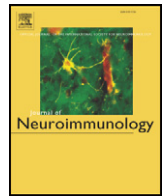




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Identification of α -fodrin as an autoantigen in experimental coronavirus retinopathy (ECOR)



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ABSTRACT

The coronavirus, mouse hepatitis virus (MHV), JHM strain induces a biphasic disease in BALB/c mice that consists of an acute retinitis followed by progression to a chronic retinal degeneration with autoimmune reactivity. Retinal degeneration resistant CD-1 mice do not develop either the late phase or autoimmune reactivity. A mouse RPE/choroid DNA expression library was screened using sera from virus infected BALB/c mice. Two clones were identified, villin-2 protein and α -fodrin protein. α -Fodrin protein was used for further analysis and western blot reactivity was seen only in sera from virus infected BALB/c mice. CD4 T cells were shown to specifically react with MHV antigens and with α -fodrin protein. These studies clearly identified both antibody and CD4 T cell reactivities to α -fodrin in sera from virus infected, retinal degenerative susceptible BALB/c mice.

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1. Introduction

Experimental coronavirus retinopathy (ECOR), an animal model of a retinal degenerative disease triggered by a virus, was established to examine the contributions of host genetics and host immune response to retinal degeneration (Robbins et al., 1990b). When retinal degeneration susceptible (BALB/c) mice are injected intravitreally with a neurotropic strain (JHM) of mouse hepatitis virus (MHV), a biphasic retinal disease develops. The acute phase (days 1–7 post-infection) is marked by inflammation and the presence of infectious virus and viral proteins, and a late/chronic phase (day 10–several months post-infection) that is characterized by the absence of infectious virus and retinal degeneration (Robbins et al., 1990b, 1991). In contrast, when retinal degeneration resistant (CD-1) mice were infected in the same manner, they developed only the acute phase of the disease (Wang et al., 1996).

In subsequent studies we examined the host immune response to retinal degeneration and the effects of cytokines and cytokine receptors in this disease process. We noted that IFN- γ plays a critical role in clearing the virus from the retina and we identified a correlation between retinal degeneration and TNF- α and TNF- α signaling in the susceptible coronavirus-infected mice (Hooks et al., 2003; Hooper et al., 2005). We next investigated very early cytokine and chemokine profiles as a measure of intensity of immune reactivity in the infected mice. These studies identified a distinct difference in the early innate immune response between the two mouse strains.

The retinal degeneration susceptible BALB/c mice had augmented innate responses that correlated with the development of autoimmune reactivity and retinal degeneration. These findings suggest a role for autoimmunity in the pathogenesis of ECOR (Detrick et al., 2008; Detrick and Hooks, 2010).

Our group also reported that autoantibodies to the retina and retinal pigment epithelium (RPE) developed in the BALB/c mice during the late phase of the disease (Hooks et al., 1993). However, no retinal autoantibodies were detected in the retinal degenerative resistant CD-1 mice who also failed to develop a retinal degeneration. It is known that anti-retinal antibodies can participate in retinal damage (Hooks et al., 2001). A few of the targets for these anti-retinal antibodies have been identified, but only antibodies against three of these targets, recoverin, α -enolase and heat shock cognate protein 70 (hsc70), have been shown to cause retinal cell death (Adamus et al., 1997, 1998; Ren and Adamus, 2004).

In this present study we identified retinal autoantigens from a mouse RPE/choroid cDNA expression library. We demonstrated that only sera from virus infected retinal degeneration susceptible mice reacted to one of the autoantigens, α -fodrin. We also show that incubation of T cells from virus infected retinal degeneration susceptible mice with α -fodrin protein caused the cells to proliferate. Thus, this virus infection triggered both a humoral and cellular responses to the α -fodrin protein.

2. Materials and methods

2.1. Animals and tissue

Male BALB/c (Harlan Sprague Dawley, Indianapolis, IN) and CD-1 (Charles River, Raleigh, NC) mice (8–13 weeks old, 25–30 g) were

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used for these studies. Lewis and Sprague Dawley rats as well as eyes from Brown Norway rats were purchased from Harlan Sprague Dawley (Indianapolis, IN). Bovine eyes were a gift from Theodore Fletcher (NEI). All experimental procedures conformed to the Association for Research in Vision and Ophthalmology (ARVO) resolution for the use of animals in ophthalmic and vision research.

2.2. Virus

Mouse hepatitis virus (MHV), strain JHM, was obtained from the American Type Tissue Collection (Manassas, VA). Viral stocks were propagated in mouse BALB/c 17CL1 3T3 or mouse L2 cells. Briefly, infected cultures were frozen and thawed, centrifuged at 2000 rpm for 20 min to remove cellular debris and the supernatant was centrifuged at 15,000 rpm for 2 h to pellet the virus. The viral pellet was resuspended in DMEM with 2% heat-inactivated fetal bovine sera (HI FBS), divided into small aliquots and stored at -70°C . Viral titers were determined by plaque assay on mouse L2 cells with serial dilutions of the virus.

2.3. Mouse inoculations

Eyes were injected intravitreally with $5\ \mu\text{l}$ of either 1.35×10^6 PFU/ml of MHV (virus-infected) or with MEM containing 2% HI FBS (mock-infected). Blood was collected in Microtainers (Becton Dickinson, Franklin Lakes, NJ) from un-injected, mock-injected and virus-injected mice 20 days after inoculation. Sera was separated from the cells and stored at -70°C until analyzed. The mice were euthanized by cervical dislocation, and eyes were removed and fixed in 10% buffered formalin for hematoxylin and eosin staining.

3. Immunohistochemistry

Methods used for immunohistochemistry were described previously (Hooks et al., 2006). Briefly, cryosections of rat eyes were fixed in acetone/methanol (1:1) and rinsed with phosphate buffered saline (PBS), pH 7.4. Endogenous peroxidase activity was quenched by incubating sections in 0.6% H_2O_2 , followed by washes with PBS. Sections were incubated in blocking solution (10% normal horse serum, 2% bovine serum albumin, 1% glycine, 0.4% Triton X-100, 5% cold water fish gelatin in PBS) at room temperature. Sera were pooled from groups of three animals for each condition: un-injected, mock-injected and virus-injected mice. The pooled sera were diluted 1:40 and 1:80 in blocking solution and applied to tissue sections and incubated overnight at 4°C . Biotinylated horse anti-mouse IgG and horseradish peroxidase conjugated streptavidin were used at a 1:200 dilution. The slides were developed with 3,3'-diaminobenzidine following the manufacturer's instructions (Vector Laboratories, Burlingame, CA).

4. Western blotting

Eight to 12 week old BALB/c mice (Harlan Sprague Dawley, Indianapolis, IN) were euthanized, and eyes were enucleated. Retinas were isolated and homogenized in 50 mM Tris, pH 7.6, 10% glycerol, 0.2 mM EDTA, 10 mM MgCl_2 , 0.5 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride. Soluble and membrane fractions were separated by centrifugation of the tissue suspension at 14,000 rpm for 30 min at 4°C . The soluble fraction was stored at -70°C in small aliquots. Brown Norway rat eyes were purchased from Harlan Sprague Dawley (Indianapolis, IN). After the retina was removed, the RPE layer was peeled off of the choroid, homogenized and processed as described above. Bovine retinal and RPE soluble protein fractions were generated following the procedure as described above. All protein samples were mixed with sample buffer and boiled for 10 min prior to loading on a protein gel. Electrophoresis was performed using 12% NuPAGE Bis-Tris gels (Invitrogen, Carlsbad, CA; 1.5 h, 150 V, 80 mA, 20 μg retinal and RPE proteins per lane and 25 ng of α -fodrin

peptide per lane). Separated proteins were transferred to nitrocellulose membranes (1.6 h, 30 V). Membranes were blocked in PBS containing 5% dried milk and 1% cold-water fish gelatin, followed by a 1 hour incubation with either sera from control or JHM infected mice (1:20 or 1:40). The membranes were then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD) for 1 h. Blots were washed in Tris-buffered saline with 0.05% Tween 20 (TBST) between incubations with sera/antibodies. Western blots were developed using Luminol (Amersham Pharmacia Biotech, Piscataway, NJ) or DuoLux (Vector Laboratories, Burlingame, CA) as the substrate.

4.1. Screening a mouse RPE/choroid cDNA expression library with sera from JHM infected BALB/c mice

The cDNA library (BioScience, MD) was plated at approximately 50,000 plaque-forming units (PFU)/150-mm NZY agar plate. Nitrocellulose filters soaked in IPTG were applied to the agar plates, and the plates were incubated at 37°C for 3.5 h. Duplicate filters were prepared by applying a second IPTG-treated filter to the agar plates after the first filters were removed. After filters were washed in TBST, they were placed in blocking solution (5% dried milk and 1% cold-water fish gelatin in PBS) for 1 h at room temperature. Filters were incubated with pooled sera from BALB/c mice 20 days post-infection with MHV JHM diluted 1:40 in blocking solution for 1 h at room temperature. Filters were processed identically as described above for the protein blots. Agar plugs containing plaques corresponding to signals found on both the first and second lifts were cored and placed in SM buffer with chloroform to allow phage particles to diffuse from the agar plug. The immunoscreening process was repeated until all plaques produced a positive signal.

5. DNA sequencing and expression of the cloned genes

An isolated plaque was cored from the agar plate with 100% positive signals and placed in SM buffer with chloroform. The single-clone excision protocol, plating of the excised phagemids and generation of plasmids were performed as directed by Stratagene (La Jolla, CA). Plasmid purification was accomplished with the QIAfilter plasmid maxi kit (Qiagen, Valencia, CA). Sequencing primers used included the pBluescript Reverse and M13 – 20 primers, rev2 (5'-AGAACTCCAGGCTGCT-3') and rev3 (5'-TCCGCGGTTCAAAGTCA-3') primers. All sequencing primers were custom synthesized by BioSynthesis (Lewisville, TX). DNA sequencing was performed by the Molecular Technology Laboratory at the NCI-Frederick Cancer Research and Development Center on an ABI PRISM 377 DNA sequencer.

A truncated mouse α -fodrin protein was expressed as a His-tagged fusion protein using the pET100/D-TOPO vector from Invitrogen (Carlsbad, CA). Protein expression was induced with IPTG, and protein purification was carried out following the manufacturer's instruction for the Ni-NTA purification system from Invitrogen (Carlsbad, CA). The histidine tag for the fusion protein was removed by digestion with enterokinase (New England Biolabs, Inc., Beverly, MA), followed by removal of enterokinase with EK-away resin from Invitrogen (Carlsbad, CA).

5.1. Proliferation assay

BALB/c mice were intravitreally inoculated with MHV JHM as described above. Ten days post-infection, three to six virus infected mice and three to four uninfected mice were euthanized and the spleens were removed and placed in MEM + 2% HI FBS. The spleens were individually dissociated and the cell suspensions were layered over Lymphoprep (Axis-Shield, Oslo, Norway) to separate splenocytes from red blood cells and fibroblasts. Splenocytes from individual animals were plated at a density of 2×10^5 cells/well in a 96 well plate and cultured in HyQ RPMI-1640 (Hyclone, Logan, UT) supplemented with

non-essential amino acids (Invitrogen, Carlsbad, CA), gentamycin (50 µg/ml), β-mercaptoethanol (1×10^{-5} M), L-glutamine (2 mM) and 10% heat-inactivated fetal bovine serum (HI FBS) overnight at 37 °C. The next day media was changed to fresh culture media or fresh culture media containing the following compounds for stimulation: retinal protein (50 µg/well), purified truncated α-fodrin protein (10 µg/well), phytohemagglutinin (PHA, 1 µg/well) or UV-inactivated MHV JHM (2×10^5 PFU/well). Each condition was set up in triplicate. After cultures were incubated at 37 °C for 72 h, cell proliferation was quantified with Alamar Blue (Biosource, Rockville, MD) following the manufacturer's instructions.

5.2. B cell, CD4⁺ T cell and adherent cell enrichment

BALB/c mice were infected with MHV JHM as described above and animals were euthanized 10 days post-infection and the spleens were harvested. Splenocytes were prepared from un-pooled spleens as described above. Cultures were enriched for adherent cells (macrophages) by incubating the splenocyte suspensions in Costar 96 Well cell culture dishes for 2 h at 37 °C and then removing the media with the non-adherent cells. Following the manufacturer's directions, mouse CD19 MicroBeads and the Miltenyi Biotec MidiMacs kit (Auburn, CA) were used for B cell enrichment. CD4⁺ T cells were enriched using the unlabeled splenocytes from the B cell enrichment step and the CD4⁺ T cell Isolation Kit and the MidiMacs kit (Miltenyi Biotec, Auburn, CA). After enrichment, B cells and CD4⁺ T cells were resuspended in HyQ RPMI-1640 supplemented with non-essential amino acids, gentamycin (50 µg/ml), β-mercaptoethanol (1×10^{-5} M), L-glutamine (2 mM) and 10% HI FBS, plated at a density of 2×10^5 /well and incubated overnight at 37 °C. The next day media was changed to fresh culture media or fresh culture media containing purified truncated α-fodrin protein (10 µg/well). Each condition was set up in triplicate. The cultures were incubated at 37 °C for 72 h, and cell proliferation was quantified with Alamar Blue (Biosource, Rockville, MD) following the manufacturer's instructions.

5.3. Statistical analysis

Numerical values for cell proliferation experiments were evaluated by using the Student's *t* test.

6. Results

6.1. Immunocytochemical staining of retinal tissue with sera from BALB/c mice 20 days post-infection (DPI)

Indirect immunohistochemical staining of cryosections from normal rat eyes was performed with a 1:40 dilution of sera from virus infected BALB/c mice. Fig. 1 represents the patterns of immunoreactivity to retinal tissues with sera from individual control (A), mock-injected (B) or virus-injected (C–F) BALB/c mice. Sera from BALB/c mice injected with MHV JHM reacted strongly to cells in the retinal ganglion layer (RGL), the inner nuclear layer (INL) and outer nuclear layer (ONL). Sera from some of the mice infected with virus reacted with cells of the ONL (E and F) and of the retinal pigment epithelium (RPE) (C and E). The staining pattern spanning the inner retina seen in panel F is reminiscent of that with immunostaining associated with Müller cells. In contrast, an identical dilution of sera from un-injected (control) BALB/c and mock-injected BALB/c did not demonstrate reactivity to cells in the RGL, INL, ONL or RPE. Fig. 2 is representative of the pattern of immunoreactivity to retinal tissues with pooled sera from control, mock-injected or virus-injected CD-1 mice. Sera from un-injected (control) CD-1 and mock-injected CD-1 mice did not demonstrate reactivity to cells in the RGL, INL or ONL. These sera were used for further analysis in this report.

6.2. Characterization of retinal antigens by Western blot analysis

Sera from control and virus infected BALB/c mice were reacted against protein western blots of mouse retina, bovine retina, rat retinal pigment epithelium (RPE) and bovine RPE (Fig. 3). Sera from infected BALB/c mice demonstrated reactivity to five proteins in mouse retinal extracts (20, 38, 43 and 48 kDa), four proteins in bovine retinal extracts (37, 43, 45, 47 and 56 kDa) and one protein in rat retinal extracts (37 kDa). With rat and bovine RPE extracts, sera from infected BALB/c mice reacted with 25, 26, 38, 50 and 71 kDa and 38 and 60 kDa, respectively.

6.3. Identification of immunoreactive cDNA clones

A mouse RPE/choroid cDNA expression library was screened, using sera pooled from BALB/c mice infected with MHV JHM 20 dpi. Two clones were isolated and purified. Sequencing confirmed that two unique clones were isolated. One clone demonstrated 82% identity with mouse α-fodrin protein (Fig. 4A) and the other demonstrated 100% identity with mouse villin-2 protein (Fig. 4B).

6.4. Immunoreactivity of sera to α-fodrin

A truncated mouse α-fodrin protein (approximately 125 kDa) was expressed as a His-tagged fusion protein. Protein purification using a Ni-NTA column, and the histidine tag was removed by digestion with enterokinase. The resulting protein was used to generate protein blots. Reactivity of sera to α-fodrin from un-injected, mock-injected and MHV JHM-injected BALB/c and CD-1 mice was examined by Western blot analysis. None of the sera from CD-1 mice demonstrated reactivity to the α-fodrin peptide. Only sera from MHV JHM-injected, but not un-injected or mock-injected, BALB/c mice reacted with the α-fodrin peptide (Fig. 5). These studies clearly identify immune reactivity against retinal antigens and α-fodrin protein in sera from virus infected BALB/c mice.

6.5. α-Fodrin triggers proliferation of T cells from virus infected BALB/c mice

In order to monitor cellular immune reactivity, proliferation assays were employed to identify activation of splenocytes and T cells from virus infected BALB/c mice. The effect of α-fodrin peptide, retinal protein, PHA and UV-inactivated MHV JHM on proliferation of splenocytes isolated from either un-injected or virus infected BALB/c mice was determined using an Alamar Blue assay. As shown in Fig. 6A, treatment with PHA resulted in approximately a two-fold increase in splenocyte proliferation from both MHV infected and un-infected BALB/c mice when compared to splenocytes receiving no treatment. When splenocytes were incubated with the α-fodrin peptide, cells isolated from MHV infected animals demonstrated a two-fold increase in proliferation ($p = 0.04$), whereas cells isolated from uninfected animals did not significantly proliferate when exposed to the α-fodrin peptide. As expected, splenocytes from MHV infected mice proliferated (2.4-fold) when exposed to UV-inactivated MHV ($p < 0.0001$), and splenocytes from uninfected mice showed no significant difference in proliferation when compared to splenocytes receiving no treatment.

After the splenocytes from uninfected and MHV infected mice were sorted into populations of cells enriched for B cells, CD4⁺ T cells or adherent cells, the enriched cell populations either received no treatment or were incubated with 10 µg/well of the α-fodrin peptide (Fig. 6B). The CD4⁺ T cell enriched population from MHV infected mice, had approximately a two-fold proliferation response to α-fodrin ($p = 0.01$). The population enriched for adherent cells from the spleens of MHV infected mice had smaller response to α-fodrin protein when compared to the same population of cells from uninfected mice exposed to α-fodrin ($p = 0.04$). However, exposure to α-fodrin did not result in proliferation of the B cell enriched population from either MHV infected mice

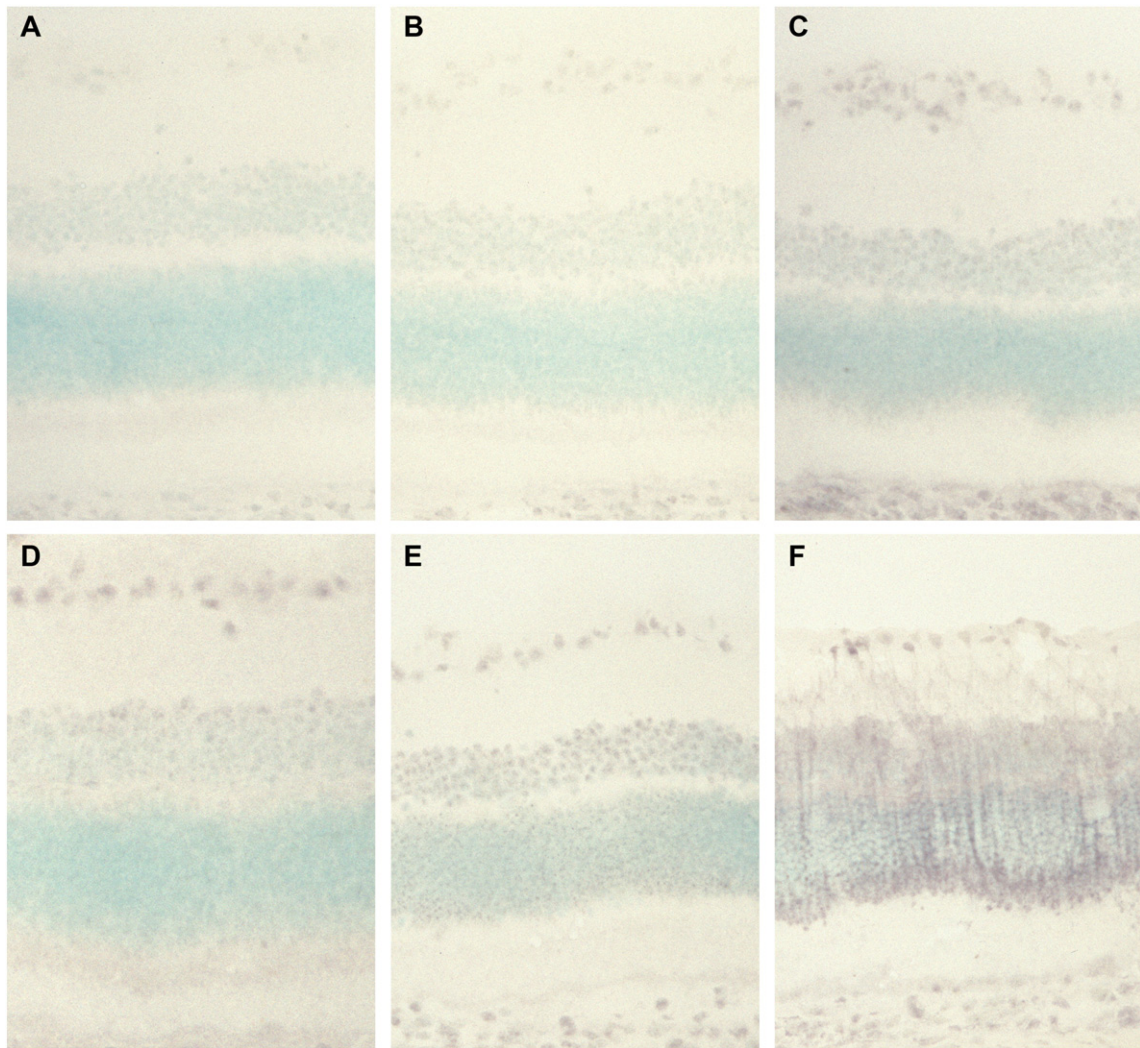


Fig. 1. Immunoperoxidase staining of rat retina with non-pooled sera from uninjected, mock injected or MHV JHM injected BALB/c mice. Cryosections of normal rat retina were fixed and permeabilized with acetone/methanol and reacted with 1:40 dilutions of sera from individual (A) un-injected, (B) mock-injected BALB/c and (C–F) MHV JHM injected BALB/c mice. Sections were counter-stained with methyl green producing blue-green colored nuclei in the retina.

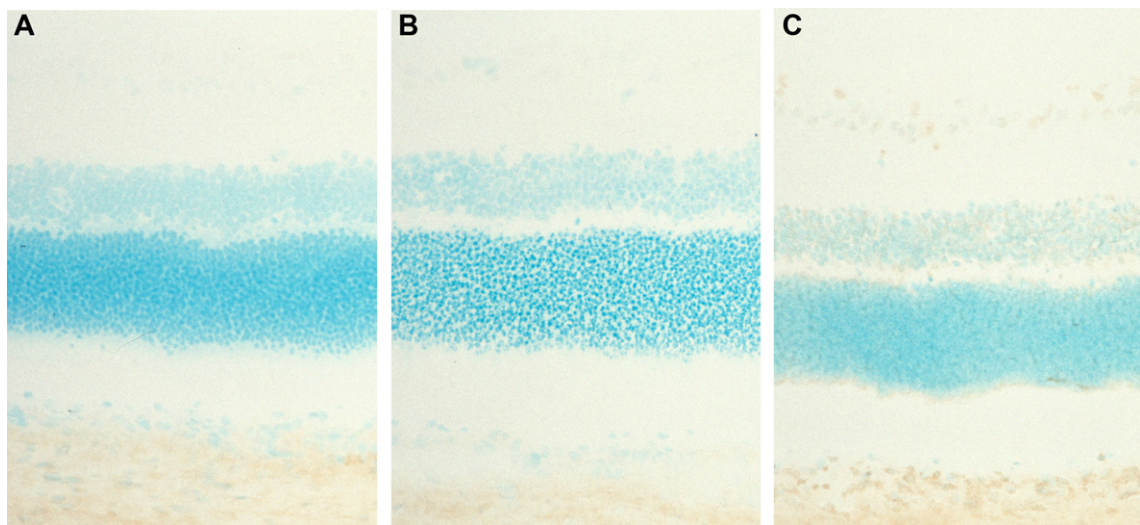


Fig. 2. Immunoperoxidase staining of rat retina with pooled sera from uninjected, mock injected or MHV JHM injected CD-1 mice. Cryosections of normal rat retina were fixed and permeabilized with acetone/methanol and reacted with 1:40 dilutions of pooled sera from (A) un-injected, (B) mock-injected CD-1 mice or (C) MHV JHM injected CD-1 mice. Sections were counter-stained with methyl green producing blue-green colored nuclei in the retina.

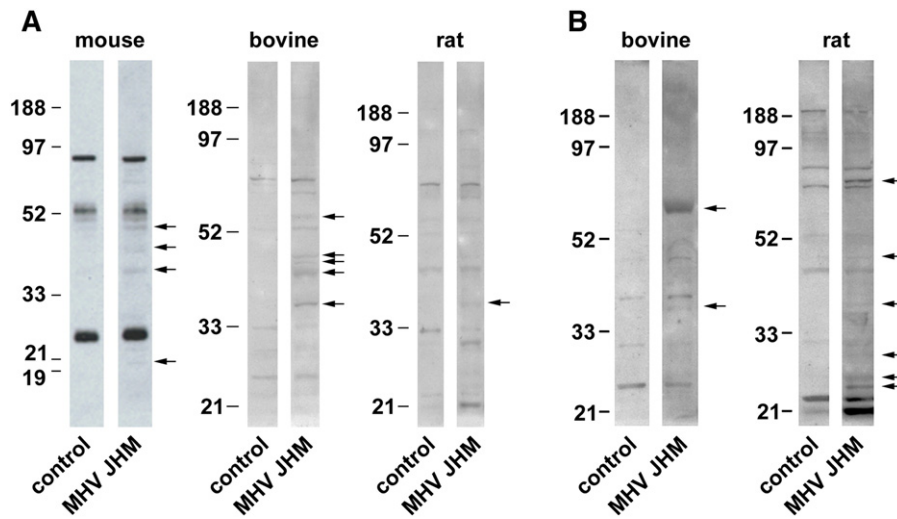


Fig. 3. Western blot analysis of murine, bovine and rat retinal proteins (A) or rat and bovine RPE proteins (B) with sera from control or MHV JHM infected BALB/c mice. Twenty micrograms of a retinal protein homogenate derived from either BALB/c mice or bovine eyes (A) or 20 μ g of RPE protein homogenate derived from either Brown Norway rat or bovine eyes (B) was subjected to SDS-PAGE electrophoresis and then transferred to a nitrocellulose membrane. The protein blots were then reacted with pooled sera from control BALB/c mice or pooled sera from MHV JHM infected BALB/c mice with retinal degeneration. The arrows indicate proteins reactive with sera from mice infected with virus but not reactive to sera from control mice.

or uninfected mice. There also was no difference in proliferation of the population of cells enriched for CD8⁺ T cells, and NK cells (data not shown). These studies demonstrate that the virus infection in BALB/c mice results in specific activation of CD4⁺ T cells to MHV antigen and to α -fodrin peptide.

7. Discussion

Two of the autoantigens that react with sera from virus infected, retinal degeneration susceptible mice (BALB/c) were identified as α -fodrin and villin 2.

A truncated form of α -fodrin was expressed and purified. This purified α -fodrin was shown to react only with sera from virus infected BALB/c mice. Moreover, incubation of CD4⁺ T cells from virus infected BALB/c mice specifically responded to α -fodrin peptide.

Autoantibodies have been detected in a variety of autoimmune diseases such as myocarditis (Caforio et al., 2005; Rose, 2006) and type 1 diabetes (Pihoker et al., 2005), and the presence of autoantibodies is useful for the diagnosis of these diseases. Production of anti-retinal antibodies is associated with selected retinal degenerative disorders (Hooks et al., 2001). Sera from patients with cancer-associated retinopathy have been found to react with recoverin (Thirkill et al., 1992), α -enolase (Adamus et al., 1996), hsc 70 (Ohguro et al., 1999a), neurofilament (Kornguth et al., 1986) and tubby-like protein (Kikuchi et al., 2000). However, only antibodies to recoverin, α -enolase and hsc 70 have been shown to cause retinal cell death by apoptosis (Ohguro et al., 1999b; Ren and Adamus, 2004; Adamus et al., 2006).

We have previously demonstrated the presence of anti-retinal autoantibodies in pooled sera from MHV infected BALB/c mice (Hooks et al., 1993). Here we examine the staining pattern obtained with sera from individual animals. The reactivity seen in the majority of sera from MHV infected BALB/c mice is localized to nucleated cells, such as the cells in the nuclear layers, ganglion cells and RPE cells. The cell body of Müller cells is found in the INL and the processes of the Müller cell span from the photoreceptors to the vitreous (Newman and Reichenbach, 1996). Some sera stained fibers spanning the neural retina, a pattern reminiscent of a Müller cell, and is similar to the pattern seen 4 dpi when staining with virus specific antiserum (Robbins et al., 1990a). This result agrees with earlier observations that the anti-retinal and anti-RPE antibodies localized to areas of the retina that were infected by virus.

The sizes of the retinal proteins reacting with the sera from MHV infected BALB/c mice (25–26 and 47–48 kDa) are similar to the size of

known retinal autoantigens such as recoverin (23 kDa) (Thirkill et al., 1992; Whitcup et al., 1998; Heckenlively et al., 2000) and α -enolase (46 kDa) (Adamus et al., 1996; Heckenlively et al., 1999). Sera from MHV infected BALB/c mice reacted strongly with a 71 kDa rat protein and a 60 kDa bovine protein from rat and bovine RPE extracts. There are no identified retinal autoantigens within this molecular weight range (60–71 kDa). However, there is an RPE protein, RPE65, with a size within this molecular weight range. It has been reported that defective functioning of the RPE65 protein causes photoreceptors to die (Hooks et al., 1989; Redmond et al., 1998). We examined the sera from MHV infected BALB/c mice and found no immunoreactivity to RPE65 (data not shown). Using sera pooled from BALB/c mice infected with MHV to screen a mouse RPE/choroid cDNA expression library, two unique clones were isolated. The DNA sequence of these clones showed high homology to the genes for α -fodrin/spectrin 2A and villin 2. A truncated form of α -fodrin was expressed and purified, and only sera from MHV infected BALB/c mice demonstrated reactivity to this protein. We are in the process of expressing villin 2.

α -Fodrin/spectrin 2A is a member of the spectrin super family (Dhermy, 1991). Spectrin was first discovered as a major component of the erythrocyte cytoskeleton. Later Goodman et al. (1981) found that spectrin-like proteins were found universally in nonerythroid cells and tissues, including neuronal tissues such as the brain and retina (Isayama et al., 1991; Goodman et al., 1995). Like erythroid spectrin, nonerythroid spectrin, also called fodrin, consists of heterodimers of α and β subunits. Two forms of fodrin have been found in the retina of mice, differing only in the β subunits. Immunoreactivity to fodrin occurred in the cytoplasm of cell bodies in the INL and ganglion cell layer. Fodrin has been localized to the apical plasma membrane of RPE in vivo but to both the apical and basolateral membranes in cultured RPE (Davis et al., 1995).

The presence of antibodies to α -fodrin has been described in three human diseases namely, glaucoma, Alzheimer's disease and Sjogren's syndrome. According to Frus et al., detected autoantibodies to α -fodrin were detected in sera from patients with normal-pressure glaucoma. These investigators also discovered that the autoantibodies had high reactivity to a 120 kDa fragment of α -fodrin and less activity to a 150 kDa fragment of α -fodrin. The 120 kDa α -fodrin breakdown product results specifically from cleavage by caspase-3 (Janicke et al., 1998), and the 150 kDa α -fodrin fragment results from breakdown of α -fodrin by calpain (Dutta et al., 2002). Tahzib and co-workers demonstrated that caspase-3 was activated in a chronic ocular hypertensive rat

A

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c1 11G          HFDAENIKKKQEALVARYEALKEPVMARKQ
                : . . . . . : . . . . .
SPTA2 RHQEHRTETIDARAGTFQAFEFQGGQLLAHGHYASPEIKEKLDILDQERTDLEKAWVQRRM

c1 11G KLADSLRLQQLFRDVEDEETWIREKEPIAASTNRGKDLIGVQNLKKHQALQAEIAGHEP
                : . . . . . : . . . . . : . . . . . : . . . . . : . . . . . : . . . . .
SPTA2 MLDHCLELQLFHRDCEQAENWMAAREAFLNTEDEKGDSDLDSVEALIKKHEDFDKAINVQEE

c1 11G RIKAVTQKGNAMVEEGHFAAEDVKAKLSELNQKWEALKAKASQRRQDLEDLSLQAQQYFAD
                : . . . . . : . . . . . : . . . . . : . . . . . : . . . . . : . . . . .
SPTA2 KIAALQAFADQLIAVDHYAKGDIANRRNEVLDRWRRLLKAQMI EKRSKLGESQTLQQQFSRD

c1 11G ANEAESWMREK-----EP-----IVGSTDYGKD----
                : . . . . . : . . . . . : . . . . . : . . . . .
SPTA2 VDEIEAWISEKLTASDES YKDPNTIQSKHQKQAFEAELHANADRI RGVIDMGNSLIER

c1 11G -----EDSAEALL-----KKLKEANKQQNFNTGIKDFDFWLSEVEA
                : . . . . . : . . . . . : . . . . . : . . . . . : . . . . .
SPTA2 GACAGSEDAVKARLAAALADQWQFLVQKSAEKSQKLEANKQQNFNTGIKDFDFWLSEVEA

c1 11G LLASEDYGD LASVNNLLKKHQ LLEADISAHEDRQKDLNSQADSLMTSSAFDTSQVKEKR
                : . . . . . : . . . . . : . . . . . : . . . . . : . . . . . : . . . . .
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c1 11G DTINGRFQIKSMATSRRAKLSESHRLHQFFRDMDEESWIKEKKLLVSEDYGRDLTGTV
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SPTA2 TVHKDRVNDVCTNGQDLIKKNNHHEENISSKMKGLNGKVS DLEKAAAQRKAKLDENSAFL

c1 11G QFNWKADVVE SWIGEKENSLKTDDYGRDLSVQTLTQKETF DAGLQAFQOEGIANITAL
                : . . . . . : . . . . . : . . . . . : . . . . . : . . . . . : . . . . .
SPTA2 QFNWKADVVE SWIGEKENSLKTDDYGRDLSVQTLTQKETF DAGLQAFQOEGIANITAL

c1 11G KDQLLAAKH IQSKAIEARHASLMKRWTQLLANSATRKKKLL EAQSHFRKVEDLFLTFAKK
                : . . . . . : . . . . . : . . . . . : . . . . . : . . . . . : . . . . .
SPTA2 KDQLLAAKH IQSKAIEARHASLMKRWTQLLANSATRKKKLL EAQSHFRKVEDLFLTFAKK

c1 11G ASAFNSWFENAEEDL TDPVRCNSLEEI KALREAHDAFRSSLSA QADFNQLAELDRQIKS
                : . . . . . : . . . . . : . . . . . : . . . . . : . . . . . : . . . . .
SPTA2 ASAFNSWFENAEEDL TDPVRCNSLEEI KALREAHDAFRSSLSA QADFNQLAELDRQIKS

c1 11G FRVASNPYTWFTMEALEETWRNLQKIIKERELQLKEQR RQEENDKLRQEF AQHANAFHQ
                : . . . . . : . . . . . : . . . . . : . . . . . : . . . . . : . . . . .
SPTA2 FRVASNPYTWFTMEALEETWRNLQKIIKERELQLKEQR RQEENDKLRQEF AQHANAFHQ

c1 11G WIQETR TYLLDGSCMVEESGTLESQLEATKRKHQEIRAMRSQLKKIEDLGAAMEEALILD
                : . . . . . : . . . . . : . . . . . : . . . . . : . . . . . : . . . . .
SPTA2 WIQETR TYLLDGSCMVEESGTLESQLEATKRKHQEIRAMRSQLKKIEDLGAAMEEALILD

c1 11G NKYTEHSTVGLAQQWDQLDQLGMRMQHNLEQQIQARN TTGVTEEALKEFSMMFKHFDKDK
                : . . . . . : . . . . . : . . . . . : . . . . . : . . . . . : . . . . .
SPTA2 NKYTEHSTVGLAQQWDQLDQLGMRMQHNLEQQIQARN TTGVTEEALKEFSMMFKHFDKDK

c1 11G SGRLNHQEFKSCLRSLGYD LPMVEEGEPDPEFEAILD TVDPNRDGHVSLQEYMAFMISRE
                : . . . . . : . . . . . : . . . . . : . . . . . : . . . . . : . . . . .
SPTA2 SGRLNHQEFKSCLRSLGYD LPMVEEGEPDPEFEAILD TVDPNRDGHVSLQEYMAFMISRE

c1 11G TENVKSSEEIESAFRALSSEGKPYVTKEELYQNL TREQADYCVSHMKPYVDGKGRELPTA
                : . . . . . : . . . . . : . . . . . : . . . . . : . . . . . : . . . . .
SPTA2 TENVKSSEEIESAFRALSSEGKPYVTKEELYQNL TREQADYCVSHMKPYVDGKGRELPTA

c1 11G FDYVEFTRSLFVN
                : . . . . . : . . . . .
SPTA2 FDYVEFTRSLFVN

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Fig. 4. Alignments of amino acid translation of the cDNA clones with α-fodrin and villin 2. (A) Alignment of the amino acid sequences for mouse α-fodrin and clone 11G (c1 11G). (B) Alignment of the amino acid sequences for mouse villin 2 and clone 8 (c1 8). Colons indicate identical amino acid residues and dots indicate conservative substitution of amino acid residues.

B

c1	8	MPKPINVRVT	TMDAELEFAI	QPNTTGKQLF	DQVVKTIGLR	EVWYFGLQYV	DNKGFPTWLK
	
vill		MPKPINVRVT	TMDAELEFAI	QPNTTGKQLF	DQVVKTIGLR	EVWYFGLQYV	DNKGFPTWLK
c1	8	LDKKVSAQEV	RKENPVQFKF	RAKFYPEDVA	EELIQDITQK	LFFLQVKDGI	LSDEIYCPPE
	
vill		LDKKVSAQEV	RKENPVQFKF	RAKFYPEDVA	EELIQDITQK	LFFLQVKDGI	LSDEIYCPPE
c1	8	TAVLLGSYAV	QAKFGDYNKE	MHKSGYLSSE	RLIPQRVMDQ	HKLSRDQWED	RIQVWHAHR
	
vill		TAVLLGSYAV	QAKFGDYNKE	MHKSGYLSSE	RLIPQRVMDQ	HKLSRDQWED	RIQVWHAHR
c1	8	GMLKDSAMLE	YLKIAQDLEM	YGINYFEIKN	KKGTDLWLGV	DALGLNIYEK	DDKLTPKIGF
	
vill		GMLKDSAMLE	YLKIAQDLEM	YGINYFEIKN	KKGTDLWLGV	DALGLNIYEK	DDKLTPKIGF
c1	8	PWSEIRNISF	NDKKFVIKPI	DKKAPDFVfy	APRLRINKRI	LQLCMGNHEL	YMRRRKPTTI
	
vill		PWSEIRNISF	NDKKFVIKPI	DKKAPDFVfy	APRLRINKRI	LQLCMGNHEL	YMRRRKPTTI
c1	8	EVQOMKAQAR	EEKHQQLER	QOLETEKKRR	ETVEREKEQM	LREKEELMLR	LQDYEQKTKR
	
vill		EVQOMKAQAR	EEKHQQLER	QOLETEKKRR	ETVEREKEQM	LREKEELMLR	LQDYEQKTKR
c1	8	AEKELSEQIE	KALQLEEEER	RAQEEAERLE	ADRMAALRAK	EELERQAQDQ	IKSQEQIAAE
	
vill		AEKELSEQIE	KALQLEEEER	RAQEEAERLE	ADRMAALRAK	EELERQAQDQ	IKSQEQIAAE
c1	8	LAEYTAkIAL	LEEARRRKED	EVEEWQHRAK	EAQDDLvKTK	EELHLvMTAP	PPPPPPvYEP
	
vill		LAEYTAkIAL	LEEARRRKED	EVEEWQHRAK	EAQDDLvKTK	EELHLvMTAP	PPPPPPvYEP
c1	8	VNYHVQEGlQ	DEGAEPmGYS	AELSSegILD	DRNEEKRITE	AEKNERvQRQ	LLTLsNELSQ
	
vill		VNYHVQEGlQ	DEGAEPmGYS	AELSSegILD	DRNEEKRITE	AEKNERvQRQ	LLTLsNELSQ
c1	8	ARDENKRTHN	DI IHNENMRQ	GRDKYKTLRQ	IRQGNTKQRI	DEFEAM	
		
vill		ARDENKRTHN	DI IHNENMRQ	GRDKYKTLRQ	IRQGNTKQRI	DEFEAM	

Fig. 4 (continued).

model of glaucoma resulting in cleavage of α -fodrin to a 120 kDa fragment (Tahzib et al., 2004).

Autoantibodies to α -fodrin have been detected in sera from patients with Alzheimer's disease (Vazquez et al., 1996). Fernández-Shaw and co-workers hypothesized that anti-spectrin antibodies in the sera of patients with Alzheimer's disease resulted from increased production of spectrin breakdown products in areas of neurodegeneration in the brain that leaked into the systemic circulation and led to the production of anti-spectrin antibodies (Fernandez-Shaw et al., 1997). The increase in spectrin breakdown products is attributed to increased activation of calpain activity (Dutta et al., 2002).

Autoantibodies to α -fodrin have been detected in sera from patients with Sjögren's syndrome (Haneji et al., 1997; de Seze et al., 2003). In the NFS/*sld* mouse model of primary Sjögren's syndrome (SS), mice developing the disease were found to make autoantibodies to a 120 kDa autoantigen purified from salivary glands that was latter found to be identical to human α -fodrin (Haneji et al., 1997). This 120 kDa α -fodrin fragment has been shown to occur during apoptosis (Martin et al., 1995). Haneji et al. demonstrated that purified-fodrin antigen activated specifically sensitized T cells in human SS and in the animal model.

Both glaucoma and Alzheimer's disease are chronic neurodegenerations and apoptosis has been found to play a role in neuronal death in both diseases (Zhang et al., 2000; Tahzib et al., 2004). It has been hypothesized that caspases are chronically activated in neurons in glaucoma and Alzheimer's disease resulting in the slow accumulation of breakdown products, such as the 120 kDa or 150 kDa fragments of α -fodrin, followed by delayed apoptosis and neuronal death. ECOR susceptible mice may produce antibodies to α -fodrin due to damage caused by the viral infection which could include chronic activation of caspases and calpain. Thus, one possible mechanism of retinal degeneration in ECOR could involve chronic activation of caspases or calpain leading

to the accumulation of toxic breakdown products, followed by delayed apoptosis and retinal cell death.

In ECOR, we found that splenocytes from the MHV infected BALB/c mice proliferated when exposed to purified α -fodrin antigen. A splenocyte population that was enriched for CD4 T cells also proliferated when exposed to purified antigen. This population of CD4 T cells could also contribute to retinal degeneration in ECOR possibly by altering the cytokine/chemokine environment in the retina to either cause direct damage to retinal cells or by increasing infiltration into the retina by other immune cells. CD4 T cells have been found to contribute to the development of disease in other models of autoimmune diseases such as multiple sclerosis (MS) (Wu et al., 2001) and autoimmune type 1 diabetes (Anderson and Bluestone, 2005). The same virus used to induce ECOR is also used to study a mouse model of MS. In this virus triggered MS model system, CD4 T cells appear to contribute more to the severity of clinical disease than CD8 T cells. Wu et al. also showed that macrophage infiltration into the CNS was greater in CD4 T cell enriched recipients. Studies by Lane et al. (2000) indicated that CD4 T cells played a role in demyelination by regulating the expression of RANTES and thus augmenting the infiltration of macrophages into the CNS.

The other antigen identified in our library screen was villin 2, also called ezrin. Villin 2 expression in vivo is usually restricted to the apical microvilli of epithelial cells and acts to link actin filaments to plasma membrane proteins (Berryman et al., 1993). In the eye, villin 2 is found in the apical microvilli of both Müller and RPE cells (Bonilha et al., 2006). Autoantibodies to villin 2 have not been found in any human autoimmune diseases, but autoantibodies to villin have been detected in patients with colon cancer (Rimm et al., 1995). Photoreceptor loss was noted in ezrin knockout mice and this loss was probably due to morphological defects in RPE and Müller cells since ezrin is not found in the photoreceptors (Bonilha et al., 2006). It is possible that

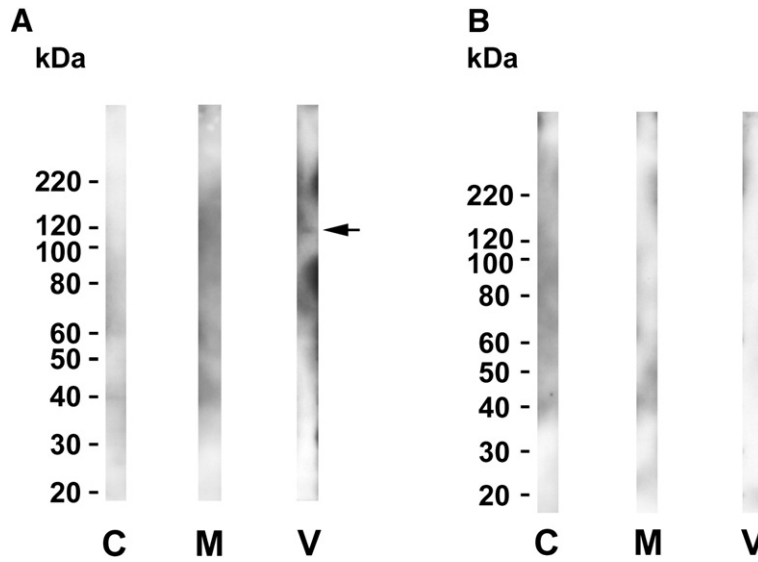


Fig. 5. Western blot analysis demonstrating immunoreactivity of sera from MHV JHM infected BALB/c mice with an α -fodrin peptide. Twenty-five nanograms of purified mouse α -fodrin peptide was loaded in each lane and subjected to SDS-PAGE and then transferred to a nitrocellulose membrane. The protein blots were then reacted with sera from control (C), mock-injected (M) and virus-injected (V) BALB/c (A) and CD-1 (B) mice. Molecular weight standards are indicated on the left of the blots. The black arrow indicates reactivity of the α -fodrin peptide with sera from virus-injected BALB/c mice.

autoantibodies to villin 2 could cause morphological changes in both the RPE and Müller cells and thereby could affect the function of the photoreceptors. We are presently working on expressing villin 2 in large quantities so that we can determine whether there is a population of splenocytes that will react to this antigen.

In summary, we have identified two retinal autoantigens, α -fodrin and villin 2, in ECOR. After ECOR susceptible mice were infected with

MHV JHM, they produced antibodies to α -fodrin and their CD4 T cells were specifically activated by α -fodrin. These data suggest that both antibodies to α -fodrin and CD4 T cells specifically sensitized to α -fodrin may contribute to the retinal degeneration seen in the ECOR susceptible mice. Identification of the mechanism of retinal degeneration in our ECOR model may provide new targets for therapeutic intervention in human retinal degenerative disorders.

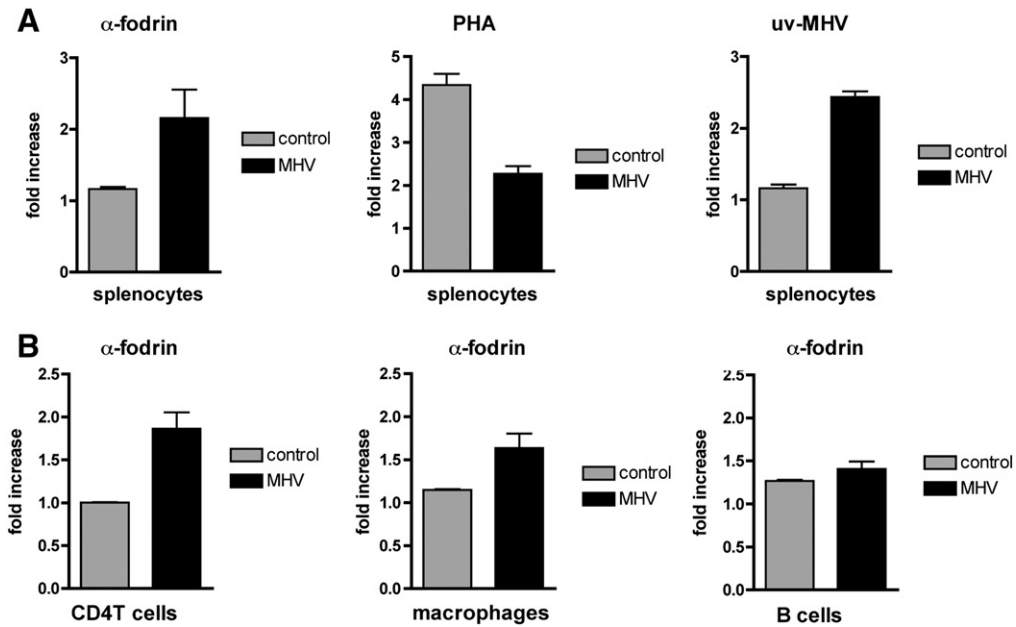


Fig. 6. (A) Effects of phytohemagglutinin (PHA; 1 μ g/well), UV-inactivated mouse hepatitis virus (UV-MHV; 2×10^5 PFU/well) and purified α -fodrin peptide (10 μ g/well) on proliferation of splenocytes from MHV infected BALB/c mice (black bars) and uninfected BALB/c mice (gray bars). The bars represent the mean fold change in splenocyte proliferation \pm SEM for each group. The data shown here is representative of two separate experiments, and three to six animals were in each group for each experiment. Treatment with PHA resulted in significant increases in splenocyte proliferation for both MHV infected ($p = 0.02$) and uninfected ($p = 0.004$) BALB/c mice. Incubation with UV-MHV significantly increased splenocyte proliferation only for MHV infected BALB/c mice ($p < 0.0001$). Splenocytes from MHV infected BALB/c mice ($p = 0.04$) responded to a peptide of α -fodrin, whereas, splenocytes from uninfected BALB/c mice did not respond. (B) Effects of a peptide of α -fodrin (10 μ g/well) on proliferation of splenocyte populations enriched for CD4⁺ T cells, adherent cells or B cells from MHV infected BALB/c mice (black bars) and uninfected BALB/c mice (gray bars). Treatment with an α -fodrin peptide resulted in increases in proliferation of CD4⁺ T cells ($p = 0.01$) and adherent cells ($p = 0.04$) but not of B cells. Results were normalized by arbitrarily setting the change in proliferation of untreated cells to 1.0.

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