screening dilution of 1:64. Two were negative even at dilution 1:32 and would have been designated seronegative. Very low serum titers or seronegativity in patients with ocular toxocariasis have been reported previously. Therefore, it has been suggested that sera should be tested at dilutions as low as 1:2 and that any positive result in combination with clinical correlation is relevant in ocular toxocariasis.[22] Moreover, Hagler et al. found a positive result at a 1:8 serum dilution or higher highly accurate in association with typical clinical findings.[23] By screening at lower dilutions, the seroprevalence in patients with ocular toxocariasis may be higher than reported thus far.[4,24] Interestingly, the seroprevalence in patients with ocular toxocariasis was reported to be higher in children than in adults. This is most likely attributable to waning antibody titers, as was demonstrated in a follow-up study of 20 patients with ocular toxocariasis, where 85% showed a decrease in serum titers.[22] Therefore, patients with a low or undetectable serum titer against *Toxocara*, including two of our GWC-positive children, may have had higher titers in the past.

Still, the presence of serum IgG against *Toxocara* does not unambiguously

prove ocular involvement even in the presence of typical clinical findings, as is exemplified by six seropositive patients in our study who had no detectable intraocular antibodies against *Toxocara*. Therefore, determination of intraocular antibody production can help to establish the diagnosis of ocular toxocariasis.

All three GWC-positive patients had low or undetectable serum IgG titers, but very high AH titers. Similar antibody distributions have been reported previously.[14, 25] This most likely is a reflection of the localized nature of an intraocular *Toxocara* infection, with extensive intraocular immunostimulation, but a systemic decrease in antibody titers.[22]

Although ocular toxocariasis has been described in adults,[14,15,20] none in our study, including the five seropositive patients, had intraocular antibody production against *Toxocara canis*. The significantly higher incidence of GWC proven ocular toxocariasis cases in juveniles (P = .012), is in agreement with ocular toxocariasis being mainly a pediatric disease.[1-3,13]

It is difficult to establish the diagnosis of ocular toxocariasis based on clinical manifestations solely, because ocular symptoms may be diverse and inflammatory signs such as redness and pain are not always present. The diagnosis of ocular toxocariasis is often made coincidentally in eyes without inflammation, for instance, during an evaluation for strabismus, in cases of decreased vision, or while undergoing a routine examination.[13] Our first GWC-positive patient presented with a decrease of visual acuity, intermittent redness, and cataract in combination with severe vitritis. The second patient had a low-grade uveitis and a peripheral retinal lesion and the third presented with posterior uveitis and a chorioretinal scar. Posterior focal lesions were found in two patients and lead to the suspicion of ocular toxoplasmosis or toxocariasis. However, ocular toxocariasis can also cause severe vitreous inflammation mimicking endophthalmitis, which applies to our first case.[13]

Taking into account that establishing the diagnosis of ocular toxocariasis based on clinical features and serologic results is unreliable, we suggest the addition of *Toxocara canis* GWC determination to the diagnostic repertoire in patients with unexplained focal chorioretinitis or vitritis. Moreover, toxocaral granuloma might be mistaken for retinoblastoma, because both diseases can clinically present with leukocoria, strabismus and loss of visual acuity.[1,2] In 1950, Wilder reported 24 patients whose eyes were enucleated because of suspected

retinoblastoma.[26] The enucleated eyes were found to have nematodes, four of which later appeared to be *Toxocara canis*.[27] *Toxocara* GWC determination might play a role in the differentiation between retinoblastoma and toxocaral posterior pole granuloma in children. However, the decision to perform paracentesis should be made reluctantly, attributable to the risk of spreading malignant cells in case of retinoblastoma.

Summarizing, intraocular IgG production against *Toxocara canis* was demonstrated by GWC determination in three children with posterior focal lesions or vitritis, despite negative or very low serum IgG titers. *Toxocara* GWC analysis might be of value when diagnosing patients with posterior focal lesions or vitritis of unknown etiology.

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Chapter 6

The importance of intraocular fluid analysis in ocular toxocariasis

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Submitted for publication

Abstract

A 54-year-old Caucasian male with a diagnosis of posterior uveitis with a focal retinal infiltrate did not improve after treatment with doxycyclin or corticosteroids. Despite earlier negative serologic testing for *Toxocara canis*, aqueous humor (AH) and serum analysis with Goldmann-Witmer coefficient calculation was performed with a highly positive result. Due to the delay in diagnosis, treatment with albendazole and oral corticosteroids was initiated 10 months after presentation. The retinal infiltrate decreased in size, but proliferative vitreoretinopathy with relapsing retinal detachment occurred with loss of visual function. The present case highlights the importance of AH analysis in suspected ocular toxocariasis and the importance of early diagnosis and treatment.

Case

A 54-year-old Caucasian male presented at the University Medical Center St. Radboud Nijmegen because of a recent decrease in visual acuity of his right eye (RE). He had no ophthalmic history. On examination, the best corrected visual acuity was 20/60 in the RE. The anterior chamber revealed no cells, whereas the vitreous exhibited 2+ cells and mild opacities. In the posterior pole a white retinal infiltrate was observed (Photo 1). The left eye had a visual acuity of 20/20 and no abnormalities on examination. Fluorescein angiography showed early blockage and late hyperfluorescence indicating a chorioretinal infiltrate (Photo 1). Additional examinations including angiotensine converting enzyme, chest X-ray, anti-neutrophilic cytoplasmic antibodies, anti-nuclear antibodies, complete blood count and complete metabolic panel were within normal range. PPD testing was positive at 11 millimeters, but the ELISPOT test for *Mycobacterium tuberculosis* was negative. Vitreous culture was negative for bacteria and fungi. *Toxocara canis* serology was negative (titer <1:40) as well as serologic testing for *Toxoplasma gondii*, *Treponema pallidum*, *Bartonella henselae*, *Borrelia burgdorferi* and HIV. Aqueous humor (AH) analysis revealed negative PCR results for Herpes simplex virus and Varicella zoster virus. Because there was a low titer against all *Rickettsia* species doxycyclin was administered for a month without any clinical response. Oral prednisone (60 milligram daily tapered in 6 weeks) was started.

Despite this treatment, the retinal infiltrate increased in diameter and elevation (Photo 2). The clinical suspicion for *Toxocara canis* infection remained and therefore second AH and serum samples were collected and examined for intraocular antibody production by Goldmann-Witmer coefficient (GWC) as described previously.[1] The *Toxocara* IgG titer in the AH was 1:165 and exceeded the serum titer of 1:150. The resulting GWC was 187 (>3 is considered positive). These results are indicative for ocular toxocariasis.

Questions

1. Describe the fundoscopic and angiographic findings on Photo 1 and Photo 2.
2. What is your differential diagnosis based on the clinical features?
3. How would you manage this patient?

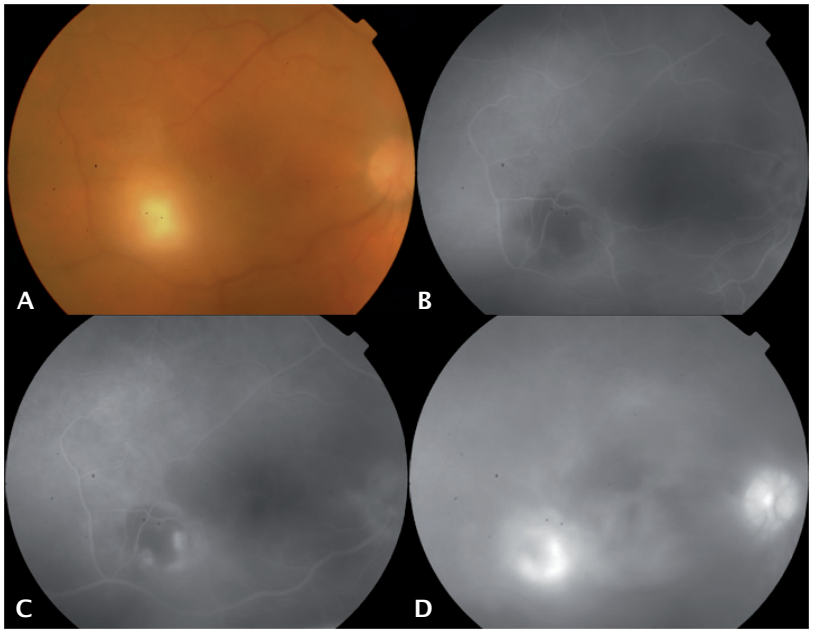

Photo 1

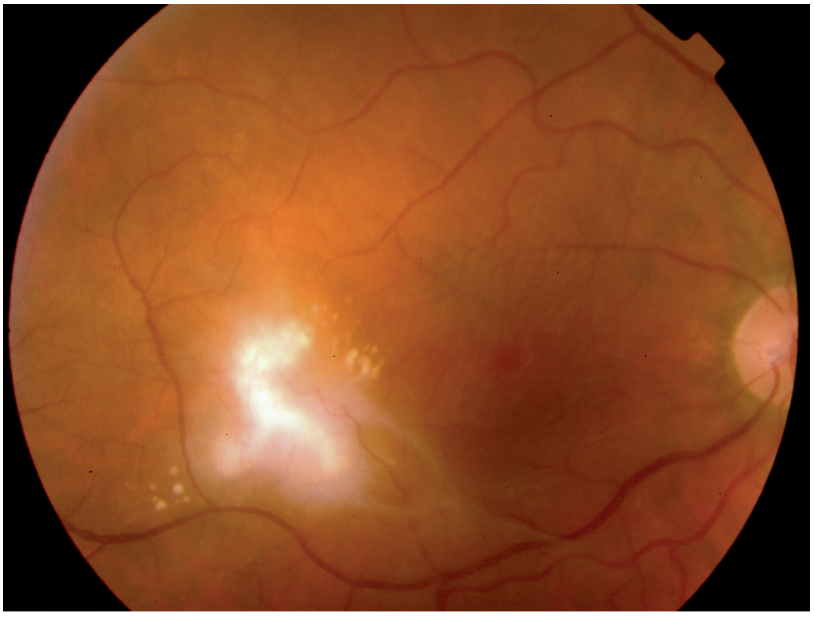

Photo 2

Answers

1. Describe the fundoscopic and angiographic findings on Photo 1 and Photo 2.

Photo 1 demonstrates a white retinal lesion in the posterior pole with early hypofluorescence and late hyperfluorescence on fluorescein angiogram and diffuse leakage in the posterior pole. Photo 2 demonstrates an increase of the lesion with epiretinal membrane formation.

2. What is your differential diagnosis based on the clinical features?

A whitish (sub)retinal infiltrate could be associated with sarcoidosis, syphilis (*Treponema pallidum*), *Mycobacterium tuberculosis*, other bacteria, *Toxoplasma gondii*, *Toxocara canis*, other nematodes, fungi and yeast like *Candida albicans*.

3. How would you manage this patient?

Ten months after presentation the patient received albendazole 10 mg/kg of body weight/day twice daily for 2 weeks in combination with oral corticosteroids to reduce the immune response expected when killing the nematode. The retinal infiltrate decreased in size, but unfortunately the patient developed proliferative vitreoretinopathy with tractional retinal detachment which relapsed after vitrectomy with silicone oil tamponade. Early analysis for *Toxocara canis* by GWC determination would have led to a correct diagnosis and proper treatment earlier in the disease. This could have prevented the proliferative vitreoretinopathy and loss of visual acuity.

Discussion

Our case illustrates that GWC analysis for intraocular antibody production against *Toxocara canis* should be performed in case of posterior uveitis of unknown etiology, even when *Toxocara canis* routine screening serology is negative.[1]

Toxocara canis is a roundworm which has the dog as its natural host. Humans can become infected by ingestion of soil or contaminated meat containing *Toxocara* larvae. Although ocular toxocariasis is mainly a pediatric disease, it should also be considered in adult patients, like in this case.[2,3]

Recently, the importance of testing intraocular fluid for *Toxocara canis* has been described. Establishing the diagnosis of ocular toxocariasis based on

clinical features and serologic results alone is unreliable.[1,4,5] Very low serum titers or undetectable serum IgG against *Toxocara canis* have been reported previously in infected patients.[1,6,7] Low serum titers against *Toxocara* may be attributable to waning antibody titers, which was demonstrated in a follow-up study where 85% of patients showed a decrease in serum titers. This might be explained by the localized nature of ocular *Toxocara* infection.[6] Therefore, when *Toxocara canis* is suspected as the cause of uveitis or when patients present with posterior focal lesions or vitritis of unknown etiology, *Toxocara* GWC should be performed. To increase the sensitivity of serology, it has been suggested to test serum at lower dilutions, however, the presence of serum IgG against *Toxocara* does not prove ocular involvement, not even in the presence of clinical findings that might imply ocular toxocariasis.[1,8,9]

Several treatment options of ocular toxocariasis have been described.[10] The correct assessment of treatment can be particularly difficult because of the variable natural course of the disease. Therefore, no commonly accepted treatment regimen for ocular toxocariasis exists.[10,11] Medical treatment includes the administration of antihelminthica, such as mebendazole, albendazole and diethylcarbamazine. To prevent or minimize serious complications due to the severe inflammatory reactions caused by dying larvae, corticosteroids should be administered simultaneously.[10-12] Cycloplegic agents may be used in the presence of an anterior segment inflammation to prevent posterior synechia. Vitreoretinal surgery is indicated if the inflammatory response results in an epiretinal membrane, (tractional) retinal detachment or a dense vitreous membrane.[13-15] In conclusion ocular toxocariasis remains a therapeutical challenge. Early diagnosis and intervention (medical and/or surgical) provide better outcome.[4] Whenever ocular toxocariasis is suspected, *Toxocara* GWC analysis of intraocular fluid should be performed.

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Chapter 7

Identification of new pathogens associated with uveitis

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Abstract

Purpose: To determine infectious causes in patients with uveitis of unknown origin by intraocular fluids analysis.

Design: Case-control study.

Methods: Ocular fluids from 139 patients suspected of infectious uveitis, but negative for Herpes simplex virus, Varicella zoster virus, Cytomegalovirus and *Toxoplasma gondii* by polymerase chain reaction and/or antibody analysis in intraocular fluids were assessed for the presence of 18 viruses and 3 bacteria by real-time PCR. The ocular fluids from 48 patients with uveitis of known etiology or with cataract were included as controls.

Results: Positive PCR results were found for Epstein-Barr virus, for Rubella virus and for Human herpesvirus-6 each in 1 patient and for Human parechovirus in 4 patients. Of the Human parechovirus-positive patients, one was immunocompromised and had panuveitis. The other three patients were immunocompetent and had anterior uveitis all with corneal involvement.

Conclusions: Human parechovirus may represent a novel cause of infectious (kerato)uveitis.

Introduction

Uveitis can be of infectious or non-infectious origin. Infections are thought to cause approximately 20-25% of cases; about 30% is associated with a non-infectious systemic disease. Although for patient management and the efficacy of treatment, the differential diagnosis is crucial, in more than half of the uveitis patients the underlying cause remains unknown.

The pathogens most commonly associated with infectious uveitis in immunocompetent patients are *Toxoplasma gondii*, Herpes simplex virus (HSV) and Varicella zoster virus (VZV). In recent years, a few other infectious agents have been implicated in the etiology of uveitis, most notably Rubella virus and Cytomegalovirus (CMV).[1-7] CMV is currently recognized as the most common cause of uveitis in immunocompromised patients.

In this study we performed an extensive search for infectious agents that cause uveitis but so far have escaped attention. Aqueous humor samples from 139 uveitis patients were tested retrospectively by real-time PCR analysis for a variety of viruses and bacteria. Our findings identify human parechovirus as a possible novel cause of infectious (kerato)uveitis.

Methods

Patients and samples

Ocular fluid samples analyzed in this study were from 629 uveitis patients who visited the ophthalmology clinic of the UMCU from October 2001 until June 2006 and were suspected of infectious uveitis. The patients were classified using the uveitis nomenclature according to the recommendations of the SUN working group 2005.[8] All patients had undergone the uveitis screening consisting of erythrocyte sedimentation rate, red and white blood cell counts, determination of serum angiotensin-converting enzyme levels, serologic tests for syphilis and chest radiography. Selected patients also underwent serological testing for *Borrelia burgdorferi*. For all 629 patients aqueous sampling was performed for diagnostic purposes. The samples were stored at -80˚C within 5 hours of collection before processing for laboratory analysis. Initial analysis was performed for HSV, VZV and in the case of posterior uveitis also for *Toxoplasma* and CMV, by PCR and by Goldmann-Witmer coefficient (GWC), to determine intraocular antibody

Table 1. General characteristics of patients and controls.

Patients	N	Immunocompromised	Gender (M:F)	Mean Age
Anterior Uveitis	49	2 (4%)	29:20	50.8 ± 16.7
Panuveitis / Posterior Uveitis	90	8 (9%)	46:44	48.9 ± 18.3
Controls				
Ocular toxoplasmosis	13	0	7:6	47.2 ± 15.4
Herpetic anterior uveitis	10	0	5:5	44.1 ± 22.1
Fuchs heterochromic uveitis	14	0	10:4	42.3 ± 16.5
Cataract	11	0	6:5	71.3 ± 15.8

production. Of the 629 patients 486 were negative for the above mentioned agents. A sufficient amount of ocular fluid remained for this study in 139 of these cases. Forty-nine patients had anterior uveitis (AU) and 90 had posterior uveitis (PU) or panuveitis (Table 1). Of the 49 AU patients, two were immunocompromised due to immunosuppressive medications (one for lethal midline granuloma and the other after allogeneic stem cell transplantation for hematological malignancy). Of the 90 patients with PU and panuveitis, 8 were immunocompromised, 5 of which had AIDS and 3 received immunosuppressive drugs (Table 1).

The remainders of ocular fluid samples from patients with PCR and/or GWC-confirmed infectious uveitis (*Toxoplasma*, n = 13; HSV, n = 10; Rubella virus, n = 14) and of patients with cataract in the absence of intraocular inflammation (n = 11) served as controls. This study was performed according to the tenets of the Declaration of Helsinki and in agreement with the regulations of the institutional review board.

Nucleic acid isolation and real-time PCR

The ocular fluid samples were analyzed for the presence of Adenovirus, Epstein-Barr virus (EBV), Human herpesvirus-6 (HHV-6) , *Mycoplasma pneumoniae*, *Chlamydia pneumoniae* and *Chlamydia trachomatis* DNA and of Coronaviruses 229E, OC43 and NL63, Enteroviruses, Human Metapneumovirus, Influenza virus A and B, Parainfluenzavirus 1 to 4, Human parechovirus (HPeV), Respiratory syncytial virus A and B and Rubella virus. If not done previously, samples from patients with anterior uveitis were also analyzed for CMV and *Toxoplasma*. DNA and RNA were extracted from 30 μl of ocular fluid using the MagNa Pure LC Total Nucleic Acid isolation kit (Roche, Mannheim, Germany). To monitor the quality of the extraction and the subsequent amplification procedure a standard dose of Phocine Herpesvirus type 1 (PhHV-1) and Encephalomyocarditis virus (EMCV) was added to each sample as an internal control prior to extraction.[9-11] Nucleic acid was collected in a volume of 240 μl. For detection of RNA viruses, copyDNA (cDNA) was produced by mixing 40 μl of extracted nucleic acid with 60 μl of reverse transcriptase mix (Taqman, reverse transcription reagents, Applied Biosystems, Foster City, CA, USA) and incubating the mixture for 10 minutes at 25ºC and 30 minutes at 48ºC. The cDNA synthesis reaction was stopped by incubating for 5 minutes at 95ºC. Per amplification reaction 10 μl of extracted nucleic acid (for DNA detection) or 10 μl of cDNA (for RNA detection) was used. Real-time PCR assays were performed on an ABI Prism 7700 sequence detection system (Applied Biosystems, Branchburg, NJ, USA). For *Chlamydia trachomatis*, 25 μl of extracted nucleic acid was analyzed using the Cobas Amplicor *Chlamydia trachomatis* detection kit according to the instructions of the manufacturer (Roche, Mannheim, Germany). All samples were examined once. In case of positive outcomes the real-time PCR reaction was repeated. Two Human parechovirus-positive samples were confirmed by nucleic acid sequencing. Samples for which the internal control was inhibited were excluded. The primers and probes used are listed in Table 2.

Antibody detection assays

Intraocular production of antibody against Rubella virus (Goldmann-Witmer coefficient) was assessed as described previously.[1] Serum and intraocular IgG titers against HHV-6 were determined using the Biotrin International Human Herpes Virus 6 IgG immunofluorescence assay (Dublin, Ireland). Serum and intraocular IgG against EBV was determined using the Panbio VCA IgG ELISA (Grenoble, France).

Table 2. Primers and probes.

Pathogen	Primers/ probe	Sequence 5' to 3'	References
Adenoviruses	Forward	TTT GAG GTG GA(C/T) CC(A/C) ATG GA TTT GAG GT(C/T) GA(C/T) CCC ATG GA	39
	Reverse	AGA A(G/C)G G(G/C)G T(A/G)C GCA GGT A AGA A(G/C)G GTG T(A/G)C GCA GAT A	
	Probe	**FAM**- ACC ACG TCG AAA ACT TCG AA-MGBNFQ -**TAMRA** **FAM**- ACC ACG TCG AAA ACT TCA AA-MGBNFQ -**TAMRA** **FAM**- ACA CCG CGG CGT CA-MGBNFQ -**TAMRA**	
Coronavirus 229E	Forward	CAG TCA AAT GGG CTG ATG CA	Na
	Reverse	CAA AGG GCT ATA AAG AGA ATA AGG TAT TCT	
	Probe	**FAM**- CCC TGA CGA CCA CGT TGT GGT TCA -**TAMRA**	
Coronavirus NL63	Forward	AAG GGT TTT CCA CAG CTT GCT AAA GGT TTT CCA CAG CTT GCT	Na
	Reverse	ATC ACC CAC TTC ATC AGT GCT AAC	
	Probe	**FAM**- TCA CTA TCA AAG AAT AAC GCA GCC TGA TTA GGA A -**TAMRA**	
Coronavirus OC43	Forward	CGA TGA GGC TAT TCC GAC TAG GT	Na
	Reverse	CCT TCC TGA GCC TTC AAT ATA GTA ACC	
	Probe	**FAM**- TCC GCC TGG CAC GGT ACT CCC T -**TAMRA**	
Enteroviruses	Forward	TCC TCC GGC CCC TGA	40
	Reverse	AAT TGT CAC CAT AAG CAG CCA GAT TGT CAC CAT AAG CAG CCA	
	Probe	**FAM**- CGG AAC CGA CTA CTT TGG GTG ACC GT -**TAMRA** **FAM**- CGG AAC CGA CTA CTT TGG GTG TCC GT -**TAMRA**	
Epstein-Barr virus	Forward	GGA ACC TGG TCA TCC TTT GC	Na
	Reverse	ACG TGC ATG GAC CGG TTA AT	
	Probe	**FAM**- CGC AGG CAC TCG TAC TGC TCG CT -**TAMRA**	
Human herpesvirus 6	Forward	GAA GCA GCA ATC GCA ACA CA	Na
	Reverse	ATG TAA CTC GGT GTA CGG TGT CTA	
	Probe	**FAM**- AAC CCG TGC GCC GCT -**TAMRA**	
Human metapneumovirus	Forward	CAT ATA AGC ATG CTA TAT TAA AAG AGT CTC	41
	Reverse	CCT ATT TCT GCA GCA TAT TTG TAA TCA G	
	Probe	**FAM**- TG(C/T) AAT GAT GAG GGT GTC ACT GCG GTT G -**TAMRA**	
	Forward	AAG ACC AAT CCT GTC ACC TCT GA	

Influenza virus A	Reverse	CAA AGC GTC TAC GCT GCA GTC C	42
	Probe	**FAM**- TTT GTG TTC ACG CTC ACC GTG CC -**TAMRA**	
Influenza virus B	Forward	AAA TAC GGT GGA TTA AAC AAA AGC AA	43
	Reverse	CCA GCA ATA GCT CCG AAG AAA	
	Probe	**FAM**- CAC CCA TAT TGG GCA ATT TCC TAT GGC -**TAMRA**	
Parainfluenzavirus 1	Forward	TGA TTT AAA CCC GGT AAT TTC TCA T	39
	Reverse	CCT TGT TCC TGC AGC TAT TAC AGA	
	Probe	**FAM**- ACG ACA ACA GGA AAT C -**TAMRA**	
Parainfluenzavirus 2	Forward	AGG ACT ATG AAA ACC ATT TAC CTA AGT GA	39
	Reverse	AAG CAA GTC TCA GTT CAG CTA GAT CA	
	Probe	**FAM**- ATC AAT CGC AAA AGC TGT TCA GTC ACT GCT ATA C -**TAMRA**	
Parainfluenzavirus 3	Forward	TGA TGA AAG ATC AGA TTA TGC AT	39
	Reverse	CCG GGA CAC CCA GTT GTG	
	Probe	**FAM**- TGG ACC AGG GAT ATA CTA CAA AGG CAA AAT AAT ATT TCT C -**TAMRA**	
Parainfluenzavirus 4	Forward	CAA ATG ATC CAC AGC AAA GAT TC	39
	Reverse	ATG TGG CCT GTA AGG AAA GCA	
	Probe	**FAM**- GTA TCA TCA TCT GCC AAA TCG GCA ATT AAA CA -**TAMRA**	
Human Parechovirus	Forward	TGC AAA CAC TAG TGG TA(A/T) GGC CC	In-house
	Reverse 1	TCA GAT CCA TAG TG(C/T) CAC TTG TTA CCT	
	Reverse 2	TCA GAT CCA CAG TGT CTC TTG TTA CCT	
	Probe	**FAM**- CGA AGG ATG CCC AGA AGG TAC CCG -**TAMRA**	
Respiratory syncytial virus A	Forward	AGA TCA ACT TCT GTC ATC CAG CAA	44
	Reverse	TTC TGA ACA TCA TAA TTA GGA GTA TCA AT	
	Probe	**FAM**- CAC CAT CCA ACG GAG CAC AGG AGA T -**TAMRA**	
Respiratory syncytial virus B	Forward	AAG ATG CAA ATC ATA AAT TCA CAG GA	44
	Reverse	TGA TAT CCA GCA TCT TTA AGT ATC TTT ATA GTG	
	Probe	**FAM**- TCC CCT TCC TAA CCT GGA CAT AGC ATA TAA CAT ACC T -**TAMRA**	
Rubella virus	Forward	CAC GCC GCA CGG ACA	In house
	Reverse 1	CAC CGG GAC TG(C/T) TG(A/G) TTG C	
	Reverse 2	CAC CGG GAC TGT TGG TTG C	
	Probe	**FAM**- AGG TCC CGC CCG AC-MGBNFQ -**TAMRA**	
Mycoplasma pneumoniae	Forward	GGT CAA TCT GGC GTG CAT CT	Na
	Reverse	TGG TAA CTG CCC CAC AAG C	
	Probe	**FAM**- TCC CCC GTT GAA AAA GTG AGT GGG T -**TAMRA**	
Chlamydia pneumoniae	Forward	TCC GCA TTG CTC AGC C	Na
	Reverse	AAA CAA TTT GCA TGA AGT CTG AGA A	
	Probe	**FAM**- TAA ACT TAA CTG CAT GGA ACC CTT CTT TAC TAG G -**TAMRA**	

Na: not available

Results

The results of the PCR analyses are shown in Table 3. In none of the ocular fluids the internal control was inhibited. Positive PCR reactions were found for Epstein-Barr virus (n = 1), Rubella virus (n = 1), Human herpesvirus-6 (n = 1) and Human parechovirus (n = 4). The PCR reactions for all other pathogens were negative. All control samples were negative except for three; two *Toxoplasma* chorioretinitis control samples were positive for EBV and one sample positive for Rubella virus intraocular antibody production also tested PCR-positive for Rubella virus RNA (Table 3).

The patients with uveitis of unknown cause and a positive PCR result for Rubella virus, HHV-6, and Human parechovirus are described below.

Case 1

A 40-year-old female complained of gradual decrease of visual acuity in the right eye (RE). Her medical history included pneumothorax and bilateral pneumonia many years ago, but she had no signs of systemic disease.

The visual acuity of the RE was 0.25. The anterior chamber and vitreous of the RE revealed cells, but no synechiae. There was a subcapsular posterior cataract, fine keratic precipitates and vitreous opacities. The retina was normal. The left eye (LE) had full visual acuity and no abnormalities on examination. Uveitis screening results were within normal limits. The clinical diagnosis of Fuchs heterochromic uveitis (FHU) was made and a cataract extraction with implantation of an intraocular lens was performed as well as pars plana vitrectomy for vitreous opacities. On examination of the vitreous, there was no evidence for systemic and/or intraocular infection using PCR and GWC for CMV, HSV, VZV, *T. gondii*, *Borrelia burgdorferi* and *Bartonella henselae*. Microbiological cultures were negative and cytologic examination revealed no malignant cells. By PCR, Rubella virus was detected in the vitreous fluid. Subsequent antibody analysis for Rubella virus revealed the presence of intraocular IgG, but the GWC was negative (2.02). However, in comparison with HSV, VZV, CMV en *Toxoplasma* intraocular antibody production against Rubella virus was elevated.

Table 3. Clinical data of patients with intraocular Human parechovirus.

Case	Gender	Age	Immune status	Location	Uni- or bilateral	Cells anterior*	Cells posterior*	Corneal involvement	Retinitis	Vasculitis	Papillitis
3	M	54	Normal	Anterior	Unilateral	1+	No	Corneal scar	No	No	No
4	M	53	Normal	Anterior	Unilateral	No	No	Corneal Infiltrate	No	No	No
5	F	73	Normal	Anterior	Unilateral	No	No	Corneal edema and keratic precipitates	No	No	No
6	M	37	HIV pos	Panuveitis**	Unilateral	3+	2+	Corneal edema and keratic precipitates	Yes	Yes	Yes

* Grading of cells was performed as recommended by Jabs et al.[Jabs, 2005]

** This patient was also diagnosed with neurosyphilis.

Case 2

A 42-year-old man was referred because of decrease in visual acuity of his RE and floaters since three months. His medical history was not contributory and the patient used no medications. Uveitis screening results were within normal range. Remarkable was the heterochromia of his eyes present since childhood. There was no serological evidence for an active infection with CMV, HSV, VZV, *T. gondii* and *Treponema pallidum. Borrelia burgdorferi* serum IgG and immunoblot were positive, however, a distinction between a past and an ongoing infection could not be made.

On ocular examination, the visual acuity of the RE was 0.8, the cornea revealed the presence of keratic precipitates, but the anterior chamber was clear. There were no synechiae, but several small noduli were present on the pupillary edge of the iris. Cataract was not observed. Funduscopy of the RE revealed vitreous cells and several peripheral snowballs. The fundoscopic findings were normal. The LE had full visual acuity, however, some peripheral vitreous opacities were observed.

Because of the possible (previous) infection with *Borrelia*, the patient was treated with intravenous ceftriaxone and additionally with periocular steroids, however with no effect. Diagnostic vitrectomy was performed and cytologic and microbiologic examinations did not reveal a cause of his uveitis. Vitreous analysis was negative for CMV, HSV, VZV, and *Borrelia*, both by PCR and by GWC. The Rubella virus GWC was negative (2.68), although intraocular IgG was detected and the GWC was elevated in comparison to CMV, HSV and VZV. Therefore, Rubella virus-associated FHU could not be excluded. Three months after vitrectomy the patient regained full visual acuity, although the keratic precipitates in his RE remained. Retrospectively, the vitreous fluid appeared to be positive for HHV-6 by PCR. Immunofluorescence assay demonstrated that the patient was seropositive for HHV-6.

Case 3

A 54-year-old male was referred to our centre with anterior uveitis of 2 years duration in his pseudophakic LE. Twenty-nine years ago the patient underwent cataract extraction and implantation of an iris-clip lens in his LE because of previous trauma. On ocular examination, the visual acuity of the

LE was hand movements (Table 3). A central corneal scar was seen, cells were present in the anterior chamber and the vitreous was clear. Fundoscopy revealed no abnormalities. The RE had full visual acuity and no abnormalities. Both eyes had normal intraocular pressure. The patient had no systemic complaints and used no medications. Uveitis screening results were within normal limits.

Uveitis was clinically attributed to irritation caused by the iris-clip lens, which was therefore surgically removed. A vitreous sample was obtained during surgery. Analysis of the ocular fluid was negative for HSV and VZV, both by PCR and GWC and for CMV and *Toxoplasma* by PCR, but was retrospectively positive for Human parechovirus by PCR. Ocular examination 4 months after removal of the intraocular lens revealed a quiet LE.

Case 4

A 53-year-old male was referred because of persistent keratitis of his LE. It was thought to be caused by HSV, but the patient did not respond to systemic and topical treatments with acyclovir and valacyclovir. His previous medical and ophthalmic histories were unremarkable. Uveitis screening did not reveal any abnormalities.

On ocular examination, the visual acuity of the LE was 20/100. An infiltrate in the upper part of the cornea with epithelial defect and sporadic cells in the anterior chamber were observed (Table 3). Corneal sensitivity was normal. The vitreous was clear and the retinal findings were unremarkable. Intraocular pressure was normal. The RE had full visual acuity and no abnormalities. Aqueous analysis was negative for HSV, VZV, and *Toxoplasma* by both PCR and GWC, however, retrospectively PCR was positive for Human parechovirus. The patient was treated with antibiotic eye ointment and the corneal lesion and anterior uveitis slowly became quiet.

Case 5

A 73-year-old female, with an ophthalmologic history of cataract extraction in both eyes at the age of 70 was referred to our institution because of secondary glaucoma in the RE. The patient had no systemic complaints and used no medications. The RE revealed pupillary seclusion with an intraocular pressure (IOP) of 50 mmHg. On examination corneal edema and keratic precipitates

were noted in the RE (Table 3). The iris revealed atrophic areas. Fluorescein angiography revealed slight optic disc leakage and cystoid macular edema in the RE. The IOP initially normalized with laser iridotomy and local treatment, however intermittent periods with IOP elevations up to 50 mmHg were regularly encountered and trabeculectomy was required. The presumed diagnosis included low grade *Propionibacterium* endophthalmitis and other various causes of hypertensive uveitis (Table 3). Screening examinations were within normal limits as well as serology for *Borrelia* and *Bartonella*. Aqueous sampling was performed and PCR was negative for HSV, VZV, CMV and *Toxoplasma* and GWC was negative for HSV and VZV. Cultures were negative for *Propionibacterium*. Retrospectively, the patient was found positive for Human parechovirus by PCR.

Case 6

A 37-year-old homosexual male was referred because of panuveitis with a focal chorioretinitis lesion in his LE since 3 months. At that time visual acuity in his LE decreased to finger counting. On examination keratic precipitates and cells in the anterior chamber and vitreous were noted with an active lesion located in the periphery of the retina (Table 3). Intraocular pressure was normal. Fluorescein angiography demonstrated optic disk leakage and vasculitis with changes of retinal pigment epithelium in the mid-periphery of the retina. The RE had full visual acuity and no abnormalities. The patient had no systemic complaints and used no medications. The presumed diagnosis of toxoplasma chorioretinitis was made. Extensive screening for panuveitis revealed positive HIV serology, an HIV RNA plasma load of 69700 copies/mL, 619 CD4 cells/ml and positive syphilis serology (TPHA >1: 2560 and a Veneral Diseases Research Laboratory (VDRL) test result of 1:256). Aqueous analysis was negative for *Treponema pallidum* by PCR and for HSV, VZV, CMV and *Toxoplasma* both by PCR and GWC. The aqueous was, however, retrospectively positive for Human parechovirus by PCR. Although both the aqueous and cerebrospinal fluid analyses were negative for syphilis, the tentative diagnosis of ocular syphilis was made and patient was treated with systemic penicillin. Antiretroviral treatment was considered not necessary at that time. Ocular inflammation subsided slowly, visual acuity increased to 20/20 and the eye remained quiet during 2 years of follow up.

Discussion

In 139 ocular fluid samples from patients with uveitis that were analyzed with a panel of 21 real-time PCRs, we found positive results in 7 cases (5%); 1 case with EBV, 1 case with Rubella virus, 1 case with Human Herpesvirus-6 and 4 cases with Human parechovirus.

EBV, the causative agent of infectious mononucleosis and several malignancies, has been implicated as a possible cause of uveitis and in primary ocular non-Hodgkin lymphoma of the central nervous system.[12-14] In our study EBV was detected in the ocular fluid of one patient with anterior uveitis of unknown cause and in two patients with toxoplasma chorioretinitis. IgG analysis in these three patients did not show any evidence of intraocular antibody production against EBV. The presence of EBV genome in the eyes of patients with various causes of uveitis was demonstrated previously and was found independent of the clinical diagnosis. The clinical significance of this phenomenon has not yet been established.[15-17] Moreover, like in our study, Ongkosuwito et al. found EBV also in ocular fluids from patients with laboratory-confirmed toxoplasmosis and in ocular fluids of patients without ocular inflammation.[16] Apparently, PCR detection of EBV in ocular fluids should be interpreted with caution, and may in most cases be considered an epiphenomenon, mostly likely due to the presence of EBV in B-cells present in the inflamed eye. Further studies combining PCR and intraocular antibody production analysis are required to determine whether EBV is a true cause of intraocular inflammation.

One patient was PCR positive for Rubella virus, the causative agent of rubella and congenital rubella syndrome.[18] Rubella virus has been associated with FHU and FHU-like uveitis.[1,5,19,20] This patient was clinically diagnosed with incomplete FHU, as she did not have iris heterochromia or atrophy.

HHV-6 is a beta-herpesvirus and the causative agent of roseola infantum (or exanthema subitum), a childhood disease.[21] In addition, HHV-6 is being recognized as an important opportunistic infection following bone marrow and/or stem cell transplantation.[22] Our HHV-6 PCR-positive patient (case 2) was neither a child, nor immunosuppressed. Antibody analysis of the ocular fluid by immunofluorescence assay did not reveal the presence of intraocular IgG against HHV-6. However, absence of intraocular antibody production does not necessarily exclude intraocular infection. Previously, we reported that by simultaneous use of PCR

and GWC in immunocompetent patients a diagnosis by PCR only was established in 9% of cases.[2] Moreover, de Boer et al. found that in patients with presumed herpetic anterior uveitis, PCR was more frequently positive than GWC.[23] HHV6 has been implicated in ocular inflammation, most notably when the posterior part of the eye was affected.[24-27] Our patient had anterior uveitis with heterochromia that had been present since childhood. Heterochromia is classically associated with FHU, but can develop in other viral infections such as HSV or VZV. [28] The detection of HHV-6 in the eye might not be a clinically relevant finding, however, like other (herpes)viruses, HHV-6 can reside latently in cells of the lymphoid and myeloid lineage, and may have entered the inflamed eye via immune cells, similar to HIV and possibly EBV).[6,15,16,27,29] The role of HHV-6 as a cause of anterior uveitis is still inconclusive and further studies are required.

Human parechoviruses belong to the genus *Parechovirus* within the family of *Picornaviridae*. They may cause gastro-enteritis, encephalitis and flaccid paralysis in young children, but rarely in adults.[30,31] Ocular disease due to other Picornavirus infections, particularly Enteroviruses, such as Echoviruses 11 and 19 and Coxsackieviruses, have been published, but an association between Parechoviruses and ocular disease has not been reported yet.[32-36] In this study the ocular fluids of four patients with undiagnosed unilateral uveitis were PCR positive for Human parechovirus. Unfortunately, intraocular antibody production could not be established as appropriate serological assays are not available and there was not enough ocular fluid left to perform viral culture.

One patient (case 6) with intraocular Human parechovirus was immunocompromised (Table 3). He had been diagnosed with active syphilis, but there were no indications for neurosyphilis or ocular syphilis. However, upon treatment with penicillin his ocular condition improved. As this patient was HIV-positive, multiple uveitis entities may have contributed to ocular disease.

The other three patients all had unilateral anterior uveitis with corneal involvement and cells in the anterior chamber, which suggested an ocular viral infection (Table 3). Further research has to be performed to determine which role Human parechovirus plays in the pathogenesis of infectious (anterior) uveitis. It is surprising to find a virus associated with disease in children, in the eyes of adults. However, other infections of childhood are known to cause intraocular disease in adults, as is the case for Rubella virus.[1,5,19]

The vast majority (93%) of ocular fluid samples was negative by PCR analysis for multiple viruses and bacteria. There are several explanations for this result. First, the uveitis might be of non-infectious origin. Second, the number of DNA or RNA copies present in the ocular samples may have been below the detection level. This may be due to the low amount of input nucleic acid, which is inherent to diagnostic assays with intraocular fluid. Alternatively, the time of sampling may have been such, that the causative agent had already been cleared from the eye. In this case, other diagnostic approaches may be more useful, such as the detection of intraocular antibody production by Goldmann-Witmer coefficient calculation. It is known, also for systemic and neurologic viral infections, that a PCR assay is most sensitive early in infection, whereas antibody can be detected over a much longer period of time and thus provide a wider window of detection.[2,23,37,38] Finally, it may be that other pathogens, not covered by our assays, were involved in these cases.

Our study addressed multiple infectious causes in patients with undiagnosed uveitis and revealed a possible new cause of infectious uveitis. Further investigations are required to narrow the diagnostic gap in patients with presumed infectious uveitis.

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Chapter 8

Intraocular fluid analysis for Cytomegalovirus, Parvovirus B19, Mumps virus and Measles virus in patients with anterior uveitis of unknown etiology

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Abstract

Purpose: To determine whether Cytomegalovirus (CMV), Parvovirus B19, Mumps virus and Measles virus are involved in the pathogenesis of anterior uveitis.

Design: Retrospective case-control study.

Methods: Paired aqueous humor (AH) and serum samples of 27 patients with unexplained anterior uveitis were examined by real-time Polymerase Chain Reaction (PCR) to determine the presence of CMV and Parvovirus B19 DNA and for intraocular antibody production against CMV, Parvovirus B19, Mumps virus and Measles virus by calculating the Goldmann-Witmer coefficient (GWC). Two control groups were included: a non-inflammatory control group (n=13) and patients with herpetic anterior uveitis (n=13). Clinical records of patients with intraocular antibody production were reviewed retrospectively.

Results: Two patients with hypertensive anterior uveitis had a positive GWC for CMV. One patient had intraocular antibody production against Parvovirus B19. One patient in the herpetic anterior uveitis group exhibited a double positive GWC, for VZV (6.2) and Parvovirus B19 (7.2). None of the patients showed intraocular antibody production against Mumps virus or Measles virus. PCR results were negative in all GWC positive patients.

Conclusion: Our results suggest that CMV and Parvovirus B19 might be associated with anterior uveitis.

The treatment and prognosis of infectious and non-infectious uveitis are entirely different, making their distinction of utmost importance. The main viral infectious causes of anterior uveitis (AU) in the Western world include Herpes simplex virus (HSV), Varicella zoster virus (VZV) and Rubella virus. Cytomegalovirus (CMV) is increasingly reported as an infectious cause of AU in immunocompetent patients.[1] We hypothesize that other common viral childhood infections might also be able to incite uveitis and selected Parvovirus B19, Mumps and Measles virus as likely candidates.

The aim of this study was to determine whether CMV, Parvovirus B19, Mumps virus and Measles virus are associated with AU.

Paired aqueous humor (AH) and serum samples of 27 patients with AU of unknown etiology were included in the study. All samples were taken for diagnostic purposes and were tested for intraocular antibody production against CMV, Parvovirus B19, Mumps virus and Measles virus, in addition to standard analysis for HSV, VZV and Rubella virus.[2,3] The current examinations were performed according to the tenets of the Declaration of Helsinki and according to institutional regulations. The Goldmann-Witmer coefficient (GWC) was determined using specific enzyme-linked immunosorbent assay (ELISA) kits (Parvovirus B19 IgG: Biotrin, France; Mumps, Measles and CMV IgG; Enzygnost® Dade Behring, Germany) as previously described.[2] Intraocular antibody production was considered positive when the GWC exceeded 3. The real-time polymerase chain reaction (PCR) analyses have been described previously for HSV, VZV and CMV and were performed similarly for Parvovirus B19.[3] All patients had previously been subjected to extensive uveitis screening, which included erythrocyte sedimentation rate, red and white blood cell counts, glucose levels, determination of serum angiotensin-converting enzyme levels, syphilis serology, HLA-B27 typing and chest radiography. The results of this diagnostic work-up were within the normal limits for all. The control groups consisted of 13 non-uveitis patients who underwent an ocular surgical procedure and of 13 patients with AU and intraocular fluid positive for HSV or VZV by PCR and/or GWC.

While all the non-inflammatory controls were negative, three patients with unexplained AU showed intraocular antibody production against CMV (n = 2) or Parvovirus B19 (n = 1). In addition, one patient in the herpetic control group with a

Table 1. Intraocular fluid analysis by PCR and by Goldmann-Witmer coefficient determination for Cytomegalovirus, Parvovirus B19, Mumps and Measles virus in patients with anterior uveitis of unknown origin.

				Performed test						
				PCR			GWC			
				Positive patients/ number tested			Positive patients/ number tested			
Group	**N**	**Mean age (range) in years**	**MFR**	**CMV**	**PV**	**RV**	**CMV**	**PV**	**MuV**	**MeV**
Anterior uveitis of unknown origin	27	42.2 (19 – 69)	13/ 14	0/ 27	0/ 1	0/ 27	2/ 27	1/ 25	0/ 25	0/ 24
Herpetic anterior uveitis	13	43.8 (6 – 73)	8/ 5	np	np	np	0/ 13	1[a]/ 13	np	np
Non-inflammatory control	13	61.7 (40 – 79)	10/ 3	np	np	np	0/ 13	0/ 13	np	np

CMV = Cytomegalovirus; GWC = Goldmann-Witmer coefficient; MFR = male/female ratio; MeV = Measles virus; MuV = Mumps virus; np = not performed; PV = Parvovirus B19; PCR = polymerase chain reaction; RV = Rubella virus.

[a] This patient was initially classified as anterior uveitis due to VZV based on a positive GWC for VZV of 6.2, but who was in our study found to have also a positive GWC of 7.2 for Parvovirus B19. In a sample taken one year later, the GWC for Parvovirus B19 remained positive at 5.6, however her GWC for VZV had dropped below 3.

Table 2. Clinical characteristics at time of sampling of the patients with intraocular antibody production against Cytomegalovirus or Parvovirus B19.

Patient	Gender	GWC	Age at onset in years	Affected eye	Duration of uveitis	Visual acuity	IOP mmHg	Clinical characteristics
1	F	CMV = 3.2	35	LE	3 months	0.4	38	Corneal edema and keratic precipitates AC cells ++ Iris: no sector atrophy; no posterior synechiae
2	M	CMV = 14.9	28	RE	8 years	1.0	45	No keratic precipitates cornea AC cells + Iris: no sector atrophy; no posterior synechiae
3	M	PV = 21.3	56	RE + LE	1 year	1.0 / 1.0	normal	Keratic precipitates cornea AC cells + Iris nodules; no posterior synechiae Vitritis
4	F	PV = 7.2 VZV = 6.2	8	RE + LE	5 years	1.0 / 1.0	normal	No keratic precipitates cornea AC cells + Iris: posterior synechiae; no sectoral atrophy

GWC = Goldmann-Witmer coefficient; F = female; M = male; RE = right eye, LE = left eye; IOP = intraocular pressure; AC = anterior chamber; PV = Parvovirus B19

VZV AU also had a positive GWC for Parvovirus B19 (Table 1). None of the patients showed intraocular antibody production against Mumps or Measles virus.

Both patients with a positive GWC for CMV showed recurrent attacks of mild unilateral AU with elevated intraocular pressure, which rapidly decreased, suggestive of Posner-Schlossman syndrome (PSS) (Table 2). Recently, PSS was associated with CMV infection by detection of CMV DNA in the ocular fluids of patients with AU.[1,4]

The patient with intraocular antibody production against Parvovirus B19 (patient 3) suffered from chronic AU (Table 2). Parvovirus B19 antibodies have been detected in intraocular fluid in patients with uveitis, however, intraocular antibody production against Parvovirus B19 has never been demonstrated.[5] The remaining patient (no 4) with GWC positive for Parvovirus B19 and VZV presented with a unilateral AU, later becoming bilateral, complicated by secondary glaucoma in both eyes. The double positive GWC might be explained by a double infection as the VZV GWC became negative after acyclovir treatment.

In conclusion, we found active intraocular antibody production against CMV and Parvovirus B19 in 4/27 patients with AU, but no evidence of Mumps virus and Measles virus infection. In view of recent reports on CMV and AU, we recommend AH analysis for CMV and also to consider testing for Parvovirus B19 in patients with unexplained AU.

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Chapter 9

Searching for intraocular antibody production against Parvovirus B19, Mumps virus and Measles virus in patients with intermediate and posterior uveitis

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Abstract

As the main infectious causes of uveitis we know the constituents of the TORCH group of agents: *Toxoplasma gondii*, 'Others' (Varicella zoster virus), Rubella virus, Cytomegalovirus and Herpes simplex virus. These pathogens are the most frequent causes of congenital and childhood infections. This raises the question if other causative agents of childhood infections are involved in the pathogenesis of uveitis, such as Mumps virus, Measles virus and Parvovirus B19. Paired aqueous humor and serum samples of 15 patients with intermediate uveitis, of 14 patients with neuroretinitis, and of 17 patients with focal chorioretinitis of non-toxoplasmic origin, were tested for intraocular antibody production against Parvovirus B19, Mumps virus and Measles virus by Goldmann-Witmer coefficient determination. All patients showed negative results, of which we concluded that Parvovirus B19, Mumps virus and Measles virus are probably not involved in the pathogenesis of these uveitis entities.

Uveitis is a destructive ocular inflammation and is caused by either infectious agents or non-infectious immune reactions. The etiology is still unknown in about 50% of the patients. The distinction between an infectious and non-infectious etiology is crucial for treatment and prognosis. The main infectious agents in the West include *Toxoplasma gondii*, Herpes simplex virus (HSV), Varicella zoster virus (VZV), Cytomegalovirus (CMV) and Rubella virus, pathogens which are also the most frequent causes of congenital and childhood infections. Several case reports have mentioned uveitis following Parvovirus, Mumps or Measles infection.[1-3] Despite the MMR-vaccination programme, Mumps and Measles outbreaks continue to occur.[4] We hypothesised that other common viral childhood infections might also be able to incite uveitis and selected Parvovirus B19, Mumps virus and Measles virus as the most likely candidates. We included specific classified uveitis entities occurring at a relatively young age: intermediate uveitis, neuroretinitis and focal chorioretinitis of non-toxoplasmic origin. The classification of uveitis was based on clinical characteristics, according to the Standardization of Uveitis Nomenclature Working Group.[5] Immunocompromised patients and patients with known causes of uveitis were excluded.

Paired aqueous humor (AH) and serum samples from patients with uveitis, which were taken for diagnostic purposes, were also tested for intraocular antibody production against Parvovirus, Mumps virus and Measles virus by determination of the Goldmann-Witmer coefficient (GWC). The current examinations were performed according to the tenets of the Declaration of Helsinki and according to institutional regulations.

Specific immunoglobin G (IgG) antibodies against Parvovirus B19, Mumps virus and Measles virus were determined using specific enzyme-linked immunosorbent assay (ELISA) kits (Parvovirus B19 IgG: Biotrin, France; Mumps and Measles IgG: Enzygnost® Dade Behring, Germany) according to the instructions of the manufacturer, and titers were calculated using the Mikrowin software version 3.0 (Mikrotek Laborsysteme, Overath, Germany). Total IgG titers in serum and AH were determined by an in-house ELISA, which has previously been described.[6] Intraocular antibody production was considered positive when the GWC exceeded 3. As samples are generally collected at later stages of uveitis, we chose to detect viral infections by GWC determination and not polymerase chain reaction (PCR). All patients underwent uveitis screening, which included

Table 1. Intraocular antibody production analysis for Parvovirus B19, Mumps virus and Measles virus in patients with intermediate uveitis, neuroretinitis and focal chorioretinitis.

				Performed test												
				PCR					GWC							
				Positive patients/ number tested					Positive patients/ number tested							
Group	**N**	**Mean age (range) in years**	**MFR**	**CMV**	**HSV**	**VZV**	**TP**	**EBV**	**CMV**	**HSV**	**VZV**	**TP**	**RV**	**PV**	**MuV**	**MeV**
Intermediate uveitis	15	25.5 (4 – 54)	9 / 6	0/6	0/10	0/10	0/7	0/1	0/6	0/10	0/10	0/7	0/1	0/15	0/14	0/15
Neuroretinitis	14	39.6 (5 – 71)	6 / 8	0/7	0/14	0/14	np	0/2	0/6	0/14	0/14	0/8	0/3	0/14	0/10	0/10
Focal chorioretinitis	17	27.9 (10 – 45)	10 / 7	0/4	0/9	0/9	0/17	np	0/4	0/9	0/8	0/17	0/1	1/17	0/12	1/12

CMV = Cytomegalovirus; EBV = Epstein-Barr virus; GWC = Goldmann-Witmer coefficient; HSV = Herpes simplex virus; MeV = Measles virus; MFR = male/ female ratio; MuV = Mumps virus; np = not performed; PCR = polymerase chain reaction; PV = Parvovirus; RV = Rubella virus; TP = *T.gondii*; VZV = Varicella zoster virus

erythrocyte sedimentation rate, red and white blood cell counts, glucose levels, determination of serum angiotensin-converting enzyme levels, serological tests for syphilis and borreliosis, HLA-B27 typing and chest radiography. The results of this diagnostic work-up were within normal limits for all included patients. In addition, all patients with neuroretinitis were serologically negative for *Bartonella henselae*. Common infectious causes of uveitis were excluded by both PCR and GWC analyses in aqueous fluid (Table 1). None of the patients used systemic antibiotics and/or antiviral drugs at time of sampling.

Specific serum IgG antibodies against Parvovirus B19 were present in 61% of the patients (28/46), against Mumps virus in 75% of the patients (27/36) and against Measles virus in 95% of the patients (35/37). None of the patients with intermediate uveitis or neuroretinitis showed a GWC>3 for Parvovirus, Mumps virus or Measles virus (Table 1). One patient with focal chorioretinitis showed a double borderline GWC (4.51 for Parvovirus B19 and 3.34 for Measles virus). Since, in this sample, the total IgG in the AH was extremely elevated (0.86 mg/ml), we attributed these marginal coefficients to the massive leakage of antibodies from the circulation into the eye. None of the patients with focal chorioretinitis showed a GWC>3 for Mumps virus.

Although the majority of the patients showed serological evidence of a previous infection or vaccination with Parvovirus B19, Mumps virus and Measles virus, none of the patients showed active intraocular antibody production. In conclusion, we found no laboratory evidence that Parvovirus B19, Mumps virus and Measles virus are involved in the pathogenesis of intermediate uveitis, neuroretinitis and focal chorioretinitis.

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Chapter 10

Cytokine and chemokine profiling in ocular fluids of patients with infectious uveitis

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Abstract

Purpose: To investigate which cytokines and chemokines are involved in the immunopathogenesis of Rubella virus-associated Fuchs heterochromic uveitis syndrome (RV-FHUS), ocular toxoplasmosis (OT) and acute retinal necrosis (ARN) and which immunological pathways play a role in these uveitis entities.

Methods: Simultaneously taken serum and aqueous humor (AH) samples of 18 patients with RV-FHUS, of 20 patients with OT, and of 19 with ARN were analyzed by multiplex immunoassay. All infections were confirmed by intraocular fluid analyses. Controls consisted of 11 paired AH and serum samples of patients with age-related cataract and three patients with non-infectious quiescent uveitis. In each sample 15 cytokines, five chemokines and two adhesion molecules were detected. Various clinical characteristics were assessed, including medication with corticosteroids and time-interval between the onset of uveitis and moment of sampling.

Results: Intraocular production was established for at least 20 of the 22 mediators as their AH levels were higher than the serum. RV-FHUS and OT revealed a similar pattern of mediator production which was distinct from ARN. ARN samples had overall higher cytokine levels than RV-FHUS and OT. IL-12 levels were higher in RV-FHUS and OT than in ARN ($P = .013$ and $P = .001$) and controls ($P = .05$ and $P = .015$). IL-10 and IL-18 levels were higher in ARN compared to RV-FHUS OT and controls ($P = .000$ for all). IFNγ levels were elevated in ARN samples. The treatment with corticosteroids and the time interval between the onset of symptoms and the sampling had no effect on cytokine levels assessed.

Discussion: RV-FHUS and OT expressed a similar pattern of immune mediators, different from ARN. The higher levels of cytokines and chemokines in ARN might correlate with higher clinical disease activity and severity. Explicit T helper (Th) pathways characteristic for a specific uveitis entity were not identified.

Introduction

The eye is an immune privileged organ. When a local immune response is required, anterior chamber-associated immune deviation (ACAID) results in a response which is characterized by suppression of pro-inflammatory cytokines and down-regulation of $CD4^+$ helper T lymphocytes, $CD8^+$ cytotoxic T lymphocytes and complement fixation antibodies. The high levels of TGFβ, anti-inflammatory cytokines in the eye and antigen presenting cells (APCs) with a tolerance-promoting phenotype and function contribute to the ACAID. In addition, the eye has no lymphatic system and clearance of cellular and molecular debris is therefore performed by aqueous outflow and endocytosis. When physiological mechanisms of tolerance and immunosuppression in the eye fail, uveitis can develop.[1-5]

Uveitis can be caused by a variety of microorganisms, including viruses, bacteria, and parasites.[6] During an intraocular infection, various cytokines, chemokines, soluble adhesion molecules and macrophage-derived factors are produced which regulate the immune response and determine the outcome of the infection. These mediators influence the communication between various cell types and can alter the properties of the vascular endothelium.[7] Overexpression or imbalance of cytokines and chemokines can cause inflammatory damage to ocular structures leading to severe visual impairment.[6,8] During uveitis, mainly T lymphocytes are present in the ocular fluid, including $CD4^+$ T-helper and $CD8^+$ T lymphocytes.[9,10] These lymphocytes, and to a lesser degree monocytes, macrophages and retinal pigment epithelium, are the cellular source of intraocular cytokines and chemokines.[4,5,11,12]

T-helper lymphocytes are currently divided into three major subsets based on the pattern of cytokines secreted by these cells: Th1, Th2 and recently identified Th17 cells.[13] Th17 cells are involved in cell-mediated autoimmune inflammatory diseases and play a dominant role in some types of experimental autoimmune uveitis (EAU).[13,14] Th1 cells are implicated in delayed type hypersensitivity responses in case of infection by intracellular pathogens and secrete IL-2 and interferon-γ (IFNγ). Th2 cells are involved in humoral responses including immediate type hypersensitivity in case of for example allergies and extracellular parasites, and secrete IL-4, IL-5 and IL-13.[6,15] Cytokines from Th1 cells inhibit the actions of Th2 cells and vice versa, resulting in balance between Th1 and Th2.[15] This balance is

maintained by the action of regulatory T cells (Treg) and the regulatory cytokine IL-10. Several studies have shown that levels of specific cytokines and chemokines may be increased or decreased in the aqueous humor (AH) of patients with uveitis.[6,8,10,16-18] However, only a few patients per uveitis entity were investigated in most of these studies and/or the quantity of AH available from each patient allowed analysis of only a limited number of mediators. In addition, many studies have measured these mediators only in ocular fluid and it can not be excluded that the intraocular mediators leaked into the eye from the circulation. In order to study the complex patterns of mediators involved in these inflammatory processes and their relationship with the clinical manifestation of disease, a comprehensive spectrum of immune mediators needs to be measured, in both serum and AH, in a sufficiently large cohort of patients, comparing the various infectious uveitis entities.

The aim of this study is to investigate which cytokines and chemokines and which immunological pathways are involved in the immunopathogenesis of three important types of infectious uveitis, namely Rubella virus-associated Fuchs heterochomic uveitis syndrome (RV-FHUS), ocular toxoplasmosis (OT), and acute retinal necrosis (ARN), caused by Herpes simplex virus (HSV) or Varicella zoster virus (VZV). To that end, cytokine and chemokine expression patterns in the AH and serum were determined by multiplex immunoassay (Luminex) technology, which is a very suitable assay for high-throughput analysis in microvolumes, such as ocular fluids.[16,19-21]

Materials and methods

Patients

In this study, AH and serum samples of 18 patients with RV-FHUS, 20 patients with OT and 19 patients with ARN were included. Of the 19 patients with ARN, 4 patients had a Herpes simplex virus (HSV) infection, 14 patients had a Varicella zoster virus (VZV) infection and one patient had a positive GWC and PCR for both viruses. The infectious cause of the uveitis had been determined by PCR and/or by establishing specific intraocular antibody production using the Goldmann-Witmer coefficient (GWC), at the Department of Virology at the University Medical Center in Utrecht as described previously.[22-25] All samples were

collected for diagnostic purposes and their remainders were used for the current examinations, which were performed according to the tenets of the Declaration of Helsinki and according to institutional regulations. None of the patients were immunocompromised.

The following clinical data were assessed for each patient: gender, age at the time of sampling, time interval between onset of uveitis and sample collection, (systemic) treatment at the time of sampling, and in OT patients, primary (n=5) and recurrent disease (n=12) as well as active (n=15) and non-active (n=5) disease at the time of sampling. In ARN patients the presence or absence of retinal detachment (n=9 and n=10, respectively) was assessed, as well as differences between HSV- and VZV-associated ARN.

The control group consisted of seven paired AH and serum samples from patients with age-related cataract and three paired AH and serum samples from patients with noninfectious uveitis which were in a clinically quiet state at the time of sampling (one patient with juvenile idiopathic arthritis (JIA)-associated uveitis, one patient with HLA B27 ankylosing spondylitis associated uveitis and one patient with uveitis of unknown etiology).

Aqueous humor and serum samples were collected as described previously, stored at -80°C in sterile screw-cap tubes within five hours of collection and thawed directly before analysis to preserve the sample.[26]

This research followed the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board.

Multiplex immunoassay

Twenty-five microliters of AH and 50 μl of serum sample were analyzed by multiplex immunoassay essentially as described previously.[19] In each sample 22 mediators were analyzed; interleukin-1β (IL-1β), IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IL-15, IL-17, IL-18, interferon-γ (IFNγ), tumor necrosis factor-α (TNFα), soluble vascular cell adhesion molecule (sVCAM), soluble intracellular adhesion molecule (sICAM), monocyte chemotactic protein-1 (MCP-1; CCL2), macrophage inflammatory protein-1-α (MIP-1α; CCL3), Rantes (regulated on activation, normal T-cell expressed and secreted; CCL5), Eotaxin (CCL11), IL-8 (CXCL8), interferon-inducible 10-kDa protein (IP-10; CXCL10) and macrophage migration inhibitory factor (MIF). Concentrations above or below the detection limit were assigned as

the highest or the lowest value from the respective standard curve (see legend Table 1). For statistical analysis, concentrations below the detection limit were converted to a value of 0.5 x the lowest value of the calibration curve.

Statistical analysis

Statistical analysis of the data was performed by using SPSS (version 15.0; SPSS Inc, Chicago, Illinois, USA). The Kruskal-Wallis and the Mann-Whitney *U* test were used for nonparametric comparison of the geometric means of the different groups. Correlations were determined by the Spearman's Rho test. *P* values of less than 0.05 were considered to be statistically significant.

Results

The cytokine, chemokine and soluble adhesion molecule concentrations in serum and AH samples of all patients are given in Table 1a and 1b. Figure 1 shows the mediator concentrations in the AH samples and points out significant results. Table 2 summarizes the results of cytokine and chemokine expression of the three uveitis entities compared to the controls.

Serum levels of all cytokine and chemokines (except for IL-8), were lower than those found in the ocular fluids, indicating local mediator production. IL-18 and Rantes were expressed in all serum samples, but were only detected in a few RV-FHUS and OT aqueous samples. In contrast, sVCAM and sICAM levels were in almost all cases higher in the serum than in the ocular fluid samples.

The ocular fluids of the patients with RV-FHUS revealed significantly higher levels of IL-12p70 (P = .05), IL-15 (P = .01) and MCP-1 (P = .005) compared to the controls (Figure 1 and Table 2). Expression of IL-12 and IL-15 was significantly correlated (r = 0.51, P = .03), but both were not correlated with MCP-1 (r = 0.21, P = .411 and r = -0.12, P = .65, respectively). Cytokines IL-6 and IL-13 and chemokines IL-8, IP-10, MIF, MIP-1α, Eotaxin, sICAM and sVCAM were elevated compared to the controls. TNFα was detected in the ocular fluids of 6/18 RV-FHUS patients, IL-2 was detected in 2/18, and IL-5, IL-10 and Rantes each in one patient. IL-1β, IL-4, IL-17, IL-18 and IFNγ were not detected in any of the AH samples.

In the patients with OT, similar to those with RV-FHUS, intraocular expression of IL-12p70 (P = .015), IL-15 (P = .000) and MCP-1 (P = .039) were significantly higher

compared to the controls. Like in RV-FHUS patients, IL-12 and IL-15 were correlated (r = 0.59, P = .006). Both IL-12 and IL-15 were not correlated with MCP-1 (r = 0.11, P = .638 and r = 0.04, P = .855, respectively). IL-6, IL-13, IL-8, IP-10, MIF, MIP-1α, Eotaxin, sICAM and sVCAM expression was elevated. TNFα was detected in the AH of 8/20 patients, IL-10 in 5 and IFNγ, IL-18 and Rantes each in 4/20 patients. IL-4 was found in 2/20 samples, IL-1β, IL-5 and IL-17 each were expressed in 1/20 AH, whereas IL-2 was not detected in the AH samples. IP-10 (P = .0131), MCP-1 (P = .0114). Intraocular sICAM (P = .0114), sVCAM (P = .0068), MCP-1 (P = .011) and IP-10 (P = .013) were significantly elevated in active OT compared to nonactive OT. No differences in immune mediator levels between primary and recurrent OT were found. The mediator profiles in ocular fluids of RV-FHUS and OT patients were highly similar and no significant differences were found in expression of all mediators between these two uveitis entities. Th1 as well as Th2 cytokines were detected in the ocular fluids of both entities.

In the ocular fluids of the ARN patients 13/22 cytokines and chemokines were significantly elevated compared to the controls: IL-6, IL-8, IL-10, IL-15, IL-18, IP-10, MIP-1α, MCP-1, MIF, Eotaxin, sICAM, sVCAM and Rantes (Figure 1). IFNγ and IL-13 levels were elevated compared to the controls. Similar to RV-FHUS and OT, IL-15 and MCP-1 were increased in ARN and IL-2 was not detected. In contrast to RV-FHUS and OT, IL-12 was detected in only one/19 AH samples. TNFα, IL-4 and IL-5 was detected in 6/19, 6/19 and 5/19 ocular fluids of ARN patients, respectively. IL-1β was found in 4/19 AHs and IL-17 in one. In ARN samples expression of IFNγ was correlated with expression of IL-18 (r = 0.46, P = .050), but not with that of IL-12 (r = 0.42, P = .074). IFNγ expression was also highly correlated with that of TNFα (r = 0.71, P = .001). No differences in mediator expression were observed between patients with and without retinal detachment or between HSV- and VZV-associated ARN.

The ocular fluids of ARN patients revealed a distinctly different profile compared to the other uveitis entities: IL-6, IL-8, IL-10, IL-18, MIF, MCP-1, Eotaxin, IP-10, sICAM, sVCAM and Rantes levels were significantly increased in ARN compared to RV-FHUS and OT (Figure 1). IL-10 and IL-18 were widely expressed in ARN samples, in contrast to OT and RV-FHUS samples in which both mediators were detected in only a few patients (Figure 1). Also, ARN samples contained significantly more IFNγ than RV-FHUS samples (P = .013) and the levels were

Table 1a. Cytokine, chemokine, adhesion molecule and macrophage factor levels in serum samples of patients and controls.

Mediator	Mediator concentrations in sera				
	RV-FHUS	**Ocular toxoplasmosis**	**Acute retinal necrosis**	**Control patients**	***P*-value**
	(n = 18)	**(n = 20)**	**(n = 19)**	**(n = 10)**	
	Mean[a]	Mean[a]	Mean[a]	Mean[a]	
	Range[b]	Range[b]	Range[b]	Range[b]	
	No.[c]	No.[c]	No.[c]	No.[c]	
IL-1β	< 1	< 1	< 1	1	0,007
	1 - 1	1 - 1	1 - 14	1 - 5	
	18	20	18	7	
IL-2	2	2	3	3	0,007
	2 - 2	2 - 2	2 - 118	2 - 16	
	18	20	18	7	
IL-4	< 1	< 1	< 1	< 1	0,007
	1 - 1	1 - 1	1 - 38	1 - 4	
	18	20	18	7	
IL-5	1	2	2	1	0,693
	1 - 1	1 - 135	1 - 902	1 - 1	
	18	18	17	7	
IL-6	3	3	5	3	0,345
	2 - 41	2 - 23	2 - 3737	2 - 9	
	17	18	15	7	
IL-10	< 1	< 1	< 1	1	0,007
	1 - 1	1 - 1	1 - 79	1 - 21	
	18	20	18	7	
IL-12p70	< 1	< 1	1	2	0,007
	1 - 1	1 - 1	1 - 79	1 - 40	
	18	20	18	7	
IL-13	< 1	1	1	1	0,039
	1 - 1	1 - 28	1 - 134	1 - 10	
	18	19	18	7	
IL-15	1	2	1	3	0,007
	1 - 1	1 - 39	1 - 93	1 - 24	
	18	17	17	5	
IL-17	1	2	2	5	0,514
	1 - 14	1 - 57	1 - 71	1 - 280	
	14	14	14	6	
IL-18	24	27	26	18	0,361
	13 - 64	8 - 69	6 - 316	3 - 121	
	0	0	0	0	
IFN-γ	< 1	< 1	< 1	3	0,005
	1 - 1	1 - 1	1 - 180	1 - 290	
	18	20	18	7	
TNF-α	1	1	1	4	0,005
	1 - 1	1 - 1	1 - 31	1 - 77	
	18	20	18	7	

Mediator	Mediator concentrations in sera				
	RV-FHUS	**Ocular toxoplasmosis**	**Acute retinal necrosis**	**Control patients**	***P*-value**
	(n = 18)	**(n = 20)**	**(n = 19)**	**(n = 10)**	
	Mean[a]	Mean[a]	Mean[a]	Mean[a]	
	Range[b]	Range[b]	Range[b]	Range[b]	
	No.[c]	No.[c]	No.[c]	No.[c]	
sICAM	53183	82689	86360	79325	0,321
	29 -> 86985	31590 -> 86985	75860 -> 86985	48848 -> 86985	
	1	0	0	0	
sVCAM	62549	100216	110562	112481	0,214
	41 -> 112820	10555 -> 112820	78474 -> 112820	110472 -> 112820	
	1	0	0	0	
RANTES	1704	5541	4737	4720	0,007
	3 -> 5541	5541 -> 5541	1486 -> 5541	3805 -> 5541	
	2	0	0	0	
Eotaxin	6	43	33	37	0,146
	1 - 115	3 - 217	1 - 367	8 - 270	
	8	0	1	0	
IL-8	12	5	58	5	0,005
	3 - 102	1 - 827	1 - 2806	2 - 75	
	0	20	2	0	
IP-10	6	18	20	62	0,029
	1 - 186	1 - 73	1 - 222	1 - 4854	
	10	1	1	0	
MIP1-α	15	14	33	20	0,071
	10 - 180	10 - 149	10 - 4682	10 - 208	
	15	17	11	5	
MCP-1	72	76	97	46	0,118
	20 -212	21 - 215	37 - 312	11 - 131	
	0	0	0	0	
MIF	136	62	178	53	0,141
	14 - 2243	13 - 1814	14 - 5733	14 - 505	
	5	10	4	3	

RV-FHUS, Rubella virus-associated Fuchs heterochromic uveitis syndrome; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; sICAM, soluble intercellular adhesion molecule; sVCAM, soluble vascular cell adhesion molecule; RANTES, regulated on activation normal T-cell expressed and secreted; IP, interferon-inducible protein; MIP, macrophage inflammatory protein; MCP, monocyte chemoattractant protein; MIF, macrophage migration inhibitory factor. Sensitivity of the assay is 1.5 pg/ml for IL-1β, 1.8 for IL-2, 1.2 for IL-4, 1.2 for IL-5, 2.4 for IL-6, 2.3 for IL-10, 4.3 for IL-12p70, 1.0 for IL-13, 1.4 for IL-15, 1.1 for IL-17, 1.2 for IL-18, 9.1 for IFN-g, for RANTES (CCL5) 1.5, for Eotaxin (CCL11) 1.3, for IL-8 (CXCL8) 5.3, for IP-10 (CXCL10) 1.0, 1.2 for TNF-a, and 4.7 for MIF. Sensitivity for MCP1 (CCL2) is 1.2 pg/ml, for MIP1-a (CCL3) 9.3, for sVCAM (CD106) 22.3, and for sICAM (CD54) 26.4 (sensitivity data from de Jager et al., 2005).

P value calculated using the Kruskal-Wallis test.

[a] Geometric mean concentration (pg/ml).

[b] Range of detectable measured samples (pg/ml).

[c] Number of samples in the undetectable range.

Table 1b. Cytokine, chemokine, adhesion molecule and macrophage factor levels in aqueous humor samples of patients and controls.

Mediator	Mediator concentrations in aqueous humor				
	RV-FHUS	**Ocular toxoplasmosis**	**Acute retinal necrosis**	**Control patients**	***P*-value**
	(n = 18)	**(n = 20)**	**(n = 19)**	**(n = 10)**	
	Mean[a]	Mean[a]	Mean[a]	Mean[a]	
	Range[b]	Range[b]	Range[b]	Range[b]	
	No.[c]	No.[c]	No.[c]	No.[c]	
IL-1β	< 1	< 1	1	< 1	0,053
	1 - 1	1 - 6	1 - 17	1 - 1	
	18	20	15	10	
IL-2	3	2	2	<1	0,137
	2 - 71	2 - 2	2 - 2	1 - 2	
	16	20	19	3	
IL-4	< 1	< 1	2	< 1	0,012
	1 - 1	1 - 13	1 - 35	1 - 1	
	18	18	13	10	
IL-5	2	2	4	< 1	0,054
	1 - 36	1 - 50	1 - 75	1 - 1	
	17	19	14	10	
IL-6	27	32	3246	8	0,000
	2 - 9299	2 -> 10016	169 -> 10016	2 - 3191	
	7	6	0	6	
IL-10	< 1	1	51	1	0,000
	1 - 4	1 - 21	1 - 559	1 - 6	
	17	15	1	7	
IL-12p70	7	6	2	2	0,001
	2 - 68	2 - 81	1 - 11	2 - 5	
	9	7	18	7	
IL-13	1	1	1	< 1	0,825
	1 - 22	1 - 28	1 - 50	1 - 3	
	11	14	13	7	
IL-15	25	45	26	7	0,038
	1 - 171	12 - 112	1 - 94	1 - 50	
	3	0	3	3	
IL-17	2	2	2	2	0,036
	2 - 2	1 - 9	2 - 20	1 - 10	
	18	19	18	7	
IL-18	1	2	28	2	0,000
	1 - 1	1 - 110	1 - 155	1 - 9	
	18	16	2	6	
IFN-γ	1	3	15	2	0,004
	1 - 1	1 - 294	1 - 1246	1 - 14	
	18	16	10	7	
TNF-α	2	2	2	1	0,995
	1 - 30	1 - 22	1 - 61	1 - 27	
	12	12	13	7	
sICAM	1458	2038	34668	1175	0,000
	161 - 23999	254 -> 81387	4750 -> 81387	14 - 51603	
	0	0	0	1	

Mediator	Mediator concentrations in aqueous humor				
	RV-FHUS	**Ocular toxoplasmosis**	**Acute retinal necrosis**	**Control patients**	***P*-value**
	(n = 18)	**(n = 20)**	**(n = 19)**	**(n = 10)**	
	Mean[a]	Mean[a]	Mean[a]	Mean[a]	
	Range[b]	Range[b]	Range[b]	Range[b]	
	No.[c]	No.[c]	No.[c]	No.[c]	
sVCAM	3041	2579	36846	1067	0,000
	248 - 28986	25 -> 95705	6169 -> 95705	25 - 47451	
	0	1	0	2	
RANTES	6	9	47	6	0,001
	5 - 433	5 - 262	5 - 634	5 - 138	
	17	16	7	8	
Eotaxin	4	5	332	1	0,000
	1 - 329	1 - 1219	9 - 3975	1 - 9	
	10	10	0	7	
IL-8	10	9	784	3	0,000
	1 - 64	1 - 879	24 -> 2483	1 - 51	
	6	8	0	5	
IP-10	103	169	2102	40	0,000
	1 -> 2468	1 -> 2468	228 -> 2468	1 - 1598	
	5	3	0	4	
MIP1-α	15	14	46	3	0,013
	9 - 288	9 - 414	9 - 805	1 - 9	
	15	17	10	3	
MCP1	625	420	2738	174	0,000
	27 - 3919	72 - 3120	488 -> 4513	22 - 486	
	0	0	0	0	
MIF	90	80	1967	58	0,000
	18 - 1962	18 - 1089	240 -> 10000	66 - 450	
	8	9	0	4	

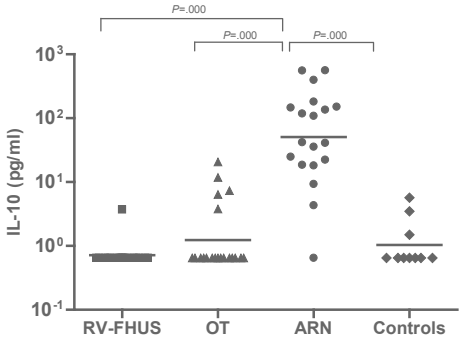
RV-FHUS, Rubella virus-associated Fuchs heterochromic uveitis syndrome; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; sICAM, soluble intercellular adhesion molecule; sVCAM, soluble vascular cell adhesion molecule; RANTES, regulated on activation normal T-cell expressed and secreted; IP, interferon-inducible protein; MIP, macrophage inflammatory protein; MCP, monocyte chemoattractant protein; MIF, macrophage migration inhibitory factor. Sensitivity of the assay is 1.5 pg/ml for IL-1β, 1.8 for IL-2, 1.2 for IL-4, 1.2 for IL-5, 2.4 for IL-6, 2.3 for IL-10, 4.3 for IL-12p70, 1.0 for IL-13, 1.4 for IL-15, 1.1 for IL-17, 1.2 for IL-18, 9.1 for IFN-g, for RANTES (CCL5) 1.5, for Eotaxin (CCL11) 1.3, for IL-8 (CXCL8) 5.3, for IP-10 (CXCL10) 1.0, 1.2 for TNF-a, and 4.7 for MIF. Sensitivity for MCP1 (CCL2) is 1.2 pg/ml, for MIP1-a (CCL3) 9.3, for sVCAM (CD106) 22.3, and for sICAM (CD54) 26.4 (sensitivity data from de Jager et al., 2005).

P value calculated using the Kruskal-Wallis test.

[a] Geometric mean concentration (pg/ml).

[b] Range of detectable measured samples (pg/ml).

[c] Number of samples in the undetectable range.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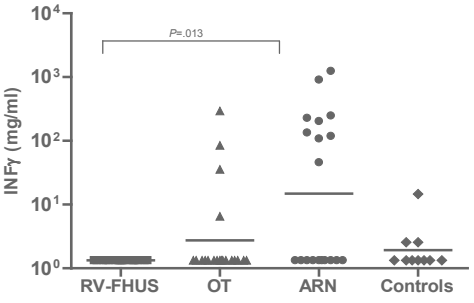
IL-10 (pg/ml)
RV-FHUS
OT
ARN
Controls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INFγ (mg/ml)
RV-FHUS
OT
ARN
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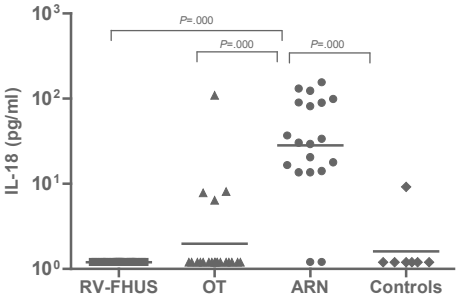
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RV-FHUS
OT
ARN
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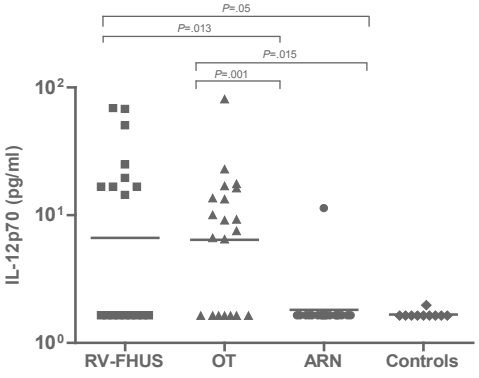
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RV-FHUS
OT
ARN
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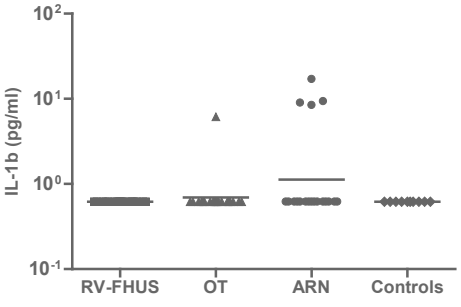
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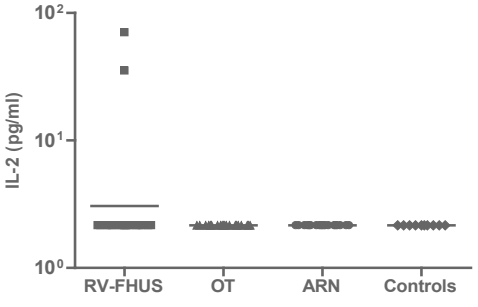
RV-FHUS
OT
ARN
Controls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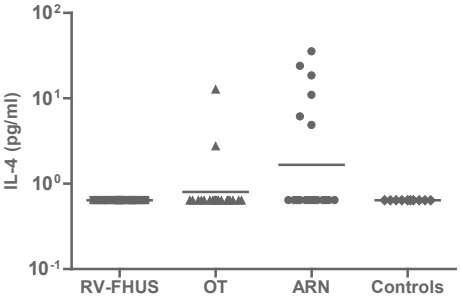
RV-FHUS
OT
ARN
Controls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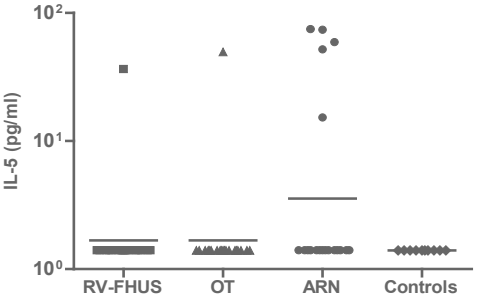
10^1
10^0
10^{-1}
RV-FHUS
OT
ARN
Controls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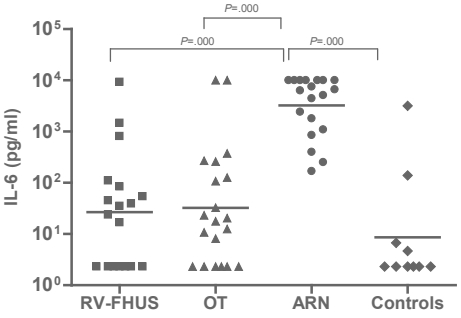
RV-FHUS
OT
ARN
Controls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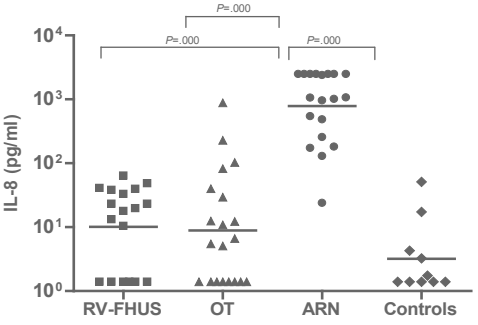
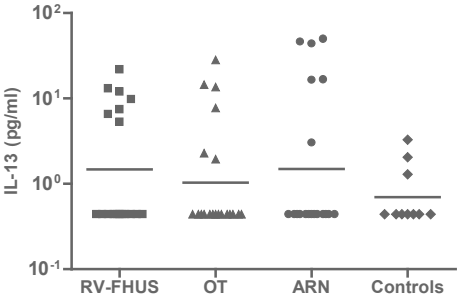
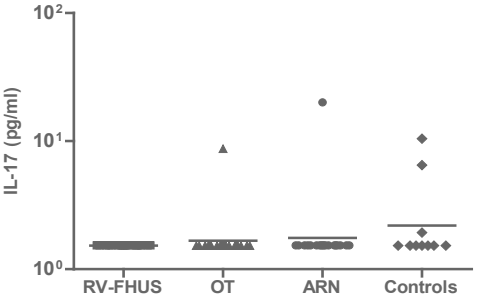
RV-FHUS
OT
ARN
Controls


IL-8 (pg/ml)
P=.000
P=.000
P=.000
10^4
10^3
10^2
10^1
10^0
RV-FHUS
OT
ARN
Controls


IL-13 (pg/ml)
10^2
10^1
10^0
10^{-1}
RV-FHUS
OT
ARN
Controls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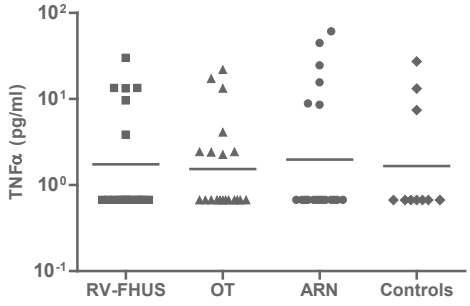
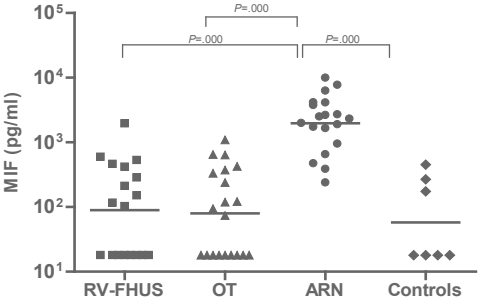
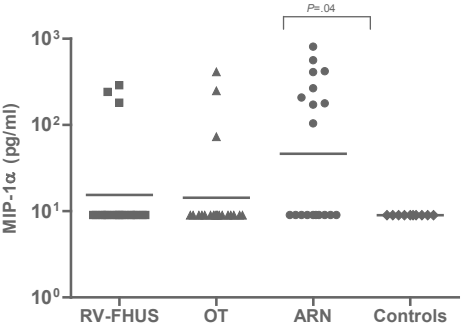
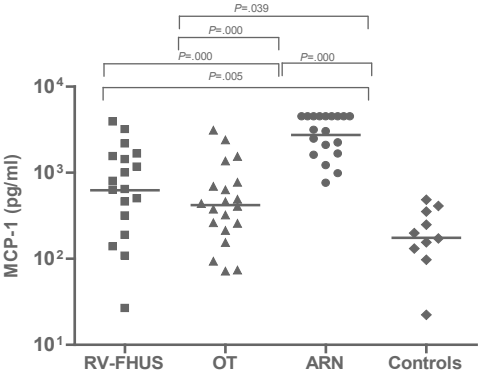
10^1
10^0
RV-FHUS
OT
ARN
Controls


TNFα (pg/ml)
RV-FHUS
OT
ARN
Controls


MIF (pg/ml)
P=.000
P=.000
P=.000
RV-FHUS
OT
ARN
Controls


MIP-1α (pg/ml)
P=.04
RV-FHUS
OT
ARN
Controls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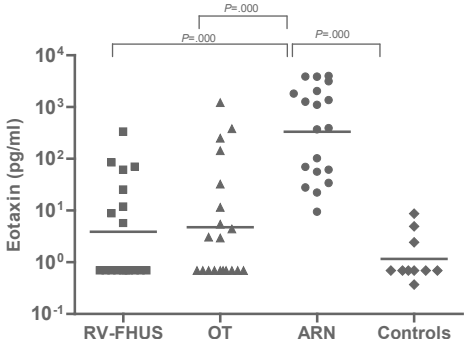
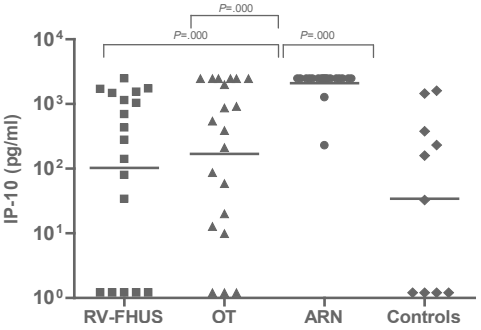
P=.039
P=.000
P=.000
P=.000
P=.005
RV-FHUS
OT
ARN
Controls


Eotaxin (pg/ml)
P=.000
P=.000
P=.000
RV-FHUS
OT
ARN
Controls


IP-10 (pg/ml)
P=.000
P=.000
P=.000
RV-FHUS
OT
ARN
Controls

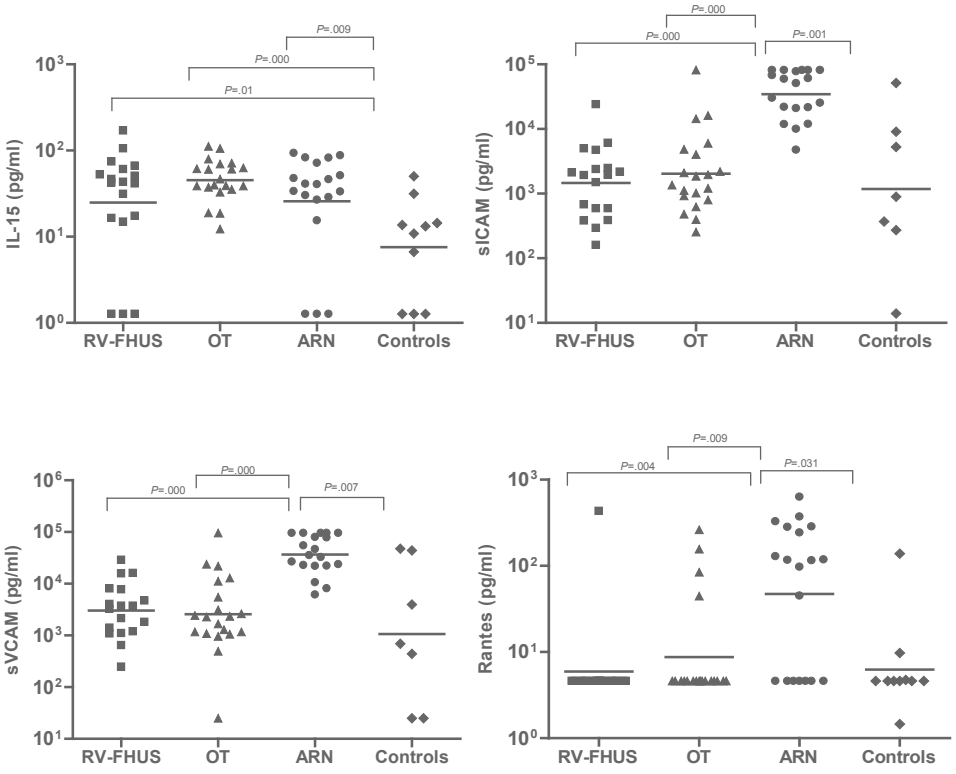


Figure 1. Cytokine and chemokine expression in the aqueous humor of patients with Rubella virus-associated Fuchs uveitis syndrome (RV-FHUS), ocular toxoplasmosis (OT), acute retinal necrosis (ARN) and of controls detected by multiplex immunoassay. On the y-axis the concentration of the immune mediator is indicated (pg/ml). Significant differences between groups, as determined by using the Mann-Whitney *U*-test, are indicated by brackets and *P* values above the each plot. A *P* value of 0.05 was considered significant.

Table 2. Cytokine and chemokine levels in the ocular fluids of patients with Rubella virus-associated Fuchs heterochromic uveitis syndrome, Ocular toxoplasmosis and Aute retinal retinal necrosis compared to the controls.

		RV-FHUS	OT	ARN
Th1	IL-2	—	—	—
	IL-12	⬆	⬆	—
	IFNγ	—	+/−	↑
	IL-18	—	+/−	⬆*
	IP-10	↑	↑	⬆
	TNFα	+/−	+/−	+/−
Th2	IL-4	—	—	↑
	IL-5	—	—	+/−
	IL-13	↑	↑	↑
	IL-10	—	+/−	⬆
Other	IL-1β	—	—	+/−
	IL-6	↑	↑	⬆
	IL-8	↑*	↑*	⬆
	IL-15	⬆	⬆	⬆
	IL-17	—	—	—
	MCP-1	⬆	⬆	⬆
	MIF	↑	↑	⬆
	Eotaxin	↑	↑	⬆
	MIP-1α	+/−	+/−	⬆
	sICAM	↑	↑	⬆
	sVCAM	↑	↑	⬆
	Rantes	—	+/−	⬆

⬆ significant
↑ not significant, at least 6 more positive patients than controls
+/− between 2 and 5 more positive patients than controls
— less than 2 positive patients detected
* possibly leakage

RV-FHUS = Rubella virus-associated Fuchs heterochromic uveitis syndrome;
OT = Ocular toxoplasmosis; ARN = Acute retinal necrosis

elevated compared to OT. IL-12 was significantly elevated in OT (P = .001) and RV-FHUS (P = .013) samples compared to ARN samples, in which virtually no IL-12 was detected (Figure 1).

No correlation was found between the use of topical and/or systemic steroids and immune mediator expression in all three types of uveitis. The analysis of the interval between the onset of symptoms and time of sampling did not reveal a correlation with the level and extend of cytokine and chemokine production for all three uveitis entities.

Discussion

In this study, AH samples of RV-FHUS and OT patients revealed a similar pattern of cytokine and chemokine production distinct from that of ARN. ARN AH samples contained a wider spectrum of mediators and these were produced in higher quantities than in RV-FHUS and OT. IL-6, IL-8, IL-10, IL-18, IP-10, INFγ, MIF, MCP-1, sVCAM, sICAM and Rantes appeared to be more important in the immunopathogenesis of ARN than in RV-FHUS and OT. The increased production of multiple cytokines and chemokines in the AHs of the ARN patients could be a reflection of the more acute character of the disease and point toward a severely imbalanced intraocular immune status, reminiscent of the cytokine storm which occurs in other acute inflammatory diseases such as acute pancreatitis.[27] The similarities in cytokine profiles between RV-FHUS and OT are remarkable, since the causative agent of these ocular diseases is notably different: virus versus parasite. However, the clinical pattern of chronic infection with periodic recurrences is present in both disorders, although the recurrences in OT have a more acute character than those observed in RV-FHUS. An explanation for these similarities might be that when the inflammation reaches the chronic state, the cytokine profile may become similar in both ocular diseases. Cytokine patterns that are specific for either RV or *T. gondii* might then no longer be obvious.

In all three infectious uveitis entities both Th1- as well as Th2-cytokines were detected, suggesting that none of these entities were explicitly mediated by a Th1- or a Th2- dominated response. However, both infiltrating leukocytes and ocular resident cells, such as retinal pigmented epithelium and glial cells, can produce cytokines. Retinal pigmented epithelium has been reported to produce

IL-1β, IL-6, IL-8, IL-10, IL-15, IFNγ, TNFα, MCP-1 and Rantes.[12] Therefore, the results presented here most likely represent the combined mediator expression from lymphocytes and ocular resident cells, possibly in response to each other.

IL-12 expression in RV-FHUS en OT is elevated while IL-12 levels in ARN are nearly absent, which is in concordance with other publications.[15,16,28-30] IL-12p70, a dimer composed of a p40 and a p35 unit, is a potent inducer of IFNγ. It is produced by many different cell types, like monocytes, B lymphocytes and connective tissue-type mast cells, but particularly by macrophages, and is able to polarize toward Th1-type cell responses. Published data on IL-12 in AH of patients with FHUS are controversial. Curnow et al. found IL-12 in only one of five FHUS patients, whereas Muhaya et al. found IL-12 in 9 of 10 FHUS patients.[10,16] These discrepancies might be explained by a defined Rubella virus-associated FHUS population in our series, whereas the causative agent of FHUS was not determined in the other studies. IL-12 was significantly elevated in OT and the expression levels were higher than in RV-FHUS. This might be explained by the property of *Toxoplasma gondii* to directly stimulate macrophages to produce IL-12.[31] Severe OT has been associated with an increase of Th1 cytokines, like IL-12.[32] Lahmar et al. found IL-12 to be specific for ocular toxoplasmosis, although RV-FHUS was not included in this study.[30] Furthermore, IL-12 has been identified as an important cytokine for the regulation of protective immune responses to *T. gondii.*[33] IL-18 is an IFNγ-inducing factor proinflammatory cytokine, which is predominantly produced by macrophages. It induces IFNγ and TNFα, and enhances NK cell activity. The ARN samples are clearly distinct from the RV-FHUS and OT samples in IL-18 levels, which were significantly elevated compared to RV-FHUS, OT and the controls. In none of the RV-FHUS and only four OT samples IL-18 was detected. So far, IL-18 was only reported in the aqueous of children with uveitis.[29]

IFNγ is a cytokine produced by $CD4^+$ Th1 lymphocytes, $CD8^+$ cytotoxic lymphocytes, and NK cells.[15] Its production is critical for innate and adaptive immunity against viral and intracellular bacterial infections and for tumor control.[15] IFNγ was not detected in the RV-FHUS samples. Absence of IFNγ in ocular fluids of FHUS patients was also reported by Ongkosuwito et al..[6] In contrast, Curnow et al. and Muhaya et al. detected IFNγ more than 90% of cases.[10,16] The differences in IFNγ detection may on the one hand be attributed to the timing of sampling. On the other hand, the absence of IFNγ in our AHs may be related to

Rubella virus as the causative agent. In none of the other studies the etiology of FHUS was established. FHUS may also be caused by Cytomegalovirus and it would be interesting to compare the cytokine and chemokine profiles of these two FHUS entities.[34] IFNγ was detected in 4/20 OT patients and 9/19 ARN patients, although the levels of expression were higher in the latter. According to Abe et al. IFNγ is one of the factors that play an important role in the clinical course of VZV-associated ARN. They found a positive correlation between the level of IFNγ in the vitreous and final visual acuity.[35] Also, the level of IFNγ has been shown to affect the severity of clinical signs of some viral infectious diseases.[36,37] It serves as an antiviral cytokine by inhibiting viral replication or eliminating viruses from infected cells.[38] Ongkosuwito et al. found significantly increased IFNγ levels in AH samples of ARN patients, which is consistent with our results.[6] Contrary to other studies, IFNγ was detected in only a few OT samples.[6,30]

Both IL-12 and IL-18 can induce IFNγ, however, in ARN and in OT the presence of IFNγ correlated only with IL-18 (r = 0.46, P = .05 and r = 0.66, P = .002, respectively), but not with IL-12 (r = 0.42, P = 0.074 and r = 0.17, P = .467, respectively). The observed correlation between IFNγ and IL-18 in ARN and OT and the lack of correlation between IFNγ and IL-12 in all three entities may indicate that IFNγ is predominantly under regulatory control of IL-18 rather than of IL-12. The observed correlation between IFNγ and IL-18 in ARN and OT and the lack of correlation between IFNγ and IL-12 in all three entities may indicate that IFNγ is predominantly under regulatory control of IL-18 rather than of IL-12.

TNFα is a proinflammatory cytokine involved in systemic inflammation and a member of a group of cytokines that stimulate the acute phase reaction. It enhances the IFNγ response, and upregulates adhesion molecules.[15] TNFα was detected in approximately 30% of the aqueous of the RV-FHUS, OT and ARN patients and was detected in viral uveitis, ocular toxoplasmosis and FHUS by others as well.[16,30] In ARN, but not in OT and RV-FHUS, TNFα and IFNγ expression was correlated, suggesting that TNFα is a good marker of activity of disease.

IL-10 is an immunosuppressive regulatory cytokine which was originally considered to be part of the Th2 pathway. However, IL-10 can be produced by a variety of T lymphocytes, including those of the Th1 and Th17 type, monocytes and macrophages and it enhances B lymphocytes survival and antibody production.[15] IL-10 can be produced by T lymphocytes that are induced by IL-12,

IL-27, TGFβ and IL-6, and it has been proposed that it downregulates protective immune responses to several intracellular pathogens, including *T. gondii*, in order to dampen T cell-mediated immune reactions and thereby control the extend of tissue damage.[39-43] In this study, IL-10 was not detected in patients with RV-FHUS, which is consistent with other reports on the absence of IL-10 in both serum and ocular fluids of patients with FHUS.[6,16,18] In contrast, Muhaya et al. did find IL-10 in 9/9 patients with FHUS.[10] As mentioned for IL-12 and IFNγ, these differences may be related to the etiological agent of FHUS, Rubella virus in our cases. In OT, only low levels of IL-10 were observed in 5/20 patients, 4 of which had active uveitis. Similar correlations between enhanced intraocular IL-10 production and uveitis activity were previously noted.[6,7]

Increased IL-10 production in herpes uveitis was previously reported.[6,16,30] In our study, all but one ARN patient expressed high levels of intraocular IL-10. The high IL-10 levels observed in the ocular fluids of the ARN patients seem paradoxal regarding the devastating course of the disease. Possibly, the expression levels of IL-10 are not sufficient to dampen harmful immune responses. Alternatively, other immune responses than those downregulated by IL-10 or other mechanism all together may contribute to the damage. In all three uveitis entities, IL-10 and IL-12 expression was not correlated. In fact, in ARN all patients except one expressing IL-10 did not express IL-12, which may reflect the negative feedback properties of IL-10 on IL-12 expression.[44]

In contrast to Th1 type cytokines, the Th2 cytokines, IL-4, IL-5 and IL-13, are less abundant in all three entities. IL-4 and IL-5 are produced by Th2 lymphocytes and provide optimal help for humoral immune responses, including IgG and IgE isotype switching and mucosal immunity, act as positive-feedback factors to stimulate T helper cells to differentiate into Th2 lymphocytes and inhibit the Th1-pathway. In RV-FHUS and OT virtually no IL-4 and IL-5 was detected, whereas in ARN only few patients expressed these cytokines in their ocular fluids. IL-13, on the other hand, was significantly elevated compared to the controls and was detected in all three uveitis entities. IL-13 is an anti-inflammatory cytokine and is an important mediator of allergic inflammation and disease. It is secreted mainly by $CD4^+$ Th2 lymphocytes, mast cells and NK cells, but was also reported to be expressed by airway epithelium.[15,45] Like in other reports, our data suggest that Th1 responses dominate in infectious uveitis.[11,16] One could further speculate that

expression of IL-13, in analogy with IL-15, is predominantly by ocular resident cells.

IL-6 is a generally expressed pro-inflammatory cytokine in uveitis. IL-6 can stimulate B lymphocytes and is also known to be produced by the RPE, T lymphocytes, and macrophages. Our data confirm earlier studies that reported elevated levels of IL-6 in RV-FHUS, OT and ARN.[6,16,30,46,47]

The IL-10 to IL-6 ratio has been suggested to be of value to distinguish intraocular lymphoma from uveitis.[48-53] Curnow et al. found IL-10/IL-6 ratios ranging from 0.003 to 0.21 (median 0.03) in the ocular fluids of uveitis patients and reported that IL-10 was only present in combination with high levels of IL-6, which is consistent with our data (IL-10/IL-6 range 0.000-0.28, median 0.027).[16]

IL-8 (CXCL8) and MCP-1 (CCL2) are important chemokines, attracting neutrophils, T lymphocytes and monocytes.[29,54-56] Moreover, IL-8 is angiogenic, and can induce surface expression of adhesion molecules and MCP-1 has been implied to play a role in inflammation of the posterior part of the eye.[55,57,58] *In vitro* experiments have shown that IL-8 and MCP-1 can be produced by ciliairy epithelial cells, infiltrating leucocytes and by human retinal pigment epithelial (RPE) cells in response to proinflammatory cytokines.[59,60] Our findings confirm previous findings on IL-8 and MCP-1 expression in the AH of patients with uveitis, including those with RV-FHUS and OT.[7,16,30,61,62]

In a Th1 immune response, secondary production of IP-10, a member of the chemokine family, occurs in response to IFNγ and TNFα. IP-10 is secreted by several cell types, such as monocytes, endothelial cells and fibroblasts and serves to direct mononuclear phagocytes to the site of inflammation and enhance migration of T lymphocytes from the peripheral blood into the eye.[15] IP-10 was elevated and locally produced in all three entities studied. The presence of IP-10 correlated with TNFα in the RV-FHUS patients (r = -0.511, P = .030) and with IFNγ in the OT patients (r = 0.523, P = .018). Elevated IP-10 and MCP-1 levels were observed in active OT, suggesting that IP-10 and MCP-1 correlate with clinical disease activity. This finding is consistent with the results of Abu El-Asrar et al., who found an association of IP-10, MCP-1 and IL-8 with severity and activity of uveitis.[63] The elevated levels of IP-10, IL-8 and MCP-1, MIF and Rantes in ARN confirm the previous studies on correlations between these cytokines and chemokines and uveitis activity and severity.[7,61,63-66]

IL-15 is a cytokine secreted by mononuclear phagocytes and constitutively produced by RPE and by monocytes following viral infection.[12,67] This cytokine induces proliferation of T and activated B lymphocytes and NK cells.[15] IL-15 was significantly elevated in all three entities, which led us to conclude that IL-15 is a general cytokine involved in infectious uveitis. IL-15 might be upregulated in the RPE in response to viral or parasitic infection or through expression of other cytokines by infiltrated immune cells and ocular resident cells. Holtkamp et al. showed an enhancement of RPE IL-15 expression by treatment with IFNγ and TNFα.[12] Indeed, in the RV-FHUS patients IL-15 and TNFα were correlated (r = 0.70, P = .001) and in the ARN patients IL-15 correlated with TNFα and IFNγ (r = 0.69, P = .001 and r = 0.70, P = .001, respectively). In the OT patients, IL-15 was not correlated to either IFNγ or TNFα.

Soluble ICAM mediates tight adhesion between all leucocytes and vascular endothelium, whereas sVCAM mediates tight adhesion between lymphocytes, monocytes and vascular endothelium prior to migration into the tissues.[7,15] In case of viral infection of the cornea and/or retina, polymorphonuclear cells will infiltrate into the site of inflammation. Adhesion of polymorphonuclear cells, mainly neutrophils, to the vascular endothelium is mediated by receptors on the granulocytes and ligands, including sVCAM and sICAM, and promoted by chemoattractants such as IL-8.[15] In our study, the cell adhesion molecules were only slightly elevated in the AH of the RV-FHUS and OT patients, but highly elevated in the AH of the ARN patients. This observation is consistent with previous studies that show significantly higher levels of sICAM and sVCAM in the AH of patients with uveitis compared to the controls.[7,29] Soluble ICAM and sVCAM were correlated with IL-8 in all cases.

Recently, Lahmar et al. suggested that cytokines may be also used as diagnostic markers as they found IL-5 and IL-12 to be specific for OT and IL-1β for viral uveitis.[30] In our study, we also found IL-12 expression in OT and not in ARN, however, the reported association with IL-5 expression was not observed and IL-1β was detected in only 4 of 19 ARN patients. In addition, the expression of other markers also differed between the Lahmar and our study. These inconsistencies might be explained by the fact that cytokine and chemokine expression patterns are part of an intricate regulatory network, which depends on many environmental stimuli, and may therefore vary considerably during the course of disease, which

limits their use for diagnostic purposes. The limitations of our study include the lack of the longitudinal follow-up of individual patients and the small number of patients with active and nonactive OT. However, studies including samples from non-inflamed eyes would obviously be very difficult to achieve.

In conclusion, we found that RV-FHUS and OT express a similar pattern of mediators, different from that of ARN. IL-12 appears to play a role in the immunopathogenesis of RV-FHUS and OT, whereas IL-10 and IL-18 appear to play a more important role in ARN. The higher levels of cytokines and chemokines in ARN might be influenced by severe inflammation characteristic for ARN. Explicit Th1 or Th2 pathways were not found in the three infections studied. Future studies of intraocular immune mediators will help to further understand the immunopathogenesis of uveitis and to determine the origin of the cytokines detected in ocular fluids of patients with uveitis.

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Chapter 11

Intraocular and Serum Levels of Vascular Endothelial Growth Factor in Acute Retinal Necrosis and Ocular Toxoplasmosis

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Abstract

Purpose: To determine the intraocular and serum vascular endothelial growth factor (VEGF) levels in acute retinal necrosis (ARN) patients and compare those with VEGF levels found in patients with ocular toxoplasmosis (OT).

Methods: Paired intraocular fluid and serum samples of 17 patients with ARN and of 16 patients with OT were analyzed by ELISA for VEGF levels and the clinical records were reviewed.

Results: The mean intraocular VEGF levels in ARN patients were higher than in OT patients (P=.004) while the serum levels did not differ. Intraocular VEGF levels exceeded the serum levels in 8/17 (47%) of patients with ARN compared to 1/16 (6%) of OT patients (P=.009). The group with high intraocular VEGF was associated with a more extensive retinitis and lower visual acuity at the 3 months follow up (P<.001 and P=.031 respectively).

Conclusions: Intraocular VEGF levels were elevated in patients with ARN compared to OT patients. Our results suggest strong intraocular VEGF production in ARN which might be of importance for the treatment of these patients.

Introduction

Acute Retinal Necrosis (ARN) represents an intraocular herpetic infection characterized by severe inflammatory symptoms and a poor visual prognosis. Currently, the treatment of ARN consists of maximal doses of intravenous or oral antiviral therapy, usually with addition of systemic steroids to minimize vitreous reaction. Retinal detachments caused by the multiple retinal holes located in the necrotic retinal areas develop in approximately 80% of ARN patients and are extremely difficult to treat. Even when the treatment is promptly initiated, the development of a retinal detachment and subsequent poor prognosis usually cannot be prevented.[1]

The pathogenesis of retinal necrosis in ARN is generally attributed to intracellular infestation by the virus and subsequent death of retinal cells. In addition, it is feasible that the vascular occlusions occurring in the early phase of the disease play also an important role in the development of retinal necrosis and optic neuropathy.

Vascular endothelial growth factor (VEGF) plays a crucial role in the intraocular ischemic processes and subsequent development of neovascularizations. In addition, it increases vascular permeability. VEGF levels in vitreous and aqueous were reported to be increased in retinal ischemia.[2] We hypothesize that the occlusive vasculitis and subsequent necrosis occurring during the early stages of ARN might be in part VEGF driven and may play a role in the development of subsequent detachments and development of neovascularizations. In this study, we determine the intraocular and serum VEGF levels in ARN patients and compare those with VEGF levels found in patients with toxoplasmic retinitis and report on highly elevated intraocular VEGF levels in patients with ARN.

Material and Methods

We determined the VEGF levels in paired aqueous and serum samples of 17 patients with ARN and of 16 patients with ocular toxoplasmosis (OT). The medical and photographic records of all included patients were analyzed for the clinical features, especially the presence and size of ischemic retinal areas, occurrence of complications and development of visual acuity.

All aqueous samples from the patients were collected for diagnostic

purposes and their remainders were used for the current examinations, which were performed according to the tenets of the Declaration of Helsinki and according to institutional regulations.

VEGF concentrations in ocular fluid and serum samples were determined by a commercially available human VEGF ELISA according to the instructions of the manufacturer (R&D Systems Europe, Abingdon, United Kingdom).[3] In each sample we analyzed the concentration of VEGF. Concentrations below the detection limit were assigned to the lowest value from the standard curve (3 pg/ml). For statistical analysis concentrations below the detection limit were converted to a value of 0.5 x the lowest point of the calibration curve.

We compared the VEGF levels according to etiology and further analyzed the possible associations between the various clinical characteristics and the levels of intraocular VEGF. For the statistical purposes, the Pearson Chi-square test and the Mann-Whitney *U* tests were used and *P* values of less than 0.05 were considered significant.

Results

The general characteristics of patients and their VEGF levels are given in Table 1. The average age at onset of ARN was 55 years (range, 14- 83) and male-to-female ratio was 6:11. The average age at the onset in OT was lower than in ARN (*P*= .005) and the gender distribution was equal (male-to-female ratio 9:7, Table 1).

The intraocular and serum VEGF levels of the ARN and OT patients are shown in Table 2. Mean serum VEGF levels in ARN patients were not distinct from OT patients (52 pg/ml versus 28 pg/ml; *P*=.295). The mean intraocular VEGF levels were significantly higher in ARN patients than in OT (77 pg/ml versus 14 pg/ml; *P*=.005). The intraocular VEGF levels exceeded the serum levels in 8/17 (47%) ARN patients in contrast to 1/16 (6%) of OT patients, which was significantly different between both groups (*P*=.009; Table 1).

Noteworthy was that in the aqueous of 8/17 (47%) patients with ARN, no intraocular VEGF was detected. No differences in clinical characteristics could be identified when the ARN patients with or without detectable levels of VEGF in aqueous were compared (included age, gender, interval between the onset of

Table 1. General characteristics of patients with herpetic and toxoplasmic retinitis.

	Acute Retinal Necrosis	Ocular Toxoplasmosis
Number of patients	17	16
Mean age at onset (range, years)	55 (14-83)	34 (16-60)
Male-to-female ratio	6:11	9:7
Prednisone medication at time of sampling	0 (0%)	1 (6%)
Etiological agent	14 VZV , 3 HSV	16 *T. gondii*
VEGF in aqueous > serum	8 (47%)	1 (6%)

VZV = varicella zoster virus
HSV = herpes simplex virus
VEGF = vascular endothelial growth factor

ocular complaints and aqueous chamber sampling, treatments and visual acuity at time of sampling, size of retinitis, visual outcome, development of retinal detachment and having previous encephalitis). Very extensive ARN with combined central and peripheral retinal lesions of the whole circumference was noted only in 2 patients with detectable intraocular VEGF. Three patients in the ARN group suffered from diabetes mellitus and none of these three had detectable VEGF in their aqueous. The three eyes which became hypotonic had all detectable VEGF in the aqueous, however this association was not significant (P=.090).

Further, all patients independent of their etiologic diagnoses were subdivided according to whether their intraocular VEGF levels exceeded that of serum levels (Table 3). The patients with intraocular VEGF levels exceeding that of serum had a more extensive retinitis and lower visual acuity at the 3 months follow-up (P<0.001 and P=0.031 respectively). No further differences were identified. In the ARN group, no association between the VEGF levels and previous associated encephalitis was observed (1/7; 14% with intraocular VEGF exceeding that of serum versus 2/9; 22% in low VEGF group). The only patient with toxoplasmosis and raised intraocular VEGF suffered from severe extended retinitis, in contrast to focal retinitis present in the remainder of OT patients.

Table 2. Aqueous and serum vascular endothelial growth factor levels in patients with acute retinal necrosis and toxoplasmic retinitis.

ARN patients	Serum VEGF in pgr/ml	Intraocular VEGF in pgr/ml	Toxoplasma retinitis patients	Serum VEGF pgr/ml	Intraocular VEGF in pgr/ml
1	81,82	235,92	1	33,43	<3,00
2	69,20	<3,00	2	19,06	<3,00
3	<3,00	<3,00	3	<3,00	<3,00
4	14,45	66,71	4	5,02	<3,00
5	77,43	<3,00	5	<3,00	<3,00
6	26,01	3,45	6	5,49	<3,00
7	101,75	<3,00	7	59,13	<3,00
8	11,08	<3,00	8	15,09	<3,00
9	5,46	<3,00	9	43,66	<3,00
10	37,90	<3,00	10	69,57	<3,00
11	186,21	293	11	30,18	196,21
12	57,49	229,57	12	<3,00	<3,00
13	31,49	121,86	13	28,67	<3,00
14	<3,00	3,48	14	15,24	<3,00
15	4,01	13,11	15	57,75	<3,00
16	29,92	<3,00	16	59,54	<3,00
17	146,66	324,38			
Mean VEGF levels	**51,99**	**76,67**		**27,89**	**13,67**

ARN = acute retinal necrosis; VEGF = vascular endothelial growth factor

Table 3. Characteristics of patients with intraocular vascular endothelial growth factor levels exceeding the serum levels compared to patients with undetectable or lower intraocular vascular endothelial growth factor levels than the serum levels.

	Patients with ARN and OT (n=33)				
	Intraocular VEGF > serum		Intraocular VEGF < serum or undetectable		*P* values
	N	%	N	%	
Number of patients	9		24		
Male	3	(33%)	13	(54%)	0.286
Average age	55	(32-79)	41	(14-83)	0.06
Diagnosis	8 ARN, 1 OT		9 ARN, 15 OT		
Antiviral medication started before sampling	2/9	(22%)	2/24	(8%)	0.276
Interval between onset and sampling less than 2 weeks	6/9	(66%)	10/24	(42%)	0.201
Unilateral retinitis	8/9	(89%)	23/24	(96%)	1.0
Retinitis at presentation: focal lesions	0/8	(0%)	15/23	(65%)	0.001
Circumference of retinitis at sampling: less than 180˚	4/8	(50%)	3/23	(13%)	0.031
Circumference of retinitis at sampling between 180˚-360˚	2/8	(25%)	5/23	(22%)	0.849
Circumference of retinitis at onset full 360˚ in combination with central lesions	2/8	(25%)	0/23	(0%)	0.013
Retinal hemorrhages	4/8	(50%)	6/24	(25%)	0.186
Retinal neovascularisation	2/9	(22%)	1/22	(5%)	0.131
Papillitis	6/7	(86%)	13/23	(57%)	0.161
Hypotony	2/8	(25%)	1/23	(4%)	0.089
Retinal detachment	4/9	(44%)	5/23	(22%)	0.199
Visual acuity after treatment < 0.1	5/9	(56%)	4/23	(17%)	0.031
Retinal vasculitis	6/8	(75%)	10/21	(48%)	0.185

ARN = acute retinal necrosis; OT = Ocular Toxoplasmosis; VEGF = vascular endothelial growth factor

Discussion

Intraocular VEGF levels in patients with ARN were higher than in OT patients while their serum VEGF levels did not differ. In particular, intraocular VEGF levels exceeded the serum levels in 47% of patients with ARN compared to 6% in OT. High intraocular VEGF levels were associated with extended retinitis and poor visual prognosis.

In our series, serum VEGF levels in patients with ARN were not different from OT (Table 2); however the number of patients was limited. Moreover, the patients were examined at different stages of their infectious ocular disease, which might also have influenced our results. High serum VEGF levels have been described in a variety of disorders including diverse autoimmune and rheumatic diseases.[4,5] In addition, others have shown that serum VEGF levels were elevated in hepatitis C virus infected patients and in patients with proliferative diabetic retinopathy.[6,7] It has been suggested that the level of serum VEGF is correlated with disease activity in systemic lupus erythematoses and in Behçet disease.[8,9] The effect of anti-VEGF treatment in these disorders is not known. In contrast, it has been reported that plasma VEGF levels in patients with uveitis are lower than in healthy volunteers.[10] The above described variability might be explained by the lack of consistency for exact diagnosis, co-morbidity, treatment and timing of the sample collection.

VEGF plays a major role in the development of intraocular neovascularizations, induces blood-retina barrier breakdown and consequently increases vascular permeability. Elevated intraocular levels of VEGF were repeatedly reported in patients with diabetic retinopathy, neovascular age-related macular degeneration, and were also noted in eyes with macular edema from various causes.[10-14] In uveitis, the intraocular VEGF levels were not yet systematically studied. High levels of VEGF in vitreous were found in a small series of 8 patients with uveitis of different origins.[15] In our study, highly elevated intraocular VEGF levels were observed in approximately one half of the patients with ARN and were associated with extended retinitis; further clinical differences were not identified in this limited series of patients. Our purpose was to determine whether the size and severity of retinal inflammation are related to intraocular VEGF formation. We did not assess intraocular and serum VEGF levels in healthy individuals without uveitis. Previously reported VEGF concentrations in aqueous in so called “healthy”

controls vary widely with a reported range of 0,072 pg/ml to 367 pg/ml.[10,16-18] In addition, our results reflect on measurements of VEGF levels at one point of time and the sequential data on the changes of VEGF levels during the different stages of the disease are not elucidated. A more comprehensive knowledge of the role of VEGF in intraocular inflammations is needed to conclude whether intraocular anti-VEGF medication might be of value when treating ARN and possibly other occlusive retinal vasculitides.

In ARN, the widespread retinal ischemia develops and the formation of neovascularizations has been reported.[1] The stimulus for intraocular VEGF production in ARN is not known; it is feasible that extensive retinal ischemia induced by occlusive vasculitis and viral destruction in ARN might stimulate its production. VEGF is thought to have a proinflammatory role and all inflammatory cell subtypes contain active VEGF receptors.[2] Vinores et al. described upregulation of VEGF in experimental herpes virus retinopathy in mice and attributed elevated VEGF levels not only to retinal ischemia, but also to severe inflammation in ARN and presence of multiple pro-inflammatory cytokines.[19] It has been reported that different cytokines, such as interleukin-1β and fibroblast growth factor, stimulate the production and secretion of VEGF, which might have also occurred in our ARN patients. In OT, where inflammation and retinal ischemia are more limited, the upregulation of VEGF probably does not reach such levels as noted in ARN. It is possible that the VEGF levels of the patients with ARN might be even higher in the samples of the retina and/or vitreous than in the aqueous as investigated in the present series. The future investigation might clarify this hypothesis. It would be also interesting to investigate intraocular VEGF levels in ocular disorders characterized by severe inflammation, but no retinal involvement. In a healthy population, serum VEGF levels are typically low and the half life is short, however multiple systemic diseases were associated with high serum levels of VEGF and it could be theoretically argued that the elevated intraocular VEGF levels might be due to leakage from serum. Intraocular VEGF levels exceeding the serum levels observed in our patients confirm the intraocular formation of VEGF.

Currently, multiple studies on positive therapeutic effect of intravitreal anti-VEGF medications on neovascularizations and macular edema were reported.[20-22] One may hypothesize that the use of anti-VEGF medications might be beneficial when treating patients with ARN. In our limited series of patients, poor visual

outcome was associated with higher VEGF levels. Our results suggest that retinal vascular occlusions in peripheral retina and at the level of optic nerve might be (at least in part) VEGF driven. One could hypothesize that the blockade of VEGF in patients with ARN might have a beneficial effect on retinal and optic disc perfusion and might limit the development of future neovascularizations. However, the exact physiologic role of VEGF in the eye is not yet fully clarified and we do not know whether high VEGF levels in ARN are a mere consequence of ischemia and severe inflammation.[2] In addition, it is not known whether its blockade would have a desirable clinical effect.

In conclusion, we report on highly elevated intraocular VEGF levels in patients with ARN, especially in patients with extensive retinitis. Our data suggest strong local VEGF production in ARN and might be of importance for future treatment of patients with ARN.

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Chapter 12

Analysis of specific protein profiles in the ocular fluids of patients with infectious intraocular inflammation using Surface Enhanced Laser Desorption/Ionization time-of-flight (SELDI-tof) technology

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Abstract

Purpose: To examine the applicability of Surface Enhanced Laser Desorption/ Ionization time-of-flight (Seldi-tof) technology in detecting disease specific protein profiles in ocular fluids from patients with post-operative endophthalmitis and in patients with Herpes simplex virus (HSV) uveitis and ocular toxoplasmosis (OT).

Methods: Paired vitreous fluids and serum samples from three patients with culture-confirmed coagulase-negative staphylococci endophthalmitis and from two culture-negative patients, and paired serum and aqueous humor (AH) samples from 16 OT patients and 10 patients with ocular HSV infection were analyzed by Seldi-tof technology. As controls three vitreous fluids and serum samples of patients with a macular hole, and seven AH and serum samples of patients with age-related cataract, were included. The protein profiles of the patient groups were scanned for ocular fluid-specific protein peaks and the results were compared to other patient groups and to the controls.

Results: The three culture-positive vitreous fluids and one of the two culture-negative vitreous fluids revealed protein peaks at 10.4, 10.8 en 11.3 kDalton, which were absent from all other sera and ocular fluids. In 14/16 OT patients a group and ocular fluid-specific peak around 5850 Da was found.

Conclusions: Our results indicate that the Seldi-tof technology can be applied to investigate protein profiles in ocular fluids and that it may eventually lead to the detection of disease-specific biomarkers to contribute to a fast diagnosis of intraocular diseases.

Introduction

In the analysis of ocular fluids the identification of the infectious agent causing intraocular inflammation can be difficult and time consuming. Ocular fluid analysis by PCR or by establishing intraocular antibody production through the Goldmann-Witmer coefficient (GWC) determination are very suitable diagnostic tools, however the amount of ocular fluid that is needed to perform the assay is often a problem, as there may be not enough ocular fluid to complete all tests.

Surface enhanced laser desorption/ionization time-of-flight (Seldi-tof) is a new high throughput technique which can be used to create specific protein profiles of several body fluids and tissues and quickly and efficiently analyze many samples simultaneously. The fluid is spotted on a biochip, which is available with various surfaces to bind proteins with different biochemical properties, and subsequently the bound proteins are ionized and shot off by a laser (Figure 1). The proteins then enter a vacuum tube and the speed by which they migrate through this tube is a measure for the mass of the protein. This results in a specific spectrum of protein peaks. This technique has appeared to be effective in the detection of several infections, such as intra-amniotic infections, SARS-coronavirus, congenital Cytomegalovirus and hepatitisviruses and appears to be highly sensitive and specific for several microbes.[1-4] Furthermore, since only 1 ml of sample is needed, this technique might be very suitable for analyzing ocular fluids.

Infectious endophthalmitis is a serious complication of intraocular surgery or perforating injury and can be caused by a variety of bacteria and fungi. The diagnosis of endophthalmitis relies on isolation of the causative organisms,

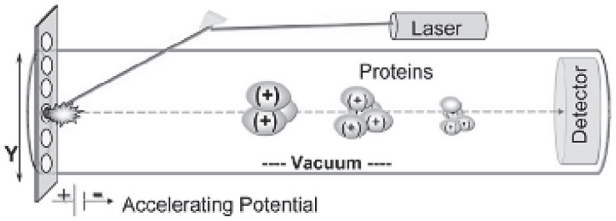


Figure 1. Schematic representation of the Seldi-tof apparatus. Proteins become ionized by the laser and enter the vacuum tube where they are detected by the detector.

which can be done by culture of an aqueous or vitreous sample.[5] In case of endophthalmitis developing within 6 weeks after cataract extraction, in 70% the cause of the infection is detected, mostly coagulase-negative staphylococci (70%), however, in 30% cultures remain negative.[6] It is not known if in case of negative culture results, the inflammation is sterile or infectious with a bacterial load too low to detect. Uveitis is an inflammation of the uvea, consisting of the iris, the ciliary body and the choroid, and adjacent structures, such as the retina, the vitreous and the optic disc. Herpes simplex virus (HSV) and *Toxoplasma gondii* are major causes of uveitis caused by an intraocular infection. Ocular HSV and *Toxoplasma* infections are readily detected by PCR and GWC analysis of intraocular humors.

In this pilot study we examine the applicability of the Seldi-tof technology in determining specific protein profiles in the vitreous of patients with post-operative endophthalmitis and in aqueous samples of patients with ocular herpes simplex virus (HSV) infection and ocular toxoplasmosis. These experiments may eventually lead to the identification of disease specific biomarkers.

Methods

Patients and samples

Simultaneously taken vitreous fluids and serum samples of five patients with endophthalmitis which developed within six weeks after cataract extraction were analyzed for specific protein profiles by Seldi-tof technology. Of these five, three vitreous samples were culture-positive for coagulase-negative staphylococci (Staph +) and two culture-negative (Staph -). Three vitreous fluids from patients with a macular hole (MG) served as controls.

Simultaneously taken aqueous humor (AH) and serum samples from 16 patients with GWC-confirmed ocular toxoplasmosis (Toxo) and 10 patients with GWC-confirmed ocular HSV infection were analyzed by Seldi-tof technology. Controls consisted of seven AH and serum samples from patients with age-related cataract. None of the patients were immunocompromised. All samples were previously taken for diagnostic purposes and the experiments were performed with permission of the institutional review board.

Seldi-tof analysis

For initial Seldi-tof analysis a Normal Phase (NP20) Protein chip array (Cyphergen), which binds proteins in general, was used.[7] The active spots on these arrays contain silicon dioxide which allows proteins to bind through hydrophilic and charged residues, including serine, threonine or lysine. All the arrays were (pre)treated according to the standard protocols of the manufacturer.[7] Each array was first spotted with 1 µl of deionized water for better diffusion. Then 1 µl of ocular fluid or serum was applied onto the array in order to mix with the water. After air drying the arrays were washed with 5 µl of deionized water two times to remove salts. When the spots were dry 1 µl of Sinapinic Acid (SPA), an energy absorbing molecule (EAM), was added twice on every spot with a pause of approximately 5-10 minutes. The EAM assists in desorption and ionization of the fluids by co-crystallizing with the proteins in the ocular fluid. It absorbs the laser energy and generates the ionized proteins, which are subsequently detected by the ProteinChip reader.[7] The mass-to-charge ratio is calculated by the time it takes the ion to go through the tube. The output of the detected proteins is visible as a series of peaks in the spectrum.

Each array was analyzed with a standard protocol where 350 shots were fired on each spot. The protocol had a laser intensity of 6000nJ, a deflector setting of 3000 Da, a detector sensitivity of 9 and a molecular mass detection range from 1000 to 200,000 Da. The optimization range was from 3,000 to 50,000 Da. The focus mass was set at 10,000 Da. For accuracy, the spectra were first calibrated with the standard external all-in-1 Protein Standard calibrant (Hirudin BHVK – 7034 Da, Cytochrome C – 12230 Da, Myoglobin – 16951 Da, Carbonic Anhydrase – 29023 Da, Enolase – 46671 Da, Albumin – 66433 Da & IgG – 147300 Da.) After the calibration the data were clustered in the different groups of the experiments; MG, Staph + and Staph – in the endophthalmitis experiment and HSV, Toxo and controls in the infectious uveitis experiment. The clusters were then manually analysed for peak profiles and differences in peak intensity between the different clusters. All arrays were tested twice to check if results were consistent with the first test.

Results

Figure 2 shows the spectra of the vitreous fluids of the patients with post-operative endophthalmitis and the corresponding controls. The three culture-positive vitreous fluids and one of the two culture-negative vitreous fluids showed protein peaks at 10.4, 10.8 and 11.3 kDa. These peaks were absent from the controls and the other culture-negative vitreous fluid. The latter had a pattern more similar to the controls than to the other endophthalmitis patients. The peaks were not found in the corresponding sera.

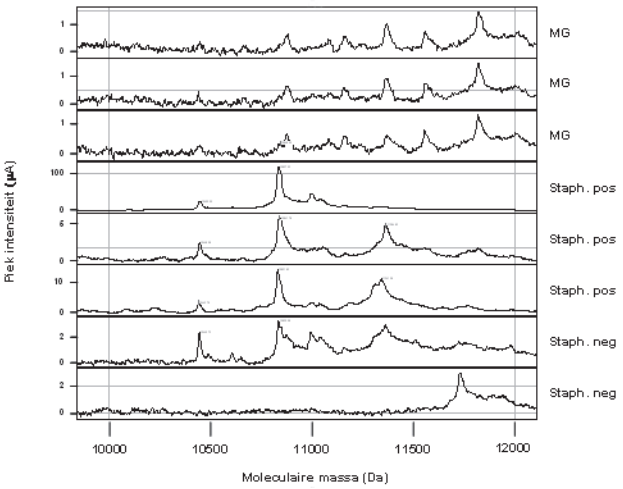
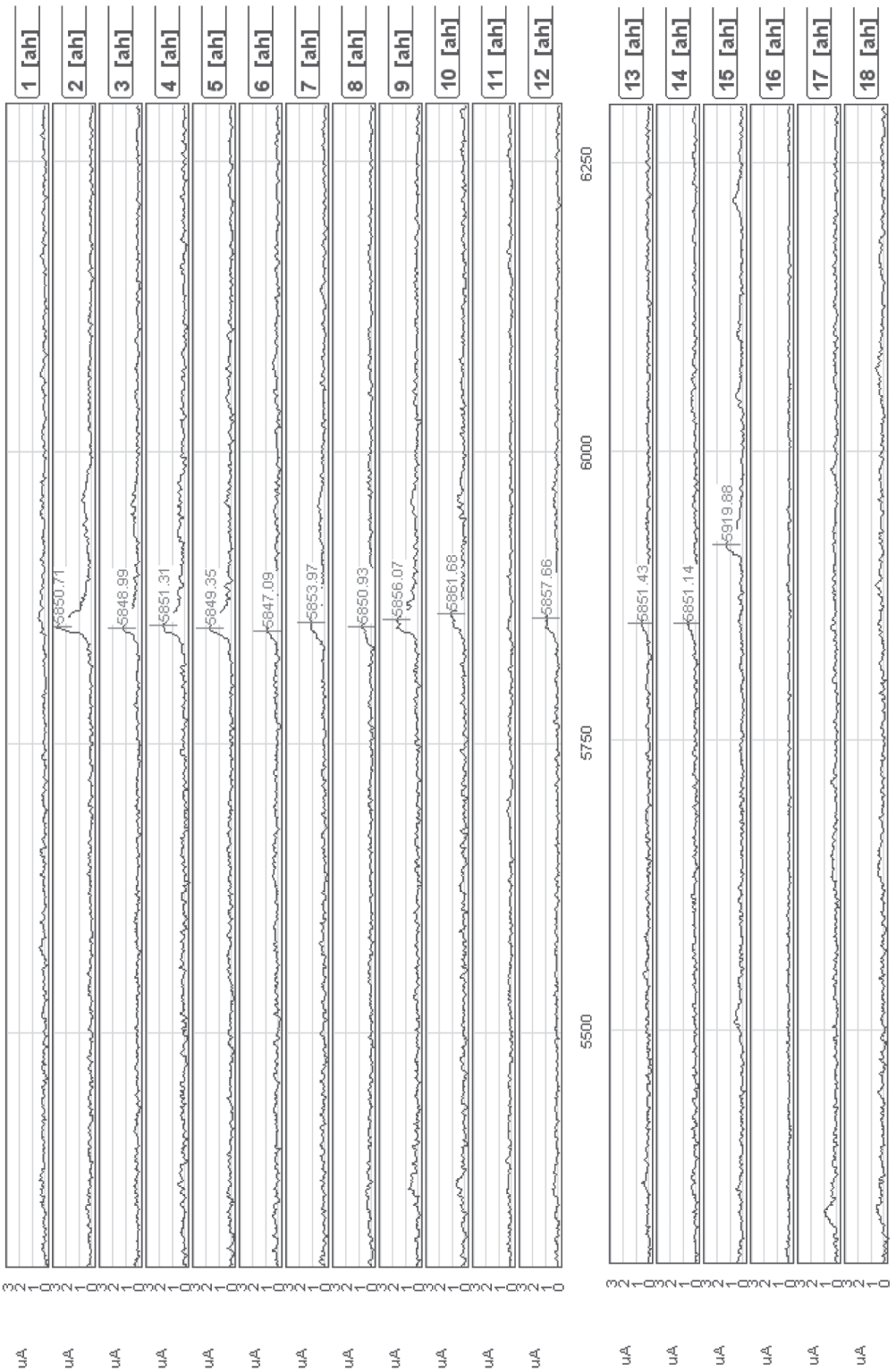


Figure 2. Seldi-tof results for the vitreous fluids of eight patients. On the X-axis the molecular mass in Dalton (Da) is shown and on the Y-axis the peak intensity. The upper three spectra are vitreous fluids of patients with a macular hole (MG). The lower five spectra are vitreous fluids of patients with endophthalmitis, of which the first three are culture-positive for Staphylococci (Staph. pos) and the last two are culture-negative for Staphylococci (Staph. neg).

Figure 3 shows the results of the aqueous humors (AH) of the patients with intraocular HSV infection, ocular toxoplasmosis and of the controls. In 14/16 toxoplasmosis patients a protein peak was detected around 5850 Da, which was not observed in the HSV patients nor in the controls. The peak was also absent from all sera. Toxoplasma patients one and 11 did not show any peaks at all. The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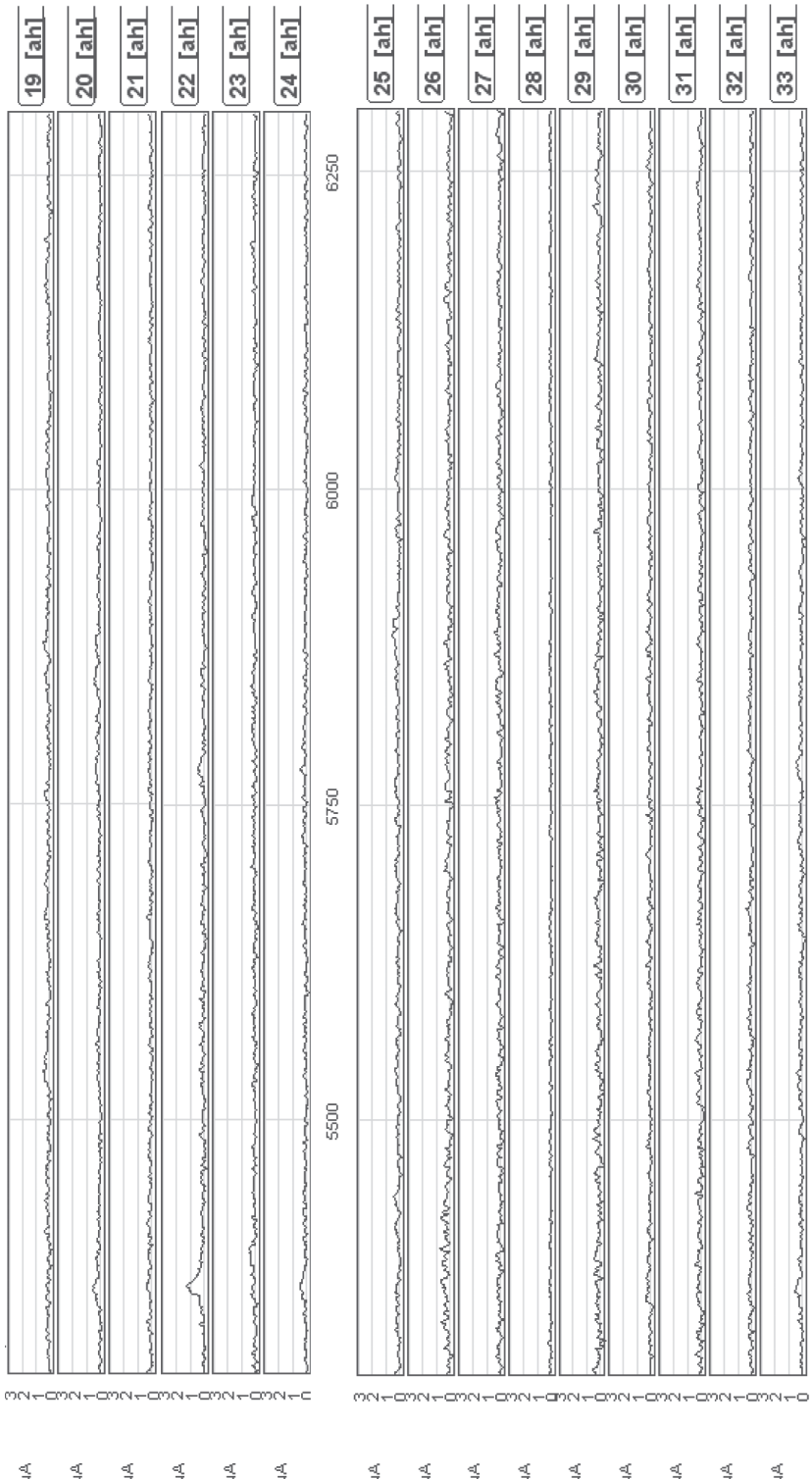

Figure 3. Spectra 1 t/m 16 are *T. gondii* positive aqueous humors. On the X-axis the molecular mass in Dalton (Da) is shown and on the Y-axis the peak intensity. Spectra 17 t/m 26 are Herpes Simplex Virus positive aqueous humors. The other spectra are the controls.

peaks detected in the vitreous of endophthalmitis patients were not detected in the aqueous of the infectious uveitis patients and vice versa.

Discussion

The data presented here, demonstrate that protein profiles can be obtained from both vitreous and aqueous humor. Moreover, specific profiles were observed for different intraocular inflammations. The detected peaks were not observed in the corresponding sera, strongly suggesting that they are ocular fluid specific.

In the endophthalmitis experiment we found that the vitreous fluids of patients with endophthalmitis had a distinct protein profile, different from that of the control patients. Surprisingly, one culture-negative vitreous showed a highly similar profile to that of the culture-positive vitreous samples. This may suggest that this patient had a culture-undetectable staphylococci endophthalmitis, however, it cannot be excluded that the proteins observed are general endophthalmitis markers.

In the infectious uveitis experiment we found a peak around 5850 Da specific for the aqueous humors of *T. gondii* patients. The absence of this peak in the serum suggests that this protein is locally produced.

We did not find corresponding protein peaks in the endophthalmitis patients and infectious uveitis patients. This may be explained by the fact that we tested vitreous fluid and aqueous humor, respectively, and that other pathogens were involved.

Proteomics of body fluids are increasingly used to find new biomarkers. Studies to investigate potential cancer biomarkers in serum using Seldi-tof are numerous, but no biomarker studies on ocular disease have been reported so far.[8-16] The absence of specific peaks in, for example, HSV patients does not exclude the presence of possible biomarkers. Moreover, the application of the technique and the interpretation of the results may be influenced other factors, such as the time of sampling after onset of disease and the use of medication. The number of tested AH and serum samples is too low to definitive exclude the presence of a biomarker. Therefore, larger numbers of sera and ocular fluid pairs need to be analyzed. It would also be interesting to determine the protein profiles of other defined infectious and noninfectious uveitis entities. In addition, the

Seldi-tof technique may be useful to categorize uveitis entities of unknown origin.

In conclusion, the Seldi-tof technology can be applied to investigate protein profiles in ocular fluids and these results suggest that it may eventually contribute to a fast diagnosis of ocular diseases. Because this technology is very suitable for high-throughput analysis in microvolumes it is therefore highly attractive for the analysis of ocular fluids. Future research of protein profiling of several types of uveitis is needed to investigate the value of Seldi-tof technology as a diagnostic tool.

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Chapter 13

Summary, conclusions and considerations

Summary

The fast identification of infectious uveitis entities is of crucial importance since their treatment and visual prognosis differ entirely from noninfectious intraocular inflammations. In Europe, the infections are thought to cause approximately 20-25% of cases, whereas about 30% is associated with a noninfectious disease. The remainder is of (yet) unknown cause and might be associated with until now undiagnosed infections. Analysis of peripheral blood does not provide conclusive evidence for the diagnosis of intraocular infections. It is therefore imperative to establish the causative agent by the examination of intraocular fluid. The aim of this thesis was to discover novel causes of infectious uveitis by implementing new diagnostic assays on ocular fluids and gain more insight in the (immuno) pathogenesis of infectious uveitis.

Chapter 1 is a general introduction and an up-to-date review of the literature on ocular fluid analysis and the etiology of infectious uveitis. Intraocular fluid analyses by means of PCR and the detection of intraocular antibody production by Goldmann-Witmer coefficient (GWC) determination are described and discussed. Furthermore, known infectious causes of uveitis and their corresponding diagnostic assays are described. Finally, possible novel infectious entities of uveitis together with their diagnostic means are addressed, including Rubella virus, Parvovirus B19, Human parechovirus and Human herpesvirus 6.

In **Chapter 2** we investigated whether Rubella virus is associated with Fuchs heterochromic uveitis syndrome (FHUS) by analyzing intraocular antibody production (GWC determination) against Rubella virus in 14 patients with clinically established FHUS, in 13 control patients with herpetic anterior uveitis (AU) and 19 control patients with ocular toxoplasmosis (OT). Intraocular antibody production against Rubella virus was found in 13/14 (93%) patients. These patients were negative for Herpes simplex virus (HSV), Varicella zoster virus (VZV) and *Toxoplasma gondii*. None of the control patients showed intraocular antibody production against Rubella virus. We concluded that Rubella virus, and not HSV, VZV and *T. gondii*, is associated with FHUS.

In **Chapter 3** we investigated the clinical profile of 30 patients with chronic anterior uveitis (AU) and a positive intraocular analysis for Rubella virus, and assessed similarities to FHUS. Clinical records were compared with the profiles of 13 patients with chronic AU of undetermined origin. Patients with Rubella virus-associated uveitis appeared to be younger at the time of initial ophthalmologic presentation and presented more frequently with unilateral ocular involvement, keratic precipitates, iris atrophy and/or heterochromia, vitreous opacities and cataract. Also, the combination of main FHUS symptoms (keratic precipitates, the absence of posterior synechiae, cataract and vitreous opacities) occurred more often in the Rubella virus-positive group. We concluded that Rubella virus causes a distinct clinical spectrum of ocular symptoms similar to the FHUS and suggest that the virus is implicated in the pathogenesis of FHUS.

Patients with FHUS have a high prevalence of (chorio)retinal lesions, reminiscent of toxoplasmic scars. In **Chapter 4** we investigated whether the clinical appearance of these focal (chorio)retinal lesions differed between patients with intraocular Rubella virus infection and patients with intraocular *T. gondii* infection. Photographic and angiographic records were masked for identification and for infectious agent and evaluated by four specialists in the field of OT. No differences were observed between the retinal lesions in Rubella virus-positive and *T. gondii*-positive patients. By at least three out of four experts, retinal lesions were considered consistent with the diagnosis of OT in 55% of Rubella virus-positive patients and in 88% of *T. gondii*-positive patients. The retinal lesions in *T. gondii*-positive patients were more frequently considered "consistent with the diagnosis of ocular toxoplasmosis" by two experts. There was a 'substantial agreement' between the four experts. We concluded that clinical features of chorioretinal lesions in patients with intraocular Rubella virus infection were not distinct from those in patients with OT, indicating that the etiological diagnosis of these lesions cannot be made on clinical grounds solely.

We found a high seroprevalence for *T. gondii* in our Rubella virus-associated FHUS (RV-FHUS) patients. However, patients with FHUS have so far never presented with active retinal lesions and other symptoms compatible with OT. Moreover, we report on a *Toxoplasma*-seronegative case of RV-FHUS who had chorioretinal scars as well. Apparently, further investigations are required to elucidate this cause of retinal lesions in FHUS.

In **Chapter 5** we investigated the contribution of *Toxocara canis* to posterior uveitis of undetermined origin by means of GWC determination in 37 adult patients and 12 children. None of 37 adults had a positive GWC, whereas three of twelve children demonstrated intraocular antibody production against *Toxocara canis*. All three had very low or undetected serum titers, and their intraocular antibody titers exceeded those in the peripheral blood. One child had vitritis, one presented with a low-grade uveitis and a peripheral retinal lesion and the third patient had posterior uveitis with a chorioretinal scar. We concluded that ocular toxocariasis is mainly a pediatric disease. Our findings underline that serological screening alone is not informative for the diagnosis of ocular toxocariasis and GWC analysis can be of value when diagnosing (young) patients with posterior focal lesions or vitritis of unknown etiology.

In **Chapter 6** we reported on an adult patient who presented with a decrease in visual acuity of his right eye, cells and mild opacities in the vitreous, and a white retinal infiltrate in the posterior pole. Aqueous analysis revealed intraocular antibody production against *Toxocara canis*, despite negative serology. After treatment with antihelmintics the retinal infiltrate decreased in size. Although ocular toxocariasis is mainly a pediatric disease (Chapter 5), one should be aware that it may also occur in adults. It is important to perform ocular fluid analysis and determine the GWC, even when *Toxocara* serology is negative, as early diagnosis and intervention provide better outcomes.

In **Chapter 7** we analyzed 139 ocular fluids samples of patients suspected of infectious uveitis, but negative for the most common inciting agents HSV, VZV, Cytomegalovirus (CMV) and *T. gondii,* for 18 viruses and three bacteria by PCR. Positive PCR results were found in seven patients: one was positive for Epstein-Barr virus (EBV), one for Rubella virus, one for Human herpesvirus 6 and four patients were positive for Human parechovirus. The latter observation is particularly interesting, as Human parechovirus infections mainly occur during childhood, whereas here all four patients were adults. One patient was immunocompromised and was suspected of ocular syphilis. The other three patients all had AU of unknown origin associated with corneal involvement and cells in the anterior chamber. We concluded that Human parechovirus may be a novel cause of infectious uveitis.

We hypothesized that like *T. gondii,* HSV and Rubella virus, other childhood infections might also be able to incite uveitis. To this end we determined whether Parvovirus B19, Mumps virus and Measles virus are associated with AU (**Chapter 8**) or with intermediate uveitis, neuroretinitis and focal chorioretinitis of non-toxoplasmic origin (**Chapter 9**) by GWC analysis. In addition, CMV as a cause of AU was investigated (**Chapter 8**). We identified two patients with unexplained AU and positive GWCs against CMV and one AU patient who was positive for Parvovirus B19. Intraocular antibody production against Mumps- or Measles virus was not detected. None of the patients with intermediate or posterior uveitis had a positive GWC for one of the investigated pathogens. We concluded that CMV and Parvovirus B19 may be associated with AU and suggested AH analysis for these pathogens in patients with unexplained AU. We found no laboratory evidence for the involvement of Parvovirus B19, Mumps virus and Measles virus in the pathogenesis of intermediate uveitis, neuroretinitis and focal chorioretinitis.

In order to identify cytokines and chemokines that may play a role in the immunopathogenesis of three important types of infectious uveitis, paired serum and aqueous humor samples were analyzed by multiplex immunoassay in 18 patients with RV-FHUS of 20 patients with OT, and of 19 patients with acute retinal necrosis (ARN) (**Chapter 10**). The results showed that RV-FHUS and OT revealed similar patterns of mediator production, which were different from ARN. ARN samples had higher overall cytokine levels than RV-FHUS and OT samples, however, IL-12 levels were significantly higher in RV-FHUS and OT patients, compared to ARN patients and the controls. On the other hand, IL-10 and IL-18 levels were significantly higher in ARN compared to RV-FHUS, OT and the controls. IFNγ levels were elevated in ARN samples. No correlation was found between cytokine levels and the interval between the onset of symptoms and the time of sampling. Also no correlation could be found between the use of corticosteroids and cytokine levels. We concluded that the differences in immune mediator expression between RV-FHUS and OT on the one hand and ARN on the other, may be related to clinical disease activity and severity. No explicit T helper (Th) pathway could be identified for either uveitis entity. Apparently, both Th1 and Th2 associated mediators are involved.

In **Chapter 11** we analyzed intraocular and serum levels of vascular endothelial growth factor (VEGF) in 17 patients with ARN and 16 patients with OT by immunoassay to determine whether the size and severity of retinal inflammation are related to intraocular VEGF formation. We found that intraocular VEGF levels in patients with ARN were higher than in OT patients while their serum VEGF levels did not differ. Intraocular VEGF levels exceeded the serum levels in 47% of patients with ARN compared to 6% in OT. Furthermore, we found that the patients with intraocular VEGF levels exceeding those in serum had a more extensive retinitis and lower visual acuity at the three months follow-up ($P < 0.001$ and $P = 0.031$ respectively). We concluded that high intraocular VEGF levels in patients with ARN are associated with extensive retinitis and poor visual prognosis. High local VEGF production in ARN might be of importance for future treatment of patients with this devastating ocular disease.

In **Chapter 12** we described a pilot study in which we attempt to detect specific protein profiles by Surface enhanced laser desorption/ionization time-of-flight (Seldi-tof) in the vitreous of five patients with acute postoperative endophthalmitis, three of whom had a positive and two had a negative culture for staphylococci. As controls we included three vitreous fluid samples of patients with a macular hole, without any inflammation. Our data showed that the patients with endophthalmitis had similar protein profiles, clearly different from those of the control patients.

In addition, we analyzed paired aqueous humor and serum samples of patients with intraocular antibody production against HSV (n=10) and *T. gondii* (n=8), and control patients with age-related cataract (n=7). These experiments revealed a peak in the aqueous humor that appeared to be specific for *T. gondii*. These preliminary data indicate that ocular fluids are suitable for Seldi-tof analysis and that this technique might be of value for detection of specific intraocular biomarkers. Further investigations are required to determine the relevance of our observations.

Conclusions and considerations

In this thesis we report on an association of Rubella virus with Fuchs Heterochromic Uveitis Syndrome (FHUS). The majority of our patients with clinically established FHUS had intraocular antibody production against Rubella virus. Furthermore, patients with Rubella virus-associated uveitis had a distinct clinical spectrum of ocular symptoms similar to patients with FHUS. However, the four classical clinical criteria of FHUS (characteristic keratic precipitates, iris atrophy and/or heterochromia, absence of posterior synechiae and cataract) were also observed in patients who had negative results for intraocular antibody production against Rubella virus, which suggests that other causes of FHUS might exist. Indeed, recently CMV was associated with FHUS. Therefore it is probable that FHUS is a clinical syndrome which might have multiple causes and additional pathogens associated with FHUS might be identified in the future.
In the past, many different names have been used to describe a Fuchs Heterochromic Uveitis Syndrome (FHUS), including Fuchs' heterochromic (irido) cyclitis, Fuchs' anterior uveitis and Fuchs' heterochromic uveitis, which explains the different names in the various chapters of this thesis. Recently however, it has been decided by the International Uveitis Study Group to address this clinical syndrome as 'Fuchs Heterochromic Uveitis Syndrome'. When a specific etiology is identified, it should be referred to as for example Rubella virus-associated Fuchs Heterochromic Uveitis or CMV-associated Fuchs Heterochromic Uveitis.

The presence of toxoplasmosis-like chorioretinal lesions in patients with Rubella virus-associated Fuchs uveitis syndrome (RV-FHUS) is intriguing. Our study clearly demonstrated that the etiological diagnosis cannot be made on clinical grounds solely and that aqueous analysis is required to establish the definitive diagnosis. The question still remains which pathogen causes the chorioretinal scars in patients with RV-FHUS.

In this thesis, we analyzed ocular fluids samples from patients with undetermined uveitis for a variety of pathogens. We found positive results for Human parechovirus, Human herpesvirus 6, for Parvovirus B19 and CMV and conclude that these pathogens might be associated with infectious uveitis. Further investigation into the role of Human parechovirus and Human herpesvirus 6 in

ocular disease has to be performed to determine whether these viruses are true causes of infectious uveitis. Furthermore, since the number of patients tested for CMV, Parvovirus B19, Mumps and Measles virus was rather small, we cannot exclude the involvement of these viruses in pathogenesis of uveitis. It would be interesting to analyze a large number of patients with uveitis of unknown etiology for these viruses and include PCR assays in these studies.

Our studies revealed that GWC analysis for ocular toxocariasis can be of value when diagnosing patients with posterior focal lesions or vitritis of unknown etiology, especially in children. However, it should be emphasized that ocular toxocariasis may also occur in adults. It is important to realize that intraocular fluid analysis is essential for the diagnosis as serological screening against *Toxocara canis* is not informative and serology can be false-negative. In future, it would be of value to investigate if other nematodes, such as *Ascaris lumbricoides*, play a role in the pathogenesis of infectious uveitis.

In a comprehensive study into the role of cytokines and chemokines in infectious uveitis, we observed that ocular fluids of patients with RV-FHUS and OT reveal a similar pattern of cytokine and chemokine production distinct from that of ARN. Ocular fluids of patients with ARN exhibited higher levels of mediators, which might correlate with higher clinical disease activity and severity. Furthermore, levels of vascular endothelial growth factor (VEGF), which plays a crucial role in the intraocular ischemic processes and subsequent development of neovascularizations, appear to be significantly higher in the ocular fluids of patients with ARN compared to OT. Analysis of a larger number of patients and a comparison with noninfectious uveitis, preferably in homogenous groups of patients, might give further insight in the immunopathogenesis of ARN, RV-FHUS and OT. Also, it would be of value to determine whether anti-VEGF treatment would contribute to a better outcome and prognosis of ARN.

The new findings described in this thesis are of value for the diagnosis of infectious uveitis and have expanded the spectrum of causative agents involved in infectious uveitis. Analysis of intraocular fluids, especially the combination of detection of intraocular antibody production and PCR, is of value in diagnosing

patients with infectious uveitis and allows the early discrimination between infectious and noninfectious uveitis entities, which is important for prognosis and treatment of the patients. The current improvement of the diagnostic procedures and implementation of new diagnostic assays will enhance the detection of yet unknown infectious causes of uveitis. Moreover, together with a better understanding of the (immuno)pathogenesis of infectious uveitis, the expansion of the diagnostic repertoire will further improve our knowledge of potentially blinding but frequently treatable intraocular infections.

Samenvatting en conclusies

Samenvatting

Uveïtis is een verzamelnaam voor alle inwendige ontstekingen van het oog en is een belangrijke oorzaak van slechtziendheid en blindheid. In Europa wordt in 20%-25% van de gevallen de uveïtis veroorzaakt door een infectie. In ongeveer 30% is het geassocieerd met een niet-infectieuze vaak onderliggende aandoening. Van de overige 50% is het (nog) onbekend wat de oorzaak is en het is mogelijk dat nog niet gediagnosticeerde infecties ook een rol spelen.
Een snelle identificatie van infectieuze uveïtis entiteiten is van groot belang, omdat de behandeling en visuele prognose totaal verschillend zijn van die van niet-infectieuze uveïtis. Bloedonderzoek alleen levert geen bewijs voor de oorzaak van de intra-oculaire infectie en het is daarom noodzakelijk om oogvocht te onderzoeken. Het doel van dit proefschrift is om nieuwe veroorzakers van infectieuze uveïtis te ontdekken door ontwikkeling en toepassing van nieuwe diagnostische testen op oogvochten en om meer inzicht te krijgen in de (immuun-) pathogenese van infectieuze uveïtis.

Hoofdstuk 1 geeft een introductie en een recent overzicht van wat tot nog toe bekend is in de literatuur over analyse van oogvochten en de oorzaken van infectieuze uveïtis. Oogvochtanalyse met behulp van PCR (aantonen van genetische informatie) en de detectie van intra-oculaire antilichaam productie door bepaling van de Goldmann-Witmer coëfficiënt (GWC) worden beschreven en bediscussieerd. Verder worden bekende infectieuze veroorzakers van uveïtis en de bijbehorende diagnostische bepalingen beschreven. Ook wordt aandacht besteed aan mogelijk nieuwe veroorzakers van infectieuze uveïtis, zoals rubellavirus, parvovirus B19, humaan parechovirus en humaan herpesvirus 6.

In **hoofdstuk 2** onderzochten wij of rubellavirus geassocieerd is met het Fuchs heterochrome uveïtis syndroom (FHUS) door bepaling van intra-oculaire antilichaam productie (GWC) tegen rubellavirus bij 14 patiënten met een klinisch beeld van FHUS, bij 13 controle patiënten met herpetische uveïtis anterior en bij 19 controle patiënten met oculaire toxoplasmose (OT). Actieve productie van antistoffen in het oog tegen rubellavirus werd gevonden in 13 van 14 (93%) patiënten. Deze patiënten waren negatief voor herpes simplex virus (HSV), varicella zoster virus (VZV) en *Toxoplasma gondii*. Geen enkele controle patiënt liet intra-

oculaire antilichaam productie tegen rubellavirus zien. Wij concludeerden dat rubellavirus, en niet HSV, VZV en *T. gondii*, geassocieerd is met FHUS.

In **hoofdstuk 3** onderzochten wij de klinische karakteristieken van 30 patiënten met chronische uveïtis anterior (UA) en een positieve oogvochtanalyse voor rubellavirus, en beoordeelden of er overeenkomsten zijn met FHUS. Wij hebben diverse klinische kenmerken van deze patiënten vergeleken met 13 patiënten met chronische UA waarvan de oorzaak onbekend is. Patiënten met rubellavirus-geassocieerde uveïtis bleken jonger bij het eerste bezoek aan de oogarts. Rubellavirus-positieve patiënten presenteerden zich vaker met unilaterale uveïtis, descemet stippen, iris atrofie en/of heterochromie, glasvochttroebelingen, en cataract. Ook kwam de combinatie van de vier hoofdsymptomen van FHUS (descemet stippen, afwezigheid van synechiae posteriores (verklevingen), cataract en glasvochttroebelingen) vaker voor in de rubellavirus-positieve groep. Wij concludeerden dat rubellavirus een klinisch spectrum van symptomen veroorzaakt dat vergelijkbaar is met het syndroom van FHUS, wat suggereert dat het virus betrokken is in de pathogenese van FHUS.

In de literatuur is vaak beschreven dat patiënten met FHUS (chorio)retinale laesies hebben, die lijken op Toxoplasmose littekens. In **hoofdstuk 4** onderzochten wij of de focale (chorio) retinale laesies bij patiënten met intra-oculaire rubellavirus infectie verschillen van de laesies bij patiënten met een intra-oculaire *T. gondii* infectie. Fundusfoto's en fluorescentie angiogrammen werden geanonimiseerd en gemaskeerd voor het veroorzakende pathogeen en geëvalueerd door vier experts op het gebied van OT. Er werden geen verschillen gevonden tussen de retinale laesies bij rubellavirus-positieve en rubellavirus-negatieve patiënten. In 55% van de rubellavirus-positieve patiënten en in 88% van de *T. gondii*-positieve patiënten vonden ten minste drie van de vier experts de retinale afwijkingen lijken op de laesies die gezien worden bij OT. Twee experts vonden de retinale laesies in *T. gondii*-positieve patiënten vaker "consistent met het beeld van oculaire toxoplasmose". Onze conclusie was dat de chorioretinale laesies bij patiënten met intra-oculaire rubellavirus infectie niet anders waren dan de laesies van patiënten met OT en dat de etiologische diagnose van deze laesies dus niet enkel op klinische gronden kan worden gemaakt. We vonden een hoge seroprevalentie

voor *T. gondii* in de rubellavirus-geassocieerde FHUS patiënten. Echter, patiënten met FHUS presenteerden zich nooit met actieve laesies en vertoonden nooit symptomen die passen bij het klinisch beeld van OT. Ook hebben wij een *T. gondii*-seronegatieve RV-FHUS patiënt gevonden, die chorioretinale littekens had. Meer onderzoek is nodig om op te helderen wat de oorzaak van deze laesies bij patiënten met FHUS is.

In **hoofdstuk 5** onderzochten wij of *Toxocara canis* een rol speelt bij uveïtis posterior waarvan de oorzaak onbekend is, door middel van GWC bepaling bij 37 volwassenen en 12 kinderen. Geen van de 37 volwassenen had een positieve GWC, terwijl drie van de twaalf kinderen wel intra-oculaire antilichaam productie tegen *Toxocara canis* vertoonden. Deze drie kinderen hadden lage of niet-gedetecteerde serum titers, en de intra-oculaire antilichaam titers waren consequent hoger dan de titers in het perifere bloed. Eén kind had vitritis, een ander presenteerde zich met een laaggradige uveïtis en een perifere retinale laesie en het derde kind had uveïtis posterior en een chorioretinaal litteken. Oculaire toxocariasis komt blijkbaar voornamelijk bij kinderen voor. Onze bevindingen onderstrepen dat alleen serologische screening niet voldoende is voor de diagnose van oculaire toxocariasis en dat GWC analyse zeer waardevol kan zijn bij de diagnose van (jonge) patiënten met posterieure focale laesies of vitritis waarvan de oorzaak onbekend is.

In **hoofdstuk 6** beschreven wij een volwassen patiënt met een afname van de visus van het rechter oog. Bij oogheelkundig onderzoek had hij cellen en troebelingen in het glasvocht en een wit infiltraat in de achterste pool van de retina. Ondanks negatieve serologie had deze patiënt intra-oculaire antilichaam productie tegen *Toxocara canis*. Na behandeling met antihelmintica werd het infiltraat kleiner. Hoewel oculaire toxocariasis voornamelijk bij kinderen voorkomt (hoofdstuk 5), moet men erop bedacht zijn dat het ook volwassenen kan treffen. Het is belangrijk om het oogvocht te analyseren op antilichaam productie tegen *Toxocara canis*, ook wanneer *Toxocara* serologie negatief is, omdat een vroege diagnose en interventie de prognose verbeteren.

In **hoofdstuk 7** analyseerden wij 139 oogvocht materialen van patiënten die

verdacht zijn voor infectieuze uveïtis, voor een groot aantal pathogenen met behulp van PCR. Alle patiënten waren negatief in het oogvocht voor de meest voorkomende veroorzakers HSV, VZV, cytomegalovirus (CMV) en *T. gondii*. We vonden positieve PCR resultaten in zeven patiënten: één was positief voor Epstein-Barr virus (EBV), één voor rubellavirus, één voor humaan herpesvirus 6 en vier patiënten waren positief voor humaan parechovirus. De laatste observatie is met name interessant, omdat infecties met humaan parechovirus vooral tijdens de kinderjaren voorkomen, terwijl de vier patiënten in onze studie volwassenen waren. Eén patiënt was immuungecompromitteerd en werd verdacht van oculaire syfilis. De andere drie patiënten hadden allemaal UA met betrokkenheid van de cornea en cellen in de voorste oogkamer. Wij concludeerden dat humaan parechovirus een nieuwe veroorzaker van infectieuze uveïtis zou kunnen zijn.

Net als *T. gondii*, HSV en rubellavirus, is het mogelijk dat andere virale kinderziekten ook uveïtis kunnen induceren. Om deze hypothese te toetsen hebben wij parvovirus B19, mazelen- en bofvirus uitgekozen als de meest aannemelijke kandidaten en met behulp van GWC analyse bepaald of deze virussen geassocieerd zijn met UA (**hoofdstuk 8**) of met intermediaire uveïtis, neuroretinitis en niet door *T. gondii* veroorzaakte focale chorioretintitis (**hoofdstuk 9**). Ook onderzochten we of CMV een veroorzaker van UA zou kunnen zijn (**hoofdstuk 8**). We vonden twee patiënten met onverklaarde UA en positieve GWC's voor CMV, en één patiënt die positief was voor parvovirus B19. Intra-oculaire antilichaam productie tegen mazelen- en bofvirus werd niet gedetecteerd. Geen van de patiënten met intermediaire uveïtis of uveïtis posterior had een positieve GWC voor de onderzochte virussen. Deze resultaten suggereerden, dat CMV en Parvovirus B19 mogelijk geassocieerd zijn met UA. We stellen voor om in patiënten met onverklaarde UA het oogvocht ook te analyseren voor deze pathogenen. Er is geen bewijs gevonden dat parvovirus B19, mazelen- en bofvirus betrokken zijn in de pathogenese van intermediaire uveïtis, neuroretinitis en focale chorioretinitis.

Omdat uveïtis is een intra-oculair ontstekingsproces is, verwachtten wij dat oogvochten van uveïtis patiënten ontstekingsmediatoren, zoals cytokinen en chemokinen, bevatten. Om de cytokinen en chemokinen te identificeren die mogelijk een rol spelen in de immuunpathogenese van drie belangrijke typen

infectieuze uveïtis, hebben wij met behulp van een multiplex immunoassay, gelijktijdig afgenomen serum en oogvocht paren van 18 patiënten met rubellavirus-geassocieerde Fuchs Uveïtis Syndroom (RV-FHUS), van 20 patiënten met OT, en van 19 patiënten met acute retinale necrose (ARN) (**hoofdstuk 10**) geanalyseerd. De resultaten lieten bij RV-FHUS en OT een overeenkomstig patroon van cytokinen en chemokinen productie zien, dat duidelijk verschillend was van dat bij ARN. ARN patiënten produceerden meer ontstekingsmediatoren en tot hogere concentraties dan RV-FHUS en OT patiënten. IL-12 niveaus waren significant hoger in de RV-FHUS en OT patiënten vergeleken met de ARN en de controle patiënten. Aan de andere kant, waren IL-10 en IL-18 concentraties significant hoger in ARN vergeleken met RV-FHUS, OT en de controles. IFNγ was verhoogd in de ARN patiënten. In geen van de patiënten kon een correlatie worden gevonden tussen cytokinen productie en het interval tussen de aanvang van de symptomen en het afnemen van het oogvocht. Er kon ook geen correlatie worden gevonden tussen het gebruik van corticosteroïden en cytokine productie. Wij concludeerden dat de verschillen in cytokinen en chemokinen expressie tussen RV-FHUS en OT en ARN, mogelijk gerelateerd zijn met ziekteactiviteit en de ernst van het klinische ziektebeeld. Geen eenduidig T helper-pad kon worden aangetoond voor de uveïtis entiteiten. Zowel Th1 als Th2 cytokinen en chemokinen lijken een rol te spelen.

In **hoofdstuk 11** analyseerden wij serum en intra-oculaire vascular endothelial growth factor (VEGF) levels in 17 patiënten met ARN en 16 patiënten met OT met behulp van een immunoassay, om te bepalen of de omvang en ernst van de retinale ontsteking gerelateerd is aan het intra-oculaire VEGF. Wij vonden dat intra-oculaire VEGF concentraties in patiënten met ARN hoger was dan in OT patiënten, terwijl de VEGF niveaus in de sera niet verschilden. Intra-oculaire VEGF concentraties overschreden die van serum in 47% van de patiënten met ARN vergeleken met 6% van de OT patiënten. Verder vonden we dat de patiënten met intra-oculaire VEGF niveaus hoger dan in het serum een uitgebreidere retinitis hadden en vaker een verminderde visus bij de drie maanden follow-up (P<0.001 en P=0.031, respectievelijk).

Wij concludeerden dat hoge intra-oculaire VEGF productie in patiënten met ARN geassocieerd is met uitgebreide retinitis en een slechte visuele prognose. Hoge lokale VEGF expressie in ARN zou een belangrijk gegeven kunnen zijn voor de toekomstige behandeling van de patiënten met ARN.

In **hoofdstuk 12** beschreven wij een pilot-studie waarin we specifieke eiwitprofielen willen genereren met behulp van Surface enhanced laser desorption/ionization time-of-flight (Seldi-tof) in de glasvochten van vijf patiënten met acute postoperatieve endophthalmitis. Drie van deze vijf patiënten hadden een positieve kweekuitslag voor staphylococcen en de andere twee patiënten een negatieve. Als controles includeerden wij drie glasvochten van patiënten met een maculagat, zonder ontsteking. Onze data lieten zien, dat de patiënten met endophthalmitis andere eiwitprofielen hebben dan de controles. Ook analyseerden wij met Seldi-tof analyse, gepaarde voorste oogkamervocht en serum samples van patiënten met intra-oculaire antilichaam productie tegen HSV (n=10) en *T. gondii* (n=8). Als controles includeerden wij patiënten met ouderdoms staar (n=7). Deze experimenten lieten een piek zien in het voorste oogkamervocht, die specifiek leek te zijn voor *T. gondii* en niet gevonden werd in sera. Deze data wijzen erop dat oogvochten geschikt zijn voor Seldi-tof analyse en dat deze techniek mogelijk gebruikt zou kunnen worden voor de detectie van specifieke intra-oculaire biomarkers. Verder onderzoek nodig is om de relevantie van onze observaties te bepalen.

Conclusies

In dit proefschrift laten wij zien dat rubellavirus geassocieerd is met het Fuchs Heterochrome Uveïtis Sydroom (FHUS). De meerderheid van onze patiënten met het klinische beeld van FHUS hadden intra-oculaire antilichaam productie tegen rubellavirus. Daarnaast vertoonden de patiënten met rubellavirus-geassocieerde uveïtis een duidelijk klinisch spectrum van oculaire symptomen overeenkomend met de symptomen van patiënten met FHUS. Echter, omdat we ook de vier klassieke klinische criteria van FHUS (karakteristieke descemet stippen, diffuse iris atrofie en/of heterochromie, afwezigheid van synechiae posteriores en cataract) vonden in patiënten die geen intra-oculaire antilichaam productie tegen rubellavirus hadden, zouden er andere veroorzakers van FHUS kunnen bestaan. Zo is recent CMV geassocieerd met FHUS. Het is waarschijnlijk dat FHUS een klinisch syndroom is dat verschillende oorzaken kan hebben en mogelijk worden in de toekomst nog andere, met FHUS geassocieerde pathogenen geïdentificeerd. In het verleden zijn veel verschillende namen gebruikt om Fuchs Heterochrome Uveïtis Syndroom (FHUS) te beschrijven, waaronder Fuchs' heterochrome (irido) cyclitis, Fuchs' uveïtis anterior en Fuchs' heterochrome uveïtis. Dit verklaart de verschillende benamingen in de hoofdstukken van dit proefschrift. Onlangs is echter besloten door de International Uveitis Study Group om deze klinische aandoening 'Fuchs Heterochrome Uveïtis Syndroom' te noemen. Wanneer het wordt veroorzaakt door een geïdentificeerd pathogeen noemen we het bijvoorbeeld rubellavirus-geassocieerd Fuchs Heterochrome Uveïtis Syndroom of CMV-geassocieerd Fuchs Heterochrome Uveïtis Syndroom.

De aanwezigheid van toxoplasmose-achtige chorioretinale laesies in patiënten met rubellavirus-geassocieerde Fuchs uveïtis syndroom (RV-FHUS) is opvallend. Onze studie liet duidelijk zien dat de diagnose niet gemaakt kan worden enkel op klinische gronden en dat oogvocht analyse nodig is om de definitieve diagnose te stellen. De vraag blijft welk pathogeen deze chorioretinale littekens veroorzaakt in patiënten met RV-FHUS.

In dit proefschrift onderzochten wij de oogvochten van patiënten met uveïtis waarvan de oorzaak onbekend is, met behulp van een PCR voor een groot aantal pathogenen. Een andere groep patiënten werd geanalyseerd met behulp

van GWC voor CMV en voor virussen die vaakvoorkomende virale kinderziekten veroorzaken, zoals parvovirus B19, mazelen- en bofvirus. Wij vonden positieve resultaten voor humaan parechovirus, humaan herpesvirus 6, parvovirus B19 en CMV en concludeerden dat deze virussen mogelijk geassocieerd zijn met infectieuze uveïtis. Verder onderzoek naar de rol van humaan parechovirus en humaan herpesvirus 6 zal moeten worden uitgevoerd om te bepalen of deze virussen echte veroorzakers zijn van infectieuze uveïtis. Daarnaast kunnen we, gezien het lage aantal patiënten dat getest is, voor parvovirus B19, mazelen- en bofvirus niet bewijzen dat deze virussen al dan niet betrokken zijn bij de pathogenese van uveïtis. Het zou interessant zijn om grote aantallen patiënten met uveïtis van onbekende oorzaak te analyseren voor deze virussen en ook PCR bepalingen toe te passen in deze studies.

Onze studies lieten zien dat GWC analyse voor oculaire toxocariasis waardevol is bij het diagnosticeren van patiënten met posterieure focale laesies of vitritis waarvan de oorzaak onbekend is, voornamelijk bij kinderen. Echter, het moet benadrukt worden dat oculaire toxocariasis ook kan voorkomen bij volwassenen. Het is bovendien belangrijk te realiseren dat oogvocht analyse essentieel is voor de diagnose, omdat serologische screening voor *Toxocara canis* niet informatief is en serologie vals-negatief kan zijn. In toekomstige studies zou het interessant zijn om de rol van andere nematodes, zoals *Ascaris lumbricoides*, in infectieuze uveïtis te onderzoeken.

In een uitvoerige studie naar de rol van ontstekingsmediatoren in infectieuze uveïtis, zagen wij dat de oogvochten van patiënten met RV-FHUS en OT een overeenkomstig patroon van cytokine en chemokine productie laten zien, anders dan dat van ARN. Oogvochten van patiënten met ARN toonden hogere productie van cytokinen en chemokinen, wat mogelijk correleert met een hogere activiteit van de ziekte en een ernstiger ziektebeeld. Een overduidelijk Th1- of Th2-geassocieerd profiel kon niet worden aangetoond. Daarnaast lijken VEGF levels, welke een cruciale rol spelen in de intra-oculaire ischemische processen en de daaropvolgende ontwikkeling van neovascularisaties, significant hoger te zijn in de oogvochten van patiënten met ARN in vergelijking met OT. Analyse van een groter aantal patiënten in vergelijking met een niet-infectieuze uveïtis, liefst in

een homogene controle groep, zou mogelijk meer inzicht kunnen geven in de immuunpathogenese van ARN, RV-FHUS en OT. Ook zou het waardevol zijn om te bepalen of anti-VEGF behandeling een bijdrage zou kunnen leveren aan een betere prognose van ARN.

De bevindingen beschreven in dit proefschrift zijn waardevol voor de snelle diagnostiek van infectieuze uveïtis en hebben het spectrum van veroorzakende pathogenen betrokken bij infectieuze uveïtis uitgebreid. Analyse van oogvochten, met in het bijzonder de combinatie van detectie van intra-oculaire antilichaam productie en PCR, is waardevol in de diagnose van patiënten met infectieuze uveïtis. Hiermee kan snel onderscheid gemaakt worden tussen een infectieuze en niet-infectieuze oorzaak, wat belangrijke consequenties kan hebben voor de behandeling en de prognose van de patiënt. De beschreven uitbreiding van het diagnostische repertoire en de nieuwe inzichten in de (immuun-)pathogenese vergroten onze kennis op het gebied van infectieuze uveïtis, een aandoening die blindheid kan veroorzaken en -indien de diagnose bekend is- vaak behandelbaar is.

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Curriculum Vitae

De auteur van dit proefschrift werd geboren op 17 november 1978 te Vught, Noord-Brabant. In 1998 behaalde zij haar VWO-diploma aan het Sint-Janslyceum te 's-Hertogenbosch. Zij startte haar opleiding geneeskunde in 1998 aan de Universiteit Utrecht en behaalde haar doctoraal en artsexamen in respectievelijk 2002 en 2005. Van juli 2005 tot februari 2006 werkte zij als officier-arts bij de Koninklijke Marine. Vanaf maart 2006 werkte zij als arts-onderzoeker aan haar proefschrift op de afdelingen Virologie en Oogheelkunde van het UMC Utrecht, onder begeleiding van Prof. dr. A. Rothova en dr. J.D.F. de Groot-Mijnes. Voor het werk beschreven in hoofdstuk 4 werd zij op de 10th Congress of Intraocular Inflammation Society 2009 in Praag beloond met de prijs voor Best Oral Presentation. Vanaf december 2009 zal zij als arts in opleiding tot specialist werkzaam zijn op de afdeling Oogheelkunde van het UMC Utrecht.