Correlation Between Disease Severity and Presence of Ocular Autoantibodies in Juvenile Idiopathic Arthritis-Associated Uveitis

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PURPOSE. The pathogenesis of juvenile idiopathic arthritis-associated uveitis (JIAU) is undefined. This study intended to analyze the presence of antiocular autoantibodies in serum and their correlation with disease course.

METHODS. Serum samples from children with JIAU (n = 47); JIA without uveitis (n = 67); idiopathic anterior uveitis (IAU; n = 12); and healthy controls (n = 52) were collected. The binding patterns of serum antibodies to ocular cryosections from swine eyes were analyzed by indirect immunohistochemistry, and were correlated to epidemiological, clinical, and laboratory test results.

RESULTS. The patient groups differed with respect to their presence of antibody binding to the sections: JIAU (94%), JIA (75%), IAU (75%), and healthy controls (29%) to uveal and/or retinal structures. Serum antibodies of JIAU patients predominantly bound at iris (74%), and ciliary body (79%). Iris/ciliary body positive staining correlated with the presence of uveitis complications (P < 0.005) in JIAU patients, but not with positivity of serum antinuclear antibodies (ANA), rheumatoid factor (RF), or HLA-B27, and was independent from uveitis activity or type of anti-inflammatory therapy.

CONCLUSIONS. In JIAU patients, antiocular serum antibodies can be detected more frequently than in control groups. Binding patterns to ocular tissue correlate with complicated uveitis course but not with uveitis activity and anti-inflammatory treatment. Antibody binding is not specific for this uveitis entity, and does not correlate with ANA positivity.

Keywords: antinuclear antibodies, autoantibodies, juvenile idiopathic arthritis, pathogenesis, uveitis

Juvenile idiopathic arthritis (JIA) is the most common disease associated with uveitis of childhood.¹ Consisting of seven subgroups, JIA summarizes a heterogeneous group of chronic arthritis diseases with onset before the age of 16 years.² In population-based studies, uveitis occurred in 9% to 12% of JIA patients,³ with a predominance in children with oligoarthritis.⁴ As JIAU often runs a severe course with the development of sight-threatening complication, classical immunosuppressive drugs or biologics are frequently required.

The pathogenesis of JIAU is still undefined. In the few histopathological studies of eyes with end-stage disease, which were enucleated because of complications, a nongranulomatous inflammation was found mainly in the iris and ciliary body. The inflammatory infiltrate consisted mainly of plasma cells and B cells, and only a few T cells and monocytes were found.⁵,⁶ There is profound evidence that the presence of serum antinuclear antibodies (ANA) is a risk factor for the development of uveitis.⁵,⁷ Some studies have shown that autoantibodies directed against nuclear histone and nonhistone proteins, or against a low-molecular bovine iris antigen or soluble retinal antigen (S-Ag)⁷–¹⁰ correlated with uveitis occurrence. However, their pathogenetic role is unclear.

In two previous studies, the binding of serum autoantibodies to eye tissues was investigated. Herein, the antibody binding to eye sections was more frequent in patients with juvenile arthritis-associated uveitis than in others without uveitis, or in healthy controls.¹¹,¹² The binding sites were particularly the iris and ciliary body, which are the primary clinical sites of uveitis. One study found a positive staining to eye tissue only with serum which was gained during the active ocular inflammation.¹¹

The present study was undertaken to investigate whether binding of autoantibodies to eye sections is specific for JIAU and ANA positivity. We therefore compared serum samples from patients with JIAU-associated uveitis, idiopathic anterior uveitis, and healthy controls. The binding pattern was correlated with uveitis complications and activity, laboratory test results, and anti-inflammatory treatment.

METHODS

Patients
Children with JIAU were recruited during 2010 in the Department of Ophthalmology at St. Franziskus Hospital.
Muenster. All JIA patients fulfilled the classification of juvenile idiopathic arthritis (JIA) in agreement with the International League of Associations for Rheumatology (ILAR) criteria.13 Our JIA control group consisted of children with established diagnosis of JIA who underwent repeated full eye examinations for screening of uveitis.5 Due to the long follow-up after JIA onset (median 4 years), there was a low probability of missing the uveitis manifestation. Additional control groups consisted of patients with idiopathic anterior uveitis, or healthy volunteers. Written consent of patients and parents was obtained. The study was performed according to the tenets of the Declaration of Helsinki, and was approved by the local ethics committee.

Rheumatologic examinations and diagnostic procedures included a review of systems, laboratory tests (e.g., antinuclear antibodies, rheumatoid factor, and HLA-B27), urine analysis and chest x-ray, if necessary. An infectious etiology was excluded if serological tests for Epstein-Barr virus, cytomegalovirus, herpes simplex virus, herpes zoster virus, treponema, and tuberculosis skin test were negative.

A standardized ophthalmic database was applied for the analysis. The epidemiological patient characteristics were collected. Uveitis was defined and anatomically classified according to the Standardization of Uveitis Nomenclature Working Group recommendations.14 The ophthalmic assessment included best-corrected visual acuity (BCVA), slit-lamp examination, tonometry (Goldmann), and funduscopy. Laser flare values were measured using a KOWA FM-500 device.15 The presence of ≥1+ cells in the anterior chamber was defined as active uveitis. Any topical and systemic anti-inflammatory treatments and uveitis-related complications (e.g., cataract, ocular hypertension, glaucoma, synechiae formation, central band-keratopathy, vitreous opacities, macular edema or macular atrophy) were documented.

Serum Sample Collection and Immunohistochemical Staining Procedure

A peripheral venous blood sample was collected from each patient, centrifuged at 700×g for 10 minutes, and serum aliquots were stored at −80°C until examination.

Swine eyes from healthy animals (aged 3–6 months) were enucleated, immediately frozen in liquid nitrogen, and embedded in optimum cutting temperature compound. Cryostat sections (7 μm) were mounted on poly-Lysin-coated slides and stored at −20°C until usage. Tissue sections were dried at room temperature (RT) for 30 minutes, and fixed in acetone. Unspecific binding sites were blocked by incubating slides for 20 minutes with 5% rabbit serum, 1% bovine serum albumin, and 1% Fc-blocking reagent (Miltenyi Biotec; Bergisch-Gladbach, Germany) in PBS with 1% fetal calf serum (FCS). After incubation with patient’s or control’s serum at 1:100 or 1:200 in PBS or PBS as a negative control in a moist chamber for 30 minutes, the sections were washed with PBS/1% FCS, incubated with H2O2 3% for 5 minutes, washed again, and were incubated with horseradish peroxidase (HRP)-conjugated rabbit-anti-human-IgG-antibody (Dako Germany, Hamburg, Germany) diluted 1:100 in PBS/1% FCS with 5% swine serum for 20 minutes. After washing, slides were incubated for 15 minutes with 3-amino-9-ethylcarbazol diluted in acetate buffer, previously activated with H2O2, fixed with acetate buffer and 4% formaldehyde for 10 minutes, and rinsed in acetic acid (1%). Nuclei were stained with hematoxilin solution, Gill No. 3 (Sigma-Aldrich, Taulkirkchen, Germany). Finally, tissue sections were embedded in Aquatex (Merck, Darmstadt, Germany), covered with cover slides, and dried at RT.

Presence of positive staining on the eye sections was analyzed independently by two observers in masked fashion. Staining patterns were classified as nuclear or nonnuclear. No quantification of tissue staining was attempted.

Statistics

Statistical significance of patient’s characteristics and clinical data was analyzed by Student’s t-test, Mann-Whitney U test, χ2 test, one-way ANOVA, and Fisher’s exact test as appropriate. Normal distribution of data was tested by Kolmogorov-Smirnov test. Statistical correlation between staining and clinical data was analyzed by Cramer’s V and point-biserial correlation. Influence of clinical parameters on staining pattern and vice versa was tested by linear regression analysis (stepwise). For analysis, we used statistical software (SPSS PASWStatistics version 20.0; SPSS, Inc., Chicago, IL, USA). P < 0.05 was counted as statistically significant.

RESULTS

Patient Groups and Characteristics

The study included patients groups with JIAU (n = 47), JIA without uveitis (n = 67), idiopathic anterior uveitis (IAU; n = 12), and 52 children without rheumatic or ocular diseases. The patient characteristics are summarized in Table 1. Patients with JIA, JIAU, and IAU had similar age. The JIA children with associated uveitis were younger at arthritis onset than those without uveitis (P < 0.0001), were more often ANA positive (P < 0.0001), and suffered more often from oligoarthritis (P < 0.0001; Table 1).

There were no significant differences between the uveitis groups respective the frequency of ocular complications, patients with active uveitis, bilateral uveitis, previous eye surgery, and BCVA. Children with JIAU were more often treated with systemic anti-inflammatory therapy than IAU patients (P = 0.036; Table 1).

Staining Patterns at Uvea and Retina

Anti-ocular staining patterns were similar in all groups. Compared with the negative control specimen (Figs. A–C), the positively stained uveal tissue showed an epithelial, vascular/perivascular, and stromal staining. The staining was cellular and extracellular. The staining at the iris was detected both at the pigmented and nonpigmented epithelium, and at the stroma (Figs. D, G). The staining at the ciliary body had a similar distribution (Figs. E, H). The choroid had predominantly a vascular staining pattern (Figs. F, I).

Two patterns of retinal staining were detected: first, a cellular staining of cells within the inner retinal layer, while the remaining retina was mainly unstained (Fig. F); second, a cellular and diffuse staining of all layers of the retina (Fig. I).

These above-mentioned general staining patterns were found in all groups of patients as well as in the healthy control group. No distinct staining pattern was found in a particular patient group. Mostly, the staining was not restrained to the nucleus, and this finding was independent from ANA positivity of the sera. Only 17 patients had a predominantly nuclear staining pattern, and this was present at all of the intraocular anatomical regions. Sixteen of them were ANA positive. 11 belonged to the group of JIA-associated uveitis, five to the group of JIA patients without uveitis, and one to the IAU group.
Positive Tissue Staining in Different Patient Groups

We did not find specific staining patterns for each patient group, but we found significant differences between patient groups concerning the frequency of positive staining. Antibody binding was most frequent in the JIAU group. Patients with JIAU (94%), JIA (75%), or IAU (75%) significantly more often showed antibody binding to the ocular tissues than healthy controls (29%; Table 2).

In the patient groups with JIAU, JIA and IAU, a positive staining was found more often at the iris and ciliary body— which are the predominant anatomical sites of inflammation in anterior uveitis—as compared with the choroid. However, positive staining of the retina was also common (Table 2).

The frequency of positive staining at the uveal target tissues in the patient group with JIAU was approximately doubling that in JIA cases without uveitis. Notably, the binding of serum antibody to iris or ciliary body was more common than to choroid (Table 2). Although retinal staining was also slightly more frequent in JIAU than in JIA patients, the difference between these two groups was not significant (Table 2). When comparing only the children with oligoarthritis, who are at particular risk for uveitis development, similar proportions of staining were found (data not shown).

By univariate analysis, the percentages of and the probability for positive tissue staining at iris, ciliary body, and choroid were higher in the group of JIAU as compared with the IAU group (Table 3). The binding frequency to iris and ciliary body in the IAU group was slightly higher than in the group of JIA patients without uveitis. Taken together, the frequency of antibody binding to ocular sections compared with the reference group of healthy controls was moderately increased in IAU and JIA cases, and was the highest in patients with JIAU.

Correlation of Positive Staining With Uveitis Course

There was a statistically significant correlation between occurrence of antibodies directed against iris, ciliary body, and/or retina, and the prevalence of ocular complications in children with JIAU (all \( P < 0.05 \)), and between the prevalence of higher laser flare values (>20 photon units/ms) and antibodies directed against ciliary body in JIAU patients (\( P = 0.009 \)). No correlation was found between antibody binding against one or each of the anatomic regions and patient age at serum collection, patient age at uveitis or arthritis onset, sex, ANA positivity, RF positivity, HLA-B27 positivity, JIA subtype, anti-inflammatory treatment, prior eye surgery, clinical uveitis activity (≥1+ grade anterior chamber cells), uni- or bilateral, or BCVA. Only the IUAU group showed a statistically significant negative correlation between intake of systemic immunosuppressive medication and antibody binding to choroid (\( P = 0.028 \)).

Using linear regression analysis, the only factor associated independently with the presence of antibodies directed against ciliary body was the presence of ocular complications in children with JIAU, even when adjusted for known risk factors for presence of complications, as age, sex, age at diagnosis of uveitis or arthritis, uveitis activity, ANA, RF or detection of HLA-B27 positivity (regression coefficient \( \beta = 0.6; 95\% \) confidence interval [CI] 0.29–0.87, \( P < 0.001 \)).

Discussion

The pathogenesis of JIAU is still unclear. The few previous studies found a nongranulomatous uveal inflammation, with an inflammatory cell infiltration mainly consisting of B cells. JIAU is commonly occurring in serum ANA-positive preschoolers.
Raised ANA levels have been found in the aqueous humor of children with JIA uveitis, even in some patients without systemic ANA detection, suggesting that local antibody production is possible.\textsuperscript{16}

Previous attempts to define the role of ANA positivity and B cells have not been successful. Several studies were performed in order to identify the likely targets of antibodies in arthritic children with or without uveitis, demonstrating reactivity against nucleosomes, histone or nonhistone proteins, high mobility group proteins, as well as against several nuclear proteins.\textsuperscript{8,10,17–20}

The presence of specific antiocular serum antibodies has been investigated in patients with JIAU. Herein, serum antibodies directed against a low molecular weight antigen extracted from bovine iris or against lens crystallins, and against retinal S-antigen have been described\textsuperscript{9,21,22}; the latter is being used for induction of uveitis in several animal models.

The binding of serum antibodies to ocular tissues has been investigated previously by means of immunofluorescence staining. Uchiyama et al.\textsuperscript{11} tested serum samples from 12 ANA-positive children with JIA-associated uveitis as well as from children with JIA only and from healthy controls for their binding to human donor eye sections.\textsuperscript{11} Of healthy children, 25% had antibodies binding to iris, ciliary body, and retina, whereas in JIA-associated uveitis, binding was detected in approximately two-thirds, and in JIA without uveitis in approximately one-third of patients. Bloom et al.\textsuperscript{12} detected antiocular binding of serum antibodies at basement membrane

### Table 2. Frequency of Positive Staining of Serum Antibodies at Different Anatomical Regions of the Eye Sections

<table>
<thead>
<tr>
<th>Positive Staining</th>
<th>Healthy Controls, n = 52</th>
<th>JIAU, n = 47</th>
<th>IAU, n = 12</th>
<th>JIA, n = 67</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uveal and/or retinal tissue, n (%)</td>
<td>13 (29)</td>
<td>44 (94)</td>
<td>9 (75)</td>
<td>50 (75)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Iris, n (%)</td>
<td>4 (8)</td>
<td>35 (74)</td>
<td>7 (58)</td>
<td>24 (36)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Ciliary body, n (%)</td>
<td>4 (8)</td>
<td>37 (79)</td>
<td>5 (42)</td>
<td>23 (34)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Choroid, n (%)</td>
<td>0 (0)</td>
<td>18 (38)</td>
<td>2 (17)</td>
<td>11 (16)</td>
<td>0.023</td>
</tr>
<tr>
<td>Retina, n (%)</td>
<td>12 (23)</td>
<td>42 (89)</td>
<td>7 (58)</td>
<td>48 (72)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Number of patients (%).
of iris and ciliary body epithelium, Bruch's membrane of the retina, lens epithelium, lens fibers, and in blood vessels of iris and retina. Antibody binding was detected in 82% of patients with JIA uveitis, 30% of patients with JIA without uveitis and none of the healthy controls.

We now tested the sera of a large number of JIAU patients for antibodies binding to eye tissue. In contrast to the former studies, we also analyzed the correlation between occurrence of antibodies and clinical course of uveitis. We additionally tested sera from patients with IAU to obtain information about disease specificity of antibody binding. Sera from children with JIA without ocular involvement as well as healthy individuals served as controls.

The clinical characteristics from our JIAU patients are typical for this entity with respect to female predominance, bilaterality, chronic course of insidious onset anterior uveitis, young age at onset of arthritis, and oligoarthritis subgroup. Also, the high degree of ANA positivity (96%), as well as the low frequencies of HLA-B27 and RF positivity, are characteristic for this JIA patient group. As to selection bias to more severe disease courses at our tertiary care center, the complication rate of both uveitis groups was high as compared with population-based numbers for JIAU. The frequent need of systemic immunosuppression is in accordance with recently published observations.

Our data now support the notion that antiocular antibodies can be detected in JIA patients. Accordingly, binding was more frequent in JIA patients with uveitis than without. However, antiocular serum antibodies are also present in healthy controls, which documents that the sole presence of such antibodies is not an independent prerequisite to uveitis development. Kubicka-Trzaska et al. described similar binding patterns and frequencies of sera from healthy individuals on retinal tissue compared with sera of patients with age-related macular degeneration. This binding was related to the presence of natural autoantibodies. Why children with JIA without ocular inflammation display such high frequencies of antiocular antibody binding remains unclear. Bloom et al. suggested that those children might be at risk to develop inflammatory eye disease over time. Considering the relatively late onset of arthritis (mean age at onset 8.1 years) in our JIA control group, and a mean arthritis duration of 5.1 years at the time of blood sample collection, the risk for these patients for developing uveitis is rather low according to epidemiological studies. Nevertheless, we cannot exclude this possibility entirely.

Our further investigations showed that a high proportion of patients with idiopathic chronic anterior uveitis also present with antiocular serum antibodies, which suggests that they may somehow be involved in the pathogenesis of intraocular inflammation, and that they are not specific for JIA.

As to the statistical significance of those differences in antibody detection between several groups of patients and a control group, the problem of multiple comparisons increasing the per-experiment Type I error rate must to be considered.

Nevertheless, we assess the experimental evidence for differences between patients and controls as distinct.

We now for the first time also investigated the association between antibody binding profiles and other laboratory test results and the clinical course of uveitis. The positivity of ANA, RF, or HLA-B27 did not correlate with antibody binding in our study, no differences in the antibody binding frequency were seen between clinically active and inactive stages of disease, which is in accordance with the findings by Bloom and colleagues. In our study, systemic immunosuppressive treatment had no significant influence on antibody binding. As arthritis activity was not documented in our JIA patients, its impact on antibody production is undefined.

Interestingly, there was a significant correlation between antibody binding to iris and ciliary body and the presence of ocular complications. As those anatomic regions are the primary sites of inflammation in anterior uveitis, this finding may be of particular relevance. Since no time course of antibody binding is available, we cannot determine whether presence of antibodies is a result or cause of the tissue damage.

Considering the high frequency of antibodies directed against retinal tissue, we hypothesized that this might correlate with occurrence of papillary or macular leakage, which is a frequent problem in juvenile uveitis. However, the relationship between antibodies directed against retinal S-antigen, which have been described in uveitis patients earlier, and the high frequency of retinal staining in our study is curious, as the cellular staining of cells within the inner retinal layer that we observed is not congruent with the physiological localization of retinal S-antigen, which is within the photoreceptor layer.

Interestingly, no differences in staining patterns between patient groups and healthy controls were noted, confirming the previous observations. Sixteen of our 17 patients, who had a predominantly nuclear staining pattern, were ANA positive, 11 belonged to the group of JIAU. However, the staining of tissue sections was commonly nonnuclear, which is also in accordance with the previous findings, and antibody detection did not correlate with ANA positivity. Therefore, it is tempting to speculate that antibodies might not primarily belong to the group of well-known ANA, but probably represent a separate subgroup of autoantibodies yet to be characterized.

Antibodies might be directed against a mutual autoantigen expressed in the eye as well as in the joint, explaining the high rate of antiocular antibody-positive children with JIA only. Collagen type II seemed one likely antigenic structure, as it is a well-characterized autoantigen in human arthritis as well as in several animal models of experimental arthritis. We observed an extracellular staining, but also a cellular staining. However, collagen type II did not induce any cellular or humoral immune response as well as trigger inflammation similar to mechanisms described before in human arthritis.
Autoantibodies in JIAU

This mechanism could possibly also be relevant to the course of disease in children with IAU, who display a high proportion of autoantibody development as well. These issues must be investigated in the future with other proteomic and genomic research tools.

It might be speculated that our observations in the immunohistochemical analysis are hampered by the use of swine eye sections instead of the human donor eye tissue, which was available in the previous two studies. First, the staining patterns that we observed are in conformity with the previous observations with human tissue. And second, donor eyes are nowadays inaccessible immediately postmortem due to current legality issues. Therefore, tissue degradation processes in human donor eyes available after prolonged preservation time are inevitable, and negatively influence the tissue integrity and subsequent access of autoantigens by autoantibodies. Indeed, heterologous target tissue is used routinely for the detection of diverse autoantigens as the autoantibodies. Indeed, heterologous target tissue is used for the detection of diverse autoantigens as the autoantibodies.

Given the high frequency of antiocular antibodies in children with chronic severe uveitis course, determination of the antiocular antibody status in children with early onset JIA and uveitis at several time points during the course of disease should be considered in future studies in order to clarify whether antibodies develop prior to or after uveitis manifestation and uveitis-related complications.

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