Immunochemical recognition of A2E, a pigment in the lipofuscin of retinal pigment epithelial cells

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The autofluorescent lipofuscin pigment A2E accumulates in retinal pigment epithelial cells with age and is particularly abundant in some retinal disorders. To generate a polyclonal antibody that recognizes this pyridinium bisretinoid molecule, we immunized rabbits with bovine serum albumin (BSA) conjugates in which the protein was linked to the A2E molecule via its pyridinium ethanolamine moiety. Analysis by matrix-assisted laser desorption ionization/time of flight mass spectrometry (MALDI-TOF MS) of the A2E–BSA conjugate indicated the presence of five intact A2E molecules covalently linked to BSA, thus deeming it a suitable antigen for immunization. By immunocytochemical staining, the rabbit polyclonal antibody recognized A2E that had accumulated in cultured cells, whereas dot-blot analysis revealed binding to both A2E and A2E-rabbit serum albumin (A2E–RSA) conjugate but no cross-reactivity with various retinoids. Preimmune serum was non-reactive. In fluorescence spectroscopy studies, antibody-A2E binding was evidenced by a fluorescence increase and by a blue-shift in the emission maximum consistent with a change in A2E milieu upon antibody binding. The changes in fluorescence emission upon antibody binding could reflect several processes including restrictions on trans-cis isomerization and intersystem crossing of photoexcited A2E.

age-related macular degeneration (AMD) | antibody | immunocytochemistry

Excessive accumulation of lipofuscin in retinal pigment epithelial (RPE) cells (1) is considered the cause of RPE atrophy in autosomal recessive Stargardt disease (arSTGD), the juvenile form of macular degeneration caused by mutations in the ABCA4/ABCR gene (2–5). Several observations also implicate RPE lipofuscin as being involved in atrophic age-related macular degeneration (AMD). For instance, just as RPE lipofuscin is amassed with age and is of highest concentration in central retina (6), so AMD afflicts the central retina in the elderly. In vivo monitoring of RPE lipofuscin as fundus autofluorescence (FA) has shown that areas of intense FA in AMD patients correspond to sites of reduced scotopic sensitivity (7, 8) and are prone to atrophy (9, 10). Additionally, because mutations in ABCA4/ABCR lead to increased acquisition of RPE lipofuscin, it is significant that some studies report an association between heterozygous mutations in the gene and increased susceptibility to AMD in a subset of patients (11–13).

A major lipofuscin constituent, A2E (1, 14–17), is a pyridinium bisretinoid compound (Fig. 1), which is synthesized in the photoreceptor outer segments from two molecules of vitamin A aldehyde and one molecule of phosphatidylethanolamine (18, 19). The structure of A2E has been corroborated by several approaches including total synthesis via a convergent double Wittig olefination (20); one-step biomimetic synthesis, giving a 49% yield, has also been reported (21). Work with A2E in in vitro models suggests mechanisms by which lipofuscin constituents may damage the RPE cells (22–26). For instance, when A2E is exposed to blue light, singlet oxygen molecules are generated, which add to the carbon–carbon double bonds of A2E along the side arms (27–28). The highly reactive photooxidized A2E species that are generated (27, 29–32) likely account for the cellular damage ensuing from A2E irradiation (33–34).

Interest in the impact of A2E accumulation on the RPE cells raises the possibility of its assay by immunological techniques. This, in turn, raises the issue of whether the unprecedented bisretinoid structure of A2E can provoke the production of antibodies. Here, we report on the preparation of antibodies to A2E by immunization of rabbits with A2E-bovine serum albumin (A2E–BSA) conjugate (Fig. 1, 4). In addition to demonstrating the immunochemical recognition of A2E, we have used the polyclonal anti-A2E antiserum to probe spectroscopic properties of A2E.

Results

In designing the immunoconjugate to generate antibodies specific for the two polyene chains of A2E, the carrier proteins BSA and rabbit serum albumin (RSA) were linked to the A2E molecule via its pyridinium ethanolamine moiety, assuring a suitable distance from the polyene chains (Fig. 1). The epsilon-amino group of lysine is the moiety within proteins that is most commonly modified to an amide bond by reacting with N-hydroxysuccinimide esters under partially aqueous conditions (35). The A2E–BSA conjugate (Fig. 1, 4) was used to immunize rabbits for the generation of anti-A2E polyclonal antibodies and both A2E–BSA and A2E–RSA conjugates (Fig. 1, 4 and 5), were used for testing the specificity of antibody binding to the A2E moiety.

The average number of A2E molecules per carrier protein in A2E–BSA and A2E–RSA conjugates was calculated by matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI-TOF MS) (36, 37). Fig. 2 shows the MALDI-TOF MS spectra of BSA, RSA, A2E–BSA conjugate and A2E–RSA conjugate. The molecular mass of authentic BSA was determined to be 66,534 Da. The mass spectral (MS) peak of the A2E–BSA conjugate was shifted toward a higher-molecular-mass region (69,930 Da) and thus corresponded to an average of five molecules of A2E-hapten covalently bound to one molecule of BSA. Similarly, RSA exhibited a mass peak at 66,570 Da, whereas that of A2E–RSA was 69,774 Da, indicating that


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Abbreviations: RSA, rabbit serum albumin; RPE, retinal pigment epithelium.

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approximately five A2E haptenic groups were linked to one RSA molecule.

The A2E–BSA and A2E–RSA conjugates displayed UV-visible spectra that corresponded to the absorbance spectrum of A2E (λmax = 336 nm and 439 nm) (19) except for the weak absorbance at 280 nm attributable to serum albumin (Fig. 3).

To determine the effect of conjugation and dialysis on the conformation of the proteins in the A2E–BSA and A2E–RSA conjugates (Fig. 1, 4 and 5), we measured the circular dichroism (CD) spectra of these conjugates and compared the latter with native BSA and RSA at similar concentrations (Fig. 4). Because there were no significant changes in the CD spectra of A2E–RSA and A2E–BSA compared with the spectra of RSA and BSA, respectively, it was concluded that the secondary structure of the protein remained intact despite conjugation within the hapten-carrier adduct. Namely, the amino groups in the protein participate in the binding without changing the overall structure (37). These results are similar to the previous work demonstrating that the CD spectra of (4-hydroxy-3-nitrophenyl) acetyl-BSA and hen-egg lysozyme conjugates were similar to those of unmodified BSA and hen-egg lysozyme (37).

Fluorescence spectroscopy was used to determine whether the two polyene chains of A2E in the A2E–BSA and A2E–RSA conjugates were exposed on the outside of the protein, a situation that would facilitate an immune response specific for A2E. Fig. 5 illustrates the fluorescence spectra generated in PBS (pH 7.4). A2E–RSA, A2E–BSA, and A2E exhibited similar fluorescence spectra with emission maxima at 605, 607, and 610 nm, respectively; these maxima are characteristic of A2E in a highly polar environment, such as PBS (22). If the A2E moiety of A2E–BSA or A2E–RSA was buried inside the BSA or RSA structure, a significant hypsochromic shift of the fluorescence would have been expected. For example, A2E in the less polar solvents methanol and N-butyl chloride shows fluorescence maxima at 600 and 585 nm, respectively (22).

The specificity and sensitivity of antibody binding to A2E was tested by adopting a dot-blot strategy using nitrocellulose as the binding matrix and synthesized A2E, A2E–RSA conjugate or various retinoids as the target (Fig. 6 A–E). After incubating with diluted rabbit anti-A2E antiserum (1:1,000 and 1:2,000), binding was detected by the addition of biotinylated secondary antibody, conjugated horseradish peroxidase (HRP) and the use of an Enhanced Chemiluminescent (ECL)-based detection system. Incubation of rabbit anti-A2E antiserum with blotted A2E and A2E–RSA resulted in intense staining in association with the spots of adsorbed A2E and A2E–RSA, whereas preimmune serum showed negligible immunoreactivity (Fig. 6 A–C). When serial dilutions of A2E were blotted onto the nitrocellulose, the antiserum (1:1,000) detected as little as 0.5 picomoles of A2E (results not shown). The robust binding of the rabbit antibody to the blotted A2E–RSA conjugate, along with weak reactivity of unconjugated RSA, demonstrated that the antibody recognized A2E rather than the protein moiety because antibody generated in a rabbit would not be expected to bind to rabbit albumin (Fig. 6D). The absence of reactivity with unconjugated BSA also indicated that the extent of conjugation of BSA with A2E masked the immunogenic characteristics of native BSA (Fig. 6D). The lack of staining in association with blotted all-trans-retinal (atRAL), all-trans-retinyl palmitate (palmitate), and all-trans-retinol (atROL) indicated an absence of cross-reactivity with these retinoids (Fig. 6E).

Antibody–antigen interaction was also observed by immunocytochemical staining (Fig. 6 F–H). After incubating human ARPE-19 cells with synthesized A2E, uptake by the cells was evidenced by the acquisition of yellow intracellular granules that were visible by microscopy with white-light illumination (Fig. 6 **An induced CD was not observed in the 330–440 nm region of the A2E maxima (data not shown), presumably because of weak intensity of the induced CD.
F and H). These yellow granules were absent from cells not incubated with A2E (Fig. 6G). These results indicated that the exogenously delivered A2E was internalized by the RPE cells in culture, as we have shown previously (22). We have also previously demonstrated that within cultured RPE cells, A2E accumulates in the lysosomal compartment, as it does in vivo. In the present work (Fig. 6F), the magenta precipitate indicative of alkaline phosphatase activity at sites of antibody–antigen binding, colocalized with A2E-containing organelles, some of which were aggregated and others of which were more evenly distributed. Only the ARPE-19 cells containing A2E exhibited alkaline phosphatase staining. Immunostaining was not observed in cultures that had not accumulated A2E (Fig. 6G). In A2E-containing cultures, immunostaining was also prevented by omission of primary antibody (Fig. 6H).

Fluorescence spectroscopic studies were performed to investigate the binding of primary rabbit anti-A2E antibody to A2E–RSA in PBS (Fig. 7A). The maximum emission of A2E–RSA at 608 nm is characteristic of A2E fluorescence in a highly polar environment, such as PBS (22). Addition of A2E-antiserum to samples of A2E–RSA resulted in an increase in fluorescence intensity and a shift in the fluorescence maximum to shorter wavelengths (Fig. 7A). The blue shift of the fluorescence spectrum is consistent with the less-polar environment provided by the antibody as compared with PBS. In control experiments in which preimmune serum was added, no changes in the A2E–RSA fluorescence were observed (Fig. 7B). This finding indicates that the A2E-antibodies specifically interact with the A2E–RSA.

**Discussion**

Here, we report the production of specific antibodies that recognize the two polyene chains of A2E. By design, the carrier protein was linked to the A2E molecule via its pyridinium ethanolamine moiety, so that the protein was positioned at a distance from A2E. Mass spectral and UV analysis of the A2E–BSA conjugate confirmed that A2E was covalently bound to BSA, thus establishing the suitability of the conjugate as an effective antigen. CD spectra indicated that the protein structure remained intact. The UV and fluorescence studies showed that, after covalent conjugation to BSA, the polyene side-arms of A2E were exposed on the exterior surface of the protein. Once the antiserum was generated to the A2E-carrier adduct, antigen–antibody interaction was observed by dot-blot and immunocytochemical staining. As part of our effort to demonstrate the specificity of the antibody, we tested immunoreactivity using cultured cells that had accumulated the lipofuscin pigment A2E. This approach provided an internal negative control cultured cells that were devoid of A2E. These antibodies will be valuable for the immunohistochemical detection of A2E in tissue samples obtained from aged and diseased donor eyes and for additional fluorescence spectroscopic studies.

Fluorescence spectroscopic studies revealed that binding of A2E–RSA to antibody leads to a blue shift in the emission maximum of A2E, likely because of a repositioning of A2E from a polar milieu to a more hydrophobic environment. This spectral shift is consistent with the previously reported solvatochromy of A2E fluorescence (22). Binding of antibody to A2E–RSA also led to an increase in the fluorescence intensity of A2E. This hyperchromism is indicative of an increase in fluorescence quantum yield. It is also plausible that the interaction of the two retinoid arms of A2E with adjacent amino acids of the antibody and preimmune serum restricts motion and thus the trans-cis isomerization at the polyene chain. Trans-cis photoisomerization, fluorescence emission, and intersystem crossing into the triplet state are competing processes by which energy is dissipated after photoexcitation. Therefore, reduced deactivation by trans-cis isomerization could be expected to occur in conjunction with an increase in fluorescence yield. It is perhaps interesting that placing the pyridinium ethanolamine moiety of A2E within a protein environment via linkage to BSA or RSA did not change the UV-visible and fluorescence spectra of the A2E portion of the conjugates. This was apparently because the polyene side-arms of A2E remained outside the protein.
Materials and Methods

General Information. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO). 1H-NMR was recorded at 400 MHz on a DMX spectrometer (Bruker, Billerica, MA), and chemical shifts were expressed in ppm relative to TMS (0.00 ppm). FAB mass spectra were measured on a JMS-HX110A mass spectrometer (JEOL, Tokyo, Japan) using a m-nitrobenzyl alcohol (NBA) glycerol matrix and Xe ionizing gas. MALDI-TOF MS was performed on a Voyager spectrometer (Applied Biosystems, Foster City, CA). UV-visible spectra were recorded on a V-530 spectrophotometer (Jasco, Easton, MD). Pyridine was distilled from KOH under argon before use. Flash chromatography and TLC were carried out with silica gel 60 (230–400 ASTM mesh) (Merck, Darmstadt, Germany) and Merck 60F254 (0.25-mm thick) sheets, respectively. All air- and water-sensitive reactions were performed in flame- and vacuum-dried glassware under argon atmosphere.

A2E Hemisuccinate 2. For the synthesis of A2E hemisuccinate (Fig. 1, 2), A2E (21) (100 mg, 0.169 mmol) (Fig. 1, 1) was dissolved in freshly distilled anhydrous pyridine (8.0 ml). Succinic anhydride (33.8 mg, 0.337 mmol) and 4-dimethylamino-pyridine (DMAP) (7.5 mg, 0.0614 mmol) were added and stirred overnight at room temperature under argon in the dark. The reaction was monitored by TLC and HPLC. After the mixture was concentrated in vacuo, the residue was purified by silica gel column chromatography with 4.5% MeOH in EtOAc to afford 78 mg of 2 as an orange-colored solid. 1H-NMR (CDCl3, 400 MHz) δ 1.05, 1.06 [each 6H, s, C5-(CH2)3, and C5’-(CH2)3], 1.5 (4H, m, C4-H2, and C4’-H2), 1.65 (4H, m, C3-H2, and C3’-H2), 1.75, 1.76 (each 3H, s, C1-CH3, and C1’-CH3), 2.1 (9H, s, C9-CH3, C9’-CH3, C13-CH3), 2.2 (4H, m, C2-H2, and C2’-H2), 3.25 (2H, m, CH2-COO), 3.3 (2H, m, CH2-COO), 4.5 (2H, m, OCH2), 4.75 (2H, m, N-CH2), 6.15–6.80 (9H, overlapped m, C7-H, C7’-H, C8-H, C8’-H, C10-H, C10’-H, C12-H, C12’-H, C14-H), 7.1–7.2 (1H, m, C11-H), 7.85 (1H, d, C13’-H), 7.95–8.05 (2H, m, C11’-H, C14’-H), 8.65 (1H, d, C15’-H). FAB-HRMS: calculated for C46H62NO4 M+, 692.4658; observed m/z 692.4658.

A2E N-Hydroxysuccinimide Ester 3. To synthesize A2E N-hydroxysuccinimide ester (Fig. 1, 3), a mixture of hemisuccinate (Fig. 1, 2) (70 mg, 0.101 mmol) and N-hydroxysuccinimide (29.0 mg, 0.252 mmol) in anhydrous DMF (7.0 ml) was stirred in the presence of N,N’-dicyclohexylcarbodiimide (DCC) (62.5 mg, 0.303 mmol) at 0°C under argon in the dark, and the temperature was gradually increased to room temperature. The reaction was monitored by TLC. After 48-h stirring, the mixture was concentrated in vacuo. Because of the instability of this compound, it was used directly for the next step. FAB-HRMS: calculated for C50H68N2O6 M+, 789.4843; observed m/z 789.4869.

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**Fig. 6.** Detection of A2E-antibody by dot blot (A–E) and immunocytochemical analysis (F–H). (A and B) A2E and A2E–RSA were applied to nitrocellulose and probed with rabbit anti-A2E antibody (1:1,000 dilution) (A) or preimmune serum (1:1,000) (B). HRP-catalyzed enhanced chemiluminescence with image development on film was used for the detection system. (C) The nitrocellulose membrane blotted with A2E and A2E–RSA is also presented. (D and E) A2E–RSA, nonconjugated rabbit serum albumin (RSA) and BSA (D) and A2E–RSA, all-trans-retinal (atRAL), all-trans-retinyl palmitate and all-trans-retinol (atROL) (E) were also probed with rabbit anti-A2E antibody by using the same detection system. (F) ARPE-19 cells that had accumulated A2E were immunocytochemically stained by incubation with anti-A2E antibody (1:100 dilution), followed by secondary antibody. (G) and (H) For controls, ARPE-19 cells that had not accumulated A2E were probed with anti-A2E and secondary antibody (G), and ARPE-19 cells that had accumulated A2E were reacted with secondary antibody in the absence of anti-A2E antibody (H).
A2E–BSA 4 and A2E–RSA 5 Conjugates. To generate A2E–BSA conjugate (Fig. 1, 4), an excess of A2E N-hydroxysuccinimide ester (Fig. 1, 3) (5.0 mg, 6.33 µmol) dissolved in dry pyridine (600 µl) in the dark with anhydrous DMSO (60.0 µl) to improve the solubility. It was then added drop-wise to a stirred solution of BSA (14.5 mg, 0.21 µmol, Sigma–Aldrich) in 0.1 M NaHCO3 (1 ml) over a period of 10–15 min. Some turbidity was observed during the addition, and the stirring was continued for 4 h to allow the formation of A2E–protein conjugates. The mixture was dialyzed (regenerated cellulose, Nominal MWCO 12,000–14,000; Fisher Scientific Pittsburgh, PA) against PBS (pH 7.4) at 4°C in a cold room for 24 h during which the buffer solution was changed several times. Conjugates were stored at 4°C in PBS buffer or kept frozen at −78°C. A2E–RSA conjugate (Fig. 1, 5) was synthesized in the same way.

Mass Spectrometry. MALDI-TOF MS analysis was performed on a Voyager Applied Biosystems spectrometer equipped with a 337-nm nitrogen laser and pulsed ion extraction. The crystal matrix, sinapinic acid (Sigma–Aldrich) was prepared at a concentration of 10 mg/ml in a 2:1 (vol/vol) mixture of 0.1% trifluoroacetic acid (TFA) (100 µl)/water (567 µl): acetronilite (333 µl). Spectra of BSA, RSA, A2E–BSA, and A2E–RSA conjugates were obtained in linear-positive mode with an accelerating voltage of 20 KV.

CD. CD spectra of BSA, RSA, A2E–BSA (Fig. 1, 4) and A2E–RSA (Fig. 1, 5) were measured by using a 1-cm cell, in PBS (pH 7.4) at 25°C on a Jasco J-810 spectrometer. Spectra were obtained with scanning speed of 100 nm min−1, time response of 1 s, bandwidth of 1 nm, and averaged over eight scans.

Antibody Generation. Polyclonal antibody was generated by contract with IMGENEX (San Diego CA) by using A2E–BSA (Fig. 1, 4) as immunogen. Two rabbits were immunized by ventral s.c. injection. For primary immunization, 200 µg of immunogen was administered in Complete Freund’s Adjuvant (CFA) and for boosters 100 µg of immunogen was given in Incomplete Freund's Adjuvant (IFA). The immune response was monitored by IMGENEX by using ELISA and A2E–RSA conjugate (Fig. 1, 5) as the target antigen. Wells were coated with 200 ng of A2E–RSA conjugate, and antisera were diluted 1/5,000. Robust binding to A2E–RSA was observed (~1.2–1.4 absorbance units). Immune titres in both rabbits after the first and third bleeds were elevated 12- to 15-fold relative to preimmune bleeds. The IgG concentration of A2E-antiseraum was measured (UV absorbance at 280 nm) after ammonium sulfate fractionation, followed by extensive dialysis and was determined as 3.1 mg/ml. Protein concentrations of both antisera and preimmune serum were measured by protein assay (Bio-Rad, Hercules, CA).

Fluorescence Spectroscopy. Fluorescence emission spectra were recorded at room temperature on a Fluororlog-3 spectrometer FL3–22 (J. Y. Horiba, Edison, NJ) by using quartz cuvettes with path length of 1 cm and an excitation wavelength (λex) of 430 nm. The absorbance of A2E–RSA in PBS (pH 7.4) was 0.26 at 430 nm. The fluorescence spectra were corrected for the autofluorescence of the antibody by subtracting the luminescence spectra of PBS buffer solutions containing the equivalent amounts of antibody in the absence of A2E–RSA. The spectra were recorded after at least 10-min equilibrium time after addition of antibody. Identical experimental conditions were used for the control with preimmune serum.

Dot-Blot Analysis. A2E (5 µl of 5 µM in PBS with 0.03% DMSO), RSA (0.25 µg in 5 µl), A2E–RSA (0.25 µg in 5 µl), BSA (0.25 µg in 5 µl), all-trans-retinal (1.4 µg in 5 µl), all-trans-retinol (2.6 µg in 5 µl), and retinyl palmitate (1.3 µg in 5 µl) were blotted onto nitrocellulose membrane (LC2001; Invitrogen Life Technologies, Carlsbad CA). Three successive blocking reagents were used: Tris-buffered saline (TBS) with 0.1% Tween-20 TBST (1× TBS, 0.1% Tween-20), and 5% nonfat dry milk (NonFat-Dried Milk bovine; Sigma–Aldrich); 1× Casein solution (Vector Laboratories, Burlingame, CA); and Vector avidin/biotin blocking Kit (Vector Laboratories). The membrane was incubated with primary antibody (rabbit anti-A2E antibody) or preimmune serum (diluted 1:1,000 and 1:2,000) in blocking buffer at 2–8°C overnight, and unbound antibodies were removed by washing the membrane three times with TBS–Twee 20 for 10 min each. The membrane was then incubated with secondary antibody (goat anti-rabbit IgG, Vector Laboratories) conjugated with biotin (diluted 1:10,000 in 1× Casein solution), followed by VECTASTAIN ABC (peroxidase), (Avidin-Biotinylated enzyme Complex). Reagent (Vector Laboratories) at room temperature for 30 min. The membrane was washed with TBS–Twee 20 and then briefly rinsed with TBS. Images were developed on film by using the ECL kit (Amersham Biosciences, Piscataway, NJ).

A2E Accumulation in ARPE-19 Cells. Human ARPE-19 cells (ARPE-19, American Type Culture Collection, Manassas, VA), lacking endogenous A2E (21) in 10% serum was synthesized A2E (21) in PBS with 0.03% DMSO, RSA (0.25 µg in 5 µl), A2E–RSA (0.25 µg in 5 µl), BSA (0.25 µg in 5 µl), all-trans-retinal (1.4 µg in 5 µl), all-trans-retinol (2.6 µg in 5 µl), and retinyl palmitate (1.3 µg in 5 µl) were incubated in Dulbecco’s Modified Eagle Medium (DMEM; Fisher Scientific, Pittsburgh, PA) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT), amino acids, antibiotics/gentamicin, and 5% CO2 at 37°C. Once the cells were confluent, 10 µM synthesized A2E (21) in 10% serum was delivered to the cells in media for 15 days. Cultured cells (with and without A2E) were then subjected to immunocytochemistry studies.

Fig. 7. Fluorescence spectra of A2E–RSA (1.38 µM) in PBS buffer (pH 7.4) before (black line) and after (red line) addition of primary rabbit anti-A2E antibody (0.0309 μg/µl, protein) (A) and before (black line) and after (blue line) addition of preimmune serum (0.0309 μg/µl, protein) (B). λex = 430 nm.
Immunocytochemistry. The cultured RPE cells were washed twice with PBS to remove the serum-containing medium. The cells were fixed with 300 μl of 2% paraformaldehyde in DPBS (GIBCO, Grand Island, NY) for 20 min at room temperature and washed five times with 400 μl of PBS for 5 min. The cells were then permeabilized with 300 μl of freshly prepared 0.1% Triton X 100 in 0.1% sodium citrate for 15 min at room temperature. After washing with PBS, nonspecific binding sites were blocked as described above for dot-blot analysis. Subsequently, 150 μl of primary antibody (rabbit anti-A2E antibody; 1: 100 dilution in blocking buffer; 40 min, room temperature). After washing five times with PBS, each for 5 min, the cells were incubated with secondary antibody (goat anti-rabbit IgG) conjugated to biotin (1: 200 dilution in blocking buffer) was added, and incubation was carried out at room temperature for 40 min. After washing five times with PBS, nonspecific binding sites were blocked as described above, the cells were treated with Vectastain ABC- Alkaline Phosphatase (Vector Laboratories) for 30 min at room temperature. After washing, alkaline phosphatase activity was detected by using an Alkaline Phosphatase Magenta 3 detection system (Leinco Technologists, St. Louis MO). Controls included ARPE-19 cells that had not accumulated A2E and samples in which primary antibody was omitted. Microscopic images were captured by an automated Axioplan II fluorescent microscope (Zeiss, Thornwood, NY) equipped with an AxioCam HRc digital camera and Axiovision 4.3 software (Zeiss).

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