Identification and Functional Validation of Novel Autoantigens in Equine Uveitis*

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The development, progression, and recurrence of autoimmune diseases are frequently driven by a group of participatory autoantigens. We identified and characterized novel autoantigens by analyzing the autoantibody binding pattern from horses affected by spontaneous equine recurrent uveitis to the retinal proteome. Cellular retinaldehyde-binding protein (cRALBP) had not been described previously as an autoantigen, but subsequent characterization in equine recurrent uveitis horses revealed B and T cell autoreactivity to this protein and established a link to epitope spreading. We further immunized healthy rats and horses with cRALBP and observed uveitis in both species with typical tissue lesions at cRALBP expression sites. The autoantibody profiling outlined here could be used in various autoimmune diseases to detect autoantigens involved in the dynamic spreading cascade or serve as predictive markers. Molecular & Cellular Proteomics 5: 1462–1470, 2006.

Uveitis is a remitting-relapsing autoimmune disease of the inner eye characterized by breakdown of the blood-retinal barrier, infiltration by inflammatory cells, and tissue destruction ultimately leading to blindness (1). Although a diverse set of initiating events have been proposed to cause uveitis, the pathogenesis is finally perpetuated by CD4+ Th1 effector cells (2). Equine recurrent uveitis (ERU), a spontaneous, T cell-mediated disease with high prevalence among horses, serves as a suitable model for human autoimmune uveitis due to the common sharing of at least two different autoantigens, S-antigen (S-Ag) and interphotoreceptor retinoid-binding protein (IRBP), between equine and human uveitis patients (3, 4). Furthermore both autoantigens are capable of inducing experimental uveitis in horses (5, 6), and in both, peripheral blood-derived, autoaggressive T cells specific for S-Ag and IRBP epitopes have been detected (3, 4). In most autoimmune diseases, several autoantigens participate in the pathogenesis (7), and epitope spreading is accountable for disease induction, progression, and inflammatory relapses (8–12). Knowledge of the targeted autoantigens is therefore indispensable for understanding the nature of acute autoimmune responses during recurrent attacks. Furthermore identifying persons at risk to develop several autoimmune diseases is based upon the presence of an autoreactive response to several autoantigens prior to disease state (13–16). The accuracy of prediction increases by combining autoantigens (14, 15). The profiling of autoantibodies serves best in predicting T cell-mediated autoimmune diseases (13). Although autoantibodies play a subordinated role in the pathogenesis of Th1-mediated diseases, they are detectable in both ERU cases and human autoimmune uveitis patients (4, 17). To identify potential novel candidate autoantigens in autoimmune uveitis, we tested the IgG binding profile to the retinal proteome from horses with spontaneous ERU.

EXPERIMENTAL PROCEDURES

Animals

Spontaneous Uveitis—For the analysis of cellular retinaldehyde-binding protein (cRALBP) as an autoantigen in the spontaneous ERU model, we sampled a total of 344 ERU cases and 137 controls for Western blotting, ELISA, and T cell assays. In detail, sera of 35 ERU cases and 32 healthy control horses were used for the autoantibody profiling on the retinal proteome. Sera of 289 additional ERU cases and 95 control horses were used in the cRALBP autoantibody screening with ELISA. All cRALBP-positive samples were verified using one-dimensional Western blots with 1 μg of cRALBP/lane (84 cRALBP-positive ERU sera, 20 cRALBP-negative ERU sera as negative control, 11 cRALBP-positive healthy control horses, and 20 cRALBP-negative, healthy control horses). We examined the autoreactive T cell response of 20 additional ERU cases and 10 healthy control horses; 10 ERU cases and five control horses were repeatedly tested for their T cell response in a long term study (22 months) on epitope spreading. All cases were selected randomly; we tested the
next cases brought to the Equine Clinic in Munich without further selection in a respective experimental condition (autoantibody profiling, large scale autoantibody screening, T cell response, and epitope spreading).

**Induced Uveitis**—We used 20-week-old Lewis rats (Elevage Janvier; 14 rats, Table I) and German standard outbred horses for the induction of uveitis (six horses, Table I). Initial uveitis induction experiment in rats involved immunization of six Lewis rats subcutaneously with 100 μg of recombinant human cRALBP emulsified in 50 μg of complete Freund’s adjuvant (Sigma). As a negative control, three Lewis rats were immunized with an unrelated protein (chicken IL-12p40 in 50 μg of complete Freund’s adjuvant (CFA)) expressed and purified using the same bacteria and protocols. In a second experiment, Lewis rats were immunized with 75 μg of cRALBP in 50 μg of CFA (n = 3); separate animals (n = 2) were immunized with the same amount of IL-12p40 in 50 μg of CFA as a negative control. For the induction of uveitis in horses, three horses received 500 μg of cRALBP in 500 μl of CFA subcutaneously at the neck. Two identical booster immunizations were given in 28-day intervals for potential disease reinduction. Three control horses received identical injections whereby cRALBP was replaced by an irrelevant protein (BSA). Animal studies were approved by the upper Bavarian Government Review Board (AZ 209.1/211-2531-86/02).

**2D Gel Electrophoresis**

Porcine retinas were homogenized, stabilized with protease inhibitors (Roche Applied Science), lyophilized, and stored. The protein pellets were solubilized in 2DE lysis buffer (9 m urea, 2 m thiourea, 1% dithioerythritol, 4% CHAPS, 2.5 μM EGTA, and 2.5 μM EDTA). Immobilized dry strips (pH 3–10 non-linear, 24 cm; Amersham Biosciences) were immersed overnight in lysis buffer containing 150 μg of protein sample, additional 1% Pharmalyte pH 3–10 (Amersham Biosciences), and 0.5% bromphenol blue. Isoelectric focusing was done on a Multiphor (Amersham Biosciences) for 100 kV-h at 20 °C followed by separation on gradient SDS-PAGE gels (9–15%) at a constant 3 watts/gel (Ettan, Amersham Biosciences). One set of gels was silver-stained for mass spectrometry, and the second was transferred onto PVDF membranes (Amersham Biosciences) for Western blot analysis.

**Autoantigen Detection**

PVDF membranes were blocked with PBS-Tween 20-polyvinylpyrrolidone and incubated with sera from ERU cases and controls. Autobody binding was detected with rabbit anti-horse IgG-horse-radish peroxidase (Sigma) and enhanced chemiluminescence (Amersham Biosciences). To assign visible spots to those detected on silver-stained gels, we subsequently stained the PVDF membranes with colloidal gold (Bio-Rad). Autoantibody binding was detected with rabbit anti-horse IgG-horse-radish peroxidase (Sigma) and enhanced chemiluminescence (Amersham Biosciences). To assign visible spots to those detected on silver-stained gels, we subsequently stained the PVDF membranes with colloidal gold (Bio-Rad).

**Mass Spectrometry**

Selected spots were excised from silver-stained gels, destained, dehydrated in 100 μl of 40% acetonitrile (3 × 15 min), and subjected to overnight tryptic proteolysis in 5–10 μl of 1 mM Tris-HCl, pH 7.5, containing 0.01 μg/μl trypsin (Promega). MALDI-TOF peptide mass fingerprints were obtained on a Bruker Reflex III mass spectrometer (Bruker) as described before (18). Briefly samples were cocrystallized with a matrix consisting of 2,5-dihydroxybenzoic acid (Sigma) (20 mg/ml in 20% acetonitrile, 0.1% TFA) and 2-hydroxy-5-methoxybenzoic acid (Fluka) (20 mg/ml in 20% acetonitrile, 0.1% TFA) in a 9:1 ratio (v/v) on 400-μm AnchorChip targets (Bruker). Database searches were performed using the Mascot software package (Version 1.9.05, Matrix Science, London, UK) allowing one cleavage and 100-ppm mass accuracy in all mammalian entries in the Mass Spectrometry Protein Sequence Database (MSDB). Scores are given as probability-based MOWSE scores.

**Purification of cRALBP**

Human cRALBP (plasmid kindly provided by John W. Crabb, Cole Eye Institute, Cleveland, OH) was expressed in BL21DE3 (Invitrogen) Escherichia coli bacteria and purified on a nickel-agarose column (Qiagen). Endotoxin was removed on an Endotrap column (Profs) and then controlled by a Limulus amebocyte lysate test (Sigma; levels below 0.8 endotoxin units/ml). The purified preparation was confirmed by mass spectrometry as human cRALBP (Mascot score, 183).

**Measurement of cRALBP-specific Antibodies**

For large scale autoantibody screening, an indirect ELISA was used to measure anti-cRALBP autoantibodies. Maxisorb plates (Nunc) were coated with 1 μg of cRALBP/ml of coating buffer. Sera were tested in a 1:1000 dilution. A positive control serum (cRALBP-immunized horse number 1) was tested in a dilution series on every plate. Positive samples were defined as absorbance values greater than the mean absorbance value of negative controls with low titers plus the 3-fold standard deviation. All positive results of the cRALBP ELISA were verified with Western blots using 1 μg of cRALBP/lane. Twenty cRALBP autoantibody-negative samples from ERU and healthy control group were also retested by Western blotting. An anti-human cRALBP antibody (Cayman Chemical) was used as a positive control.

**T Cell Proliferation Assays**

We carried out T cell proliferation assays using the following models: spontaneous ERU (n = 20) and healthy control horses (n = 10), cRALBP-induced uveitis (three horses and nine rats), BSA-immunized negative control horses (n = 3), and chL-12p40-immunized negative control rats (n = 5). Peripheral blood-derived lymphocytes (PBLs; horses) or spleen-derived lymphocytes (rats) were separated by density gradients (Amersham Biosciences), and 5 × 10⁶ cells were cultivated for 5 days with or without cRALBP, IRBP-derived peptides R14 (bovine IRBP; aa 1169–1191), PDIIRBP (bovine IRBP; aa 1174–1187), PI731 (human IRBP; aa 731–745), PI1137 (human IRBP; aa 1137–1153), and S-Ag-derived peptides S-Ag 281 (bovine S-Ag; aa 281–296), S-Ag 286 (bovine S-Ag; aa 303–320), PDSAg (bovine S-Ag; aa 342–355), Peptide M (bovine S-Ag; aa 303–320) or complete bovine S-antigen. Cultures were pulsed with 2 μCi of [3H]thymidine (Amersham Biosciences)/well for 18 h, and incorporated radioactivity was subsequently counted (Packard Instrument Co.).

**Uveitis Induction**

Uveitis in horses was induced by subcutaneous injections of 500 μg of cRALBP emulsified in CFA (Sigma) and an intravenous injection of 10⁷ dead Bordetella pertussis bacteria. Negative control horses (n = 3) received the same immunizations, but cRALBP was replaced by BSA. One group of rats (Experiment 1, n = 6) received 100 μg of cRALBP in CFA; the second group of rats (Experiment 2, n = 3) received 75 μg of CFA. Control rats (Experiment 1, n = 3; Experiment 2, n = 2) received the same amount of chL-12p40 instead of cRALBP.

**Disease Evaluation and Scoring**

We graded clinical signs between 0 and 4 (no disease and severe disease, respectively) based on conjunctivitis, anterior chamber inflammation, miosis, opacification of the lens, vitreal inflammation, and retinal changes. Histopathological studies were also graded on a scale from 0 to 4 based on infiltration by inflammatory cells, vascu...
granuloma formation, retinal detachment, and destruction of the retinal architecture.

Histology and Immunostaining

Bouin (Sigma)-fixed eyes were embedded in Paraffin (Microm). Antigen retrieval was performed at 99 °C for 15 min in 0.1M EDTA-NaOH buffer, pH 8.8. The rat anti-CD3-12 antibody was a kind gift from E. Kremmer (GSF Grosshadern). We used mouse antibodies specific for glutamine synthase (Pharmingen), vimentin (Sigma), and glial fibrillary acidic protein (Dako) as Mueller glia cell markers; rabbit anti-human van Willebrand factor (Linaris) and mouse anti-human vascular endothelial growth factor (Pharmingen) were used to label blood vessels. Sections were stained with the VECTASTAIN Elite ABC horseradish peroxidase kit (Linaris) coupled to Vector VIP (Linaris) or Histogreen (Linaris) or with Vectastain ABC alkaline phosphatase kit (Linaris) coupled to Vector red or black alkaline phosphatase.

Statistical Significance

Frequency of cRALBP autoantibodies in ERU and control groups was compared using the χ² test.

RESULTS

Identification of Novel Autoantigens—The remitting-relapsing character of several autoimmune diseases can be explained by epitope spreading. During inflammation of the target tissue, cryptic epitopes become visible to the immune system and induce a new autoaggressive reaction. To determine the number of involved autoantigens in the spontaneous disease ERU, we first tested the specificity of sera from affected horses on the porcine retinal proteome. Total protein was resolved by 2DE (Fig. 1a), transferred to PVDF membranes, and probed with sera containing autoantibodies from 35 ERU cases and 32 controls. Only those protein spots detected by ERU sera and not by controls were selected for further identification by MALDI-TOF mass spectrometry. As expected, marked positive reactions to the well characterized autoantigens S-Ag (Fig. 1b) and IRBP were detected. Two additional proteins were selectively bound by autoantibodies of ERU cases (8 of 35) that we identified as recoverin (Fig. 1c) and cRALBP (Fig. 1d). The uveitogenicity of recoverin had already been demonstrated in the Lewis rat (19). Furthermore because cRALBP is a novel autoantigen not described previously, we first evaluated the pathological relevance of our findings in a larger group of horses with spontaneous disease.

Frequency of Autoantibodies and T Cells Specific for cRALBP—We tested the frequency of cRALBP autoantibody-positive horses with ERU by sampling sera from 289 ERU-affected horses against 95 controls and applying an indirect ELISA with human recombinant cRALBP as antigen (Fig. 2a); positive reactions were verified with Western blots (Fig. 2b). Twenty-nine percent of ERU cases (84 of 289) demonstrated a positive reaction to human cRALBP (Fig. 2a) compared with 12% of control sera (Fig. 2a; 11 of 95; frequency of cRALBP autoantibodies in ERU was greater than that in controls, p < 0.001). As most autoimmune diseases are T cell-mediated, we next examined the cRALBP recognition by autoaggressive T cells. PBLs from 20 ERU cases and 10 control horses were stimulated in vitro with 5 µg of human cRALBP in triplicate experiments. For these assays, we used an endotoxin-free cRALBP preparation to avoid false positive reactions to lipopolysaccharide derived from the E. coli expression system. We found that lymphocytes from five horses with spontaneous uveitis showed an exclusive positive reaction to human cRALBP stimulation (Fig. 2c). One additional case, also reacting to human cRALBP, also showed a reaction to an IRBP-derived peptide (Fig. 2c, ERU case 11). PBL from healthy control horses did not proliferate.
Epitope Spreading as a Pathogenic Mechanism in ERU—We next directed our attention to the effect of cRALBP as autoantigen in a long term study on epitope spreading in 10 ERU-affected horses. Blood samples were drawn every 8 weeks, and the in vitro cellular response pattern of PBLs were tested to human cRALBP and several peptides representing various epitopes of the retinal autoantigens S-antigen and IRBP. These same horses were closely monitored during this period for signs of inner eye inflammation. In the event of an overt uveitic attack, an additional blood sample was drawn at that particular time point to obtain data on the immune response during relapses. The total time course of observation ranged from 18 to 36 months, and analysis of cRALBP was included during the last 6 months. The immune response in five visually healthy horses was also tested concurrently with experimental cases. We found a positive T cell reaction to cRALBP in three ERU horses during the study (Fig. 2, d and e), two of which are presented here (Fig. 2, d and e), that was either preceded or proceeded by a reaction shift to other retinal antigens (Fig. 2, d and e, respectively). These data substantiate epitope spreading in ERU horses and the participation of cRALBP. One particular ERU case, “Roger,” demonstrated a positive cRALBP response during a uveitic attack in July 2004 (Fig. 2 e).

Histopathology for cRALBP-positive ERU Horse Roger—One ERU case, Roger, was euthanized in September 2004 due to a broken leg. This event enabled us to collect the eyes for histological analysis. Histopathological examination revealed complete destruction of the retinal cytoarchitecture of the right eye with sparing of the retinal pigment epithelium, which itself appears to undergo an inflammatory process. Complete destruction of photoreceptor outer segments in the left eye with accompanying infiltration of the nerve fiber layer. h, up-regulation of GFAP (green) in remaining Mueller glial cells of the right eye. i, similar GFAP up-regulation was noted in the left eye. Ab, antibody.

Fig. 2. cRALBP association with spontaneous disease. a, large scale cRALBP autoantibody screening using an indirect ELISA. Red dots represent positive reactions of ERU cases (gray bar, left) or healthy control horses (white bar, right). Results are given as absorbance units at 450 nm (OD 450 nm). b, example of result verification in Western blot. cRALBP-positive reaction at 36 kDa (left) and negative reaction (right) are shown. c, lymphocyte reaction profile for ERU cases (n = 20) examined as a function of stimulation index. Six cases showed striking T cell responses after stimulation with cRALBP (red), whereas five independent cases responded to either S-antigen (blue) or IRBP (white); two cases shared an immune response to IRBP. d and e, long term analysis of T cell response to various epitopes. d, stimulation index for ERU case Cricket. cRALBP (red) analysis was introduced 5 months prior to the end of the study. Here a shifting profile was evident with a striking response to cRALBP. e, stimulation index for ERU case Roger. A shifting profile was also observed in which a sudden cRALBP response was detected during a uveitic attack. f–i, histopathology of Roger’s eyes sampled following unexpected death: f and g, H&E stain; h and i, Histogreen. f, complete destruction of the retinal cytoarchitecture of the right eye is evident with sparing of the retinal pigment epithelium, which itself appears to undergo an inflammatory process. g, complete destruction of photoreceptor outer segments in the left eye with accompanying infiltration of the nerve fiber layer. h, up-regulation of GFAP (green) in remaining Mueller glial cells of the right eye. i, similar GFAP up-regulation was noted in the left eye. Ab, antibody.
The immunization dosage of cRALBP or the respective control antigen is given in column 3 for each individual animal (columns 1 and 2). The results of the cRALBP autoantibody ELISA are given as $A_{450\text{nm}}$ (column 4) using 1 $\mu$g of cRALBP/ml as coating antigen and a 1:1000 dilution of the tested sera. Column 5 shows the stimulation index in the in vitro proliferation assay. PBLS from horses and spleen-derived leucocytes from Lewis rats were used for this assay.

| Species            | Imunization antigen and dosage | $A_{450\text{nm}}$ cRALBP ELISA | T cell stimulation index cRALBP |
|--------------------|-------------------------------|----------------------------------|--------------------------------|
| Horses             |                               |                                  |                                |
| 1                  | 500 $\mu$g of cRALBP          | 0.336                            | 2.4                            |
| 2                  | 500 $\mu$g of cRALBP          | 0.301                            | 4.0                            |
| 3                  | 500 $\mu$g of cRALBP          | 0.210                            | 3.0                            |
| 4                  | 500 $\mu$g of BSA             | 0.078                            | 1.0                            |
| 5                  | 500 $\mu$g of BSA             | 0.089                            | 1.0                            |
| 6                  | 500 $\mu$g of BSA             | 0.079                            | 1.0                            |
| Lewis rats         |                               |                                  |                                |
| Experiment 1       |                               |                                  |                                |
| 1                  | 100 $\mu$g of cRALBP          | 0.403                            | 23.0                           |
| 2                  | 100 $\mu$g of cRALBP          | 0.427                            | 26.0                           |
| 3                  | 100 $\mu$g of cRALBP          | 0.442                            | 26.7                           |
| 4                  | 100 $\mu$g of cRALBP          | 0.382                            | 1.2                            |
| 5                  | 100 $\mu$g of cRALBP          | 0.447                            | 26.7                           |
| 6                  | 100 $\mu$g of cRALBP          | 0.457                            | 40.3                           |
| 7                  | 100 $\mu$g of IL-12p40        | 0.174                            | 1.0                            |
| 8                  | 100 $\mu$g of IL-12p40        | 0.145                            | 1.1                            |
| 9                  | 100 $\mu$g of IL-12p40        | 0.168                            | 1.1                            |
| Experiment 2       |                               |                                  |                                |
| 10                 | 75 $\mu$g of cRALBP           | 0.433                            | 13.7                           |
| 11                 | 75 $\mu$g of cRALBP           | 0.419                            | 8.6                            |
| 12                 | 75 $\mu$g of cRALBP           | 0.449                            | 9.0                            |
| 13                 | 75 $\mu$g of IL-12p40         | 0.115                            | 0.9                            |
| 14                 | 75 $\mu$g of IL-12p40         | 0.142                            | 2.3                            |

S-Ag-induced uveitis in the horse, inflammation was restricted to the posterior part of the eye accompanied by lesser clinical signs of pain (clinical scores = 0.6, 0.8, and 0.9). Relapse of uveitis occurred in all horses within 7 days following reapplication of cRALBP.

Histopathological analysis of cRALBP-induced equine uveitis demonstrated marked focal destruction of the retinal architecture with complete structural (cellular and layer) loss (Fig. 3, a (healthy) and b (cRALBP-immunized)) and disruption of the blood-retinal barrier (Fig. 3b). The infiltration was mainly by CD3$^+$ cells (Fig. 3d, green) that were localized in the choroid. In sites spared of tissue destruction, we observed a striking increase in the number of retinal blood vessels, as demonstrated by van Willebrand factor (vWF) staining (Fig. 3e, purple), that were delineated with VEGF immunoreaction (Fig. 3f, dark brown). Moreover analysis of the Mueller glia cell markers GFAP and glutamine synthase (GS) (Fig. 3g: GFAP, green; GS, red) revealed marked GFAP up-regulation (Fig. 3h) accompanied by a GS down-regulation (Fig. 3h) within retinal scars (histological scores = 4, 2, and 4).

Three control horses were treated with BSA alone at the same time points as the cRALBP-treated group. We could not detect signs of uveitis in the eyes of these animals (clinical and histological scores = 0, 0, and 0).

Uveitogenicity of cRALBP in the Lewis Rat—To complete the characterization of cRALBP as autoantigen, we next tested the uveitogenic potential of human cRALBP in the Lewis rat. Six rats were immunized subcutaneously with 100 $\mu$g of recombinant human cRALBP emulsified with 50 $\mu$g of CFA. In the control group, cRALBP was replaced with an unrelated protein (chicken IL-12p40) expressed and purified using the same bacteria and protocols. The time course for this experiment was 32 days, and the eyes were then examined histologically. Five of six cRALBP-immunized rats showed signs of uveitis with lesions typical for posterior uveitis involving the choroid and retina. Near complete destruction of the retinal architecture was observed in cRALBP-treated rats accompanied by loss of photoreceptor outer segments (Fig. 4, cf. a and b, healthy and immunized, respectively) and the outer nuclear layer. We found the majority of inflammatory cells located beneath the inner nuclear layer (Fig. 4b) as well as minor infiltration in the nerve fiber layer and inner plexiform layer (CD3; Fig. 4d, dark brown). VEGF expression was found near disintegrated retinal parts (Fig. 4e, dark brown). The retinae of cRALBP-treated rats had enlarged blood vessels as shown by vWF staining (Fig. 4f, purple). Immunostaining for GFAP and GS showed differential expression within inflamed parts: namely GFAP-positive was restricted to Mueller glia end-feet in spared retinal areas (Fig. 4g, green) and up-regulated in destroyed areas (Fig. 4h; GS expression was down-regulated throughout the lesioned areas (Fig. 4h, red). The average histological score of the cRALBP-immunized rats was 1.2 (0.5, 2, 1, 0, 3, and 0.5) with a five of six incidence.
In a second experiment, the cRALBP immunization dosage per rat was reduced (75 μg). Nevertheless, we observed 100% incidence in this group (three of three) with histological alterations similar to those in the higher dosage group and an average score of 1.8 (2, 0.5, and 3). No signs of uveitis were detected in animals comprising the control groups.

A marked autoreactive T cell response was detectable in eight of nine cRALBP-immunized rats using spleen cells for the in vitro proliferation assay (Table I). Spleen cells from chicken IL-12p40 control-immunized rats from both experiments did not react to in vitro stimulation with cRALBP (Table I). Autoantibodies to cRALBP were evident in all rats from the cRALBP immunization groups (Table I) in contrast to the IL-12p40 negative control rats (Table I).

**DISCUSSION**

The identification of disease-specific autoantigens is essential for understanding the pathogenesis of autoimmune diseases in general and defining markers for detection of preclinical autoimmune disorders (20). Epitope spreading accounts for recurrent episodes characteristic of many autoimmune syndromes and has been extensively studied in experimental autoimmune encephalitis (21, 22), leading to discovery of a hierarchical order of determinants occurring throughout the inflammatory process (21). In the same model, the shift of the autoimmune response is followed by a new attack of the target organ and a subsequent diminished response to the initial autoantigenic repertoire (23). Successful inhibition of disease progression induces tolerance to a peptide at the third position of the reaction chain (10). Experimental autoimmune encephalitis progression was exclusively stopped by tolerance induction to an encephalitogenic spreading determinant instead of to unrelated encephalitogenic peptides uninvolved in the spreading cascade (10). Additionally, tolerance to an intermolecularly derived determinant was essential in blocking disease progression.

Patient studies have meanwhile confirmed existing T and B cell neoautoreactivity in different autoimmune diseases.
as rheumatoid arthritis (24), type I diabetes mellitus (12), multiple sclerosis (23), and autoimmune uveitis (3). Intermolecular reaction patterns underline the necessity to identify the complete arrangement of individual participating autoantigens (9).

Using a proteomic approach, we attempted to determine additional autoantigens in a spontaneous autoimmune disease occurring in outbreed horses. This method generated a broad antigenic repertoire comprising a wide range of proteins from the target tissue. The two-dimensional separation of proteins resulted in clear differentiation of ~3000 proteins, including post-translational modifications. The problem of matching immunoreactive protein spots from Western blots to overall protein pattern on silver-stained gels for identification of potential autoantigens by mass spectrometry was circumvented by staining the blots with colloidal gold (25). The procedure displays the complete protein pattern transferred to PVDF membranes after probing with sera, enabling the alignment of marked proteins to silver-stained gels and the confirmation of the efficiency, magnitude, and quality of the protein transfers. We found mass spectrometry to be an excellent method for autoantigen identification allowing us to identify not only already described autoantigens (S-Ag and recoverin) but also a previously unknown autoantigen in autoimmune uveitis, cRALBP. This retina- and pineal gland-specific protein interacts with several retinoid-processing enzymes, thus supporting retinoid supply to the retina. cRALBP participates in the visual cycle and is expressed in retinal Mueller glia cells and retinal pigment epithelium (26). Mutations in the human gene encoding cRALBP (RLBP1) cause autosomal recessive retinitis pigmentosa, a condition characterized by progressive photoreceptor degeneration and delayed dark adaptation (27). After identifying cRALBP-specific autoantibodies in some ERU cases (Fig. 1d) by 2DE/Western blotting, further evidence for the relevance of cRALBP in ERU was gained by carrying out a large scale screening for cRALBP autoantibodies in horses (spontaneous uveitis, n = 289; healthy controls, n = 95). We found a clear difference in autoantibody-positive results between ERU cases and

![Fig. 4. cRALBP also mediates uveitis in the Lewis rat. Representative sections are shown from nine cRALBP-immunized and five chlL-12p40-immunized control rats. a, H&E reference staining of a control rat showing normal retinal architecture. b, despite moderate loss of retinal architecture, a complete loss of photoreceptor outer segments with accompanying infiltration was observed. c, normal immunohistological counterstaining reference. d, infiltration of the retina by CD3-positive cells (brown) in cRALBP-treated rats. e, VEGF expression in cRALBP-treated rats was accompanied by alteration of retinal layers and scattered VEGF staining (brown). f, vasculitis demonstrated by von Willebrand factor staining (violet) in cRALBP-treated rats. g, reference double staining of GFAP (green) and GS (red) in control retina. h, as in the horse, an up-regulation of GFAP (green) was observed in the rat retina. However, a coincident and striking down-regulation of GS (red) was found.](image-url)
healthy controls (29 and 11\%, respectively). Additionally autoreactive, cRALBP-specific T cells were also demonstrated in six ERU cases (Fig. 2c) but not in healthy control horses. This finding substantiates the value of the autoantibody profiling strategy in identifying relevant autoantigens in primarily T cell-mediated diseases such as uveitis. Moreover we could establish a first link between the reactivity to cRALBP and epitope spreading in two horses (Roger and “Cricket”) from a long term T cell spreading study. Although it was impossible to follow Roger’s immune response profile to cRALBP over extended time, we could nevertheless observe a neoreactivity to cRALBP at the time point of the last uveitic attack (Fig. 2f–i). No autoaggressive, cRALBP-specific lymphocytes were apparent 3 months preceding this final uveitic episode (Fig. 2e), whereas previously the response pattern was restricted to IRBP-derived epitopes. Because cRALBP reactivity coincided with the last observed recurrence of uveitis, this attack could have been caused by the cRALBP-autoreactive reaction. A further long term study including several ERU cases is necessary to evaluate the possible role of cRALBP in the uveitis spreading cascade.

In our complete characterization of cRALBP as autoantigen, we could demonstrate clear uveitogenic potential in the horse and Lewis rat. In both models, the Mueller glia cells were partially destroyed and showed marked up-regulation for GFAP at transition sites between severely damaged and mildly inflamed retina (Figs. 3 and 4, g and h). Another marker, GS, was down-regulated (Figs. 3 and 4, g and h), indicating Mueller glia cell proliferation (28). The overexpression of VEGF, a major cytokine causing vascular leakage and angiogenesis, could be demonstrated in retinal vessels and Mueller glia cells in the equine model (Fig. 3f). In diabetic retinopathy, VEGF plays a role in the neovascularization of proliferative retinopathy and in breakdown of the blood-retinal barrier characterized by hyperpermeability of retinal vessels (29).

In the ERU case Roger, we found histopathology comparable to the cRALBP-induced uveitis model in horse and rat (Fig. 2, f–i). Roger was part of a long term study on epitope spreading and was euthanized unexpectedly 8 weeks following a uveitic attack with a shift to cRALBP as newly recognized autoantigen (Fig. 2e). The histopathology of Roger’s eyes underscores a possible role of cRALBP autoimmunity in ERU as there is a clear Mueller glia pathology visible (Fig. 2, f–i).

Additionally knowledge of all relevant autoantigens is useful for discriminating individuals at risk of developing autoimmune disease. This is best predicted when autoantibodies to different autoantigens are already present in the preclinical phase (9). Type I diabetes is one such example and best studied in this context. The presence of autoantibodies against three different autoantigens, the 65-kDa isoform of glutamate decarboxylase (GAD65), tyrosine phosphatase IA-2, and insulin, represents a positive predictive value for developing diabetes between 46 and 80\% in the general population that increases to 100\% in first degree relatives (9).

Additional studies indicate the effectiveness of a population-based diabetes screening (16, 30, 31), and a placebo controlled study is underway testing nasal tolerance induction to insulin in islet cell antibody-positive children (32).

Autoantibody positivity prior to clinical manifestations has also been demonstrated between 0.7 and 4.5 years before onset of systemic lupus erythematosus (33) and between 0.3 and 13.8 years in rheumatoid arthritis (34). Combining anti-cyclic citrullinated peptide antibodies and IgM rheumatoid factor increases both the positive predictive value and specificity to 100\% of developing rheumatoid arthritis within 5 years in double positive persons (34).

The predictive value of cRALBP autoantibodies for autoimmune uveitis remains to be tested in additional control samples. Investigation of a number of autoantigens in type I diabetes has shown that the three major islet autoantigens are most relevant for diagnosis and prediction (9). Because cRALBP autoantibodies have been shown in some control horses, we plan to extend these observations for several years because multiple reports have demonstrated the considerable value of autoantibodies for disease prediction, even in T cell-mediated autoimmune diseases (9, 13, 34). In addition, it has also been reported that the autoimmune process precedes overt clinical symptoms for many months or years (9). As such, cRALBP autoantibody-positive healthy horses (Fig. 2a) could be at risk for developing uveitis attacks in the future. Our findings suggest that the proteomic approach for autoantibody profiling of various autoimmune diseases is a convenient and effective tool for detecting autoantigens involved in the dynamic spreading cascade and may be applied as a tool for predictive screening.

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§ The on-line version of this article (available at http://www.mcponline.org) contains supplemental material.

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Novel Autoantigens

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