Definition of Ocular Antigens in Ciliary Body and Retinal Ganglion Cells by the Marker Antibody pANCA

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PURPOSE. A subset of patients with anterior uveitis express the marker, perinuclear anti-neutrophil cytoplasmic antibody (pANCA). In this study, recombinantly isolated pANCA monoclonal antibodies were used to search for ocular cells expressing the pANCA antigen.

METHODS. Paraffin sections of human ocular tissues obtained after death were analyzed by immunohistochemistry to identify cell types expressing pANCA antigen. Microdissected eye-bank ocular tissue was characterized by western blot analysis to confirm antigen expression and identify candidate protein species.

RESULTS. Immunohistochemical analysis with pANCA monoclonal antibodies revealed cytoplasmic antigen expression in retinal ganglion cells and ciliary body epithelium. pANCA antigen expression was restricted to tissues bearing these cell types by western blot analysis. A common set of epitope-positive protein species was shared by the two tissues (28 kDa, 80 kDa, and 90 kDa). Comparison of ocular tissues from seven subjects revealed no heterogeneity in antigen expression.

CONCLUSIONS. In this study, novel cytoplasmic antigens of the pANCA marker antibody expressed in ciliary body and retinal tissue were identified. Validation of these antigens as targets of inflammation in pANCA+ uveitis requires further biochemical and immunologic analysis.

Antigenic targets of marker antibodies offer insights into the genesis of immune-mediated disease. Immunopathogenesis in chronic inflammatory diseases is generally attributed to CD4+ T-cell activation.1 B-cell activation and clonal expansion occurs in tandem with such T-cell responses.2-5 These clonally expanded B cells have the same antigenic specificity as the pathogenic T cell, and their antibodies offer a potentially important tool for characterizing the target antigen driving the immune response. From a conceptual standpoint, disease-associated marker antibodies may reflect the specificity of activated B cells in sites of the disease-specific immune response. The best evidence for this view comes from the effort to build a comprehensive panel of serologically defined antigens derived from patients with diabetes mellitus.6 From this panel, some antigens have now been validated as targets for disease-specific T-cell responses and used as tools to delineate clinically meaningful immunogenetic subgroups and to perform direct immunotherapeutic interventions.

A distinctive pattern of marker anti-neutrophil autoantibody—anti-neutrophil cytoplasmic antibody, perinuclear pattern (pANCA)—is found in most patients with ulcerative colitis (UC) and sclerosing cholangitis and in clinically distinct subsets of patients with uveitis or Crohn’s disease.7-11 pANCA is distinguished from other ANCA by nuclear membrane localization and DNase I sensitivity.12-13 In UC and sclerosing cholangitis, elevated serum titers of pANCA are independent of clinical status and are expressed among healthy relatives of the patients.14-17 Accordingly, pANCA has been recognized as an immunogenetic susceptibility trait in UC and sclerosing cholangitis. Inflammatory diseases of the eye and ocular adnexa, including uveitis, are associated with inflammatory bowel disease and are observed in 3.5% to 11.8% of patients.18-19 In another study, an increased incidence of chronic intestinal inflammation was shown in patients with anterior uveitis.20 However, the pANCA marker antibody is found in a subset of patients with anterior uveitis who do not have a personal or family history of inflammatory bowel disease. It is hypothesized that the presence of pANCA in a subset of patients with uveitis may reflect a shared immunopathogenesis of UC and a uveitis subset. It is possible that novel pANCA antigens are expressed in the eye and are involved in the extracolonic manifestations of UC.

Our laboratory has recently cloned two representative pANCA Fab monoclonal antibodies using combinatorial libraries and antibody phage display technology.21 The purpose of this study was to use the monoclonal pANCA antibodies as a tool for identifying pANCA antigens that are expressed in ocular tissues.

METHODS

Human Recombinant Fab and Serum pANCAs

Fab 5-2 and 5-3 are human UC-pANCA Fab monoclonal antibodies cloned and characterized as previously described.21 The P313 anti-tetanus toxoid Fab monoclonal antibody was a gift obtained as a recombinant clone from Carlos Barbas III, Scripps Institute.22 Recombinant Fab proteins were purified as hexahistidine-tagged products and directly biotin labeled for use in immunohistochemical study (Pierce Chemicals, Rockford, IL). Human sera from normal subjects and patients with UC were selected based on UC-pANCA activity, as previously defined.11

Immunohistochemistry

Paraffin-embedded ocular tissue specimens were sectioned (5 μm), transferred to slides, and heated at 60°C for 60 minutes (Fisher Plus; Fisher, Hampton, NH). The sections were deparaaffinized with xylene, rehydrated with different concentrations of ethanol, and washed with distilled water. Tissue hydrogen
Identification of ocular pANCA-positive cells. Immunohistochemical identification of ocular tissue pANCA antigen was performed using biotinylated primary antibodies. Biotinylated antibody was detected using a staining method in which the VIP chromogen produced a deep violet hue. Nuclei were detected with methyl green counterstain. Ciliary body (A, C) and retina (B, D) are shown. The biotinylated primary antibodies were Fab 5-3 (A, B) and Fab anti-tetanus toxoid (C, D). Arrowheads, Fab 5-3 reactivity (purple).

 Peroxidase was inactivated by preincubation of the slides with hydrogen peroxide. Antigen retrieval was performed using the microwave technique with a citrate buffer (Biogenex, San Ramon, CA). The slides were blocked with 4% bovine serum albumin (Gibco, Grand Island, NY), incubated for 60 minutes at room temperature with biotinylated Fab, washed, and detected with a stain and chromogen yielding a violet precipitate (Vectastain and Vector VIP; Vector Laboratories, Burlingame, CA). The tissues were counterstained with methyl green, washed, dehydrated, and permanently mounted.

As a positive control for immunohistochemistry and preservation of human ocular structures, detection of either neurofilaments or endothelium was performed. Preparation of slides was as noted followed by an initial antibody reaction using mouse anti-neurofilament protein clone 2F11 (Dako, Carpenteria, CA), rabbit anti-factor VIII-related antigen (Dako) or no primary antibody. Visualization of bound antibody was achieved by using a horseradish peroxidase-conjugated rabbit anti-mouse or swine anti-rabbit antibody (as appropriate for the detection of the mouse or rabbit primary antibody) (Dako) with colorimetric detection using diaminobenzidine yielding a brown color. Counterstaining of nuclei was performed with hematoxylin.

**Ocular Specimens**

Human eyes were procured from the University of California Los Angeles Eye Bank 4 to 24 hours after death, stored in a moist chamber at 4°C until dissection, and processed immediately on arrival. The eyes were bisected in the horizontal plane adjacent to the macula. Half of each eye was fixed for 24 hours in formalin, paraffin embedded, and processed for immunohistochemistry. The remaining portions of the eye were microdissected so that samples of the cornea, sclera, iris plus ciliary body, retina, and retinal pigment epithelium plus choroid were saved. The dissected tissues were snap frozen and stored at −80°C. In preparation of the cell extracts, tissues were homogenized in glass in 2% sodium dodecyl sulfate (SDS), 100 mM dithiothreitol, and 50 mM Tris (pH 6.8). The homogenized sample was heated to 100°C for 5 minutes. Remaining tissue fragments were removed by centrifugation; the supernatant was frozen at −80°C until use.

**Western Blot Analysis of pANCA Tissue Antigens**

Tissue extracts were fractionated on 12% gels by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. Proteins were transferred to nitrocellulose membranes (Amersham Life Sciences, Buckinghamshire, UK) in Tris glycinse buffer (National Diagnostics, Atlanta, GA), and verified by Ponceau S red staining (Sigma, St. Louis, Missouri).
FIGURE 3. Identification of candidate ocular pANCA antigens by western blot analysis. Tissue extracts were prepared and separated on 12% gel by SDS-PAGE, transferred to a nitrocellulose filter, and analyzed for Fab 5-3 reactivity. Fab anti-tetanus toxoid showed limited reactivity in western blot analysis with these cell extracts. Tissues used included retina (R), retinal pigment epithelium plus choroid (RP-C), ciliary body plus iris (CB-I), cornea (C), and sclera (S). Samples were applied to the gel in equal protein concentration, judged by Coomassie staining. Molecular weight markers are listed on the right. Candidate pANCA tissue antigens are indicated by arrows on the left.

MO). The positions of the proteins used as molecular weight markers were noted and marked. The membrane was then blocked in 5% nonfat milk in Tris-buffered saline (TBS)-0.05% Tween 20 (Pierce). Fab 5-3 at a concentration of 2 μg/ml or human serum diluted in TBS-0.05% Tween 20 was incubated for 1 hour and washed three times in TBS-0.05% Tween 20. Nitrocellulose membranes, after protein transfer, were cut into strips before incubation with the primary antibody to evaluate reactivity with human pANCA+ or pANCA− serum. An alkaline-phosphatase-conjugated goat anti-human IgG (Fab′)2 was exposed to the blots at a 1:1000 dilution. 5-bromo-4-chloro-3-indolyl-phosphate (BCIP)/nitroblue tetrazolium (NBT) was used to visualize the location of the bound antibody (Sigma).

RESULTS
Identification of Ocular Cells Expressing pANCA Antigen
The Fab 5-3 monoclonal antibody was used to identify pANCA reactivity in formalin-fixed, paraffin-embedded ocular specimens. For specificity control, an anti-tetanus toxoid Fab (Fab TT) was used in all experiments. In the spleen, pANCA+ cells were limited to the neutrophil, which expressed a nuclear antigen (data not shown). This result is consistent with the immunofluorescent pattern of pANCA reactivity observed in isolated blood neutrophils. Immunohistochemical reactivity of the Fab 5-3 antibody in the eye was remarkable for an intense reactivity with the cytoplasm of the nonpigmented layer of the ciliary body epithelium (Fig. 1A) and retinal ganglion cells (Fig. 1B). Identical reactivity was observed with both the Fab 5-2 and the Fab 5-3 antibodies (data not shown). The ocular specimens showed appropriate staining of vascular endothelium (Fig. 2B, 2E) and neurofilaments (Fig. 2C).

Candidate Ocular pANCA Antigens
A panel of ocular tissue extracts (retina, retinal pigment epithelium plus choroid, ciliary body plus iris, cornea, and sclera) was analyzed by western blot to identify pANCA-reactive ocular proteins (Fig. 3). In accordance with the findings in immunohistochemical analysis, the specific proteins detected by Fab 5-3 were expressed in the retina and the ciliary body plus iris. One protein species was a 32- to 33-kDa doublet, similar or identical with the neutrophil antigen recently identified as histone H1 (Eggena M et al., unpublished results, 1998). Minor bands migrating at approximately 35 kDa to 40 kDa and 67 kDa in the retinal extracts were also observed. A second set of proteins was detected in retina and in ciliary body plus iris extracts (Fig. 3) but was absent in the neutrophil (Eggena et al., unpublished results, 1998). These multiple ocular-associated
pANCA+ protein species predominantly migrated at 20 kDa, 28 kDa, and 80 kDa and at 90 kDa to 94 kDa.

Ocular tissues from seven subjects were evaluated by western blot analysis to determine whether pANCA expression was a polymorphic trait (Fig. 4). Retina and ciliary body plus iris exhibited the identical pattern of reactive protein species, indicating that pANCA antigen expression in these tissues is nonpolymorphic.

One concern about the use of Fab 5-3 to identify the candidate pANCA antigen is whether the monoclonal Fab is representative of serum pANCA. Serum pANCA is a low-titer antibody and is difficult to analyze by western blot because of problems with nonspecific background reactivity. A high-titer pANCA-positive serum from a patient with uveitis was therefore chosen and compared with Fab 5-3 in western blot analysis of retinal cell extracts (Fig. 5). This comparison showed that the serum pANCA detected the same protein species as Fab 5-3. This finding validates the use of Fab 5-3 as a tool for identifying the cognate pANCA antigen.

**DISCUSSION**

In this study we investigated the prediction that the marker antibody pANCA would identify a pANCA-positive cell type in ocular tissues. Immunohistochemical experiments using the pANCA monoclonals Fab 5-2 and Fab 5-3 showed prominent reactivity with cytoplasmic proteins in retinal ganglion cells and ciliary body epithelium. Previous studies of U C-pANCA have characterized a nuclear antigen, recently identified as histone H1 (Eggena et al., unpublished results, 1998) or related family members.24 The reactive epitope of histone H1 is localized to the COOH-terminal linker domain, which is concealed in most other cell types because of intact nucleosome structure. This epitope is exposed during western blot analysis because of SDS-PAGE dissociation of chromatin protein complexes and detected as a 32- to 33-kDa doublet (Fig. 3). Because histone H1 expression is ubiquitous in mammalian cells, it was surprising that these protein species were not observed in some ocular tissues. In sclera and cornea, this may have been because of the abundance of extracellular matrix and therefore a relative paucity of cellular nuclei. This explanation does not account for the low level of histone H1 in the highly cellular retinal pigment epithelium-choroid. An alternate possibility is that the histone H1 is degraded secondary to tryptic protease activity. Tryptic protease levels are highly variable and tissue specific and can result in rapid postlysis degradation of histone H1 in such tissue extracts.26

In a recent immunohistochemistry study, investigators have described a novel set of pANCA antigens, distinguished by their cytoplasmic localization and restricted tissue-specific expression (Gordon LK, Eggena M, Targan SR, Braun J, unpublished results, 1998). In a broad survey of systemic tissues, this cytoplasmic family of pANCA antigens was only detected in mast cells, pancreatic islets, and cerebellar neurons. Western blot analysis of the mast cell revealed that the predominant cytoplasmic antigens were 24- and 28-kDa proteins. In the present study, we identified two new cell types derived from the ocular compartment: retinal ganglionic neurons and ciliary body epithelium. By western blot analysis, these share with mast cells a 28-kDa protein species. In addition, the ocular tissues uniquely express higher molecular weight reactive proteins (80 kDa and 90-94 kDa). The structural relationship between these different protein species is uncertain, and the possibility that some may be related or identical peptides merits investigation.

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**Figure 4.** Expression of pANCA antigens in retina and ciliary body is nonpolymorphic. Tissue extracts from seven specimens were analyzed for pANCA antigen expression by western blot analysis. Retina (A) and ciliary body plus iris (B) extracts were prepared and separated on 12% gel by SDS-PAGE, transferred to a nitrocellulose filter, and analyzed for Fab 5-3 reactivity. Samples were applied to the gel in equal protein concentration, judged by Coomassie staining. Molecular weight markers are listed on the right. Candidate pANCA tissue antigens are indicated by arrows on the left.
What is the relationship between these cell types and antigens to uveitis? There are elegant precedents for antigen polymorphisms of somatic proteins that form a genetic susceptibility trait for autoimmunity. However, in a limited survey of seven subjects, the present study failed to detect polymorphism in pANCA antigen expression.

The detection of retinal associated cytoplasmic pANCA antigens was surprising, because there is no ganglion cell dysfunction in patients with pANCA+ UC or anterior uveitis. Determining the functional significance of this observation requires further evaluation. Although there is evidence for divergent accessibility of retinal ganglion cell layer and ciliary body inflammatory processes, the large body of work with the retinal S antigen model indicates retinal antigen presentation and immunologic activity in the setting of simultaneous inflammation of the anterior and posterior segment.

The observation of pANCA antigen in ciliary body epithelium is provocative, because this microanatomic site is a nidus of inflammation clinically observed in human anterior uveitis. In experimental uveitis, infiltration of the ciliary body and iris by inflammatory cells is an early observation. It is possible that local antigen expression, in combination with the ciliary body microenvironment plays a role in uveitis pathogenesis. At present there is no confirmation that reactivity against the pANCA ciliary body antigens plays a role in pANCA+ uveitis.

Identification and purification of these ciliary body-associated pANCA antigens with subsequent analysis of immunity directed against these antigens will allow further investigation into their potential role in anterior uveitis.

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**References**


Loss of Heterozygosity in Pseudoxefoliation Syndrome

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PURPOSE. Pseudoxefoliation (PEX) syndrome is characterized by the accumulation of a material of unknown origin in the anterior structures of the eye. Loss of heterozygosity (LOH) in a genetic locus indicates the presence of a gene located in the same region that could be implicated in the development or the progression of a disease. In this study, the occurrence of LOH in tissues involved in PEX and the possible correlation of LOH incidence with clinical parameters were evaluated.

METHODS. Twelve iris specimens, 12 anterior capsule specimens, and respective blood samples were obtained from 17 patients with PEX (13 men), who were undergoing glaucoma and cataract surgery. Sixteen anterior capsule specimens and four iris specimens were obtained from 16 patients with PEX (13 men), who were undergoing glaucoma and cataract surgery. Sixteen anterior capsule specimens and four iris specimens were obtained from 16 patients with PEX (13 men), who were undergoing glaucoma and cataract surgery. Sixteen anterior capsule specimens and four iris specimens were obtained from 16 patients with PEX (13 men), who were undergoing glaucoma and cataract surgery.

RESULTS. Overall, 83.3% (20/24) of PEX specimens and 94.11% (16/17) of patients with PEX had LOH. The highest incidence of LOH was observed in marker D13S175 (41.6%) followed by D7S478 and D7S479 (37.5%). Only one non-PEX specimen showed LOH. The number of loci lost was directly related to the altitude of the patients’ present residence, but the number lost did not differ significantly between the iris and capsule samples.

CONCLUSIONS. The occurrence of LOH in tissues involved in PEX implies a genetic role in PEX pathogenesis at a cellu-