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Autoantibodies to retinal astrocytes associated with age-related macular degeneration*

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Abstract. Sera from 128 patients with age-related macular degeneration (AMD) were examined and profiles of a variety of serum constituents, including immunoglobulins, alpha and beta globulins and autoantibodies, were tabulated. A similar series of tests were carried out on 20 control sera. The results indicate a higher incidence of serum abnormalities, particularly involving alpha-2 globulin, in patients with disturbance of pigmentation of the retinal pigment epithelium (RPE). The sera were further tested for the presence of autoantibodies with specificity for retinal tissue, and five major staining patterns were observed. Many sera produced patterns of labelling on human retina identical to that observed using labelled monoclonal anti-glial fibrillary acid protein (GFAP) antibodies, which are an established marker of retinal astrocytes. Although anti-retinal autoantibodies have been reported in association with a number of ocular pathologies, the observation of anti-astrocyte autoantibodies is new. Astrocytes are involved in the maintenance of the blood-retinal barrier (BRB) and also appear to be the facultative antigen-presenting cells of neural tissue. The present results indicate that the formation of anti-astrocyte autoantibodies may be an early feature of the pathogenesis of AMD.

Introduction

Age-related macular degeneration (AMD) is now recognised to be a leading cause of registered blindness in England [17], the United States of America [7] and China [20], affecting one in four of the elderly population according to some estimates [7]. Loss of central vision may develop gradually, following atrophy of the retinal pigment epithelium (RPE) or rapidly following the complications of subretinal neovascularisation [5, 21]. The primary lesion in AMD is often assumed to reside in the RPE, although proof of this hypothesis is lacking and the pathogenesis remains unclear [3]. Immunocompetent cells appear to influence the pathogenesis of both the neovascular and atrophic lesions [13–15]. In addition, it has been shown that elevated levels of an alpha globulin, ceruloplasmin, are associated with AMD [12], and a correlation between white blood cell count and the neovascular complications of AMD has been reported [2].

Anti-retinal autoantibodies have previously been reported in a number of ocular disorders, including retinitis pigmentosa, cataract [4, 10] and retinal vasculitis [6]. However, the relationship of anti-retinal autoantibodies to the pathogenesis of AMD has not been examined in detail. The present study demonstrates the existence of autoantibodies to a variety of retinal constituents in sera from patients with AMD.

Sera from patients with disturbance of RPE pigmentation (a primary ophthalmological sign of AMD [21]) frequently produced patterns of labelling on human retina identical to that observed using labelled monoclonal anti-glial fibrillary acid protein (GFAP), a marker of astrocytes in many species [16]. A functional relationship appears to exist between retinal astrocytes and the RPE, since both tissues contribute to the maintenance of the blood-retinal barrier (BRB) [1, 11, 18]. The present data suggest that the formation of anti-astrocyte autoantibodies may be an early feature of the pathogenesis of AMD.

Materials and methods

Clinical diagnoses of patients with AMD were made in the Outpatients Department of Sydney Eye Hospital. Fluorescein angiography and fundus photography were carried out where indicated, and consent for venepuncture was obtained. Blood samples (20 ml) were collected and allowed to clot at room temperature, and the serum fraction was then separated. On the basis of their clinical notes, patients were classified as belonging to one of the following categories: controls (including normal and normal aged individuals); pigmentary disturbance; drusen; geographic atrophy, end stage; or disciform, end stage. Sera were divided into three fractions; one was used for anti-retinal antibody testing, a second was analysed by a range of routine tests for serum abnormalities and the remainder was frozen and stored. Additionally, 50 control sera (from donors aged 55 years and older) were obtained from The Sydney Red Cross Blood Bank and tested for the presence of anti-retinal autoantibodies.

Serum protein electrophoresis was carried out on cellulose acetate membranes and, after staining with Ponceau S dye and scanning with a Beckman Appraise densitometer

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Table 1. Serum and autoantibody profiles

Test	Pigmentary disturbance ($n=40$)	Geographic atrophy ($n=21$)	Disciform ($n=44$)	Drusen ($n=13$)	Normal ($n=20$)
IgG	4	5	3	3	2
IgA	3	7	1	3	–
IgM	2	1	3	1	–
Albumen	8	2	10	2	4
Alpha-1 globulin	–	–	4	1	1
Alpha-2 globulin	17	6	16	6	4
Beta globulin	9	4	6	4	3
Gamma globulin	1	1	1	2	1
Total protein	13	1	5	3	4
Complement C3	3	1	1	3	1
Complement C4	1	1	2	2	1
Properdin B	5	3	5	3	1
Antitrypsin	5	2	2	3	2
Rheumatoid Factor	3	1	6	2	–
Anti-nuclear Ab	16	5	12	2	1
Mitochondrial Ab	1	–	1	–	–
Smooth-muscle Ab	3	1	2	1	–
Parietal Ab	5	1	4	1	–
Thyroid microsomal Ab	3	1	2	–	1
Thyroglobulin Ab	4	–	1	–	–

Ab, Antibodies

(Brea, Calif., USA), individual levels of albumen, alpha-1, alpha-2, beta and gamma globulins were obtained as percentages of the total serum protein. Immunoglobulins G, A and M, complement components C3 and C4, properdin factor B and alpha-1 antitrypsin were measured by rate nephelometry (Beckman Auto ICS; Brea, Calif., USA). Thyroid microsomal and thyroglobulin autoantibodies were measured by haemagglutination (Thymune M, Thymune T; Wellcome Diagnostics, England). Rheumatoid factor was assayed by a latex slide test (Ortho RA test; Beerse, Belgium). Other autoantibodies were detected by indirect immunofluorescence on frozen sections containing rat and mouse stomach, rat kidney and rat liver. Antinuclear antibodies were also sought by indirect immunofluorescence on methanol-fixed HEp-2 (Modern Diagnostics Inc.; Sacramento, Calif., USA). Results of individual tests that fell outside the normal range were tabulated and related to the patient's clinical diagnosis.

Anti-retinal antibody tests were carried out on fresh, unfixed retinae from human eyes obtained from the Sydney Eye Bank and from laboratory animals, including the rat and mouse. Sera were diluted 1:10 in phosphate-buffered saline and tested for the presence of anti-retinal autoantibodies on cryostat sections of unfixed normal human and animal retinae using the streptavidin-biotin fluorescent labelling technique (Amersham Australia). Slides were coded and read independently by two observers without reference to the diagnostic categories. Results were scored positive only when anti-retinal staining occurred in the absence of, or could be distinguished from non-retinal autoantibodies apparent in routine serological tests, described above. Additional sections were treated with commercially available monoclonal antibodies raised in mice against GFAP and vimentin (Amersham Australia). The protocol used for detection of anti-retinal monoclonal antibodies was similar to that used for human sera. Comparison of the pattern of binding of anti-GFAP and anti-retinal autoantibodies was made using 10-μm serial sections.

Results

The results of the serum analyses and clinical diagnoses are summarised in Table 1. Broadly, they indicate a greater frequency for abnormal levels of serum constituents, including autoantibodies, in all four categories of AMD compared with controls. Two features of the serum analyses of patients in the pigmentary disturbance and disciform groups stand out; firstly, the frequent appearance of anti-nuclear antibodies, and secondly, the high incidence of raised levels of alpha-2 globulin. The mean values for alpha-2 globulin levels in the pigmentary disturbance and disciform end-stage groups were compared with alpha-2 globulin levels in the control group, using Student's t-test. The results indicated a significant difference in levels of alpha-2 expression between groups with pigmentary disturbance and normal controls ($t=2.1$, $P<0.05$) and a marginal difference between groups presenting the disciform scar and normal controls ($t=1.1$, $P<0.1$).

Five distinct patterns of anti-retinal autoantibody staining were observed when sera were applied to human retina (Table 2). The majority of sera produced one staining pattern in the retina. A few sera demonstrated more than one staining pattern; in these cases, the dominant staining pattern was tabulated (Table 2). Where sera were tested against three or four different retinae, binding patterns were found to be consistent. Figures 1a–e illustrate the photographic appearance of the staining patterns of monoclonal antisera, autoantisera and controls.

The presence of anti-retinal autoantibodies of all types was more frequent in sera from patients from each of the AMD groups (pigmentary disturbances, drusen, geographic atrophy and disciform lesions) than in controls (Table 2).

Table 2. Anti-retinal autoantibody patterns

Pattern	Pigmentary disturbance ($n=40$)	Geographic atrophy ($n=21$)	Disciform ($n=44$)	Drusen ($n=13$)	Normal ($n=50$)
Filamentous	23	3	8	3	1
Microfilamentous	3	0	1	2	7
Cell body	3	0	1	1	2
Photoreceptor	0	1	3	0	0
Diffuse	2	2	10	2	0
Negative	9	15	21	5	40

The gender ratios (F:M) and mean ages of the groups were as follows: pigmentary disturbance (F:M=1.2), 71.4±8.6 years (mean±SD); geographic atrophy (F:M=1.1), 75.4±8.8 years; disciform (F:M=1.6), 73.8±7.4 years; drusen (F:M=1.6), 71.6±8.9 years; aged controls (F:M=0.7), 60.4±3.5 years. Of the control sera, 20% contained anti-retinal autoantibodies, vs 58% of sera in the four AMD groups.

From the pigmentary disturbance group, more than half (57%) of the sera contained antibodies that resulted in the labelling of fine filaments, corresponding to the distribution of astrocytes (see below), in the inner retinal layers. This pattern is subsequently referred to as arising from 'filamentous' autoantibodies (Fig. 1a). The mean age of patients with pigmentary disturbance who were positive for filamentous antibodies was 71.3±8.9 years. A second, 'microfilamentous', pattern of labelling was identified, which was confined to the nerve fibre layer but occurred at a low rate of incidence (Fig. 1b). The pigmentary disturbance group had the highest overall incidence of all types of anti-retinal autoantibody (78%), including anti-ganglion cell autoantibodies (Fig. 1c). Sera from the drusen group also showed a high overall incidence (61%), although fewer specimens were analysed. Of the disciform sera, 52% contained antiretinal antibodies, but the most frequent staining pattern was the diffuse form (Fig. 1d). The geographic atrophy group showed the lowest overall incidence of autoantibodies, with 29% of sera displaying anti-retinal reactivity.

The pattern of distribution for filamentous anti-retinal autoantibodies was compared with that for monoclonal anti-GFAP antibodies on serial frozen sections. Both the filamentous and GFAP antisera showed almost identical distributions on serial sections of a number of human retinal specimens (Figs. 1g, h), suggesting that monoclonal anti-GFAP and filamentous autoantisera stain the same population of retinal astrocytes. The pattern of binding for filamentous autoantibodies on rat and mouse retinae was similar to that observed on human retina, indicating that the antigen concerned was not species-specific.

Discussion

The present report represents the first detailed study of the relationship of anti-retinal autoantibodies to the pathogenesis of AMD. IgM anti-retinal autoantibodies have previously been noted in two sera from patients with AMD [4, 10], but the correlation of patterns of anti-retinal antibody reactivity with clinical manifestations of AMD has not been attempted. Earlier reports of anti-retinal autoantibodies have referred only to antibodies directed against photoreceptors [4, 10], which were also observed in this study.

The present descriptions of antibodies reactive with cells of the ganglion cell layer and filamentous antibodies are new, although similar specificities have been described for monoclonal antibodies directed against retinal cells [8]. We defined some of the characteristics of the filamentous antibodies. They appear to stain the same population of cells as do monoclonal anti-GFAP antibodies, strongly suggesting that they are directed towards retinal astrocytes, and the antigen does not appear to be species-specific. Studies to localise the binding site at the ultrastructural level and define the class of the antisera are in progress.

It has previously been shown that elevated levels of an alpha globulin, ceruloplasmin, are associated with AMD [12], and Blumenkranz et al. [2] have shown that there is a correlation between white blood cell count and the neovascular complications of AMD. Together with previous results that implicate immunocompetent cells in the pathogenesis of AMD [13–15], the present study, which reveals a generally higher incidence of serum abnormalities in patients with pigmentary disturbance, further supports the hypothesis that the pathogenesis of AMD may involve an early inflammatory phase.

The aetiology of the anti-retinal autoantibodies described here remains to be determined. They may be produced in response to the release of retinal antigens associated with loss of the integrity of the RPE. Alternatively, the anti-retinal autoantibodies may have arisen against another tissue (for example, the brain) and be cross-reactive with retinal antigens. It has been pointed out that astrocytes are the facultative, inducible antigen-presenting cells of neural tissue. In a recent review, Werkerle et al. [19] drew two main conclusions regarding cellular immune reactivity within the CNS: firstly, only activated lymphocytes can cross the blood-brain barrier, and secondly, these T cells then cooperate within the CNS with astrocytes. Accordingly, the generation of anti-retinal autoantibodies may be related to the presentation of previously sequestered retinal antigens by astrocytes.

Although it is known that autoantibodies occur more frequently in elderly persons, the present findings appear to be related to the pathogenesis of AMD, since a significant increase in the frequency of anti-retinal autoantibodies was found in patients with pigmentary disturbances, which was not seen to the same degree in the controls and other groups and was not simply related to increasing age. Conceivably, these are an epiphenomenon and do not influence the pathogenesis of AMD. However, serum autoantibodies with specificity for retinal antigens, arising in response to

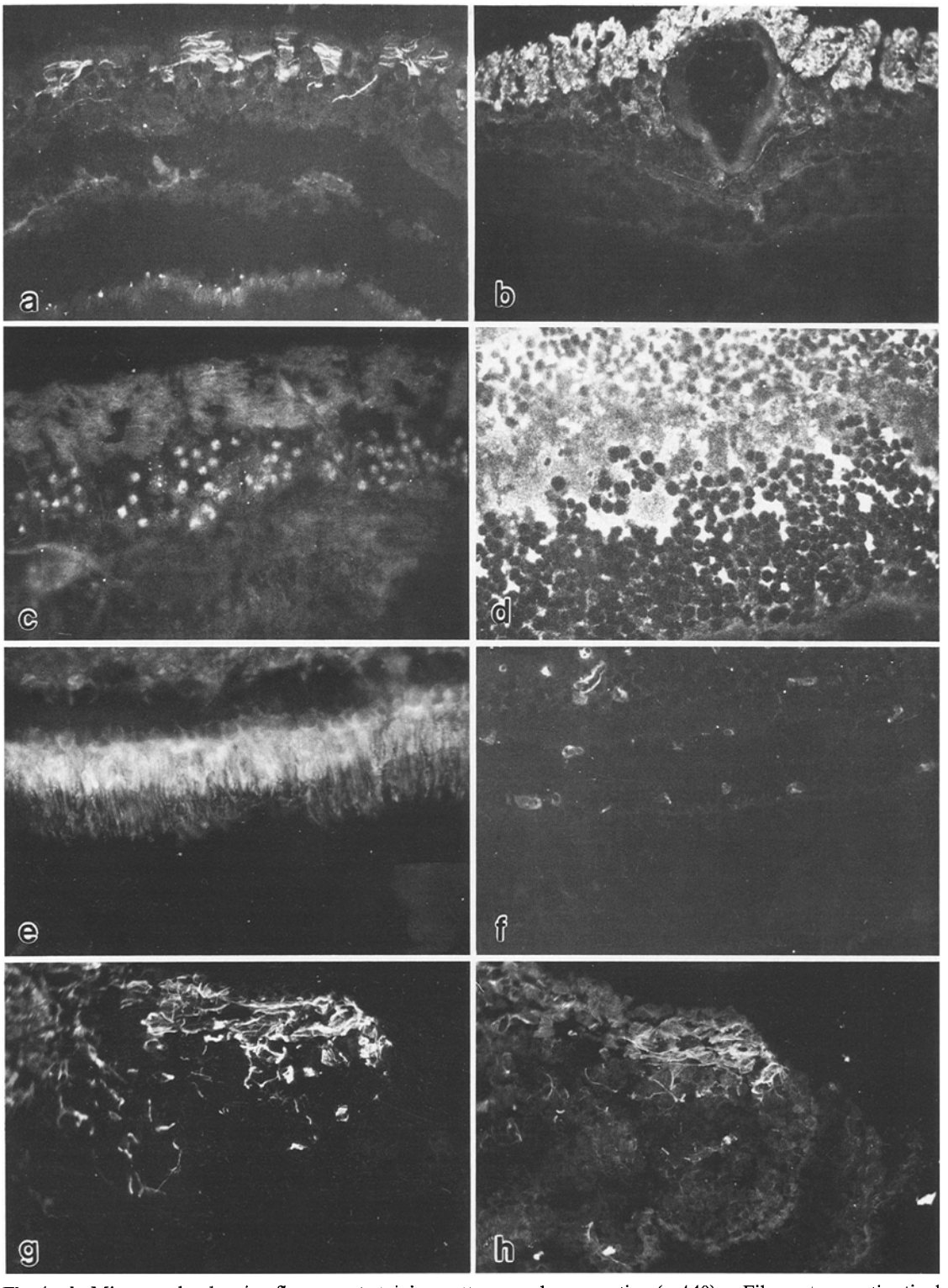

Fig. 1 a–h. Micrographs showing fluorescent staining patterns on human retina (× 140). **a** Filamentous anti-retinal autoantibodies apparently directed against retinal astrocytes [in *h* (below) the filamentous staining pattern is shown on a different specimen]. **b** The microfilamentous staining pattern shown appears to be directed against an as yet unidentified constituent of the nerve fibre layer of the retina. **c** Anti-ganglion cell autoantibodies were found in a small percentage of sera. **d** The diffuse pattern of staining shown in this micrograph was consistently observed in sera from patients with neovascular lesions. **e** Photoreceptor cell-body autoantibodies were also commonly observed. **f** A control section incubated with serum from a donor with no apparent eye disorder, treated in the same way as the previous sections, shows no significant anti-retinal reactivity. **g** The distribution of retinal astrocytes is illustrated in frozen sections of the human retina by staining with fluorescein-labelled anti-GFAP monoclonal antibodies raised in the mouse. Monoclonal antibodies produce more intense fluorescence and reduced background staining as compared with filamentous autoantibodies (cf. **h**). **h** Consecutive serial sections, following the previous section, were incubated with patient's sera containing filamentous autoantibodies. The staining pattern appears to be almost identical to that seen using anti-GFAP monoclonal antibodies, indicating that the monoclonal antibody and the autoantibody stain the same population of cells

certain carcinomas, have been shown to be capable of crossing the BRB and promoting visual dysfunction, the so-called paraneoplastic syndrome [9]. This finding indicates that the retina is not impermeable to antibodies, and anti-retinal autoantibodies in the sera of patients with AMD may be pathogenic in a similar way.

Additionally, where AMD develops unilaterally, anti-retinal autoantibodies may promote retinal degeneration in the fellow eye. Disturbance of RPE pigmentation and focal hyperpigmentation are classical ophthalmological signs of AMD [21]. The cause of these changes has not been established, but it is possible that they may be associated with the expression of autoimmunity directed towards the retina, including the RPE. The possible existence of anti-RPE autoantibodies is currently under investigation in our laboratories.

Both the RPE and retinal astrocytes appear to play a role in the maintenance of the BRB. It has been suggested that retinal astrocytes influence the permeability of the retinal vasculature, which forms the innermost barrier, whereas the outer barrier is formed by the RPE [1, 11, 18]. Disturbance of the RPE may not be the result of a primary lesion in the RPE [3], but may be a response to a compromise of barrier function associated with the formation of anti-astrocyte antibodies. Finally, if the relationship between pigmentary disturbance and anti-astrocyte autoantibodies is confirmed, their presence in the serum of patients with AMD may form the basis of a diagnostic test.

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