Naturally occurring antibodies to bovine and human retinal S antigen: a comparison between uveitis patients and healthy volunteers

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SUMMARY The antibody responses to human and bovine retinal S antigen in the sera of patients with uveitis from various causes were compared with those of a group of healthy volunteers who were fully screened for signs of eye disease. Antiretinal antibodies were found with equal frequency and through the same range of titres in patients and controls, irrespective of the nature or activity of the uveitis. These findings were confirmed by spectrotypic analysis of sera from patients and controls where the predominant serum antibody response was polyclonal. In a small group of patients with retinal vasculitis there was an additional monoclonal response, indicating clonal expansion of a single lymphocyte subset. The prevalence of serum antibodies to retinal antigens in the normal population may indicate a protective role for 'natural' autoantibodies, as has recently been suggested for autoimmune diseases generally.

Autoimmunity to retinal antigens, particularly retinal S antigen,^{1,2} has been implicated in the pathogenesis of idiopathic uveitis. Gregerson, Abrahams, and Thirkill³ reported that antibodies to S antigen and to retinal cell membrane proteins (P antigen) occurred in patients with various forms of uveitis, though antibody levels bore no direct correlation with phasic activity of the disease.^{4,5} Using an immunoblotting procedure, this group also found patients with uveitis had serum antibodies to several soluble retinal proteins, including a 40 K and a 33 K protein, as well as S antigen (50–55 K^{6,7}). Dumonde *et al.*⁸ used an immunofluorescent antibody technique to show that patients with retinal vasculitis had raised antibody titres to retinal S antigen and that there was an inverse correlation between the level of circulating immune complexes and severity of disease. They suggested that immune complexes, possibly involving anti-S antibodies, serve a protective immunomodulatory role via the idiotypic network.

The lack of correlation between autoantibody titres and disease activity in uveitis has raised questions on their relevance to the disease. Antibodies to various self-antigens occur widely in the

normal population, and Murray⁹ has recently shown that, contrary to previous reports, there is no difference in the titres of non-organ-specific autoantibodies in patients with uveitis compared with normals. In fact, B and T cell autoreactivity is considered by some to be the normal state, and indeed is a sine qua non for antigen recognition via the major histocompatibility complex (MHC) system.¹⁰ The degree of autoreactivity, however, is held in check by a variety of controlling (suppressor) mechanisms. Autoimmune disease (AID) may therefore be the result of an imbalance in this state—that is, it is the degree of autoreactivity which determines whether the organism is adversely affected (for review see Smith and Steinberg¹¹). This suggests that autoantibodies found in both normal people and patients may simply represent an epiphenomenon, which might occur, for example, as a result of polyclonal B cell activation during bacterial or viral infection. In contrast, if clonal restriction of antibody secretion occurred in certain disease states, then this might indirectly support the view that they were aetiologically important either in the initiation or in the perpetuation of the disease. Mono- or oligoclonal restriction of autoantibody against defined antigens (the acetylcholine receptor, IgG, thyroglobulin, DNA) has been reported in human and experimental

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models of autoimmune disease,¹²⁻¹⁴ including S-antigen-induced uveitis in guinea-pigs.¹⁵

In this report we present data on secretion of antibody to retinal S antigen in patients with various forms of uveitis, including chronic idiopathic uveitis affecting the posterior segment of the eye, and compare the results to antiretinal antibody titres in a group of normal controls who were screened for evidence of subclinical retinal or uveal disease. In addition we have studied the antibody spectrotpe of antiretinal antibodies by reverse immunoblotting.¹³ Our results indicate that there is no difference in the occurrence of antiretinal S antigen antibodies between uveitis patients and normal controls, and that high levels of anti-S antibody can occur in apparently healthy people as well as uveitis patients. Spectrotype analysis indicates that in most people (patients and controls) antibody secretion is polyclonal. However, monoclonal antibodies were observed in two patients both of whom had retinal vasculitis as part of their chronic intraocular inflammatory disease.¹⁶

Materials and methods

Patients. Patients with various forms of uveitis and uveoretinal inflammation who presented to the Uveoretina Clinic, Aberdeen Royal Infirmary, were initially classified by diagnosis (see Table 1) and severity of disease. As part of their examination for uveitis 10 ml of whole blood were collected from each patient, and the serum was separated and stored at -20°C before use. Twenty-four healthy volunteers of similar age range and sex distribution who were free of uveoretinal disease (active or past inflammation) as determined by slit-lamp biomicroscopy and dilated indirect ophthalmoscopy were accepted into the study as controls.

Preparation of retinal S antigen. Bovine retinal S antigen was prepared by the method of Al-Mahdawi *et al.*⁶ with minor modifications. Human and guinea-pig S antigens were prepared by similar techniques and also by high-performance liquid chromatography with a TSK-DEAE column. The purified antigen migrated as a single band after SDS-polyacrylamide gel electrophoresis. Protein concentration was measured by a dye-binding assay.¹⁷ Cross-reaction between the species of S antigen was detected by double immunodiffusion using polyclonal rabbit anti-serum against bovine S antigen. Reactions of partial identity were obtained for all three proteins. Human and guinea-pig S antigens were labelled with Na¹²⁵I by the method of Fraker and Speck¹⁸ to a specific activity of 0.16–0.25 MBq/μg.

Enzyme linked immunosorbent assay (ELISA). The assay system was developed using antisera from

Table 1 Anti-S antibodies (ELISA)

Patient category	n	Serum dilution (A ₄₉₀)	
		1:30	1:100
Controls	24	0.85 (0.46)*	0.42 (0.29)
Acute iridocyclitis	36	0.65 (0.35)	0.24 (0.17)
Posterior uveitis (all causes)	43	0.92 (0.33)	0.41 (0.20)
Birdshot/focal choroidretinopathy	8	0.86 (0.32)	0.42 (0.23)
Chronic intraocular inflammation	17	0.99 (0.31)	0.41 (0.20)
Retinal vasculitis	7	1.07 (0.24)	0.52 (0.21)
Sarcoid uveitis	6	0.82 (0.31)	0.36 (0.14)

*Mean with SD in parentheses.

a known positive antibody secretor with high titres of anti-retinal S antibodies (by Ouchterlony analysis and immunohistochemical analysis). Dynatech microtitre plates were coated with 100 μl purified bovine retinal S antigen (10 g/ml) in 20 mM trometamol (TRIS) HCl, pH 7.5. Preliminary checkerboard analysis indicated that higher concentrations of protein did not increase the sensitivity of the assay. Plates were washed throughout the procedure with 0.2 M trometamol-saline buffer, pH 7.4, containing 0.05% Tween 20. Blocking of free binding sites was achieved with 0.5% bovine serum albumin (in 0.02 M trometamol). Antisera for testing were diluted in 33% newborn calf serum/trometamol Tween pH7.4. Peroxidase labelled rabbit antihuman IgG was diluted 1 in 500 in normal sheep serum and incubated at room temperature for two hours. Enzyme substrate, O-phenylenediamine 0.04%, was diluted in citrate-phosphate buffer containing 1 ml H₂O₂ and incubated with the bound antibody for 30 min. The reaction was then stopped with 4 N H₂SO₄, and the absorbance read at 490 nm.

Isoelectric focusing. Human sera were analysed by isoelectric focusing (IEF) as described by Williamson.¹⁹

Reverse immunoblotting. Focused antibodies from IEF gels were transferred to nitrocellulose membranes by an adaptation of the method of Towbin *et al.*²⁰ as described previously.¹³ Blockage of free binding sites on the membrane was achieved with 10% sheep serum in phosphate buffered saline prior to autoantibody detection using homologous labelled S antigen (10⁶ cpm/ml). After extensive washing the membranes were dried and bound antigen detected by exposure to Kodak X-omatic S1 x-ray film with an Ilford fast tungstate intensifying screen at -70°C, followed by development in Kodak LX 24 developer.

Results

ELISA

A total of 79 patients with uveitis (36 anterior, 43

posterior uveitis) were studied (Table 1) in addition to 24 normal healthy controls. No significant differences were observed in antiretinal S antibody titres

Fig. 1 Serum anti-S antibodies in uveitis patients and controls as measured by ELISA (see 'Materials and methods'). Ordinate: A_{490} readings from wells of microtitre plate, indicating increasing amounts of bound anti-S antibodies. Abscissa: dilutions of serum. Shaded area = mean $\pm$ SD of A_{490} values for the group of 24 controls.

between controls and any of the groups. In general, patients with acute iridocyclitis had anti-S antibody titres lower than the controls, while within the subgroups of patients with posterior uveitis the patients with retinal vasculitis had values towards the higher range detected in controls. The most interesting finding, however, was that within the controls, titres of antiretinal S antibody as high as those found in patients with active posterior uveitis were found, indicating that autoantibodies to retinal S antigen occur within the normal population (Fig. 1). No significant differences were detected on dilution of the sera, indicating that these results were not due to non-specific binding of irrelevant immunoglobulin to the microtitre plates.

SPECTROTYPE ANALYSIS

Polyclonal antibody responses appear as diffuse non-separable bands of immunoglobulins on isoelectric focusing gels whereas monoclonal immunoglobulin focuses as discrete (3–6) bands of protein.¹⁹ The technique of reverse immunoblotting, where the focused immunoglobulin is transferred to a nitrocellulose membrane and reacted with labelled antigen,²¹ has allowed clonal analysis of the antibody response to high molecular weight antigens (>10 K). Fig. 2 shows that sera from patients and controls produced a predominantly polyclonal pattern of antibodies against human retinal S antigen, focusing mainly between pH 7.0 and 8.5. Most sera were

Fig. 2 Isoelectric focusing gel using reversed immunoblotting procedure (see 'Materials and methods') showing antibody reactions against 125 I-labelled human S antigen. Polyclonal staining is indicated by diffuse, variably dark pattern in each of the serum samples (tracks 1–19). Track 6 from a retinal vasculitis patient shows superimposed monoclonal, three banded pattern (arrows). (Tracks 1, 2, 21, 22 controls; tracks 3–20 uveitis patients).

Fig. 3 Isoelectric focusing gel using the same focused human serum immunoglobulins as in Fig. 2 but against ^{125}I -labelled bovine S antigen. Note similar immunoreactive monoclonal bands in track 4.

weakly polyclonal, the variation in intensity of antigen binding usually reflecting their corresponding ELISA results. Sera from two patients showed dominant clones (one is shown on track 6, Fig. 2) both in the basic region of the fluorograph against a polyclonal background. When the same sera were overlaid with ^{125}I -labelled bovine retinal S antigen similar patterns, including the two sera with dominant clonotypes, were obtained although with higher background (Fig. 3). This indicates that the clones of lymphocytes responding to human S-antigen recognise the same epitope on bovine S-antigen.

Discussion

The presence of natural autoantibodies is well established in normal adult humans and laboratory

mice,²²⁻²⁵ but their function and relevance to autoimmune disease are not clear. Recent studies suggest that there may be a separate class of B lymphocytes (Lyb 1⁺ B cells) which are involved solely in secreting autoantibodies.²⁶ Autoantibodies may be directed towards ubiquitous cellular proteins, for example, anti-DNA antibodies in SLE or to specific targets such as the acetylcholine receptor (AChR) in myasthenia gravis. However, high levels of anti-AChR antibodies may occur in the absence of myasthenia gravis,²⁷ while active disease may occur in the absence of AChR antibodies.²⁸ The results of the present study indicate that a similar situation obtains in idiopathic uveitis. Recent studies have indicated that idiopathic endogenous uveitis may have a large autoimmune component²⁹ with various retinal antigens, particularly S antigen, identified as target antigens for autoreactivity.³⁰ However, antibodies to retinal S antigen occur equally in patients with endogenous posterior uveitis as well as acute iridocyclitis, as in normal healthy controls (Table 1, Fig. 1). Furthermore, the titre of retinal S antibodies does not correlate either directly or indirectly with disease activity.⁴

In their study of retinal vasculitis Dumonde *et al.*⁸ suggested that antiretinal antibodies may have a protective role, since they observed an inverse correlation between disease activity and circulating immune complexes. Cohen and Cooke³¹ have taken the concept of natural autoantibodies one stage further and suggest that autoantibodies may serve a protective function generally by acting as a filter to 'blind the immune system' to invading foreign antigens which may fortuitously possess epitopes cross-reactive with self. Evidence for cross-reactivity between bacterial antigens and self-antigens is accumulating,^{32,33} and natural autoantibodies which recognise the cross-reacting epitope could block the autoimmune response before it could be augmented by the other immune enhancers (for example, adjuvants, foreign determinants) present on the microbe. Indirect links between uveitis and microbial diseases are well known, and, if Cohen and Cooke are correct, a search for common antigenic determinants may yield valuable data.

An important distinction between natural autoantibodies and autoantibodies associated with disease may be the class of immunoglobulin. Natural autoantibodies tend to be IgM which have a short half-life and could therefore be continuously secreted, while disease-associated autoantibodies tend to be IgG.³⁴ Study of the class of autoantibody may therefore be helpful in determining its relevance to the disease. Another approach is to study clonal secretion of antibody, for instance, by spectrotypic analysis. Previous studies have shown that clonal

restriction of antibody secretion is more likely to occur in non-organ-specific than in organ-specific autoimmune disease.¹³ In the present study auto-antibody secretion in uveitis was also predominantly polyclonal, though monoclonal responses were observed in two patients with retinal vasculitis, and in both cases the restriction was to an epitope common to both human and bovine S-antigen (Figs. 2, 3). Experimental studies indicate that the antibody response to S-antigen in guinea-pig uveoretinitis is highly restricted, and shared spectrotypes were observed in some animals, suggesting that the antibodies are derived from a limited V-gene pool.¹⁵ A similar restriction may account for the variable susceptibility of humans to uveitis.

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