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The Need for Standardization of Antiretinal Antibody Detection and Measurement

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AUTOIMMUNE RETINOPATHY

Autoimmune retinopathy (AIR) is an immunological process whereby retinal antigens are aberrantly recognized as autoantigens, leading to retinal degeneration. The diagnosis is based in part on the detection of circulating antiretinal antibodies in the setting of clinical and electrophysiological evidence of retinal degeneration. The clinical spectrum of AIR is heterogeneous and diverse. Onset has been described as acute, subacute, or chronic[1]. Electroretinographic abnormalities have been observed using both the full-field and multifocal electroretinogram (ERG) with abnormal rod, cone, Müller cell, and bipolar cell responses being described[1–3]. Fundus findings can appear normal or may demonstrate findings of retinal degeneration such as attenuated retinal vessels, waxy disc pallor, and retinal pigment epithelial (RPE) mottling or atrophy[4, 5]. Signs of an inflammatory response are classically absent[6]. AIR was first described as a paraneoplastic syndrome termed cancer-associated retinopathy (CAR)[7]. Since then, retinal autoimmunity in the absence of cancer has become increasingly

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recognized as a cause of vision loss[1, 2, 8, 9]. Serum antiretinal antibodies have also been reported in patients diagnosed with retinitis pigmentosa (RP), and a pathogenic link between retinal autoimmunity and RP has been suggested[10–13]. Various laboratory techniques, including immunohistochemistry (IHC), Western blot, and enzyme-linked immunosorbent assay (ELISA) have been described for the detection of circulating antiretinal antibodies in patient sera.

Numerous retinal antigens have been described as targets of retinal autoantibodies in patients with suspected AIR, and many putative targets of antiretinal antibodies remain to be identified. A summary of the retinal proteins identified as targets of antiretinal antibodies, along with the assay techniques used to detect and measure the antiretinal antibodies, is shown in Table 1. Antibodies against recoverin and α-enolase have been extensively studied in the context of AIR. Anti-recoverin and anti-enolase retinopathies are believed to be clinically and electrophysiologically distinct from each other; anti-recoverin retinopathy has a strong though non-specific association with systemic malignancy, whereas the cancer association with anti-enolase retinopathy is much less[1]. The majority of this article will focus on examples from the literature describing the laboratory measurement of autoantibodies against recoverin and α-enolase, as these antiretinal antibodies have been the most widely studied with respect to pathogenicity as well as detection and measurement. Although these autoantibodies are the focus of this article, it should be noted that several other retinal autoantibodies have been described as putative mediators of AIR, and other potentially pathogenic antiretinal antibodies remain to be discovered. Hence, our discussion is in no way inclusive with respect to important antibody mediators of retinal autoimmunity.

ANTI-RECOVERIN AND ANTI-ENOLASE ANTIBODIES

Cancer-associated retinopathy (CAR) was first described in 1976 as a photoreceptor degeneration which was presumed to represent a remote effect of malignancy[7]. Early studies demonstrated that serum from patients with CAR labeled photoreceptors using immunohistochemical methods[30, 31]. The serum from CAR patients was also shown to react with a 23 kDa protein using Western blot which was later identified as recoverin[16, 32–34], a calcium-binding protein found in photoreceptors. Recoverin has been shown to be aberrantly expressed in tumors of CAR patients[35, 36]; leading to the hypothesis that CAR is secondary to antibodies generated against tumor-expressed recoverin which cross-react with photoreceptors. The diagnosis of anti-recoverin retinopathy has been described in the setting of various tumors, including small cell lung carcinoma[36], cervical cancer[15], mixed Müllerian tumor[37], endometrial carcinoma[38], and uterine sarcoma[5]. In addition, anti-recoverin retinopathy in the absence of cancer has also been reported[9, 12].

Investigations into the pathogenic mechanisms underlying anti-recoverin retinopathy have been both revealing and interesting. Cell culture experiments and animal models have shown that, following internalization into the cell, anti-recoverin antibodies induce apoptotic cell death mediated by caspase-dependent pathways along with intracellular calcium influx[39–45]. Caspase inhibitors and calcium channel blockers have been demonstrated to ameliorate anti-recoverin antibody toxicity in various experimental models[40, 44–46].

Enolase is a ubiquitously expressed 48 kDa glycolytic enzyme which exists in three isoforms: α-enolase, found in many tissues; β-enolase, found predominantly in muscle; and γenolase, found specifically in neurons and neuroendocrine tissue[47]. In 1996, CAR secondary to autoantibodies targeting α-enolase was reported in patients with various tumors[19]. Increased tumor expression of α-enolase, along with circulating anti-enolase antibodies, has been described in a number of patients with lung cancer[48]. These observations suggest that, similar to anti-recoverin autoantibodies, CAR secondary to anti-enolase antibodies is mediated by

autoantibodies that cross-react with tumor and photoreceptor proteins. Since the initial report, more reports of proposed anti-enolase retinopathy have been reported in CAR and in patients without cancer[1, 13–15]. Analogous to the proposed pathogenic mechanism of anti-recoverin antibodies, anti-enolase antibodies are also believed to induce apoptosis of retinal cells following cellular internalization[18 20]. Furthermore, this apoptotic effect is proposed to be mediated by caspase pathways and intracellular calcium influx[18, 49]. In addition to AIR, antibodies against a-enolase have been described in various systemic autoimmune diseases where they are believed to have a pathogenic role by mediating cell death[50, 51].

CURRENT DIAGNOSTIC APPROACHES

A) IMMUNOHISTOCHEMISTRY

Immunohistochemical detection of antiretinal antibodies involves incubation of varyingdilutions of patient sera with normal retina sections, followed by incubation with a secondary (anti-human immunoglobulin) antibody. The binding of the secondary antibody is then detected and localized using a colorimetric or fluorescent reaction. The first described laboratory method for the detection of antiretinal antibodies in patient sera involved immunostaining of patient sera against retinal sections[30]. Since then, IHC techniques have been used to detect the presence of circulating antiretinal antibodies in several patients with proposed AIR[2, 9, 17–19, 52]. IHC detection of antiretinal antibodies has also been described in various inflammatory, infectious, and age-related ocular syndromes[53–56]. In the setting of inflammatory, infectious, and age-related disease of the retina, it is unclear if antiretinal antibodies precede the retinal disease or if retinal autoimmunity is simply the consequence of the retinal degenerative process[57]. In either case, however, antiretinal antibodies have the potential to be pathogenic and IHC provides valuable information on possible disease associations by allowing visualization of the location of autoantibody binding.

One of the advantages of the IHC assay is the ability to demonstrate cellular localization of antiretinal antibody binding within the retina. Furthermore, the intensity of staining observed on IHC sections may provide clues to relative titers of antiretinal antibodies when multiple serum samples are compared. IHC methods are limited by their inability to detect reactions with specific proteins using patient sera. Because of this limitation, IHC may serve as a good screening assay for the detection of specific antiretinal antibodies, but may not be appropriate as a confirmatory assay. Several factors need to be taken into consideration when standardizing IHC testing for antiretinal antibodies. The binding of antibodies to tissue sections can be affected by whether the section is fresh, frozen, or fixed prior to incubation with patient sera. Furthermore, the type of fixative agent can affect the strength and specificity of binding interactions between patient sera and the tissue section. The non-specific binding of the secondary (anti-human immunoglobulin) antibody to retinal proteins and normal human immunoglobulins in the tissue section needs to be minimized and taken into consideration with proper controls. As normal human serum can also bind non-specifically to normal retina sections, controls consisting of serum from healthy individuals are also required.

B) WESTERN BLOT

Another common method employed for the detection of antiretinal antibodies is the Western blot technique. With this technique, retinal extract or purified retinal proteins are separated into discrete bands based on their molecular weight in a gel using electrophoresis. The separated proteins are then transferred to a nitrocellulose membrane and incubated with varying dilutions of patient sera. A secondary (anti-human immunoglobulin) antibody is then used to detect the bound antiretinal antibodies in the patient sera. Binding of the secondary antibody is detected using a chemiluminescent reaction with exposure to photographic film. Retinal proteins which have bound antiretinal antibodies from patient sera will appear as dark bands on the

photographic film. This technique has been described for the detection of autoantibodies against recoverin[1, 12–17] and αenolase[1, 13–15] using various dilutions of sera from patients with AIR. Western blot methods which have been described in the literature for detecting antiretinal antibodies, however, have not routinely used positive and negative controls.

The interpretation of Western blots relies on the intensity of protein bands visualized on photographic film. This is influenced by multiple factors including adequacy of protein transfer to the nitrocellulose membrane, exposure time to primary and secondary antibodies, the length of colorimetric development, and exposure time to photographic film. For this reason, Western blots should be performed with a positive loading control which consists of an antibody known to react with a specific protein in the protein sample[58, 59]. The intensity of protein bands resulting from specific binding between retinal antigens and antiretinal antibodies in patient sera is then compared to that of the loading control, allowing for an accurate estimate of the amount of autoantibody of interest. The use of a positive loading control also allows for quantification of the protein bands on the blot using densitometry[58, 59]. Western blot techniques describing the presence of recoverin or a-enolase autoantibodies have not reported the use of such controls[1, 12–17]. Analogous to IHC testing, in Western blots the secondary (anti-human immunoglobulin) antibody can occasionally bind to the antigen of interest and give a false positive result. The secondary antibody can also bind normal immunoglobulin which is present in retinal extracts or purified protein samples. This false-positive binding can result in protein bands of molecular weight 25 kDa and 50 kDa, which are very close to the molecular weights of recoverin (23 kDa) and α-enolase (48 kDa). When performing Western blots it is thus advisable to have a negative control consisting of only the secondary antibody. Several reports describing the measurement of serum antiretinal antibodies against recoverin and a-enolase did not report the use of such negative controls[12, 13, 15–17]. The lack of use of positive loading controls and negative controls in the available literature makes it difficult to interpret the presence of the observed bands. Finally, it is important in Western blot techniques to employ the use of human serum from unaffected individuals as a control. The use of serum from unaffected individuals was only described in a few reports[13, 14, 16]. Given the fact the serum from healthy human controls has been shown to produce several bands when blotted against human retinal extract[58, 59], the use of control serum from unaffected individuals is essential in order to interpret the result of an affected patient.

C) ENZYME-LINKED IMMUNOSORBENT ASSAY

Enzyme-linked immunosorbent assay (ELISA) is a highly sensitive technique for measurement of serum proteins. The principles of ELISA are very similar to those of IHC and Western blot. Small wells are coated with a specific retinal protein, various dilutions of patient sera are added and allowed to bind to the protein, and then the binding is detected using a secondary (anti-human immunoglobulin) antibody. A colorimetric reaction measured using a spectrophotometer is used to measure the amount of secondary antibody binding. Several groups have reported the use of direct ELISA techniques for the detection of anti-recoverin[5, 9, 12, 14, 16, 17] and anti-enolase[14, 20] antibodies using patient sera. However, these did not incorporate standard curves, positive controls, or the use of replicates. Furthermore, the use of serum from unaffected individuals was reported in only a few reports[14, 16, 20]. Since the relationship between the measured spectrophotometric absorbance and antibody titer in an ELISA can not be assumed to be linear, a standard curve is needed to determine the titer of the antibody of interest in relation to the measured absorbance. Positive controls are required to prove that the assay is measuring what it is intended to measure. Standard curves and positive controls employ an antibody with known specificity against the retinal protein in place of patient sera. The use of replicates allows for the determination of the standard deviation associated with a particular antigen-antibody reaction, as well-to-well variation is known to occur in ELISA. Replicate wells are essential in order to show if the difference between two

reactions is real and not due to normal well-to-well variation. The lack of standard curves, positive controls, unaffected serum controls, and replicates make it difficult to draw conclusions based on many of the antiretinal antibody ELISA results reported in the literature.

ANTIRETINAL ANTIBODY TITERS: A THERAPUETIC ENDPOINT?

In addition to having diagnostic value, the measurement of antiretinal antibodies may prove useful in the therapeutic monitoring of patients with AIR. Various therapies, including immunosuppresants, corticosteroids, plasmapheresis, and intravenous immunoglobulin, have been reported to stabilize or improve vision in some patients with AIR[15, 27]. Although visual outcomes are the most important endpoint to monitor in the therapeutic management of AIR, a surrogate serum marker of disease activity such as antiretinal antibody titer could be clinically relevant in monitoring these patients. Titers of antiretinal antibodies have previously been shown to decline in response to therapeutic intervention[5, 15, 17, 28, 60]. However, due to assay limitations described earlier in this report, these results are difficult to interpret. In order to accurately measure the response to therapeutic intervention, the development of a validated and reproducible assay is warranted.

TOWARDS A STANDARDIZED ASSAY

Historically, the detection of autoantibodies has served as an invaluable laboratory tool for the diagnosis, monitoring, and management of patients with autoimmune diseases. Since the first report of AIR in 1976[7], a great deal of knowledge regarding this syndrome has accumulated. Numerous clinical and scientific observations have led to an increased understanding of this entity. Despite these advancements in knowledge and understanding, significant obstacles hamper our ability to accurately diagnose and manage patients with AIR. The main obstacle appears to be the lack of standardized, validated, and reproducible assays to measure the presence and titers of antiretinal antibodies in patient sera, and specific knowledge about which antiretinal antibodies are pathologic. The field of AIR is still in its infancy. Development of standardized assays and multi-center collaborations will allow the sharing of knowledge and expertise in order to establish standardized testing and more reliable interpretations.

A critical component for establishing the appropriate assay system is the availability of sera from patients with well-characterized diseases. Clinical diagnosis will allow the data to be analyzed for sensitivity, specificity, as well as the positive and negative predictive value. The collection and availability of accurate and vigorously defined positive disease controls and negative controls are one of the first steps to consider when developing an assay for autoantibodies. In order to ensure the accuracy and reproducibility of assays for antiretinal antibodies, rigorous control measures must be observed within these assays. These should include the masking of laboratory personnel to the patient diagnosis in order to minimize potential bias. Western blots should incorporate the use of negative controls which consist of only secondary antibody, as well as the use of positive loading controls with known specific antiretinal reactivity in order to allow relative quantification of proteins bands. ELISA methods must employ the use of negative controls, standard curves, and replicates. The use of control sera from healthy individuals should also be employed in Western blot and ELISA assays. Serial dilutions of patient and control sera need to be performed within these assays in order to establish a dilution that allows discrimination between normal individuals and patients with AIR. Furthermore, serial dilutions of the secondary (anti-human immunoglobulin) antibody need to be performed in order to establish a dilution that results in minimal non-specific background. Laboratory assays for antiretinal antibodies require a source of protein/tissue. Western blot, ELISA, and IHC techniques described in the literature for the detection of antiretinal antibodies have used protein and tissue from various sources including bovine[13, 19], rodent[22, 61], and monkey[9]. Although proteins from these sources are likely to be

homologous to human proteins, the use of human tissue in assays for antiretinal antibodies with patient sera may be preferable. Having fresh tissue (within 6 hours postmortem) is optimal as degradation of protein/tissue with time can lead to erroneous results in assays for antiretinal antibodies. One drawback of using human protein/tissue is the difficulty in obtaining fresh tissue, and the potential for variation in protein/tissue quality between different donor eyes. The use of tissue from laboratory rodents, which are inbred and genetically homogeneous, has the potential to circumvent these drawbacks. When purified antigens are required the use of human recombinant proteins, when available, would be preferable. Although IHC and Western blots using retinal extract are able to identify binding of patient sera to retina proteins or tissues, respectively, these methods are unable to detect and quantify specific antigen-antibody interactions. These techniques may thus serve as good screening methods, but confirmatory testing using Western blots and ELISAs with specific purified retinal proteins is a useful adjunct.

Regardless of the method or methods selected for detecting and measuring antiretinal autoantibodies, there is a clear need for standardization and internal validation before an assay system is adopted in the clinical diagnostic laboratory. Until such standardized testing is achieved, the use of antiretinal antibody testing will have limited clinical value. Regulatory review through the Clinical Laboratory Improvement Amendments (CLIA) process could assist in the standardization and validation process. To this end, we encourage collaboration and discussion of these standardization issues among clinicians, scientists, and regulatory groups in order to develop a valid and accurate assay system for the detection of antiretinal antibodies.

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Table 1

Proposed Targets of Antiretinal Antibodies

Retinal Protein	Assay Method(s) Described
Recoverin	WB, IHC, ELISA[1, 5, 9, 12–17]
α-Enolase	WB, IHC, ELISA[1, 13–15, 18–20]
Tubby-like protein 1 (TULP-1)	WB[21]
Lens-epithelium-derived growth factor (LEDGF)	IHC, WB[22]
Transducin	IHC, WB[23]
Carbonic anhydrase II (CAII)	WB[13]
Arrestin	WB[13]
Interphotoreceptor retinal binding protein (IRBP)	WB[13]
Heat shock cognate protein 70 (hsc70)	WB[24]
Unknown (22 kDa)	WB, ELISA[25]
Unknown (34 kDa)	WB[26]
Unknown (35 kDa)	WB, IHC[2]
Unknown (40 kDa)	WB[27]
Unknown (46 kDa)	WB[15]
Unknown (60 kDa)	WB[28]
Unknown (70 kDa)	WB[29]

Retinal proteins which are proposed to be targets of antiretinal antibodies are shown, as well as the assay method(s) used to detect their corresponding antiretinal antibodies. WB = Western blot, IHC = immunohistochemistry, ELISA = enzyme-linked immunosorbent assay.