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Complex autoantibody repertoires in patients with glaucoma

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Purpose: Glaucoma is one of the leading causes for blindness in the world. It is characterized by a progressive loss of retinal ganglion cells. An elevated intraocular pressure cannot explain the disease in all subjects. Autoimmune mechanisms maybe involvemed in the pathogenesis of the disease. The aim of our study was to analyze the IgG autoantibody repertoires in sera of glaucomatous subjects against optic nerve antigens.
Methods: Nintey-four subjects were included in this comparative cross-sectional study of healthy (CTRL), primary-open-angle-glaucoma (POAG), ocular-hypertension (OHT), and normal-tension-glaucoma (NTG) volunteers. Sera of subjects were tested against western blots of optic nerve antigens. For each western blot, a densitograph was built by digital image analysis and subsequently a multivariate analysis of discriminance was performed.
Results: Complex IgG autoantibody repertoires could be found in all subjects, even in healthy subjects. The multivariate analysis of discriminance can test for statistical differences between the groups using the whole complex staining pattern for the calculation. A significant difference between all groups against optic nerve antigens was found. The NTG group had the highest variance from controls ($p<0.01$).
Conclusions: This study demonstrates immunological effects in POAG and NTG and may provide further evidence for an involvement of autoantibodies in the pathogenesis of both NTG and POAG. Using the techniques presented in this study, the differences in the complex autoantibody repertoires were assessed by means of statistical analysis. Further studies are needed to determine whether these changes in autoantibodies could be helpful in the diagnosis of glaucoma.

Glaucoma, one of the leading causes of blindness in the world [1], is a group of ocular disorders that are characterized by a loss of retinal ganglion cells and their axons, damage to the optic nerve, and progressive loss of visual field. People of all ages can be affected by this disease. At the age of 70, about 7% of the population suffers from glaucoma. The most common form of glaucoma, primary open-angle glaucoma, is associated with an elevation of the intraocular pressure. However, more than 30% of glaucoma patients do not have an elevated intraocular pressure (normal tension glaucoma). However, the most common therapy even in those cases is to lower the intraocular pressure towards unphysiological values.

Several recent findings support the hypothesis that autoimmune mechanisms may be involved in the pathogenesis of normal tension glaucoma [2]. Several antibodies against ocular antigens could be found that play a possible neurodegenerative role in glaucoma, including antibodies against heat shock proteins (HSPs) [3], rhodopsin [4], γ-enolase [5], glutathione-S-transferase (GST) [6], tumor necrosis factor-α [7], and γ-synuclein [8]. Patients suffering from normal tension glaucoma have an elevated serum titer of autoantibodies against small heat shock proteins (25 to 30 kDa) [9,10].

Although some autoantibodies in the sera of glaucoma patients have been identified and correlated with glaucoma, as described hereinbefore, many are still unknown. Western blotting has surfaced as a powerful tool for detecting specific autoantibodies in autoimmune diseases. Complicating the straightforward identification of pathogenically relevant antigens, however, is that normal sera contain large amounts of natural antibodies which manifest themselves in complex staining patterns [11-14]. This can complicate the differentiation of disease-associated autoantibodies from the complex background of “auto-immune noise” (naturally occurring autoantibodies). The role of these natural autoantibodies is still unclear. Nonetheless, it is widely accepted that an individual repertoire remains stable for a specific individual [15].

Most studies evaluate one or a few specific disease-related antibodies and screen only for a limited number of purified homologous or heterologous proteins (antigens) by means of ELISA or RIA [4]. However, in most autoimmune diseases, a diagnosis based on a single autoantibody was impossible to establish. On the other hand, the western blotting technique permits simultaneous screening for a wide spectrum of different autoantigens. Using digital image analysis techniques, these complex banding patterns of antigen-antibody reactions can be quantified and statistically analyzed, allowing the detection of minor changes in the antigenic composition of autoantibody repertoires [15]. In this approach, pattern matching algorithms that are based on statistical techniques (like discriminant analysis) or artificial neural networks can be used to differentiate between the mean banding patterns of groups, and to measure the extent to which multiple dependent variables (like the intensity of a antigen-antibody reaction at a specific molecular weight region) contribute to differences between the groups [16-19]. This technique has been successfully used in studies of myasthenia gravis, Graves’ disease, experimental autoimmune uveitis, Guillan-Barré, and Tourette syndrome [20-27].

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In this study we analyzed complex IgG autoantibody repertoires against optic nerve antigens in glaucoma patients and healthy subjects. We attempted to quantify those changes by inclusion of the complete repertoire and not just single antibody-antigen reactions. Furthermore, we tried to quantify which groups are statistically the most different from controls.

METHODS

Subjects: Ninety-four subjects (51 male, 43 female), mean age 67.7 years (range 24-94), were included in the study. The subgroups of this study population were: 34 non-glaucomatous control subjects (IOP<21 mm Hg, no primary or secondary glaucomatous disease; CTRL), 17 normal tension glaucoma subjects (IOP<21 mm Hg, optic disc cupping and visual field defect; NTG), 17 subjects with ocular hypertension (IOP>21 mm Hg, no optic disc cupping and visual field defect; OHT), and 27 primary open-angle glaucoma subjects (IOP>21 mm Hg, optic disc cupping and visual field defect; POAG). Subjects with any kind of corneal or retinal pathology or autoimmune diseases were excluded from this study. The investigation was conducted in accordance to the tenets of the Declaration of Helsinki. The sera of subjects were tested against western blots of optic nerve antigens. After giving their informed consent blood was taken from all subjects. The samples were centrifuged and the serum stored for later examination.

Biochemical procedures: Optic nerves are dissected from bovine eyes. They were homogenized in sample buffer (1 M Tris, pH 7.5; 10% SDS; 20 mM DTT; bromophenol blue, pH 6.8), and centrifuged (15000 rpm for one hour). The samples were cooked for 10 min at 100 °C and homogenized several times. The pellet was discarded and the supernant was stored for later analysis.

The optic nerve extracts were used for 13.5% sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE) using MultiGel-Long (Biometra, Goettingen, Germany). After electrophoresis, the gels were transferred to nitrocellulose membrane Protran BA 83 (Schleicher and Schuell, Dassel, Germany) by using a Semi-Dry Blotter (Biometra). After blotting the membranes for 1 h, the quality of the transfer was checked by staining the nitrocellulose with Ponceau S solution (Sigma, Munich, Germany). The blots were blocked with blocking buffer (5% non-fat dry milk in phosphate-buffered saline (PBS)) for one hour. The nitrocellulose was cut into 4 mm wide strips. One strip was used per subject. The strips were incubated overnight with subject serum (1:40 dilution, in washing buffer). 1000 µl volume per strip was used. After washing the strips with Tris-buffered saline (TBS) three times, they were incubated with secondary antibody solution, which was peroxidase-conjugated Immuno Pure® Goat Anti-Human IgG (H+L) diluted 1:500 (Pierce, Illionois, USA) for 1 h. After several washing steps the bands were developed by 0.05% 4-chloro-1-naphthol (Sigma, Munich, Germany) with 0.015% hydrogen peroxide in 20% methanol in TBS for 20 min.

Molecular weights were estimated for each band based on the relative mobility of ten known molecular weight standards (BenchMark, Invitrogen, Karlsruhe, Germany).

Digital image analysis: The data was acquired using the video documentation system BioDocAnalyze (Biometra). All lanes were defined by start, end, and their width. For each particular Rf-region (relative mobility), a gray value was calculated by averaging the values within that width of the lane. For each electrophoretic lane, a densitometric data file was created by showing the gray-intensity values versus the Rf values. BioDocAnalyze evaluates the area, the relative mobility (Rf), and the molecular weight for all peaks in all these densitographic data files [28]. Only those peaks on the western blots were included in the calculation that exceeded a specific cut-off value to assure that only "real" peaks and not artifacts were taken into account. The separated proteins could be identified by comparing the relative mobilities of unknown proteins of a sample to the relative mobilities of known proteins of molecular weight standards (Broad Range, BioRad, Munich). These data vectors of peak data of each subject were compiled into a database and assigned to a clinically predefined group (CTRL, POAG, OHT, or NTG).

Statistical procedure: The western blot analysis techniques used in this study were developed in our group and described elsewhere in detail [17,19]. They were successfully used as standard protocols in several other studies in autoimmune diseases [21,22,24].

All these studies have in common that the densitographic data files need to be transformed into normalized data vectors to be used as input data to the multivariate discriminant analysis. Therefore, the x-axis (molecular weight values) was divided into 50 classes of molecular weight ranges. For each western blot, a data vector was created and the Rf range (Rf between 0 and 1) was divided into 50 different classes. Every variable of the data vector thus represents 1/50 of the complete Rf-range. For all peaks of each western blot vector it was calculated into which particular Rf-class this peak falls. The volume of that peak was added to the corresponding variable of this Rf-class in the data vector of this western blot. The overall band intensity of each individual blot was set at 100% and the remaining intensities were transformed into relative percentages, reducing the influence of different absolute staining intensities on individual blots. From these data vectors, a multivariate analysis of discriminance was performed. This analysis of discriminance not only tests the zero hypothesis that mean data vectors of the different groups derive from a multivariate normally distributed population, but also shows which of the various groups are statistically different. Based on this, discriminant function analysis can be used to determine which variables (Rf ranges) caused the mean value comparison to become significant or which variables can discriminate between groups.

Using discriminant analysis allows one to calculate a parameter, the so called canonical roots, as a kind of similarity index of each western blot. This can be used to illustrate the quality of discriminance between the samples and to demonstrate the sharpness of a diagnostic criterion. It can be understood as an indivual repertoire clustering. The closer the canonical roots of the banding patterns of western blots the more similar the blots were.

This calculation procedure has been described in detail elsewhere [13,22]. The statistical calculations were performed by STATISTICA™ (Version 6.0; Statsoft, Tulsa, OK).

RESULTS

The western blots of all sera including those of healthy subjects showed complex banding patterns against optic nerve antigens. No molecular weight range revealed measurable antibody reactivity exclusive to only one of the four groups (e.g., POAG or NTG) for the antigenic tissue.

Figure 1 shows some of the western blots of autoantibodies in subject sera against optic nerve antigens.

Figure 2 reveals the mean number of antibody reactivity peaks on all blots for each group. None of the mean peak numbers were significantly different between the groups (CTRL=11.8; POAG=10.9; NTG=9.1; OHT=10.8).

The mean area under the peaks reflecting the overall staining intensities of each blot were not different between any groups for optic nerve antigen.

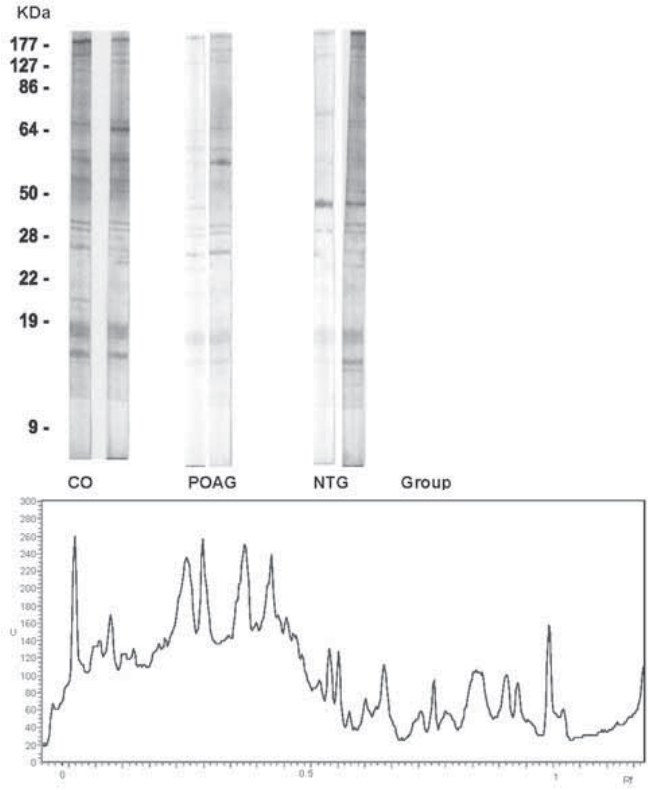

Figure 1. Photographs and densitographs of Western blots against optic nerve antigens. Top: Western blots of IgG autoantibody repertoires in subject sera against optic nerve antigens. There is a complex pattern of antibodies in all groups (CTRL, POAG, NTG). Each lane was incubated with subject serum (diluted 1:40) against bovine optic nerve over night. Secondary antibody, peroxidase-conjugated goat anti-human IgG (diluted 1:500), was applied for one hour. Bottom: Densitograph of a subject with primary open-angle glaucoma (POAG). In the densitograph scanner units ("U" represents optic density) were plotted against relative mobility (Rf-values) that correspond to the molecular weight of the antigenic tissue.

Figure 3 shows the mean antibody reactivities of all groups for each molecular weight region.

The multivariate analysis of discriminance, which includes the complete complex banding pattern of each western blot in the calculation, revealed a significant difference between all groups (Wilks' Lambda=0.15; $p<0.01$). The analysis can also provide a measure of the overall similarity between the groups. These are the statistical distances (Mahalanobis distances) between the groups and the canonical roots which can reveal the quality of separation for each patients' banding pattern.

Against optic nerve antigens, several molecular weight regions could be found elevated, especially in the NTG group at approximately 22, 27, and 75 kDa. However, several more complex regions could be found with reactivities in all groups or even much lower reactivities in one of the glaucoma groups compared to all other groups at approximately 39 kDa (Figure 3).

In terms of statistical distances, the NTG group was the most different from all other groups ($p<0.002$; distance NTG-CTRL=5.76). Furthermore, the POAG group showed the smallest difference from the control group (POAG-CTRL=2.91; OHT-CTRL=3.83). The statistical distance of the antibody reactivity of the OHT group in comparison to the control group was expressed greater? than in POAG, but less? than in NTG patients.

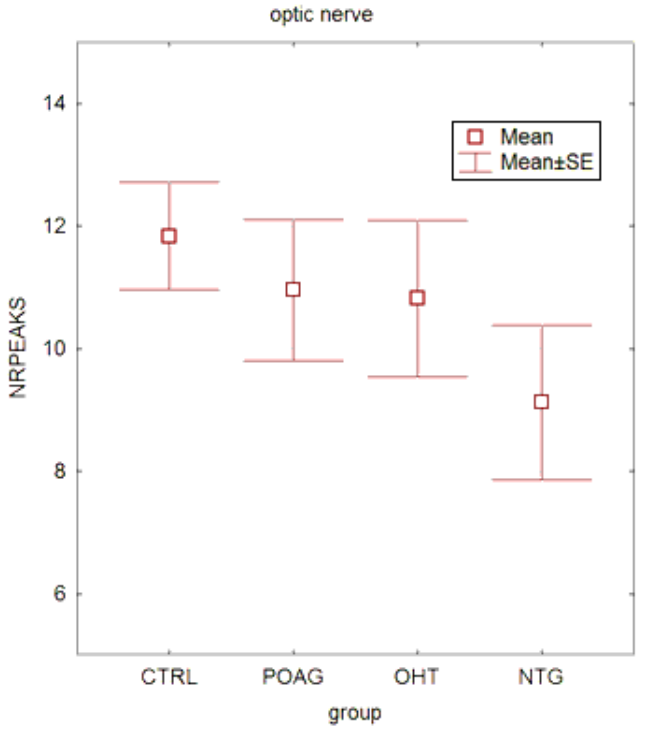

Figure 2. Mean number of peaks in Western blots against optic nerve antigens. Mean number of peaks in sera of subjects with primary open-angle glaucoma (POAG), normal tension glaucoma (NTG), ocular hypertension (OHT), and healthy volunteers (CTRL). Against optic nerve antigens, no significant difference in the number of peaks can be found in POAG, OHT, or NTG compared to controls.

Figure 4 shows the canonical roots of IgG antibodies of the four different groups for optic nerve antigens. Most of the IgG reactivity patterns of NTG subjects lay outside of the accumulation of the patterns of the other subjects from all other groups. This confirms the statistical results that the NTG group revealed the largest distance to the control group compared to OHT and POAG.

DISCUSSION

The aim of this study was to analyze and compare quantitatively and simultaneously a broad spectrum of antibodies in the sera of glaucoma subjects using a pattern matching technique of western blots. This is an important further step in comparison to earlier studies that analyzed only a single or a few antibody reactivities. Our results suggest that an autoimmune mechanism might play a role in glaucoma subjects with and without an elevated intraocular pressure.

In this study, complex IgG autoantibody repertoires in all four groups could be demonstrated against optic nerve antigens. In the complex group data vectors that we found in our study (Figure 3), several antibodies could be detected at molecular weights that likely represent antibodies that have been already described in other studies, such as heat shock proteins [3], rhodopsin [4], gamma-enolase [5], glutathione-S-transferase [6], tumor necrosis factor alpha [7], gamma-synuclein [8], and glycosaminoglycans [29]. Furthermore, at approximately 22 and 27 kDa, a highly elevated titer of antibody reactivity could be found in the sera of normal-tension glaucoma subjects compared to all other groups. The 27 kDa peak is described as a member of the heat shock proteins that are implicated in the development of a number of human autoimmune disorders [3]. These antibody peaks in the complex profiles of this study are consistent with the findings of the group of Martin Wax, who proposed an autoimmune mechanism to be involved prominently in subjects suffering from normal tension glaucoma [2,30,31].

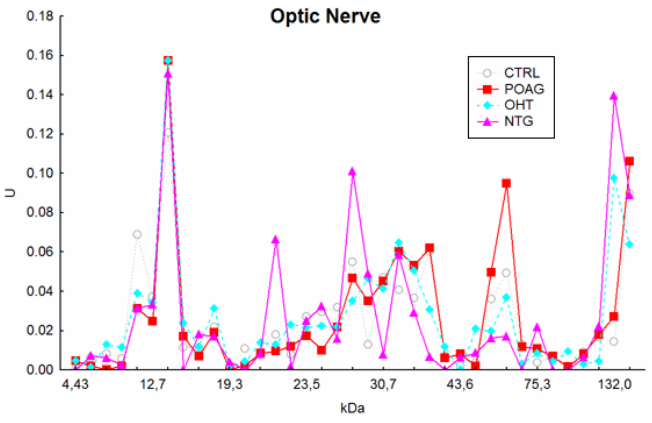

Figure 3. Antibody profiles against optic nerve antigens. The mean antigen-antibody reactivity of all groups were plotted vs. the corresponding molecular weight of the antigens. Complex staining patterns could be found in all groups. The NTG group revealed the highest difference from controls ($p<0.002$).

Beside those already known antibody reactivities, we could demonstrate in this study that the IgG reactivity in glaucoma subjects compared to healthy subjects and ocular hypertension subjects is much more manifold. There are many more regions showing lower or higher reactivities in glaucoma subjects as those known from preceding studies. This is not unexpected, because we used a quantitative statistical approach to investigate these reactivities. The image analysis system BioDocAnalyze could precisely quantify the peaks. Thus, more than only one single antibody-antigen reaction could be found to be strongly correlated with glaucoma. This is consistent with the findings in other established autoimmune diseases. In myasthenia gravis for example, it is known that antibodies against the acetylcholine receptor are involved in the pathogenesis of the disease. However, this does not explain the disease in all myasthenia patients,because there are many myasthenia patients that don't have anti-acetylcholine receptor antibodies [32].

Furthermore, analyses of autoantibodies in autoimmune diseases are always hampered by the fact that natural autoantibodies occur even in the sera of healthy subjects and that there is a large variability of those repertoires from subject to subject [11,18]. Our data indicate that even normal individuals have complex autoantibody reactivities against optic nerve antigens. The role of those naturally occurring autoantibodies remains unclear, but they may be non-specific, have low affinity, and could thus be of little physiological importance.

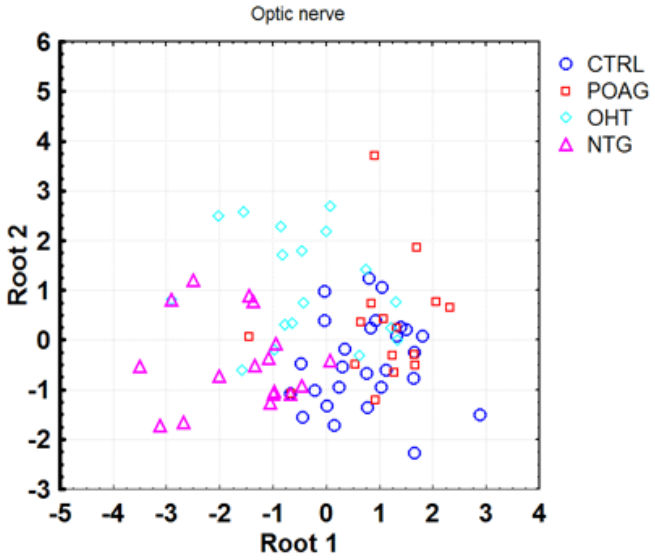

Figure 4. Canonical roots of IgG serum antibodies against optic nerve. Canonical roots of IgG serum autoantibodies of subjects with primary open-angle glaucoma (POAG), normal tension glaucoma (NTG), ocular hypertension (OHT), and healthy volunteers (CTRL). The analysis of discriminance is able to calculate a parameter, the so called canonical roots, as a kind of similarity index of each electrophoretic lane. This can be used to illustrate the quality of discriminance between the samples and to demonstrate the sharpness of a diagnostic criterion. The closer the canonical roots of samples are the more similar the electrophoretic patterns were.

Using this multivariate approach, we could detect molecular weight regions with specific higher or lower reactivities in the glaucoma groups. The analysis was able to calculate statistical distances which revealed that the antibody reactivities in NTG group were the most different compared to all other groups using optic nerve antigens. Conversely, we found in previous studies the highest difference in autoantibody reactivities against retinal antigens compared to controls in the POAG group [33]. These findings support the hypothesis that autoimmune mechanisms might be involved even in glaucoma subjects with an elevated intraocular pressure. Nevertheless, we are not able to conclude whether the differences in antigen-antibody reactions between glaucoma and non-glaucoma groups are causative for the development of the disease or develop as a consequence of the course of the disease. Although both NTG and POAG antibody patterns are different from controls, we must speculate that there are large underlying differences in the pathogenesis of the diseases in both groups that are independent from the occurrence of an elevated intraocular pressure, because the autoantibody repertoires are strongly different in NTG against optic nerve and in POAG against retinal antigens [33]. Additionally, this study could demonstrate regions with decreased antigen-antibody-reactivies in glaucoma. This phenomenon is not yet explained, but provide further hints for changes in the autoimmunity in glaucoma patients.

Further studies have to elucidate the role of the autoantibody reactivities demonstrated in this study for the pathogenesis of the disease and have to identify the antigenic targets of those reactivities that are not already known from prior studies. However, if they are an epiphenomenon or not, autoantibodies can serve as markers in diagnosis and can lead to prognosis and treatment in autoimmune diseases. The application of the analysis of discriminance or artificial neural networks as pattern matching algorithms might be a benificial approach to establish a diagnosis of the glaucoma disease based on the autoantibody repertoires.

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