Disruption of the Blood-Brain Barrier in Experimental Optic Neuritis: Immunocytochemical Co-Localization of $H_2O_2$ and Extravasated Serum Albumin

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Purpose. To probe the role of endogenous hydrogen peroxide ($H_2O_2$) in the pathogenesis of disruption of the blood-brain barrier (BBB) associated with experimental allergic encephalomyelitis (EAE), an animal model for primary central nervous system demyelination.

Methods. Strain-13 guinea pigs were sensitized for EAE with central myelin in complete Freund's adjuvant. Magnetic resonance imaging with Gd-DTPA was performed twice a week for 2 weeks to assess disruption of the BBB, in vivo, by the enhancement of the optic nerves. Two weeks after antigenic sensitization, ultracytochemical localization of endogenous $H_2O_2$ was performed using the cerium perhydroxide method, with co-localization of endogenous serum albumin extravasation using gold-labeled antibodies against serum albumin. Examination of blood vessels for perivascular immunogold-labeled serum albumin and $H_2O_2$ derived reaction product began in the optic nerve head and proceeded toward the retrobulbar optic nerve until a total of 20 vessels were evaluated per animal.

Results. Magnetic resonance imaging revealed Gd-DTPA enhancement of the optic nerves in all animals sensitized for EAE. Optic nerve ultrastructure revealed colloidal gold-labeled antibodies against serum albumin in the perivascular and adjacent interstitial spaces of capillaries and small venules in which $H_2O_2$ derived cerium perhydroxide reaction product was also simultaneously evident. Immunogold-labeled serum albumin was predominantly confined to the intravascular compartment of the optic nerve in the absence of perivascular $H_2O_2$ and/or perivascular foci of inflammatory cells. The difference between the mean percentage of blood vessels (61.8%) with co-localization of perivascular immunogold-labeled serum albumin and $H_2O_2$ reaction product, to the mean percentage of blood vessels (9.5%) with perivascular immunogold-labeled serum albumin in the absence of cerium perhydroxide, was statistically significant ($P = 0.0019$).

Conclusions. Endogenous $H_2O_2$, found at the foci of BBB disruption, may be one of the mediators involved in the alteration of vascular permeability in experimental optic neuritis. Invest Ophthalmol Vis Sci. 1994;35:1114-1123.
vasculature by accumulation of extravasated tracers in the perivascular and interstitial spaces. Reactive oxygen species such as superoxide, hydrogen peroxide (H$_2$O$_2$), and hydroxyl radical, among others, are known to affect endothelial cell permeability and have been implicated in BBB disruption. Because most reactive oxygen species are extremely reactive, they are difficult to demonstrate directly. The role of reactive oxygen species has been implicated by the suppression of EAE by detoxification of H$_2$O$_2$ with antioxidants. To simultaneously probe the role of H$_2$O$_2$ in vascular permeability associated with EAE, we used ultracytochemical localization of endogenous H$_2$O$_2$ followed by immunogold co-localization of endogenous serum albumin.

**MATERIALS AND METHODS**

**Induction of EAE**

Strain-13 guinea pigs were purchased from Crest Caviary (Sacramento, CA). Eleven guinea pigs, weighing between 200 to 1100 g, were sensitized for EAE with an emulsion (1.0 ml/kg body weight) of spinal cord, freshly dissected from normal strain-13 guinea pigs, in complete Freund's adjuvant injected subdermally into the nuchal area. Three additional strain-13 guinea pigs served as unsensitized controls. Animals were cared for in a veterinarian supervised vivarium in accordance with ARVO guidelines on the use of animals in research.

**Magnetic Resonance Imaging**

Magnetic resonance imaging of guinea pigs sensitized for EAE was performed twice a week for 2 weeks. A 2.0 tesla 32 cm bore superconducting magnet (Oxford Instruments Limited, Oxford, England) with a SUN computer-based acquisition and processing system (Spectroscopy Imaging Systems, Freemont, CA) was used to perform magnetic resonance imaging, using a 6 cm field of view, a 256 X 192 matrix with four repetitions, and a section thickness of approximately 1.25 mm. A specially designed surface coil was placed over the head for an improved signal-to-noise ratio. Suppression of orbital fat was accomplished using a frequency selective saturation pulse method with a T1 weighting (T1w) of TR = 600 ms and a TE = 20 ms. T1w imaging was again performed immediately after intravascular administration of Gd-DTPA (Berlex Laboratories, Wayne, NJ) at a dose of 0.2 mmol/kg of body weight. Images were acquired in the axial and coronal planes with the guinea pig lying prone, after sedation with intramuscular ketamine (Fort Dodge Laboratories, Fort Dodge, IA) and xylazine (Butler Co, Columbus, OH) in a 1:1 mixture (0.4 ml/kg of body weight).

**Ultracytochemical Localization of Hydrogen Peroxide**

Two weeks after antigenic sensitization, 11 animals with EAE and three unsensitized strain-13 guinea pigs, who served as normal controls for BBB integrity and H$_2$O$_2$ production, were euthanized by intracardiac injection with sodium pentobarbital (2 ml/kg of body weight) (Butler Co, Columbus, OH) after initial sedation with intramuscular ketamine:xylazine mixture (0.4 ml/kg of body weight).

The right eyes were enucleated and the globes, and attached optic nerves were dissected and fixed in cold 5.0% acrolein (Sigma Chemical Company, St. Louis, MO) in 0.1 M sodium cacodylate-HCl buffer (pH 7.4). Specimens were washed in cold 0.15 M sodium cacodylate-HCl buffer (pH 7.4), plus 5% sucrose, and 1% dimethylsulfoxide (Sigma). Specimens were brought to room temperature in the final two buffer washes that contained 0.1 M glycine. Specimens were preincubated for 30 minutes at 37°C with agitation in the following medium: 2.0 mM cerium chloride (Sigma), 10 mM 3-amino-1,2,4-triazole (Sigma), 0.1 M Tris-maleate buffer (pH 7.5), 7% sucrose, and 0.0002% Triton X-100. They were then incubated for 1 hour at 37°C with agitation in the following complete reaction medium: 2 mM cerium chloride, 10 mM 3-amino-1,2,4-triazole, 0.8 mM NADH (Sigma), 0.1 M Tris-maleate buffer (pH 7.5), 7% sucrose, and 0.0002% Triton X-100. Cereous ions form an electron dense precipitate, cerium perhydroxide, in the presence of H$_2$O$_2$. The reaction was terminated by washing in cold 0.1 M Tris-maleate buffer (pH 7.5), and 7% sucrose, followed by a wash in cold 0.15 M sodium cacodylate-HCl buffer (pH 7.4), and 7% sucrose. Specimens were postfixed in 1% osmium tetroxide, 0.1 M sodium cacodylate-HCl buffer (pH 7.4), 7% sucrose in the cold. Specimens were then dehydrated through an ethanol series to propylene oxide, infiltrated, and embedded in epoxy resin.

**Immunogold Co-localization of Serum Albumin**

Deep gold sections on nickel grids were oxidized for 30 minutes with 1% periodic acid, then washed in deionized water. Grids were floated on 0.02 M phosphate-buffered saline, pH 7.2, 0.1 M NaCl, 2% teleost gelatin (Sigma), 2% nonfat dry milk (Carnation, Los Angeles, CA), 0.05% Tween 20, and then reacted with rabbit anti-guinea pig serum albumin antibodies (Sigma Immuno Chemicals, St. Louis, MO) in the same buffer for 2 hours at room temperature. After washes in phosphate-buffered saline, 0.5 M NaCl, plus additives, followed by washes in 0.02 M Tris-HCl buffered saline, 0.5 M NaCl, 2% teleost gelatin, 2% nonfat dry milk, 0.05% Tween 20, the grids were reacted with 10 nm gold-labeled goat anti-rabbit IgG antibodies (Sigma Immuno Chemicals) diluted with Tris-HCl buff-
ered saline plus additives, for 1 hour at room temperature. After washes in Tris-HCl buffered saline plus additives, grids were washed in deionized water. Control grids were incubated in phosphate-buffered saline, followed by the gold-labeled antibody to check for non-specific binding of the secondary antibody.

Ultrastructural Analysis

The optic nerves were examined by transmission electron microscopy without poststaining at 75 kV. For each animal, a minimum of two grids reacted with the primary and secondary antibodies were evaluated. To evaluate background labeling of each optic nerve, two control grids were reacted with the secondary antibody. Twenty blood vessels from each optic nerve were examined for the presence of perivascular cerium perhydroxide reaction product and extravasated immunogold-labeled serum albumin for each of the 11 animals with EAE. Examination of blood vessels began in the optic nerve head and proceeded toward the retrobulbar optic nerve. Care was taken to mark the x-y coordinates of each blood vessel on the grid bar.

Weighed least square regression models was used to address (a) whether the mean percentage of optic nerve head blood vessels with co-localization of perivascular H2O2 and immunogold-labeled serum albumin was equal to the mean percentage of retrobulbar blood vessels with perivascular H2O2 and immunogold-labeled serum albumin, (b) for retrobulbar blood vessels, whether the mean percentage with co-localization of perivascular H2O2 and immunogold-labeled serum albumin, and (c) for all blood vessels (optic nerve head and retrobulbar optic nerve), whether the mean percentage with co-localization of perivascular H2O2 and immunogold-labeled serum albumin was equal to the mean percentage with perivascular immunogold-labeled serum albumin in the absence of H2O2, and (c) for all blood vessels (optic nerve head and retrobulbar optic nerve), whether the mean percentage with co-localization of perivascular H2O2 and immunogold-labeled serum albumin was equal to the mean percentage with perivascular immunogold-labeled serum albumin in the absence of H2O2. The outcome variables, percentage with above-specified findings, were transformed using an arcsine transformation to stabilize the variances. Indicator variables for each animal were included in all models. The sample size upon which each percentage is based was used as the weight. For each of the above comparisons, the significance of the addition of a variable representing the type of finding was assessed.

RESULTS

Magnetic Resonance Imaging

Mild enhancement of the optic nerve sheath was seen in all animals before antigenic sensitization. In EAE animals, Gd-DTPA enhancement of the optic nerves occurred as early as 3 days after antigenic sensitization. The initial site of enhancement involved the orbital segment of optic nerve adjacent to the globe. Fourteen days after antigenic sensitization, the intensity of Gd-DTPA enhancement increased at this site and involved longer segments of the orbital optic nerves (Fig. 1).

Retrobulbar Optic Nerve Ultrastructure

Cerium perhydroxide reaction product and extravasation of serum albumin were absent in the retrobulbar optic nerves of the three normal control animals not sensitized for EAE. Control grids not reacted with the primary antibody showed scant background of 10 nm gold-labeled particles, without an intravascular or perivascular distribution for normal and EAE animals.

All animals sensitized for EAE exhibited foci of demyelination in the retrobulbar optic nerve. Perivascular demyelination and foci of inflammatory cells were often seen in association with the H2O2 derived cerium perhydroxide reaction product. At these foci, H2O2 derived cerium perhydroxide reaction product had a predominantly perivascular distribution. Extravascular gold-labeled antibodies against serum albumin indicated the sites of BBB disruption. Fifty-six percent (92 of 163) of all blood vessels counted in the retrobulbar optic nerve were identified with both perivascular cerium perhydroxide and perivascular immunogold-labeled serum albumin (Table 1). The difference between the mean percentage (58% ± 9.7%) (mean ± SEM) of blood vessels in the retrobulbar nerve, with co-localization of perivascular immunogold-labeled serum albumin and cerium perhydroxide reaction product, to the mean percentage (13% ± 5%) of blood vessels in the retrobulbar nerve with perivascular immunogold-labeled serum albumin in the absence of H2O2 was statistically significant (P = 0.014). Figure 2 illustrates the perivascular distribution of electron-dense cerium perhydroxide reaction product particles, which were irregular and pleomorphic varying from approximately 20 nm to 50 nm in size. Gold-labeled antibodies against serum albumin, identified as discrete, round 10 nm particles, were seen in the perivascular space, alongside H2O2 derived cerium perhydroxide reaction product. Cerium perhydroxide was absent at a perivascular focus of demyelination and inflammation (Fig. 3). At this site, serum albumin identified by gold-labeled antibodies against albumin was predominantly localized to the intravascular space. Although the distribution of immunogold-labeled serum albumin was predominantly intravascular, extravasated serum albumin was seen in the perivascular and extracellular spaces between axons in the absence of perivascular H2O2 derived cerium perhydroxide reaction product. However, common to all blood vessels exhibiting disruption of the BBB, in the absence of H2O2 derived reaction product there was a perivascular foci of inflammatory cells.
Blood-Brain Barrier in Optic Neuritis

Figure 1. A T1 weighted magnetic resonance imaging of a guinea pig, in the axial plane, shows Gd-DTPA enhancement (arrows) of the optic nerves adjacent to the globe 3 days (A), 7 days (B), and 10 days (C) after antigenic sensitization. By 14 days, marked Gd-DTPA enhancement of both orbital optic nerves (arrows) is evident (D).

In the presence of adjacent perivascular H$_2$O$_2$ derived cerium perhydroxide reaction product, gold-labeled antibodies against serum albumin were evident in a vesicular body located along the abluminal surface of the endothelial cell (Fig. 4A). Cerium perhydroxide reaction product was also seen within a different vesicular body of the identical endothelial cell. Clusters of gold-labeled antibodies against serum albumin were seen within vesicles close to the luminal surface of the endothelial cell, in the absence of adjacent H$_2$O$_2$ (Fig. 4B).

Extravasation of serum albumin, identified by gold-labeled antibodies against albumin, also occurred in the presence of perivascular foci of inflammatory cells and in the absence of H$_2$O$_2$ derived cerium perhydroxide reaction product. Of the retrobulbar vessels (21 of 163) counted, 13% had perivascular extravasation of serum albumin in the absence of cerium perhydroxide, seen in association with perivascular inflammatory cells. However, extravasation of serum albumin was negligible in the absence of perivascular foci of inflammatory cells and/or cerium perhydroxide reaction product.

Activated inflammatory cells were evident in the intravascular space. Cerium perhydroxide reaction product surrounded the plasmalemma of inflammatory cells within the blood vessel lumen where H$_2$O$_2$ was released into the intravascular compartment.

In addition to the perivascular distribution of inflammatory cells, activated inflammatory cells infiltrated the interstitium of the optic nerve. At foci of demyelination, cerium perhydroxide reaction product surrounded activated inflammatory cells, myelinated axons, and demyelinated axons. Inflammatory cells, devoid of H$_2$O$_2$ derived cerium perhydroxide reaction, also enveloped naked axons and myelin and phagocytized albumin. Immunogold-labeled serum albumin and cerium perhydroxide reaction product were evident in the expanded extracellular space.

Optic Nerve Head Ultrastructure

In the three normal, unsensitized animals, extravascular immunogold-labeled serum albumin was evident only at the border tissue of the optic nerve head and vascular choroid, where the BBB is normally absent. Extravasation of serum albumin was not seen within blood vessels in the optic nerve heads of the normal animals. Moreover, cerium perhydroxide reaction product was absent in the optic nerve heads of these unsensitized guinea pigs.

In the optic nerve heads of EAE animals, all blood vessels with perivascular extravasated immunogold-labeled serum albumin had co-localization of H$_2$O$_2$ reaction product. Fifty-seven of the 220 blood vessels (26%) evaluated were in the optic nerve head. Of these, 44 vessels (77%) exhibited co-localization of cerium perhydroxide and extravasated serum albumin. In the absence of H$_2$O$_2$ derived reaction product, none of the remaining 13 blood vessels (23%) counted in the optic nerve head had extravasated serum albumin. Inflammatory cell infiltration of the unmyelin-
### TABLE 1. Perivascular Albumin and H$_2$O$_2$ Localization

| Animal No./Weight/Sex (EAE) | NOH | Percentage of Vessels With Au + Ce | No. of Vessels With Au + Ce | Total | Percentage of Vessels With Au + Ce | No. of Vessels With Au + Ce | Total | Percentage of Vessels With Au + Ce | No. of Vessels With Au + Ce | Total | Percentage of Vessels With Au + Ce | No. of Vessels With Au + Ce | Total | Percentage of Vessels With Au + Ce | No. of Vessels With Au + Ce | Total | Percentage of Vessels With Au + Ce | No. of Vessels With Au + Ce | Total | Percentage of Vessels With Au + Ce |
|-----------------------------|-----|-----------------------------------|-----------------------------|-------|-----------------------------------|-----------------------------|-------|-----------------------------------|-----------------------------|-------|-----------------------------------|-----------------------------|-------|-----------------------------------|-----------------------------|-------|-----------------------------------|-----------------------------|-------|-----------------------------------|-----------------------------|-------|-----------------------------------|
| 1/283/F                    | 7   | 100%                             | 7                           | 44    | 77%                              | 57                          |       |                                   |                             |       |                                   |                             |       |                                   |                             |       |                                   |                             |       |                                   |
| 2/530/F                    | 5   | 100%                             | 5                           | 5     | 83%                              | 6                           |       |                                   |                             |       |                                   |                             |       |                                   |                             |       |                                   |                             |       |                                   |
| 3/511/F                    | 1   | 100%                             | 1                           | 1     | 83%                              | 7                           |       |                                   |                             |       |                                   |                             |       |                                   |                             |       |                                   |                             |       |                                   |
| 4/338/F                    | 5   | 100%                             | 7                           | 18    | 83%                              | 9                           |       |                                   |                             |       |                                   |                             |       |                                   |                             |       |                                   |                             |       |                                   |
| 5/310/F                    | 1   | 100%                             | 2                           | 7     | 83%                              | 9                           |       |                                   |                             |       |                                   |                             |       |                                   |                             |       |                                   |                             |       |                                   |
| 4/326/M                    | 3   | 100%                             | 2                           | 3     | 100%                             | 2                           |       |                                   |                             |       |                                   |                             |       |                                   |                             |       |                                   |                             |       |                                   |
| 7/500/M                    | 6   | 100%                             | 6                           | 6     | 100%                             | 6                           |       |                                   |                             |       |                                   |                             |       |                                   |                             |       |                                   |                             |       |                                   |
| 8/550/M                    | 9   | 100%                             | 9                           | 11    | 83%                              | 11                          |       |                                   |                             |       |                                   |                             |       |                                   |                             |       |                                   |                             |       |                                   |
| 9/600/F                    | 1   | 100%                             | 1                           | 1     | 100%                             | 1                           |       |                                   |                             |       |                                   |                             |       |                                   |                             |       |                                   |                             |       |                                   |
| 10/650/F                   | 3   | 100%                             | 3                           | 3     | 100%                             | 3                           |       |                                   |                             |       |                                   |                             |       |                                   |                             |       |                                   |                             |       |                                   |
| 11/575/F                   | 1   | 100%                             | 1                           | 1     | 100%                             | 1                           |       |                                   |                             |       |                                   |                             |       |                                   |                             |       |                                   |                             |       |                                   |
| Total (mean) vessels       | 44  | 77%                              | 57                          | 92    | 58%                              | 113                         |       |                                   |                             |       |                                   |                             |       |                                   |                             |       |                                   |                             |       |                                   |

Au = immunogold-labeled albumin; Ce = H$_2$O$_2$-derived reaction product.

At the border tissue of Elschnig, both H$_2$O$_2$ derived cerium perhydroxide reaction product particles and extravasated immunogold-labeled serum albumin were abundant (Fig. 5). In the perivascular and extracellular spaces, between the unmyelinated axons of the optic nerve head, extravasated serum albumin was seen alongside H$_2$O$_2$ derived cerium perhydroxide reaction product (Fig. 6).

#### Analysis of All Blood Vessels

For all blood vessels combined (in both the optic nerve head and the retrobulbar nerve), the difference between the mean percentage (61.8% ± 8.8%) of blood vessels with co-localization of perivascular immunogold-labeled serum albumin and cerium perhydroxide reaction product, to the mean percentage (9.5% ± 3.9%) of blood vessels with perivascular immunogold-labeled serum albumin in the absence of adjacent H$_2$O$_2$, was statistically significant ($P = 0.0019$).
FIGURE 3. Cerium perhydroxide reaction product is absent at a perivascular focus of demyelination and inflammation (X7,400). Magnification of the area surrounded by the box is shown in the inset (top); immunogold-labeled serum albumin (arrowheads) is predominantly localized to the intravascular compartment (IC = Inflammatory cell, E = endothelial cell, A = demyelinated axon) (X30,000).

DISCUSSION

The mechanism of disruption of the BBB is unclear.36–38 Despite the functional incompetence of optic nerve vasculature, the structural integrity of endothelial cells and tight junctions were maintained. Opening of interendothelial tight junctions and vesicular transport across the endothelial cell cytoplasm are two hypotheses invoked to explain BBB disruption. Neither H$_2$O$_2$ derived cerium perhydroxide reaction product nor
Immunogold-labeled serum albumin could be traced continuously across the tight junctions from the lumen to the perivascular space. However, the site of anatomic disruption of the tight junctions may have been outside the field of view of transmission electron microscopy. We did find both H$_2$O$_2$ and immunogold-labeled serum albumin within endothelial vesicles, although neither H$_2$O$_2$ nor immunogold-labeled serum albumin could be traced continuously from the vessel lumen to the perivascular space.
Integrity to the BBB has been restored by detoxification of H$_2$O$_2$, with the exogenous administration of the antioxidant enzymes catalase and glutathione peroxidase, co-localization of H$_2$O$_2$ derived cerium perhydroxide reaction product to the foci of extravasated serum albumin further suggests H$_2$O$_2$ may be one of the mediators involved in the disruption of the BBB, particularly in the optic nerve head where there appeared to be no apparent local contribution of the inflammatory cell infiltrate to the extravasation of serum albumin. At foci of perivascular inflammation in the retrobulbar nerve, the extravasation of immunogold-labeled serum albumin in the absence of perivascular H$_2$O$_2$ suggests other mediators may also be involved in BBB disruption.

Although perivascular foci of inflammatory cells appeared to be the source of H$_2$O$_2$ in the retrobulbar nerve, the origin of H$_2$O$_2$ in the optic nerve head is less clear. Diffusion of H$_2$O$_2$ from the intravascular space, where H$_2$O$_2$ is discharged by activated inflammatory cells, across endothelial cells and tight junctions into the perivascular space is one hypothesis for the perivascular accumulation of H$_2$O$_2$ in the optic nerve head. An alternative hypothesis is the immune-mediated production of H$_2$O$_2$ by endothelial cells, with deposition of H$_2$O$_2$ into the perivascular space. Certainly, diffusion of H$_2$O$_2$ and serum albumin across the border tissue of Elschnig, a site where the BBB is absent, accounted for some of the extravasation of serum albumin and H$_2$O$_2$ into the optic nerve head.

Alterations in vascular permeability may not be exclusively attributed to H$_2$O$_2$ itself. In the retrobulbar optic nerve, while perivascular H$_2$O$_2$ surrounded the majority of vessels exhibiting BBB disruption, extravasation of serum albumin also took place at the foci of perivascular inflammatory cells in the absence of H$_2$O$_2$ suggesting that other mediators not identified by our technique may also mediate disruption of the BBB. Hydrogen peroxide may be an intermediate in a cascade of highly reactive oxygen species. Dismutation of superoxide by the action of superoxide dismutase generates H$_2$O$_2$. Superoxide has been shown to alter endothelial cell permeability and structure. Administration of superoxide dismutase was not protective in EAE, although detoxification of H$_2$O$_2$ was protective, suggesting H$_2$O$_2$ and/or metabolites of H$_2$O$_2$ were mediators of BBB disruption in EAE. Though a reactive oxygen species, H$_2$O$_2$ is much less reactive than most free radicals. Hydrogen peroxide is converted to the highly reactive hydroxyl radical by the Fenton reaction. Hydroxyl radical may contribute to BBB permeability and peroxidation of adjacent myelin lipid and generation of lipid-free radicals. Consequently, disruption of the BBB and myelin peroxidation may reflect oxidative in...
jury from a cascade of reactive oxygen species, with H$_2$O$_2$ as a less reactive intermediary also serving as the fuel for hydroxyl radical and lipid radical generation.

**Key Words**
optic neuritis, experimental allergic encephalomyelitis, hydrogen peroxide, free oxygen radicals, multiple sclerosis

**References**


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