Müller Cell–Specific Autoantibodies in a Patient with Progressive Loss of Vision
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PURPOSE. To study the specificity of circulating retinal autoantibodies in a patient with progressive loss of vision resembling cancer-associated retinopathy in the absence of systemic malignancy.

METHODS. Patient's serum was tested for the presence of antiretinal antibodies by Western blot analysis, and immunohistochemical analysis was performed on cryosections of rat retina and on cultured rat retinal Müller cells.

RESULTS. Western blot analysis revealed that the serum contained a high titer of autoantibodies against a 35-kDa retinal antigen. A protein of similar molecular weight and antigenicity has been found to be present in protein extracts of bovine, rat, and fish retina. Immunohistochemical analysis suggested that the autoantibodies did not localize to retinal neural cells, as reported for several other putative autoimmune retinal disorders, but rather to the retinal Müller cells. This cell type-specificity could be confirmed using purified cultured retinal Müller cells.

CONCLUSIONS. These results suggest that an autoimmune response directed against the retinal Müller cells, in the absence of overt systemic malignancy, may lead to a slow-developing visual deterioration. (Invest Ophthalmol Vis Sci. 1998;39:1976-1979)

The role of autoantibodies in the pathogenesis of uveoretinal inflammation has been a controversial issue until now.1 However, the observation that patients with cancer-associated retinopathy (CAR) have circulating retinal autoantibodies has renewed the interest in this topic, especially since in vitro and in vivo experiments have provided firm evidence for a direct role of an autoimmune response in this noninflammatory retinal disorder.2 The expression of retinal antigens by certain tumors is held responsible for the induction of an autoimmune reaction leading to impaired retinal function with a minimal concomitant inflammatory reaction.3

A number of autoantigens have now been identified to be involved in CAR, and the reaction against recoverin, a 23-kDa photoreceptor protein, has been most extensively investigated until now.4 The determination of antirecoverin antibodies is now recognized as a valid test for the diagnosis of CAR and may provide a positive result even before the diagnosis of cancer is made.2 This latter observation has led to a new surge in the analysis of the retinal autoimmune response in patients with unexplained retinopathies. In the course of such a study we detected a retinal autoantibody in the serum of a patient with an unexplainable progressive loss of vision and a normal-appearing fundus. Evidence is presented to show that the autoimmune response in this patient differs from that of previous reports in that it is not directed to retinal neuronal components but rather to a 35-kDa protein associated with retinal Müller cells.

MATERIALS AND METHODS

Case History
A 67-year-old woman came to the Ophthalmology Department of Academic Medical Centre, University of Amsterdam, in 1991 with a complaint of intermittent decrease of vision and photopsias. Her previous ocular history was unremarkable, but she had had a chronic pulmonary disorder since an episode of tuberculosis for which she had been treated some 50 years before. In the previous 4 years she had received systemic corticosteroid therapy for serious emphysema. Ophthalmic examination showed a best corrected visual acuity of 16/20 OD and 14/20 OS. Anterior segments were normal except for a slight cataract in both eyes. There were no signs of inflammation either in the anterior chamber or in the vitreous body. Fundoscopy revealed a normal optic disc, normal vessels, normal macula, and normal periphery. Ocular pressure was 16 mm Hg in both eyes. The patient was screened for a possible diagnosis of amaurosis fugax, but no serious vascular problems could be found.

The patient was regularly followed for the next couple of years during which her visual acuity gradually deteriorated. In 1995 visual acuity was reduced to 6/20 OD and 5/20 OS, but the amount of cataract could not explain this reduced vision completely. Ophthalmic examination did not show any additional abnormality or signs of inflammation. Further examinations were performed, and visual fields showed an extremely constricted visual field in both eyes. Electroretinograms of both eyes showed greatly reduced responses that especially affected the b-waves. Photopic responses were more reduced than scotopic responses. Scotopic implicit times for both the a-wave and the b-wave were increased, and photopic implicit times were nearly normal. Oscillatory potentials were almost absent. The visual evoked response showed a normal response to flash and a slightly prolonged latency time to the pattern reversal stimulus. Fluorescein angiography was completely normal, without any sign of inflammation or pigment epithelium disturbances. All vessels had a normal appearance, without leakage.

A paraneoplastic retinopathy was suspected; however, despite the fact that the patient complained of malaise and a weight loss of approximately 20 kg in the previous year, extensive examination did not reveal any sign of malignancy. Nevertheless, serum was obtained and examined for the presence of antiretinal autoantibodies.

Control Sera
Serum from a patient known to have CAR, which contained antibodies against recoverin, was obtained from Charles A. Thirkill and was used as a positive control. Negative control sera included a pooled serum obtained from healthy control subjects and various individual sera from patients with uveitis.
and individuals undergoing cataract surgery. The study was performed according to the Declaration of Helsinki, and informed consent was obtained from all blood donors.

Preparation of Total Protein Extracts
Tissues from animals were obtained in agreement with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Human corneal and retinal tissues were obtained from donor eyes that were processed at the Eye Bank of the Netherlands Ophthalmic Research Institute. The corneas used had been donated for transplantation but had been found unacceptable because of changes in the endothelium. A soluble protein extract was obtained by adding approximately 100 mg fresh retinal tissue to 500 μl sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer (125 mM Tris · Cl, pH 6.8, 2% sodium dodecyl sulfate, 5% β-mercaptoethanol, 10% glycerol, 0.005% bromphenol blue) followed by vigorous stirring, boiling for 5 minutes, and spinning for 5 minutes at 10,000g. To obtain a total corneal protein extract, corneas were first snap-frozen in liquid nitrogen and then homogenized using a mortar.

Western Blot Analysis
Fifteen to 20 μg total protein extract per lane was size-fractionated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted to nitrocellulose filters (Schleicher and Schull, Dassel, Germany). Blots were incubated with serum from a patient or control subject (diluted 1:100), washed, incubated with a 1:1000 dilution of goat anti-human Ig alkaline phosphatase-conjugated secondary antibody (DAKO, Glostrup, Denmark), and developed with 0.3 mg/ml 5-bromo-4-chloro-3-indolyl phosphate, toluidine and 0.3 mg/ml nitroblue tetrazolium chloride (both from Research Organics, Cleveland OH) in 100 mM Tris · Cl (pH 9.5), 100 mM NaCl, 5 mM MgCl₂ for 10 minutes. Immunoblot analysis using a total human retinal protein. As a positive control, a serum from a patient with autoantibodies directed against recoverin, an immunologic marker that identifies the CAR syndrome, was included. As a negative control, pooled normal human serum was used. The immunoblots showed that our patient's serum contained antibodies to a 35-kDa retinal protein (Fig. 1). The positive control serum recognized the 23-kDa recoverin, whereas the pooled normal human serum did not produce any specific bands. In addition to normal human serum, the sera of 33 patients with uveitis (27 of whom had posterior uveitis and 5 who had anterior uveitis) and 3 patients with cataracts were tested for the presence of antibodies to anti-35-kDa autoantibodies. Although some of these control sera gave weak bands of various molecular weights, none recognized the 35-kDa protein (data not shown). To determine the titer of the anti-35-kDa autoantibodies in our patient's serum, a serial dilution of separate serum samples (taken 5 weeks apart) was tested by western blot analysis. Both serum samples displayed a similar reaction to the antigen, and the 35-kDa band remained detectable up to a dilution of 1:500 (data not shown).

RESULTS
In view of the reports describing retinal autoantibodies in patients with unexplainable retinopathy, we tested the patient's serum for the presence of these autoantibodies by western blot analysis using a total human retinal protein. As a positive control, a serum from a patient with autoantibodies directed against recoverin, an immunologic marker that identifies the CAR syndrome, was included. As a negative control, pooled normal human serum was used. The immunoblots showed that our patient's serum contained antibodies to a 35-kDa retinal protein (Fig. 1). The positive control serum recognized the 23-kDa recoverin, whereas the pooled normal human serum did not produce any specific bands. In addition to normal human serum, the sera of 33 patients with uveitis (27 of whom had posterior uveitis and 5 who had anterior uveitis) and 3 patients with cataracts were tested for the presence of antibodies to anti-35-kDa autoantibodies. Although some of these control sera gave weak bands of various molecular weights, none recognized the 35-kDa protein (data not shown). To determine the titer of the anti-35-kDa autoantibodies in our patient's serum, a serial dilution of separate serum samples (taken 5 weeks apart) was tested by western blot analysis. Both serum samples displayed a similar reaction to the antigen, and the 35-kDa band remained detectable up to a dilution of 1:500 (data not shown).

FIGURE 1. Western blot analysis of total human retinal protein with the patient serum (lane 2), the serum of a patient known to have cancer-associated retinopathy (CAR) (lane 3), and the serum from a healthy control subject (lane 1). Arrowheads point to the position of the 35-kDa protein and to the position of the 23-kDa recoverin antigen, the immunologic marker of CAR. Molecular weight standards are indicated. Endogenous phosphatase activity of the retinal protein extract produced a band at 50 kDa. MWM, molecular weight marker.
FIGURE 2. Western blot analysis of total human cornea protein and rat, human, bovine, and goldfish retinal proteins with a 1:250 dilution of the patient serum. The position of the 35-kDa protein is indicated. Only the relevant part of the blot is shown.

To analyze the tissue specificity and the evolutionary conservation of this 35-kDa protein, western blots containing protein extract of human cornea and human, bovine, rat, and goldfish retina were incubated with diluted serum from our patient and normal human control subjects. A 35-kDa protein was detected in the retinal extracts of all species tested with the patient serum and was not detected when tested with the control sera (Fig. 2). The strong reaction shown with the goldfish retinal extract suggests that the 35-kDa protein is relatively more abundant in the goldfish retina. In the human corneal protein extract no specific signal could be observed.

Immunohistochemical analysis of rat eyes was performed to determine the source of the retinal autoantigen in more detail (Fig. 3). Our patient's serum gave a very distinct staining of a region between the photoreceptor cell nuclear layer (outer nuclear layer) and the outer segments of the photoreceptors, whereas the rest of the neural retina (with the exception of the photoreceptor cell outer segments) was very diffusely stained. Interestingly, thread-like structures, which crossed the photoreceptor cell nuclear layer, were also stained. Also, several nuclei in the inner nuclear layer were found to be positive. The patient's serum could be diluted up to 1:1000 and still give detectable staining of the retina. Other parts of the eye, including the cornea, iris, choroid, and orbital muscle tissue, were all negative (data not shown). Incubation of the sections with pooled normal human serum or sera from several patients with uveitis did not show any signal above background.

The characteristic immunohistochemical staining pattern of cryosections of rat retina suggests that the retinal Müller cells were targeted by the anti-35-kDa antibodies. To confirm that the 35-kDa autoantigen is indeed expressed by these cells, retinal Müller cells were isolated from retinas of adult Wistar rats and 8-day-old Brown Norway rats according to standard procedures, and the cells obtained were analyzed for the presence of the 35-kDa antigen by immunocytochemical analysis using diluted patient serum. Normal human serum and sera of several patients with uveitis were used as controls. Retinal Müller cells of adult Wistar and newborn Brown Norway rats showed a positive staining with the diluted patient serum. The cytoplasm and nucleus both were stained. In some cells a discrete nonstained spot in the nucleus was observed, which probably represents the nucleolus. A punctate pattern in the cytoplasm was observed also. Control sera showed no labeling (Fig. 4). Cultured human retinal pigment epithelial cells did not stain with the patient serum or control sera (data not shown).

**DISCUSSION**

In this report we describe a patient with a gradually developing retinopathy who had circulating autoantibodies directed against a 35-kDa retinal Müller cell-associated antigen. Although retinal autoantibodies have been implicated in the pathogenesis of paraneoplastic retinopathy, no evidence for a malignancy was observed in our patient despite extensive medical examinations. Independently from our study, Mizener et al. recently described two patients with an autoimmune retinopathy resembling CAR in the absence of detectable cancer. The gradual decrease in vision observed in these patients was also observed in the patient presented in this study. Nevertheless there are differences. The retinopathy in the patients described by Mizener et al. was highly asymmetrical and the patients were much younger, they were affected by autoimmune diseases, and...
their visual field defects were paracentral. Furthermore, these patients had circulating antibodies that specifically stained the inner plexiform layer of the retina, whereas western blot analysis did not reveal a specific staining pattern. This is in sharp contrast to our patient in whom western blot analysis revealed a strong antibody reaction with a 35-kDa retinal protein, associated with the retinal Müller cell. The labeled Müller cell processes appeared to be more tortuous than those of another study in which Müller cells were stained for specific proteins. This tortuosity could be due to the differences in section thickness, the interference by other cells, or an unequal distribution of the 35-kDa autoantigen in the Müller cells of the intact retina. Immunostaining of different parts of Müller cell somata and processes has been shown to depend on the nature of the antigen.

Circulating autoantibodies to a 34-kDa retinal protein of similar size were recently described in a patient with retinopathy who suffered from endometrial cancer. In this patient a gradual deterioration was observed over a period of several years also. Because no attempts to characterize the antibodies have been reported, it remains speculative whether our study and that of Ohkawa et al. have identified the same autoimmune response. Evidence is now accumulating that paraneoplastic retinopathies may be divided into a number of subtypes, each associated with its own unique antiretinal protein.

Retinal autoantibody formation as observed in paraneoplastic syndromes, such as CAR and melanoma-associated retinopathy, leads to an extensive loss of vision in a relatively short period. This is in contrast to our patient who showed a slow but progressive deterioration of vision in the absence of uveitis over a period of several years. Possibly, the discrepancy in disease progression between the paraneoplastic syndrome-associated retinopathy and the patient studied in this report is related to the cell type within the retina that is targeted by the autoantibodies. In CAR and melanoma-associated retinopathy the autoantigen is part of cells such as photoreceptors, bipolar cells, or horizontal cells. These cells are all directly involved in retinal signal transduction, and degeneration of these cells is likely to have a severe and rapid impact on visual function. However, the functions of the retinal Müller cell include the maintenance of retinal structural integrity and ion uptake from the extracellular space. Impaired retinal Müller cell function may therefore not lead to a rapid loss of vision but may induce a more gradual development of retinopathy.

It is interesting that the autoantibodies in our patient serum recognized a protein of similar size in both closely related and more distantly related species such as humans, rat, cow, and fish. The highly conserved nature of this protein indicates that it may play an essential role in function of the retina. The fact that the 35-kDa protein could not be detected in corneal extracts and the fact that immunohistochemical analysis of the patient serum did not reveal specific staining of ocular tissues other than the retina indicate that at least for the eye there is a degree of tissue specificity. More studies are obviously needed to identify the 35-kDa protein and to investigate whether antibodies directed against it are an epiphenomenon or whether they are indeed causally involved in the pathogenesis of certain forms of retinopathy.

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References