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A Hapten Generated from an Oxidation Fragment of Docosahexaenoic Acid Is Sufficient to Initiate Age-Related Macular Degeneration

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Abstract The protein adduct carboxyethylpyrrole (CEP) is present in age-related macular degeneration (AMD) eye tissue and in the blood of AMD patients at higher levels than found in age-matched non-AMD tissues. Autoantibodies to CEP are also higher in AMD blood samples than in controls. To test the hypothesis that this hapten is causally involved in initiating an inflammatory response in AMD, we immunized C57BL/6J mice with mouse serum albumin (MSA) adducted with CEP. Immunized mice develop antibodies to CEP, fix complement component-3 in Bruch's membrane, accumulate drusen below the retinal pigment epithelium during aging, show decreased a- and b-wave amplitudes in response to light, and develop lesions in the retinal pigment epithelium mimicking geographic atrophy, the blinding end-stage condition characteristic of the dry form of AMD. Inflammatory cells are present in the region of lesions and may be actively involved in the pathology observed. We conclude that early immunization of mice with CEP-adducted MSA sensitizes these animals to the ongoing production of CEP adducts in the outer retina where DHA is abundant and the conditions for oxidative damage are permissive. In response to this early sensitization, the immune system mounts a complement-mediated attack on the cells of the outer retina where CEP adducts are formed. This animal model for AMD is the first that was developed from an inflammatory signal discovered in eye tissue and blood from AMD patients. It provides a novel opportunity for dissecting the early pathology of AMD and the immune response contributing to this disorder. The availability of a mouse with a mechanistically based AMD-like disease that progresses rapidly is highly desirable. Such a model will allow for the efficient preclinical testing of the much-needed therapeutics quickly and inexpensively.

Keyword Inflammation · Retina · Oxidative damage · Age-related macular degeneration · Fatty acid

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Age-related macular degeneration (AMD) is the most common cause of legal blindness in developed countries and constitutes a major health problem. Millions of the elderly are blind from AMD in Europe and North America, with over 300,000 new AMD cases being diagnosed annually [1, 2]. During aging, many individuals accumulate material in Bruch's membrane, causing this acellular lamina below the retinal pigment epithelium (RPE) to thicken [3–5], while in others, focal deposits of debris accumulate below the RPE along Bruch's membrane and are recognized in an eye exam as drusen. Clinicians have long recognized that drusen in the macula of the eye, their density, and the area covered by these deposits are early stages in the AMD disease process. Individuals with drusen are considered at risk for developing the advanced blinding forms of AMD [6–8]. Advanced AMD is subdivided into two forms: (a) geographic atrophy and (b) choroidal neovascularization. Geographic atrophy (also referred to as the "dry form" of AMD) develops

slowly but results in blindness by the focal degeneration of the RPE below the fovea [9]. Without the RPE, the foveal photoreceptors lose function and foveal blindness ensues. Choroidal neovascularization (also called the "wet form" of AMD) is characterized by new blood vessels that break through Bruch's membrane and the RPE. When these new blood vessels hemorrhage, a blood clot accumulates between the RPE and foveal photoreceptors causing immediate blindness [6, 7].

Our initial studies on AMD focused on understanding the composition and distribution of proteins in drusen [10–15]. Over 120 different proteins were identified in isolated drusen. A number of other laboratories have made significant contributions to the understanding of drusen composition in recent years, and a consistent finding in their reports is that proteins in the complement attack pathway and its regulators are present in drusen [11, 16–20]. The older literature, primarily from twin studies, pointed to the likelihood that genetic factors played a role in AMD [21–24]. Recent genetic studies also establish that mutations/polymorphisms in genes coding for complement pathway proteins (complement component C2 and factor B) and its regulators (factor H and factor H-related proteins) are present in approximately 50% of AMD patients. Collectively, these studies strongly indicate that AMD is a genetic disease and that inflammation is a likely participant in the AMD pathology [25–29].

The complement system plays an essential role in inflammation and immune responses. Soluble complement proteins are present in the blood in precursor forms and require activation to fulfill their specific physiological roles. Activated complement has diverse functions, including the initiation of inflammation, recruitment of leukocytes, clearance of immune complexes, neutralization of pathogens, regulation of antibody responses, and disruption of cell membranes. The complement cascade can be activated by three initiating pathways. The classical activation pathway depends on assembly of complement factors at sites of antigen–antibody complexes. Activation of the alternative pathway is triggered by a variety of pathogen surfaces and requires the interaction of the third complement component (C3), factor B, and factor D. The lectin pathway is initiated by mannan-binding lectin bound to pathogen surfaces. Regardless of the pathway, activation leads to the cleavage of C3. This generates the smaller, proinflammatory C3a fragment and the larger C3b fragment. C3b together with other activated proteins form the important convertases required for the terminal part of the complement cascade, culminating in the assembly of the membrane attack complex and cell lysis. Many of these complement pathway proteins are found in drusen and sub-RPE deposits [11, 16–20].

We have focused our interest on oxidative protein modifications as a potential source of an inflammatory signal in AMD. In addition to the identity of specific proteins present in drusen described above, we also found that many of the proteins present there were not normal (native), but were covalently linked into high-molecular weight aggregates, modifications that can be caused by reactive lipid and carbohydrate oxidation products. Many of the drusen proteins were covalently modified with adducts, including advanced glycation end products and pyrrole adducts generated from the oxidative damage of docosahexaenoic acid (DHA). Carboxyethylpyrrole (CEP) adducts were identified on albumin, pyruvate kinase, glutathione S-transferase, and a number of other proteins [10]. In addition, we found that these CEP adducts were more prevalent in AMD donor eyes than in RPE/Bruch's membrane/choroid tissues from normal age-matched donor tissue [11]. Plasma samples from AMD patients contained 40% higher levels of CEP-adducted protein and autoantibodies to CEP than were present in plasma from healthy age-matched donors without AMD [30]. The presence of these oxidation-generated fragments derived from DHA are particularly intriguing because of the long-recognized association of AMD with oxidative damage [31–34]. The findings that smoking is a risk factor for AMD [31, 35, 36] and that antioxidant vitamins and zinc reduce the risk of vision loss in AMD also support the notion that oxidative damage may play a fundamental role in this disease [37]. The presence of higher levels of autoantibodies to CEP in AMD patients was also a novel finding. Could these DHA oxidation fragments be involved in the initiation of inflammation in AMD?

DHA is the most oxidizable fatty acid in the body, and it is highly concentrated in the photoreceptors and RPE [38, 39]. The availability of high levels of oxygen from the choriocapillaris and the focus of environmental light on this tissue by the lens system of the eye provides the perfect conditions for the generation of reactive oxygen that can damage DHA. Once generated, these oxidation fragments can condense with amino acid groups in proteins (i.e., Cys, Lys, and His) to generate Michael, Schiff base, and pyrrole adducts. DHA is only one of many sources of lipid-derived protein adducts. Other polyunsaturated fatty acids, their phospholipids, and cholesterol ester derivatives, can also generate a complex mixture of oxidation products that are capable of reacting with protein. Pyrrole adducts are an important class of lipid-derived protein modifications, which include CEP from DHA; carboxypropylpyrrole from arachidonic acid; and carboxyheptylpyrrole from linolenic acid [40].

Our interest in this oxidation fragment of DHA is based on the following: (a) CEP can only be generated from DHA, (b) DHA is more concentrated in the outer retina (photoreceptors and RPE) than elsewhere in the body, (c)

the high oxygen levels and light in the outer retina are permissive for the oxidative damage of DHA, (d) CEP adducts are more abundant in outer retinal tissues in AMD donor eyes than in normal age-matched control eyes, (f) CEP adducts and autoantibodies are more abundant in the plasma of AMD patients than in normal age-matched controls. From these facts, we developed the following hypothesis regarding an initiating inflammatory signal and the vulnerability of those with mutations in complement pathway genes to developing AMD: CEP adducts are slowly generated during aging in the outer retina. Some of the CEP-precursor fragments or CEP-adducted proteins are deposited in Bruch's membrane and in the debris that accumulates below the RPE. The CEP adducts are antigenic, and antibodies against them are produced by the immune system. In individuals that have normal protective mechanisms against oxidative damage, only small amounts of these adducts are generated. In contrast, more adducts are likely produced in individuals that have compromised protection against oxidative damage. We hypothesize that if higher levels of CEP are generated in individuals with an altered ability to regulate the complement attack pathway, the possibility of end-stage AMD will be greatly enhanced.

To test this hypothesis, we immunized mice with CEP-adducted mouse serum albumin [41]. Since CEP adducts are generated behind the blood retinal barrier in the outer retina, we reasoned that CEP-adducted proteins are probably released slowly during aging for interaction with the immune system. By systemically immunizing with CEP, we expect to sensitize mice to endogenously generated CEP. As CEP is produced by cells in the outer retina, an immune response similar to that occurring in AMD should follow, but much quicker than in the absence of systemic immunization. A summary of the results of these studies is described below.

To generate an immune response to CEP in mice, 2-month-old C57BL mice were immunized with CEP–MSA. Standard immunization protocols were followed (Percopo et al., 1990) using 200-μg CEP–MSA or non-adducted MSA (control) in complete Freund's adjuvant (CFA) at day 0, followed by challenge at day 10 with CEP–MSA in incomplete Freund's adjuvant (IFA). In order to generate a strong immunological response and confirm that our CEP–MSA-CFA is immunogenic, a group of "short-term" immunizations were performed in which animals received three immunizations of CEP–MSA during a 2–3-month period, the final given 10 days before the mice were sacrificed for analysis. To determine the role of aging on the CEP–MSA immune response, a second set of "long-term" immunizations were initiated in which mice received only the first immunizations described above, followed by maintenance for 12–14 months before analysis.

CEP antibody levels were followed in plasma samples recovered at the time of sacrifice from CEP-immunized and control mice. In the short-term recovery animals, antibody levels were six to eight times higher in the CEP–MSA-immunized mice in contrast to naïve mice and control animals immunized with MSA or CFA only [42]. Moreover, no anti-CEP antibodies were detected in Rag −/− mice that have no mature T-cells or B-cells. Antibody titers were similar in the long-term recovery animals, with the anti-CEP antibody titers higher in the CEP–MSA-immunized mice than in any of the controls. These results demonstrate that CEP–MSA can induce an antibody-mediated response.

Analysis of the retina from the short-term recovery animals revealed focal changes in the RPE and photoreceptors (Figs. 1 and 2). In the CEP–MSA-immunized mice, localized lesions in the RPE consisted of vesiculation and swelling of individual or groups of cells (Fig. 1a, b, f, g), cell lysis (Fig. 2a–e), pyknosis (Figs. 1a and 2d–f), and the presence of invading cells in the interphotoreceptor matrix (Fig. 2h), some of which labeled with the macrophage marker F4/80, identifying them as macrophages. We also observed long expanses of contiguous RPE cells that were swollen and appeared to be in stages of cell lysis (Fig. 1g). Also present were patches of the outer retina where the RPE was degenerated and the overlying photoreceptors were greatly swollen and edematous (Fig. 1f–g). In addition to photoreceptor swelling, some also appeared to be in stages of pyknosis (Fig. 1c–f). RPE lesions described here were evident in each of the specimens examined in short-term CEP–MSA immunization protocols. Cell counts reveal 1 to 17 RPE cell lesions in each section examined. Except for the occasional appearance of a monocyte in the interphotoreceptor matrix, these RPE lesions were not observed in any of the control eyes. Invading cells (macrophages and other monocytes) were observed at a frequency of 0 to 5 cells between the RPE and photoreceptors in each section examined.

Because of the findings that a number of proteins in the complement pathway are present in drusen and Bruch's membrane from AMD donor eyes [11, 16, 18–20], we wish to determine whether complement is deposited and fixed in the outer retina in these CEP-immunized mice. Eye tissues from the short-term protocol animals were processed for immunolocalization of complement component C3d, a degradation product of C3b, which is a key component in the generation of the C3–C5 convertases required for complement activation through the classical, lectin, and alternate pathways [43]. C3d immunolocalization was observed in Bruch's membrane below the RPE in the mice receiving CEP-immunization, but not in control mice, except for minor fluorescence observed in the non-adducted MSA-treated animals (Fig. 3). The localization

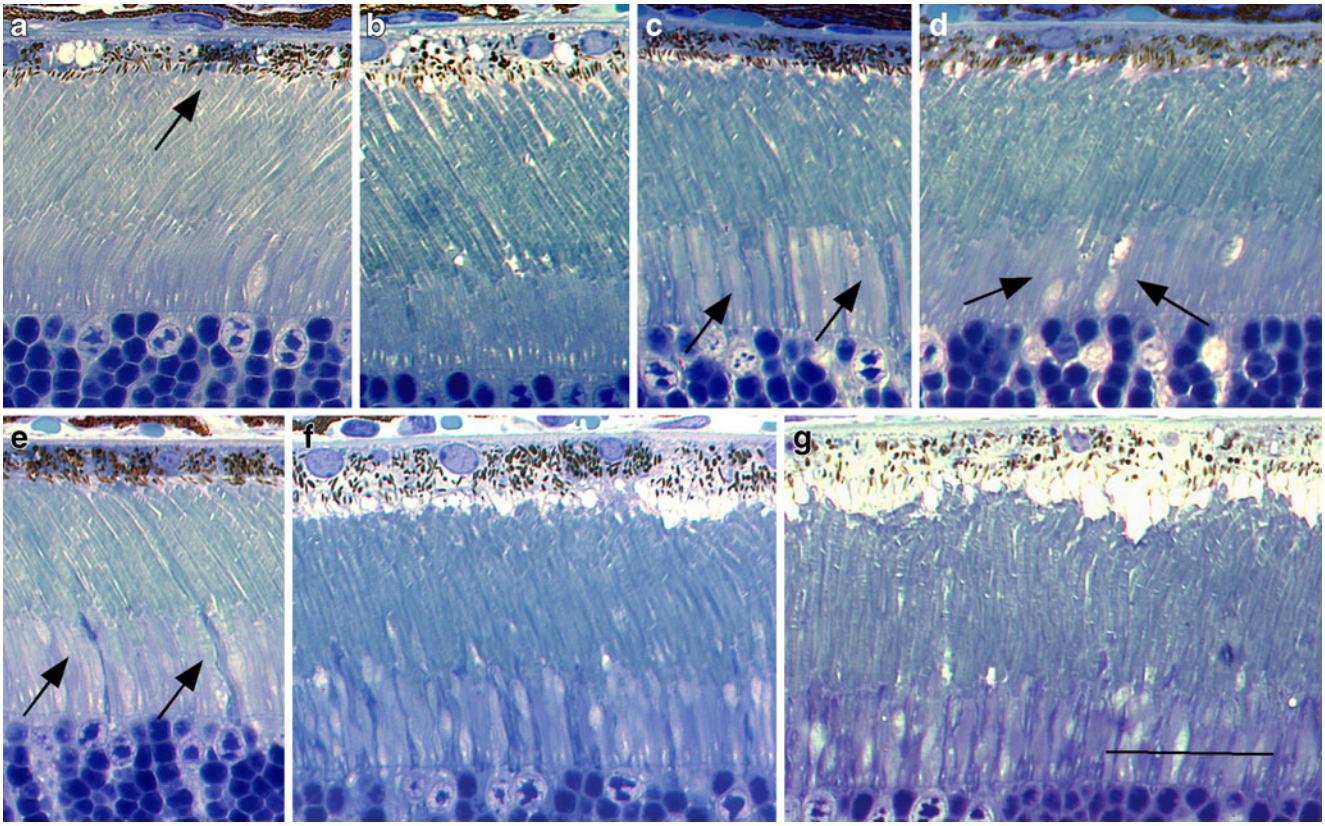

Fig. 1 Outer retina of mice immunized with CEP–MSA from the short-term protocol illustrating degenerative changes in photoreceptors and RPE. The RPE is at the *upper margin* and the photoreceptor nuclei are along the *lower border* of each image. A vesiculated RPE cell is at the *upper left* while a pyknotic, darkly staining RPE cell is just to the *right of center* (*arrow*) (**a**). Extensive vesiculation of the RPE is evident (**b**). Photoreceptors also show pathological changes as evident in the darkly staining individual photoreceptors in (**c**) and (**e**) and the swelling of cone inner and outer segments in (**d**) indicated by *arrows*. Extensive vesiculation and lysis of the RPE present in (**f**) and (**g**) is also accompanied by photoreceptor swelling and pyknosis, but photoreceptor changes are also evident (**c**–**e**) below areas where the RPE appears normal. All images photographed and displayed at the same magnification. *Bar length* in (**g**) represents 20 μm

of this C3 degradation product in Bruch's membrane suggests that the complement pathway is targeting this interface in these CEP-immunized mice. The deposition of C3d in Bruch's membrane and the RPE lesions in the short-term recovery animals suggest that these changes may be complement-mediated.

The CEP–MSA immunization effect in response to aging was assessed in mice at 12–14-month post-immunization. Histology revealed a dramatic buildup of sub-RPE deposits (in Fig. 4, compare (a) with (b)). The sub-RPE deposits were extensive, fluffy in appearance, and stain less intensely with toluidine blue in the plastic embedded sections than does the RPE cytoplasm. This debris formed a near continuous band 3–5 μm in thickness below the RPE. No newly formed blood vessels were evident in the outer retina in any of the long-term CEP-immunized mice, suggesting that this is a model for the dry form of AMD. Buildup of this sub-RPE material was not noted in the control eyes; however, there was some increased thickening of the RPE basal infoldings beyond the thickness observed in the short-term recovery animals.

To further define the changes occurring below the RPE in these long-term recovery animals, electron microscopy was used to evaluate selected tissues. A comparison between the Bruch's membrane interface in a naïve mouse and a CEP–MSA-immunized mouse, both from the long-term protocol, is presented in Fig. 4c–e. In naïve mice, Bruch's membrane is approximately 0.5 μm in thickness and the basal infoldings have a height of approximately 1.5 μm. This is in stark contrast to the dimensions of these areas in the CEP–MSA-immunized mice. Bruch's membrane was consistently swollen, approximately three to five times its normal thickness. The basal infoldings are evident, but these membrane extensions are displaced from Bruch's membrane and surround extracellular flocculent material that greatly expands the dimension of this sub-RPE compartment. Large vacuoles, suggesting edema and inflammation, are also evident in the RPE. Clearly, this dramatic

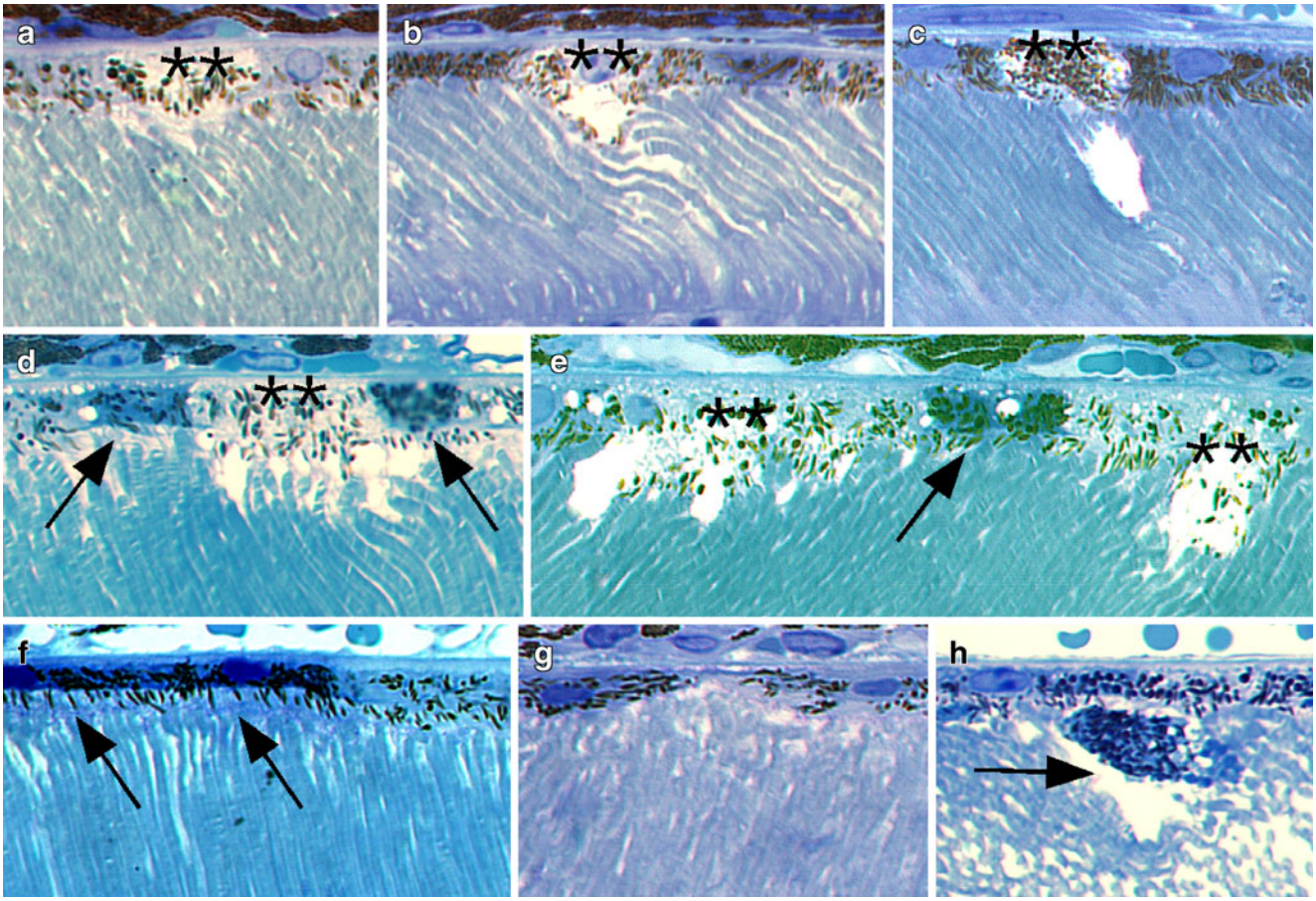

Fig. 2 Outer retina of mice immunized with CEP–MSA from the short-term protocol illustrating lesions in the RPE. Individual RPE cells or small groups of cells show evidence of lysis (*asterisks*) (**a**–**e**). Pyknotic RPE cells are indicated by *arrows* (**d**–**e**). Loss of RPE cells is evident in areas where the RPE is very thin or absent (**g**). Occasionally, inflammatory cells are present in the interphotoreceptor matrix. In the example presented (**h**), the inflammatory cell (*arrow*) is pigmented, suggesting removal of debris (melanin) lost from lysed RPE cells

buildup of this basal laminar debris in these CEP–MSA-immunized mice recovered 12 months following immunization confirms our initial hypothesis and suggests that CEP adducts are an important inflammatory signal in AMD.

Studies on this model reported recently demonstrate that mice mount an antibody-mediated response to CEP–MSA [42]. The immune system responds by the activation of complement on the RPE, as evidenced by the presence of C3d in Bruch's membrane and the development of lytic changes in the RPE that result in focal loss of these cells. This response requires an intact immune system since the Rag −/− mice that lack mature T-cells and B-cells [44] do not develop anti-CEP antibodies and show none of the changes in the outer retina observed in mice with an intact immune system.

Why is the RPE vulnerable following immunization with this hapten? It should be recalled that DHA, the source of this novel CEP-adduct, is present in the highest concentrations in the body in the phospholipid bilayer of photoreceptor outer segment membranes [38, 39, 45]. Following the onset of the light cycle daily, approximately 10% of the outer segment is shed and phagocytized by the RPE [46, 47]. Within the RPE, the phospholipids are de-esterified to release free fatty acids for recycling to the photoreceptors or for further degradation [48, 49]. This daily response of phagocytosis and degradation of photoreceptor outer segment debris result in the presence of a bolus of free DHA in the RPE. The availability of DHA in the RPE in an environment of high oxygen tension and light provides a permissive environment for oxidative damage. Furthermore, oxidative fragmentation of DHA-containing phospholipids can generate oxidatively truncated phospholipids in photoreceptor cell membranes [41, 50]. These can either form CEPs esterified in phospholipids that subsequently provide free CEPs through phospholipolysis or can first undergo phospholipolysis to release the free acid precursor of CEPs. Phospholipolysis of protein phospholipid adducts and of oxidatively truncated acyl chains in phospholipids are well known

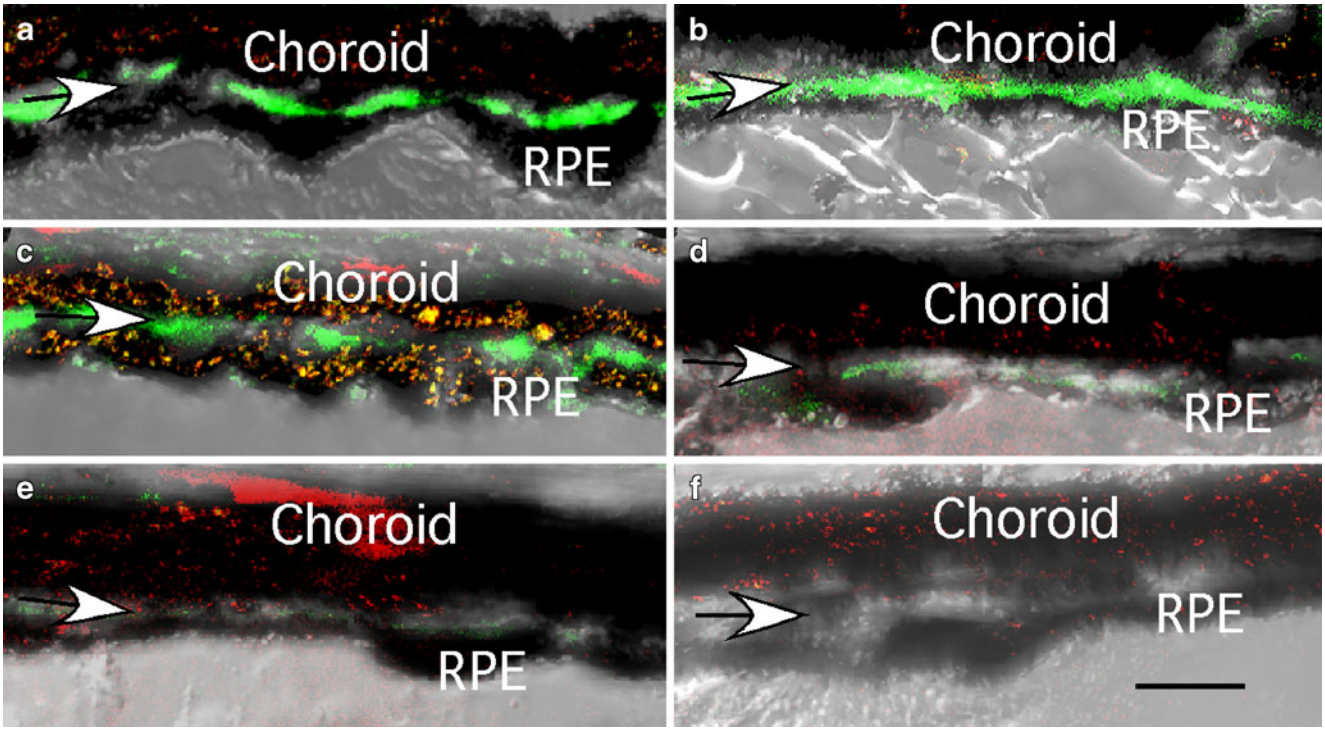

Fig. 3 Immunocytochemistry of the outer retina of mice from the short-term protocol showing fixation of complement in Bruch's membrane (the thin, horizontal, non-pigmented compartment between the choroid and RPE indicated by an *arrow* on the *left side* of each image). The tissue was probed with C3d antibody (*green*) and propidium iodide (*red*). Continuous to patchy immunolocalization with the antibody is present in CEP–MSA immunized mice (**a**–**c**), but not in control mice. Control images (**d**–**e**) are from mice immunized with non-adducted MSA and from age-matched naïve mice (**f**). *Bar length* (**f**) represents 10 μm. Quantitation of the differences in C3d immunofluorescence has been recently published [42]

[51–53]. Because the basal side of the RPE is exposed to the immune system, the generation of CEP adducts in the RPE makes it particularly vulnerable for recognition by the immune system and the development of an antibody response that could potentially activate complement and cause retinal damage, as evidenced by the RPE lesions shown in Figs. 1 and 2.

While the CEP–MSA-immunized mice showed intense accumulation of C3d immunoreactivity in Bruch's membrane, scattered C3d immunofluorescence was also observed in the non-adducted MSA-immunized mice, suggesting that minor amounts of CEP adducts were present in the MSA used in these experiments. Additional discussion regarding an immune response to MSA has recently been published [42].

Some of the invading cells present along the RPE–photoreceptor interface (Fig. 2h) are macrophages as evidenced by their labeling with the F4/80 macrophage surface marker [42]. It is of interest that aging mice deficient in the macrophage chemokine Ccl-2 or its receptor Ccr-2 also show changes along the outer retina with features of AMD [54]. Although no mutations or polymorphisms in the equivalent human genes have been found to be a risk factor for AMD, the importance of macrophages in maintaining the RPE–photoreceptor interface is strongly suggested by these studies. Because we observed numerous areas of RPE vesiculation and lysis in the absence of any nearby monocytes, it is unlikely that these inflammatory cells are involved in initiating the pathology observed in the RPE, but rather respond secondary to this damage by removing the debris released from lysed cells. Indeed, some of these cells contain melanin pigment that has likely been phagocytized following RPE lysis (Fig. 2h).

The accumulation of drusen in the form of basal laminar deposits shown in Fig. 4 was dependent on aging of the mice. Although C3d deposition was evident in the 2–3-month short-term immunization protocol, no differences in the thickness of the basal infoldings of the RPE or basal laminar deposits were evident in the CEP–MSA-immunized animals as compared to controls. Although a clear inflammatory response has occurred in these early recovery animals, additional time (aging) appears to be important for drusen accumulation below the RPE. The displacement of RPE from Bruch's membrane, as a consequence of this buildup, suggests that the deposition of this material may reflect a stress response by the RPE cells to shield the basal surface from components of the complement pathway that normally have access to this surface

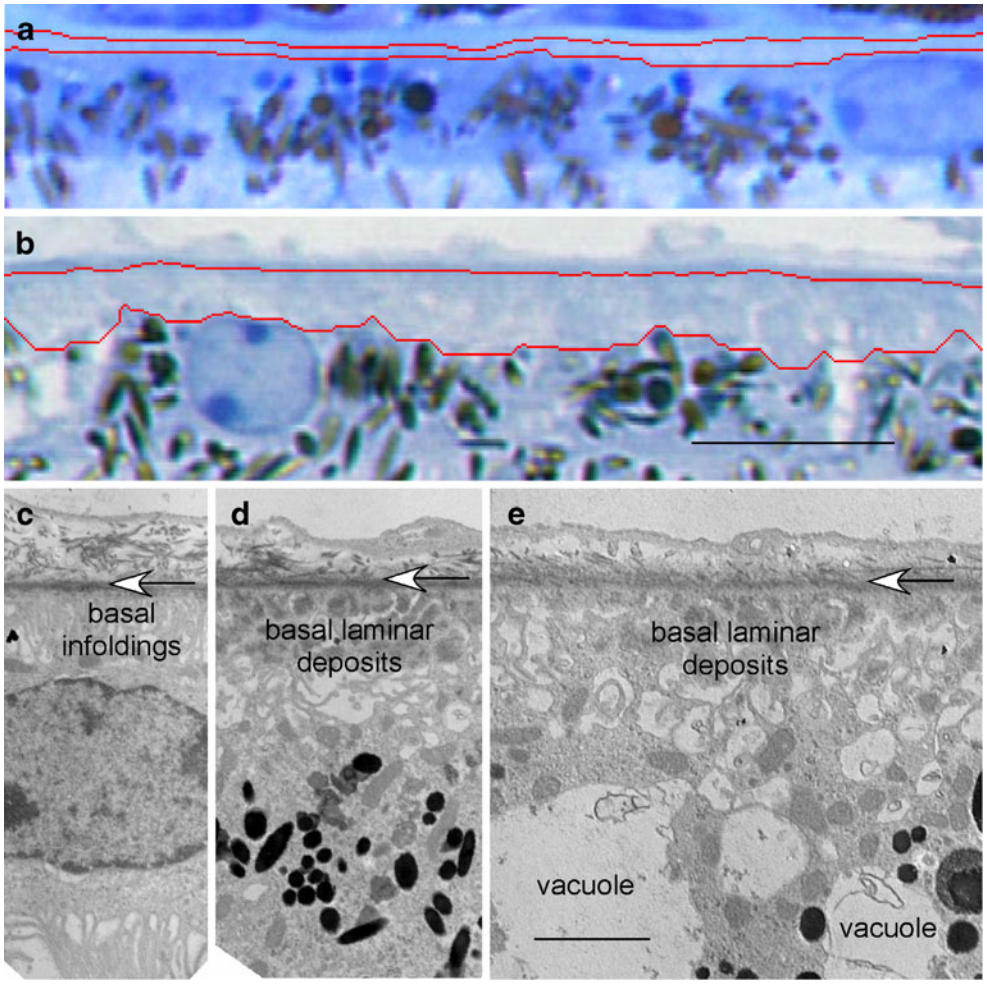

Fig. 4 Pathology of the RPE and Bruch's membrane in the long-term protocol, illustrating the extent and structure of the basal laminar deposits developing in mice immunized with CEP–MSA. Tissues in (**a**) and (**c**) are from 1-year control mice. Tissues in (**b, d–e**) are from CEP–MSA-immunized mice recovered 1 year following a single immunization with CEP–MSA. The parallel *red lines* in (**a**) define the basal infoldings of the RPE, whereas in (**b**), these lines define the extent of basal laminar deposits. The pixels between these red line boundary separation were counted in representative samples over identical linear areas in three to five samples from a single eye from three animals, with each treatment described for (**a**) and (**b**). Values for the areas occupied by basal infoldings was 10.6 $\mu m^2 \pm 2.1$ (mean± standard deviation) and for basal laminar deposits was 32.8 $\mu m^2 \pm 3.9$. These sample values were significantly different when compared using Student's *t* test ($p<0.001$). Note the extensive accumulation of basal laminar deposits in (**b**, **d–e**) and the presence of only normal basal infoldings at the interface with Bruch's membrane in (**a**) and (**c**). Light micrographs in (**a**) and (**b**) are presented at identical magnifications. *Arrows* in (**c–e**) indicate the location of the central elastin lamina of Bruch's membrane. *Bar* in (**b**) represents 10 μm. Electron micrographs are presented at identical magnifications. *Bar* in (**e**) represents 2 μm. Quantitation of the basal laminar deposit deposition has been recently published [42]

through the fenestrated capillary wall of the choriocapillaris, located just beyond Bruch's membrane.

Sub-RPE deposits are reported to develop when mice are subjected to a regimen of dietary, lighting, and/or genetic manipulations [54–57]. In these previous reports, a consistent feature was the accumulation of drusen in the form of basal laminar deposits. The deposits described in these earlier reports are virtually identical to the deposits we observe accumulating with age below the RPE in the CEP–MSA-immunized mice (Fig. 4). However, unlike these previous experiments, the pathology observed here, occurs in mice that have not been genetically manipulated, are fed normal laboratory chow, and are housed under a normal light–dark cycle. Immunization with CEP–MSA is sufficient to cause these AMD-like changes in the absence of any other genetic, dietary, or lighting stress.

While the immune system is clearly involved in mediating the outer retinal response to CEP–MSA, this inflammatory response differs substantially from that described for experimental autoimmune uveitis (EAU), where ocular inflammation follows systemic immunization to a retina-specific protein (S-antigen, interphotoreceptor retinoid-binding protein (IRBP), or cellular retinaldehyde binding protein (CRALBP)) [58–63]. Inflammation in EAU is primarily

cell-mediated involving priming and recruitment of T helper cell type 1 to cause disease. EAU responses are rapid and dramatic, involving massive inflammatory cell invasion followed by retinal and RPE necrosis. As compared to the antigens causing EAU, CEP–MSA appears to be a relatively weak antigen, and may depend more on the generation of a B-cell-dependent immune response. A relatively weak antigen like CEP, generated in the outer retina and presented to the immune system slowly over time, may explain the difference in the pathology observed here, as compared to those reported for EAU [58–63].

This is the first demonstration that a hapten generated by the oxidative fragmentation of DHA can initiate an immunological response that with time (aging) produces an AMD-like disorder. Since CEP-adducted proteins are found in eye tissue (drusen) and in plasma from patients with AMD at levels higher than in age-matched controls, the generation of AMD-like lesions in the outer retina of mice immunized with CEP–MSA strongly implicates this protein modification as being causally involved as an initiating signal for AMD. While other inflammatory initiators may also be active in AMD, our studies show that an immune response to a CEP-adducted self-protein is sufficient to cause localized AMD-like lesions in mice. The availability of this model should provide an important new resource for understanding the early changes in the outer retina in AMD as well as disease progression when initiated in mice with mutations/polymorphisms in complement pathway genes that are linked to AMD in humans.

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