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## Blood–retinal barrier breakdown in experimental coronavirus retinopathy: association with viral antigen, inflammation, and VEGF in sensitive and resistant strains

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### Abstract

Intraocular coronavirus inoculation results in a biphasic retinal disease in susceptible mice (BALB/c) characterized by an acute inflammatory response, followed by retinal degeneration associated with autoimmune reactivity. Resistant mice (CD-1), when similarly inoculated, only develop the early phase of the disease. Blood–retinal barrier (BRB) breakdown occurs in the early phase in both strains, coincident with the onset of inflammation. As the inflammation subsides, the extent of retinal vascular leakage is decreased, indicating that BRB breakdown in experimental coronavirus retinopathy (ECOR) is primarily due to inflammation rather than to retinal cell destruction. Vascular endothelial growth factor (VEGF) is upregulated only in susceptible mice during the secondary (retinal degeneration) phase. © 2001 Elsevier Science B.V. All rights reserved.

**Keywords:** Coronavirus; Blood–retinal barrier; Vascular endothelial growth factor; Retinopathy

### 1. Introduction

The neurotropic strain (JHM) of the mouse hepatitis virus (MHV) is a murine coronavirus that induces a biphasic retinal disease when injected intraocularly into BALB/c mice (Robbins et al., 1990, 1991). The early phase of the disease occurs from 1 to 7 days post-inoculation and involves a retinal vasculitis associated with the presence of infectious virus. The late phase of the disease begins at about day 10 post-inoculation and involves retinal degeneration associated with the presence of autoantibodies directed against the neuroretina and the retinal pigmented epithelium (RPE). Infectious virus, viral antigens, and inflammatory cells are absent during the late phase and virus-neutralizing antibodies are produced during both phases. When CD-1 mice are similarly inoculated, only the

early phase of the disease is induced (Wang et al., 1996); antiviral antibodies are produced, but anti-retinal autoantibodies are not (Hooks et al., 1993).

Vascular endothelial growth factor (VEGF) is well characterized as an angiogenic agent and a vascular permeability factor (Senger et al., 1983, 1986; Roberts and Palade, 1995; Dvorak et al., 1995; Ozaki et al., 1997), but recent findings show that it is also involved with the recruitment and activation of inflammatory cells and their adhesion to the vascular endothelium (Barleon et al., 1996; Clauss et al., 1996; Melder et al., 1996; Lu et al., 1999). A marked upregulation of VEGF occurs in the inner retinas of rats developing experimental autoimmune uveoretinitis (EAU) (Luna et al., 1997; Vinores et al., 1998a). In these rats, there is inflammatory cell infiltration and an autoimmune reaction with blood–retinal barrier (BRB) breakdown, but no angiogenesis. Experimental coronavirus retinopathy (ECOR) of murine retinas provides a model with inflammatory cell infiltration in the early phase and an autoimmune reaction in the late phase, when BALB/c mice are used. In CD-1 mice, the autoimmune response is

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absent; therefore, differences that may be observed in BRB breakdown and the presence and/or distribution of VEGF and its receptors, when comparing these two strains, are likely to relate to the development of retinal degenerative disease. This model will be used to determine at what phase BRB breakdown occurs, how extensive it is, and whether it is reversible. The immunohistochemical localization of extravasated albumin is an established method for visualizing the location and extent of sites of BRB breakdown (Viores, 1995; Viores et al., 1989, 1990b, 1994, 1995a,b) and it will be used to assess the integrity of the BRB. This model can also provide information as to whether VEGF may play a role in one or both of these phases.

## 2. Materials and methods

Coronavirus retinopathy was induced with the murine coronavirus, MHV, strain JHM (American Type Culture Collection [ATCC], Rockville, MD). The virus was passaged five to seven times in mouse L2 cells (a gift from Dr. Kathryn Holmes, Univ. of Colorado, Denver, CO), centrifuged at  $100,000 \times g$  for 2 h, and resuspended in Dulbecco's minimal essential medium (DMEM; Grand Island Biological Supply, Grand Island, NY) with 2% fetal calf serum (FCS). Virus infectivity was determined using L2 cells, as previously described (Wang et al., 1993). Adult male mice of the BALB/c (Harlan Sprague–Dawley, Indianapolis, IN) and CD-1 (Charles River, Raleigh, NC) strains receive intravitreal inoculations with  $10^{4.3}$  TCID<sub>50</sub>/5  $\mu$ l JHM virus or with an equal volume of DMEM containing 2% FCS (mock injections) as previously described (Hooks et al., 1993). Control mice received no treatment.

At time points ranging from 1 to 62 days post virus-inoculation, a total of 218 BALB/c and 58 CD-1 mice were anesthetized and sacrificed by decapitation. The mice are handled according to the ARVO Resolution on the Use of Animals in Research. Eyes are promptly fixed in 10% buffered formalin and embedded in paraffin. Immunohistochemistry is performed on tissue sections as previously

described (Wang et al., 1993, 2000; Viores et al., 1995a,b, 1998a; Chen et al., 1997) using a 1:500 dilution of rabbit polyclonal anti-rat albumin (Nordic, Capistrano Beach, CA), 1:20–1:50 dilutions of rabbit polyclonal anti-VEGF antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), 1:100 dilutions of rabbit polyclonal antibodies to either of the VEGF receptors, flk-1 and flt-1 (Santa Cruz Biotechnology), a 1:100 dilution of rabbit polyclonal antibodies to TGF $\beta$ 2 (Santa Cruz Biotechnology), a 1:800 dilution of rabbit polyclonal antibodies to MHV virus (provided by Dr. Kathryn Holmes), or a 1:100 dilution of rat IgG2b monoclonal antibody to macrophages and NK cells (Roche, Indianapolis, IN). Immunoreactivity is visualized with diaminobenzidine (Research Genetics, Huntsville, AL) or HistoMark Red (Kirkegaard and Perry, Gaithersburg, MD) or viewed under a fluorescent microscope.

## 3. Results

In BALB/c mice, BRB breakdown, assessed by the immunolocalization of extravascular albumin, was not demonstrated in uninjected or mock-injected eyes or in JHM-injected eyes for the first 5 days after inoculation of coronavirus (Table 1, Fig. 1A). By 6 days after inoculation, focal sites of retinal vascular leakage were evident (Fig. 1B) in six of seven eyes and their pattern resembled that of virus protein expression (Fig. 2A–D). Occasional sites where albumin had transgressed the RPE layer and entered the subretinal space were also evident. Widespread BRB breakdown was visualized in one of seven eyes at 6 days post-inoculation.

Inflammation was demonstrated by immunohistochemical staining for Mac-1, a marker for monocytes and natural killer cells (Ault and Springer, 1981), in virus-injected eyes at this time point (Fig. 3A–C). During the acute phase of intraretinal coronavirus infection (days 6–8), 70% of BALB/c mice demonstrated mild or moderate leukocyte infiltration (Fig. 4) and 89% demonstrated BRB breakdown (Fig. 5). Ten days after virus injection, areas of outer BRB (RPE) dysfunction were more extensive and

Table 1  
Blood–retinal barrier breakdown assessed by the immunolocalization of extravascular albumin

Treatment	Mice	Immunolocalization of extravascular albumin		
		Stage of infection		
		Early (days 1–5)	Acute (days 6–8)	Late (days 10–60)
Untreated	BALB/c	0/7 (0%)	0/7 (0%)	0/5 (0%)
	CD-1	0/3 (0%)	1/5 (20%)	0/3 (0%)
Mock Injected	BALB/c	0/7 (0%)	0/8 (0%)	0/8 (0%)
	CD-1	0/2 (0%)	1/7 (14%)	0/4 (0%)
Virus Infected	BALB/c	0/10 (0%)	8/9 (89%)	24/31 (77%)*
	CD-1	0/8 (0%)	8/8 (100%)	2/10 (20%)*

The number of eyes showing BRB breakdown/total number of eyes.

\* The difference between strains is statistically significant ( $p = 0.002$ ) based on Fisher's Exact Test.

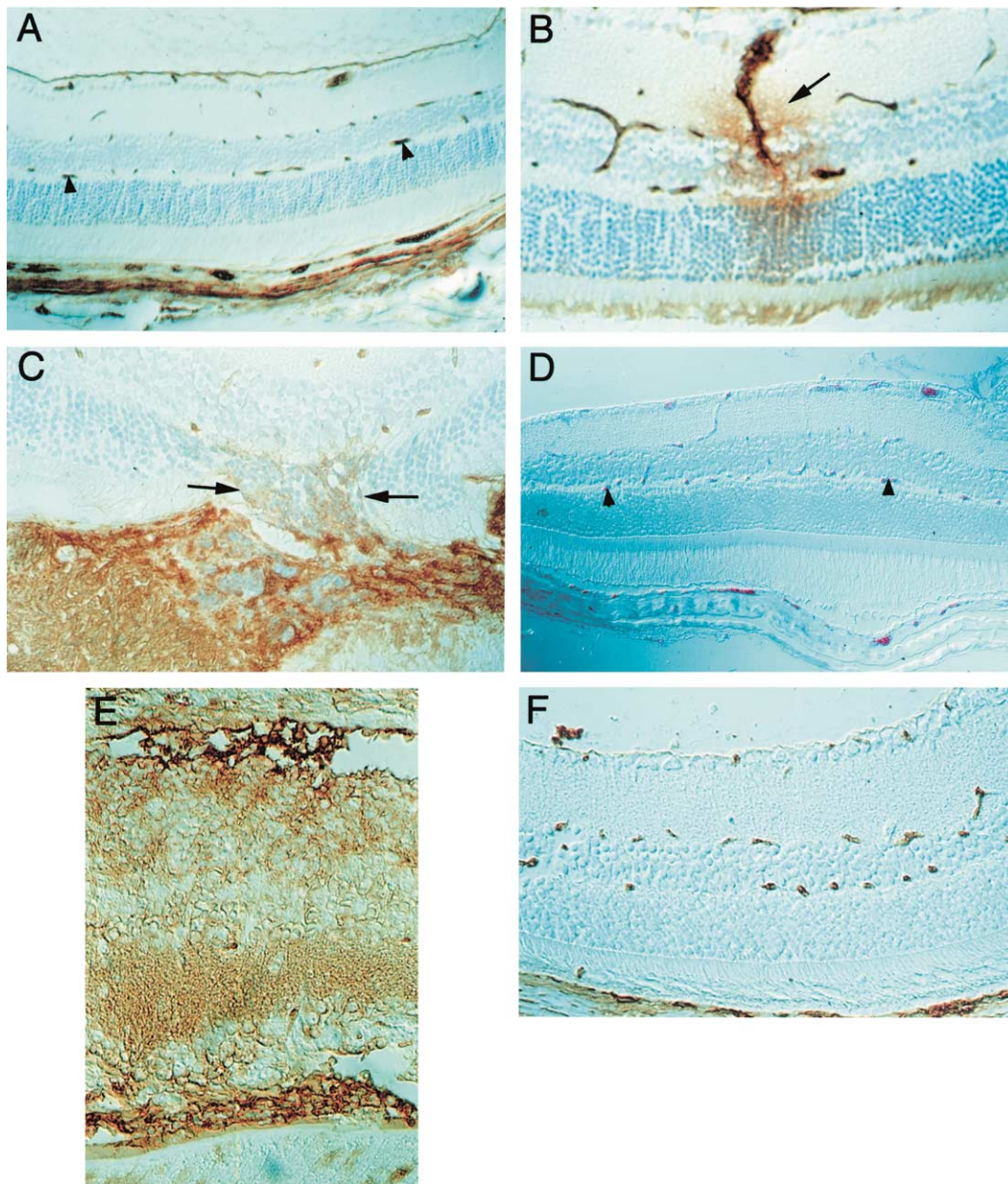


Fig. 1. Assessment of blood–retinal barrier (BRB) function determined by immunolocalization of albumin. (A) Within the retina of a 3-day control BALB/c mouse, immunostaining for albumin (brown) was confined to the vessels (arrowheads). In the choroid (bottom), diffuse staining was evident because the choroidal vessels are fenestrated and do not have a blood–tissue barrier function. (B) In a BALB/c mouse, 6 days after intraocular injection of coronavirus, leakage is seen emanating from a retinal vessel. The extravasated albumin is visualized as a perivascular diffuse brown reaction product (arrow). (C) In a BALB/c mouse, 62 days after intraocular injection of coronavirus, immunoreactive albumin is seen permeating the RPE and entering the retinal parenchyma (between arrows). This represents a breach of the outer BRB. (D) In a CD-1 mouse, 1 day after intraocular injection of coronavirus, the BRB is intact with immunoreactivity within the retina limited to the vessels (arrowheads). (E) In a CD-1 mouse, 8 days after intraocular injection of coronavirus, widespread, diffuse albumin positivity is evident throughout the retina. (F) In a CD-1 mouse, 21 days after intraocular coronavirus inoculation, albumin reactivity within the retina is confined to the vessels.

retinal vascular leakage continued, but widespread BRB breakdown was not seen. By this time point, inflammation had subsided (Fig. 3D) and the presence of viral antigens and infectious virus had diminished. Leukocyte infiltration was not detected in the late phase of coronavirus infected BALB/c mice; rather we observed a retinal degenerative process with only an occasional infiltrating cell. At 20–62

days post-inoculation, virus-mediated cell damage was clearly evident in the retina and BRB breakdown co-localized with this damage (Fig. 1C). However, in five of six eyes at 41–62 days post-inoculation, BRB breakdown was only mild or not apparent.

CD-1 mice, which like BALB/c mice developed an early transient vasculitis in the retina after coronavirus

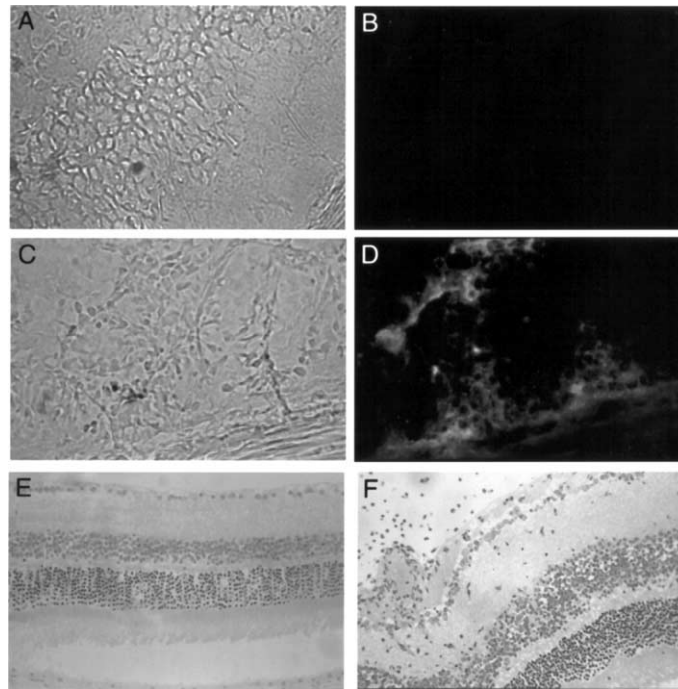


Fig. 2. Viral antigen and lymphocyte infiltration in the retinas of coronavirus inoculated eyes. (A) Micrograph of the retina of a normal BALB/c mouse taken with brightfield illumination. Choroid is at lower right. (B) Immunofluorescence labelling of the identical field with anti-JHM virus antibody conjugated to fluorescein isothiocyanate (FITC). There is no staining for viral antigens. (C) Brightfield image of the retina of a BALB/c mouse, 6 days after intraocular inoculation of coronavirus. Choroid is at the lower right. (D) Immunofluorescence labelling of the identical field with anti-JHM virus antibody–FITC shows immunoreactivity for viral antigens. (E) Hematoxylin and eosin (H & E) stained retina of a normal CD-1 mouse. (F) H & E stained retina from a CD-1 mouse, 6 days after intraocular injection of coronavirus showing infiltration of lymphocytes.

inoculation, but did not develop retinal degenerative disease as do the BALB/c mice, also did not show any BRB abnormalities for the first 4 days post-inoculation (Table 1; Fig. 1D). At 6–7 days post-inoculation, all eyes showed moderate BRB dysfunction. The extent of BRB breakdown increased in CD-1 mice to a peak at 8 days post-inoculation (Fig. 1E), when three of five eyes showed severe BRB breakdown and the remaining two showed moderate breakdown. Moderate or severe lymphocyte infiltration was demonstrated in 9 of 10 coronavirus-infected CD-1 mouse retinas during the acute phase (days 6–8) of coronavirus infection (Figs. 2E,F and 4), which was accompanied by moderate or severe BRB breakdown in all animals (Fig. 5). By 10 days after virus inoculation, the inflammation had subsided and the integrity of the BRB was restored. The retinal architecture appeared normal and infiltrating cells were not observed. Some focal staining for extravascular albumin was demonstrated in two of eight eyes at 20–21 days post-inoculation, one showed staining in the outer retina and one in the inner nuclear layer, but in the remaining eyes, the BRB was intact (Fig. 1F). BRB breakdown was not seen in BALB/c mice that were uninjected or received mock injections of saline. Focal positivity for extravascular albumin was seen in 1 of 11 untreated CD-1 mice and 1 of 13 CD-1 mice receiving mock saline injections.

VEGF staining in the retinas of coronavirus-injected BALB/c mice was negative or weak and diffuse throughout the retina and did not differ from control or mock-injected animals from 1 to 8 days after virus inoculation. At 10 days after virus inoculation, focally intense staining for VEGF was demonstrated in the outer segments of the photoreceptors in one of two mice. By 20 days, focally intense areas of VEGF positivity were evident in the nerve fiber layer, outer plexiform layer, outer nuclear layer, and the outer segments of the photoreceptors (Fig. 6A). In control BALB/c mice, VEGF staining was limited to the inner retinal surface and Müller cell processes with some weak staining in the outer plexiform layer (Fig. 6B). In CD-1 mice, constitutive staining for VEGF was somewhat stronger than in BALB/c mice, but except for one of two animals that showed more intense areas of positivity in the retina and RPE 10 days after virus inoculation, the virus-treated animals did not show an increase in VEGF staining compared to the controls (Fig. 6C,D).

In BALB/c mice, staining for the VEGF receptor, flk-1, was negative in the retina and RPE in normal and virus-treated animals. Immunoreactivity for the other iso-type of VEGF receptor, flt-1, was either absent or very weakly demonstrated in the inner plexiform layer and in the outer segments of the photoreceptors, but there was no correlation with the course of viral infection and no differ-

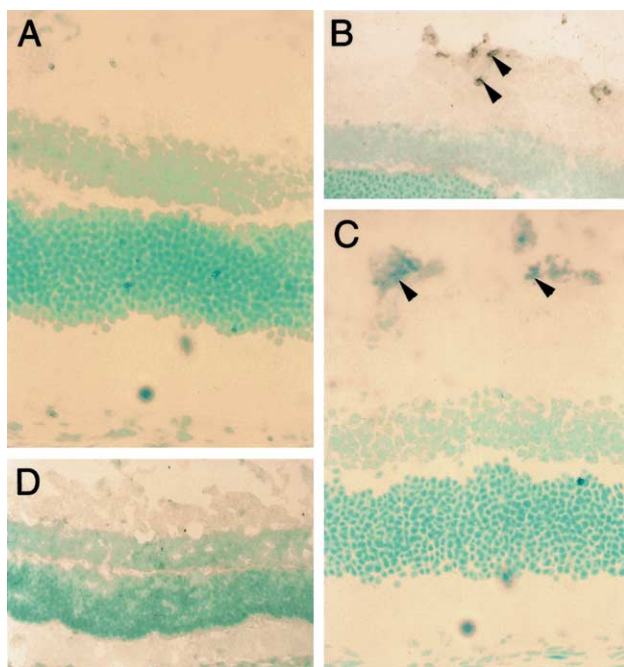


Fig. 3. Mac-1 immunoreactivity in the retinas of coronavirus-inoculated BALB/c mouse eyes. (A) Normal BALB/c mouse retina. (B) Mac-1 immunoreactivity (arrowheads) in the inner retina of a mouse, 3 days after intraocular inoculation with coronavirus. (C) Minimal Mac-1 immunoreactivity (arrowheads) in the inner retina of a BALB/c mouse, 16 days after intraocular inoculation of coronavirus. (D) Mac-1 is no longer detectable in the retina of a BALB/c mouse at 20 days post-coronavirus inoculation.

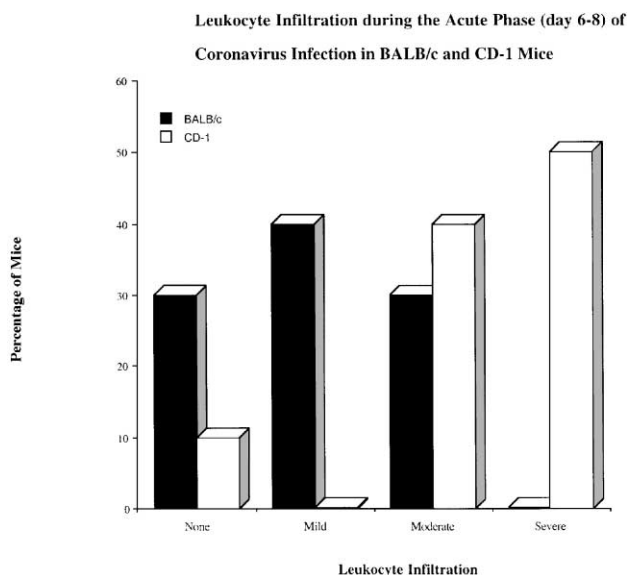


Fig. 4. A comparison of leukocyte infiltration into the retina in retinal degeneration susceptible (BALB/c) and retinal degeneration resistant (CD-1) mice during the acute phase of coronavirus infection. The intensity of the inflammatory response within the retina was graded in the following manner: none (<20 inflammatory cells), mild (20 to 50 inflammatory cells), and severe (>200 inflammatory cells). When comparing strains, the distribution shift was statistically significant ( $p = 0.016$ ) based on the Cochran–Armitage test for trend (Agresti, 1990; Margolin, 1988).

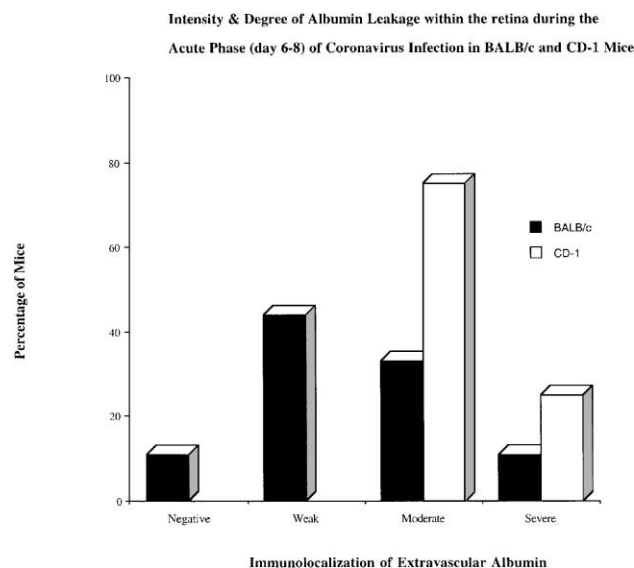


Fig. 5. A comparison of blood–retinal barrier breakdown in retinal degeneration susceptible (BALB/c) and retinal degeneration resistant (CD-1) mice during the acute phase of coronavirus infection. Immunolocalization of extravascular albumin was graded as follows: weak (isolated focus or foci of weak positivity), moderate (somewhat stronger staining limited to specific regions within the retina), and severe (widespread, intense staining throughout large areas of the retina). When comparing strains, the distribution shift was statistically significant ( $p = 0.035$ ) based on the Cochran–Armitage test for trend (Agresti, 1990; Margolin, 1988).

ence between experimental animals and controls. In addition, staining for TGF $\beta$ 2 was weak or absent in the retinas of BALB/c and CD-1 mice with no differences relating to the course of the virus infection or to the presence or absence of virus.

#### 4. Discussion

The immunolocalization of extravascular albumin has previously been shown to be a reliable means of localizing sites of BRB breakdown, at both the light and electron microscopic level, and it provides insight into the mechanisms of BRB dysfunction in ocular diseases (Vinos et al., 1989, 1990a,b, 1992, 1993a,b, 1994, 1995a,b, 1998b; Luna et al., 1997). In coronavirus infected BALB/c mouse retinas, the first evidence of vascular leakage occurred 6 days after the inoculation of virus and corresponded to the onset of inflammatory cell infiltration as demonstrated by Mac-1 staining. This is also the time when apoptosis is occurring within the retina (Wang et al., 2000). In CD-1 mice, BRB breakdown also coincided with inflammation and both BRB dysfunction and leukocyte infiltration peaked during the acute phase of the infection (days 6–8). Albumin extravasation during the acute phase was greater in CD-1 mice than in BALB/c mice, which correlated with a greater extent of leukocyte infiltration in CD-1 mice during this phase. Following the acute phase of infection, there was a decrease in inflammatory cells and virus

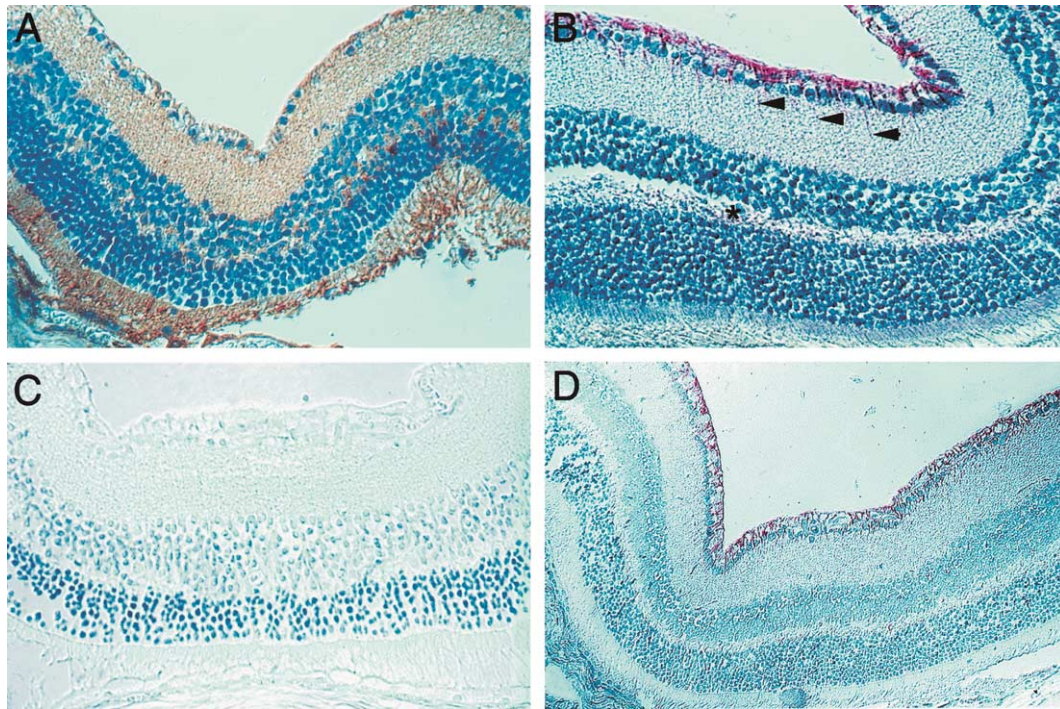


Fig. 6. VEGF immunoreactivity in ECOR. (A) Widespread, diffuse positivity for VEGF (brown) is demonstrated throughout the retina of a BALB/c mouse, 20 days after intraocular coronavirus inoculation. Immunoreactivity is stronger in the outer retina (bottom). (B) VEGF immunoreactivity (red) in a 20-day control BALB/c mouse is evident along the inner retinal surface (top) and in Müller cell processes (arrowheads). Weak positivity is also seen in the outer plexiform layer (\*). (C) VEGF staining is absent in a CD-1 mouse retina 20 days after intraocular coronavirus inoculation. (D) Control CD-1 mice show a staining pattern for VEGF that is similar to that observed in normal BALB/c mice (see B) with positivity along the inner retinal surface and in Müller cell processes.

particles and the integrity of the BRB was restored. BRB breakdown that occurred in the late phase of the infection in BALB/c mice generally corresponded to structural damage related to retinal degenerative disease.

In rats developing EAU, the marked upregulation of VEGF in the inner retina suggests that it plays a role in the pathogenesis of the disorder (Luna et al., 1997; Viores et al., 1997b, 1998a). The specific function of VEGF in the progression of EAU is unclear; however, the autoimmune disorder involves BRB breakdown and inflammatory cell infiltration, both of which can be promoted by VEGF. Likewise, with experimental herpesvirus retinopathy, which presents a unique model of a transient inflammatory response in the virus-injected eye and subsequent acute retinal necrosis associated with virus transmission to the opposite eye (Whittum et al., 1984), VEGF is upregulated in both eyes (Viores et al., 1997a). In the herpesvirus-injected eye, VEGF upregulation coincides with inflammation, with its levels diminishing as the inflammation subsides. In the contralateral eye, VEGF induction coincides with the appearance of virus particles and the onset of inflammatory cell infiltration. In experimental coronavirus infection, VEGF upregulation did not appear to coincide with inflammatory cell infiltration and/or the presence of infective virus and no differences were noted in the extent or distribution of VEGF receptors through the course of the disease; therefore, VEGF may not play a significant

role in the pathogenesis of experimental coronavirus retinopathy as it does in EAU and experimental herpesvirus retinopathy. The only significant upregulation of VEGF appears late in the course of the disease, when retinal degeneration is occurring, and may result from hypoxia related to the retinal degeneration, since VEGF is known to be induced under hypoxic conditions (Shweiki et al., 1992; Plate et al., 1992; Goldberg and Schneider, 1994; Hashimoto et al., 1994; Minchenko et al., 1994a,b; Levy et al., 1995; Pierce et al., 1995; Viores et al., 1997b).

It is possible that VEGF upregulation may play a role in the retinal degeneration that occurs in the late phase of ECOR in BALB/c mice. The mechanism for this is not clear, but retinal degeneration also occurs in a line of transgenic mice in which VEGF is produced in the photoreceptors under the control of the opsin promoter beginning approximately on day 7 (Okamoto et al., 1997). Retinal degeneration does not occur in other VEGF transgenic lines or other viral infection models, so a precise level and time of VEGF expression may be critical in promoting retinal degeneration.

One of the possible contributing factors in the role of VEGF in EAU and herpesvirus retinopathy and its apparent absence in ECOR is the predominant inflammatory cell entering the retina. EAU is characterized by a primary T cell infiltrate and herpesvirus retinopathy is characterized

by a predominance of T cells and NK cells (Tanigawa et al., 2000). In contrast, ECOR is characterized by a predominance of monocytes followed by a much smaller T cell infiltrate. Differences in the composition of the cellular infiltrates in these conditions could predispose a different spectrum of soluble factors released, thus accounting for differences in VEGF induction.

VEGF receptors have been identified on monocytes (Shen et al., 1993; Barleon et al., 1996; Clauss et al., 1996; Sawano et al., 2001) and VEGF can stimulate the activation and migration of monocytes (Clauss et al., 1990, 1996; Barleon et al., 1996; Heil et al., 2000). However, despite the fact that monocytes are the primary infiltrating cell type, VEGF does not appear to promote monocyte infiltration in ECOR, since (1) VEGF induction is not evident until the inflammation has subsided and (2) VEGF induction is not demonstrated in CD-1 mice despite the fact that the inflammatory response to coronavirus infection in CD-1 mice is even greater than that in BALB/c mice.

In models such as EAU and experimental herpesvirus retinopathy, in which VEGF is upregulated early during the course of the disorder, TGF $\beta$ 2 has been postulated to inhibit the angiogenic activity of VEGF, accounting for the absence of neovascularization (Viores et al., 1997a, 1998a). Neovascularization also does not occur in ECOR, but without the upregulation of VEGF, an angiogenesis inhibitor may not be necessary to inhibit vascular growth. Thus, there are no differences in the expression of TGF $\beta$ 2 relative to the course of the disease, suggesting that TGF $\beta$ 2 is unlikely to play a role in its pathogenesis.

In summary, BRB breakdown was observed in the acute inflammatory phase of ECOR in both retinal degeneration susceptible (BALB/c) and retinal degeneration resistant (CD-1) mice. In comparison to BALB/c mice, the CD-1 mouse BRB breakdown was more extensive and correlated with an enhanced inflammatory response. The findings indicate that BRB breakdown correlates directly with inflammatory cell infiltration and not with retinal degeneration in both sensitive and resistant strains. This study clearly demonstrates that although VEGF plays a basic role in the development of EAU and herpes simplex retinitis, it is not involved in the pathogenic processes in ECOR. Its upregulation, only in mice undergoing retinal degeneration (BALB/c mice after the acute inflammatory response), suggests that VEGF induction may be a secondary change related to retinal degeneration, rather than to inflammation or viral infection. Moreover, the lack of upregulation of VEGF in ECOR may be one of the factors which allows the CD-1 mouse retina to return to a normal appearance after the acute disease.

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