Serum antibody level to S-antigen in children with chronic uveitis

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SUMMARY Bovine S-antigen was purified by gel filtration and ion exchange chromatography according to previously described techniques. An enzyme linked immunosorbent assay (ELISA) using antiserum to bovine S-antigen raised in guinea-pigs was employed to detect S-antigen in the chromatographic fractions. The purity of the S-antigen was determined by SDS-PAGE electrophoresis, where a single band was found. The purified S-antigen in microgram quantities together with Freund's complete adjuvant induced uveitis in rats two weeks after injection into the foot pad. Serum samples from children suffering from chronic uveitis and healthy children were tested for antibodies to S-antigen by the ELISA. A statistically significant difference in the level of specific antibodies between patients and controls was found. There was no clear-cut correlation between the severity of uveitis and antibody titre, but cases with retinal involvement and aggressive uveitis all showed definite elevations of antibodies to S-antigen.

The isolation of a pathogenic antigen, S-antigen, from retina[1][2] of various species, and the demonstration of uveitis in monkeys immunised with this antigen, implicate the retina as the source of a uveitogenic autoantigen.[3] Elevated titres of antibody to S-antigen have been reported in patients with uveitis[4] as well as cellular immune responses.[5] These findings suggest that retinal S-antigen may be a causative or contributing factor in human uveitis.

Chronic iridocyclitis, which may be associated with rheumatoid arthritis, is a particularly destructive disease in children, and the prognosis for vision is often uniformly bad.[6–11] Since young tissues respond to noxious stimuli more vigorously and extensively than old ones, the changes in immunological parameters may be easily observed in children.[11] The purpose of this study was to investigate antibody titres to retinal S-antigen in children suffering from chronic uveitis. We report elevated antibody titres to S-antigen in these children.

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Materials and methods

Patients. The antibody titres reported here are based on blood samples taken from 16 patients suffering from uveitis and on blood samples from 22 normal control individuals. This series of children with chronic uveitis was selected from inpatients admitted to the Department of Paediatric Ophthalmology of the Helsinki University Central Hospital. All children with uveitis had chronic inflammation in one or both eyes that caused visual impairment. The disease was almost always in the active stage (Table 1). The control group consisted of 22 children with no signs of ocular inflammation. These children were admitted to hospital for amblyopic treatment or squint surgery. All the children with uveitis and the control children were examined and followed up by R.J.U. Blood samples were drawn from the patients and the controls at the initial examination. The patients received topical steroid and mydriatic drops. In addition many of them with active disease and ocular hypotony received parabulbar steroids—triamcinolone acetonide (Kenacort-T) 5 mg—once a week on

Table 1 *Comparison of patients with high and low titres to S-antigen*

	High antibody	Low antibody
Average age	10·4 yr	11·1 yr
Average duration	1·5 yr	4·0 yr
Clinically active	8/8 cases	5/8 cases
Binocular uveitis	5/8 cases	5/8 cases
Retinal involvement	5/8 cases	1/8 cases
HLA-B27 positive	3/7 cases	1/4 cases
JRA (Still's disease)	3/8 cases	3/5 cases

several occasions. None of the children received systemic steroids.

Each patient with uveitis had a complete medical, social, and ocular history taken, and a complete eye examination with emphasis on the following parameters: visual acuity, aqueous flare, cells in the aqueous humour and/or in the vitreous, keratic precipitates (KP), band keratopathy, synechiae, oedema in the posterior segment of the eye, and hypotony. In addition all the uveitis patients included in the study were tested for complete blood count, erythrocyte sedimentation rate, serum antistreptococcal, anti-staphylococcal, and antinuclear antibodies, rheumatoid factor in serum, HLA antigens, and serologic tests for toxoplasma and yersinia antibodies. Chest, sinus, and orthopantomographic *x*-ray examinations were also performed in all patients to exclude infectious types of uveitis.

Preparation of serum samples. Serum was drawn off 10 ml of clotted blood, centrifuged at low-speed, and stored at −20°C. Before the determination of antibody titres to S-antigen the serum was heat-inactivated (56°C for 30 min). Antiserum to bovine S-antigen raised in guinea-pigs was received from Dr D Gregerson. The preparation of this has been described earlier.[4] Rabbit anti-guinea-pig IgG was purchased from Bionetics, goat anti-rabbit IgG conjugated to alkaline phosphatase from Miles-Yeda, and rabbit anti-human IgG from Boehringer.

Preparation of S-antigen. S-antigen was isolated from bovine retinas by the technique described by Wacker *et al.*[1] with the modifications described below. A 20% (w/v) suspension of bovine retina was prepared in ice-cold phosphate buffer, pH 7·2. The retina homogenate was centrifuged 80 000 *g* at 4°C for 1·5 h, and the clear supernatant containing S-antigen was harvested by aspiration. The antigen was precipitated from harvested supernatant by half-saturated ammonium sulphate and purified by gel filtration on Sephadex G-150 followed by two passes on ion exchange columns (DEAE or QAE G-50). In gel filtration the material was eluted with phosphate buffer, pH 7·2, and in ion exchange chromatography with a linear salt gradient in Tris-Cl buffer at pH 7·9.

Protein determinations of aliquots of fractions were done by absorbance spectrophotometry (280 nm), and the presence of S-antigen was determined by the ELISA (see below).

Active fractions were pooled, dialysed, and concentrated. As a final step the antigen was passed through an immunoabsorbent column of anti-bovine serum.[12] This was done to absorb any possible remnant of bovine serum from the S-antigen purified material, which could react with antibodies in human serum at a later stage.

The S-antigen, purified as described above, gave a single band of 50 000 molecular weight in SDS-polyacrylamide gel electrophoresis (SDS-PAGE). To determine the uveitogenic activity of the S-antigen, 50 µg was emulsified in Freund's complete adjuvant and injected into the hind foot pads of albino Sprague Dawley rats.[13] Autoimmune uveitis (EAU) was induced in about two weeks in these animals.

ELISA. The ELISA (enzyme-linked immunosorbent assay) was performed according to Voller *et al.*[14] with the modifications described below.

Screening of chromatographic fractions by ELISA. Each fraction to be tested for S-antigen activity was diluted 1:100 in distilled water and a 10µl aliquot was transferred into the wells of micro-ELISA plates followed by 240 µl of coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, and 0·02% NaN_3, pH 9·6). The plates were covered and incubated at 22°C for 2h. Phosphate-buffered saline-polysorbate (Tween 20) (PBS-Tween) was used to rinse the wells three times, and 250 µl of guinea-pig antiserum against bovine S-antigen (diluted at 1:1000 in PBS-Tween) was added. After incubation at 37°C for 1 h the plates were rinsed three times with PBS-Tween. Thereafter rabbit anti-guinea-pig IgG (diluted at 1:1000 in PBS-Tween) was added. After incubation at 37°C for 1 h the plates were rinsed three times with PBS-Tween and 250 µl of goat anti-rabbit IgG conjugated to alkaline phosphatase (diluted at 1:1000 in PBS-Tween) was added. After incubation for 1 h at 37°C the wells were rinsed four times with Tris-HCl, pH 7·4, followed by addition of 250 µl of substrate solution (1M Tris base, 1M NaCl, 6 mM p-nitrophenyl phosphate, 2 mM $MgCl_2$ and 0·02% NaN_3, pH 9·0). The plates were incubated at 22°C overnight and read at 410 nm spectrophotometrically.

Determination of human antibody levels to S-antigen. In order to absorb the non-specific anti-bovine activity of human sera, preincubation of the human serum with newborn calf serum, each diluted 1:25 n PBS-Tween also containing 1 mg/ml bovine serum albumin, was carried out overnight. In three controls no preincubation was carried out.

10 µl of the antigen solution containing about 50 ng

of S-antigen was dispensed into the wells of micro-ELISA plates, followed by addition of 240 µl of coating buffer (see above). The plates were covered and incubated at 22° for 2 h. The wells were then rinsed three times with PBS-Tween. Preabsorbed human sera were diluted in PBS-Tween and the diluted serum was added in 250 µl aliquots to the wells. For each dilution a duplicate measurement was performed. The serum samples were diluted 1:25, 1:50, 1:100, 1:200, 1:400, 1:800. However, since the serum was already diluted 1:25 and added to an equal volume of 1:25 diluted calf serum for preincubation, the final dilutions of patient serum tested for S-antibody concentration were 1:50, 1:100, 1:200, 1:400, 1:800, 1:1600. The serum samples were incubated for 1·5 h at 37°C and rinsed with PBS-Tween-BSA three times. Thereafter 250 µl of rabbit anti-human IgG (diluted at 1:50000 in PBS-Tween-BSA) was added and incubated at 22°C for 1 h. The wells were rinsed three times with PBS-Tween-BSA, and 250 µl of goat anti-rabbit IgG conjugated alkaline phosphatase (diluted at 1:2000 in PBS-Tween containing a 1:400 dilution of calf serum) was added and incubated at 37°C for 1 h. The wells were then thoroughly rinsed with Tris-HCl buffer followed by incubation with substrate and the absorbance was measured spectrophotometrically. For each serum sample there was a duplicate control (diluted 1:50). This consisted of two uncoated (no S-antigen) wells in which all the subsequent incubation steps were performed. A negative control thus showed that S-antigen coating of the well as a prerequisite for reaction. A positive control would have shown non-specific sticking of the antibodies to the plastic surface.

Data analysis. Antibody titration curves were straightened and the greatest dilution showing positive reaction to S-antigen was used. A spectrophotometric reading which was at least twice the reading of the control sample (background) was considered as a positive reaction to S-antigen. A dilution of 1:50 was defined as a titre of 1, 1:100 as a titre of 2, 1:200 as a titre of 3, and so on. Both the two-tailed Student's *t* test and the Wilcoxon's rank sum test (non-parametric) were used to determine whether the response of the patient group differed from that of the normals.

Results

Serum samples obtained from children with chronic uveitis and from healthy individuals were assayed for anti-S activity with the ELISA. The results are presented in Fig. 1. Statistically significant elevation of titres to S-antigen were found in children with uveitis ($p<0.01$) (both with Student's *t* test and
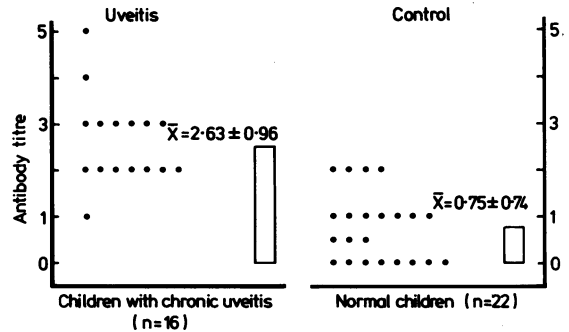

Fig. 1　*Antibody titres of children with uveitis (n=16) and controls (n=22) to S-antigen. At head of each column are arithmetical mean ± standard deviation.*

Wilcoxon's test). From the data presented in Fig. 1. it can be seen that some patients had higher titres (antibody titre of 3 or more) and others had lower titres (antibody titre of 2 or less). A comparison of these groups is seen in Table 1. In the group with high titres the incidence of retinal involvement was substantial, and the disease of the patients was in every case in an active stage. Clinically it was clear that the most severe cases with chronic uveitis also had high titres to S-antigen. No clear difference between the two groups was seen if HLA-B27 or CRA was positive in the patients.

In normal controls half of the serum samples showed no response at all or a response only in a final dilution of 1:25. Four out of 22 normal control samples showed an antibody response of 1:100 (titre: 2).

Discussion

The retinal soluble antigen (S-antigen) is a uveito-genic organ-specific antigen isolated from the retina of several mammalian species.[16-18] The first report that this soluble antigen was located in the outer segments of photoreceptor cells was by Rahi.[19] We have used previously described techniques to purify this S-antigen from bovine retinas. The S-antigen gave a single band on SDS-PAGE electrophoresis and was pathogenically active in microgram doses. It evoked a form of EAU characterised by abrupt onset of iridocyclitis in rats within two weeks after immunisation, which was followed by development of chorioretinitis. This is in agreement with previous reports[13 20] and confirms the specificity of the antigen used in the present work.

Children with chronic uveitis were selected for the study because it is known that young tissues respond to noxious stimuli more vigorously and extensively than adult ones, and changes in immunological parameters may therefore be easier to study.[11]

Uveitis in children is frequently a more serious disease than in adults.[6-8 21]

By using an indirect enzyme-linked immuno-sorbent assay the data presented in this report demonstrate that the serum antibody titres in children with uveitis are statistically significantly elevated to bovine S-antigen. This is in agreement with the findings reported by Gregerson et al.[4] in adults with uveitis. The human and bovine S-antigens have not yet been sequenced, but there are indications that the S-antigens from these species are very similar both biochemically and immunologically.[22] Although the mean value of the titres was elevated in patients with uveitis (mean antibody titre: 2·63) as compared with the controls (mean antibody titre: 0·75) there were also patients who showed only low antibody titres. In order to find out if the patients with high antibody titres (antibody titres of 3 or more) were different from those with low titres (antibody titres of 2 or less) we compared these groups for several factors described in Table 1. A comparison of the duration of the disease, or binocularity as well as association with HLA-B27 antigen or juvenile rheumatoid arthritis did not reveal significant differences between groups with high and low titres. In the group with high titres, however, the disease of all the patients was in an active stage, and most of them (5 cases out of 8) also had a retinal inflammation. In the group with low titres there was only one case out of eight with retinal inflammation. Clinically it was clear that the children with the most severe form of chronic uveitis all had significantly elevated titres. Not unexpectedly, the children with antibody titres of 4 and 5 had an exceptionally severe form of uveitis in both eyes.

A factor influencing the statistical evaluation was the patient to patient variation in antibody titre. This was also marked in the control group: 8 out of 22 control children showed no antibody titre to S-antigen, but in others it varied from 0·50 to 2. The reason for this is unclear, but it is possible that some of the control sera showed false positive reaction. Non-specific anti-bovine activity which is commonly found in human serum was absorbed by preincubating with calf serum before determination of antibody titre with ELISA. This absorption removes all the anti-bovine serum activity from the human serum.[4] In spite of these precautions it is possible that the purified bovine S-antigen may be cross-reactive with some other unidentified antigen. This non-specific binding and possibly false positive reactions can perhaps be prevented by using human S-antigen together with monoclonal antibodies in an ELISA assay. Such work is now in progress.

We have observed that the antibody titre to S-antigen is elevated in children with uveitis. However,

the correlation between the level of activity of the uveitis and the titre to S-antigen is not clear. This is similar to an observation by Rahi et al.[23] that anti-retinal autoimmunity does exist in sympathetic ophthalmitis though it is not possible to make a definitive diagnosis on immunological grounds alone. To study S-antigens in more detail in the pathogenesis of uveitis would require serial serum samples, so that the antibody level could be correlated with the clinical course of the disease. Abrahams and Gregerson[24] found no longitudinal correlation between clinical activity and anti-S-antigen titres in patients with chronic, recurrent uveitis. However, they found that the titres reached a peak and declined more predictably in those patients who presented with active lesions which subsequently resolved without recurrence. We intend to perform a longitudinal study in children suffering from uveitis with purified human S-antigen together with monoclonal antibodies using an ELISA technique.

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