Modulation of MHC Class II Expression in the Absence of Lymphocytic Infiltrates in Alzheimer’s Retinae


Abstract. This study describes the expression of MHC class II antigens in retinal flat mounts from normal donors and patients with Alzheimer’s disease (AD). We confirm previous observations of MHC class II immunoreactivity on microglia in normal retinae, while observing insignificant levels of reactivity on endothelial cells (EC). A significantly increased level of MHC class II expression was detected in AD retinae. This increased immunoreactivity was found to occur in the absence of lymphocytic infiltrates, suggesting that the pathogenesis of AD in the retina may be distinct from that reported to occur in some regions of the brain. MHC class II expression, measured using computerized optical densitometry, appeared to be increased principally as a result of induced MHC class II immunoreactivity on EC. Ramified microglia and perivascular macrophages, although hypertrophied, appeared to show unchanged levels of MHC class II expression. These findings are consistent with earlier suggestions that both aberrant MHC class II expression and suppressor activity of resident macrophages may restrict immune responses.

Key Words: Alzheimer’s disease; Human retina; MHC class II; Microglia.

INTRODUCTION

Recent studies have reported both clinical and histopathological evidence of degeneration of the primary visual pathway associated with Alzheimer’s disease (AD) (1–4). Expression of AD-related degeneration in the retina appears to reflect the close anatomical relationship of the brain and retina, although the histopathological features of AD in the cerebral cortex (neurofibrillary tangles and neuritic plaques) have not been observed in the retina (1). Some reports have identified leukocytic infiltrates in the neuropil, suggesting that inflammation may influence the pathogenesis of AD in the brain (5–7). Studies of retinae from patients with AD provide no evidence of similar infiltrates associated with ganglion cell degeneration (1, 2).

The traditional view that the retina is immunologically ‘privileged’ has arisen from structural and physiological observations of the blood–ocular barrier, the absence of a lymphatic drainage and the apparent absence of major histocompatibility complex (MHC) class II antigens in normal human retina and brain grey matter (8–12). However, we have recently described expression of both leukocyte common antigen (LCA, CD45) and MHC class II antigens by microglia in normal adult and fetal retinal flat mounts (13, 14). Additionally, MHC class II expression by perivascular macrophages or mononuclear phagocyte series (MPS) cells (15, 16) and ramified microglia has been reported in normal brain (16). Microglia have been implicated in the deposition of amyloid fibers (17–19) and proteins belonging to the complement cascade (7).

Modulation of MHC class II glycoprotein expression in response to diseases of the central nervous system (CNS) has been described previously (20). For example, increased MHC class II glycoprotein expression on microglial cells has been observed in AD brain (16, 21–23). Endothelial cells (EC) are reported to be unreactive with anti-MHC class II antibodies in normal brain (24, 25), although increased expression of MHC class II antigens by CNS EC has been observed in chronic neurological diseases such as multiple sclerosis (26).

The interaction of the T cell receptor with its ligand, a peptide in combination with a molecule of the MHC, is a central event in the initiation and propagation of most immune responses to protein antigens (27). Expression of MHC class II glycoproteins is a functional characteristic associated with antigen-presenting cells of hematopoietic origin (28). Aberrant MHC class II expression by nonhematopoietic cells has, however, been shown to promote inactivation of T cells and immunological unresponsiveness (29, 30). It has also been demonstrated that MHC class II–positive resident macrophages, in non-neural tissue such as skin, lung and gut, may have a suppressive effect on the antigen-presenting activity of dendritic cells (31, 32). In the present study we have examined the expression of MHC class II antigens in flat mounts of normal retinae and retinae from patients with AD. We describe hypertrophy of ramified microglia and perivascular macrophages together with the induction of MHC class II expression on EC in retinae with AD.
MATERIALS AND METHODS

Specimens

Thirty-five eyes, from patients with AD ranging from ages 66 to 98 years (mean age 80.9 ± 8.3 years), were donated by the Doheny Eye Institute, Los Angeles, CA. The diagnosis of AD was based on clinical criteria defined by the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) (33). The eyes were initially fixed in buffered 10% formal saline within 2–12 hours (h) postmortem (pm). Following removal of the anterior segment and the vitreous body, the eyes were further fixed in 2% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) (pH 7.4) for at least 24 h at 4°C. Normal adult eyes, ranging in age from 15 to 88 years, were obtained in 3–16 h pm through the Lion’s N.S.W. Eye Bank. Specimens were fixed in 2% paraformaldehyde in 0.1 M PBS (pH 7.4) for up to 24 h at 4°C. Eyes more than 16 h pm were not used, as a previous study (14) has shown that detectability of MHC class II antigens is significantly reduced by pm delay.

Eyes with a known history of ocular or other chronic neurological diseases were excluded from the present study. Additionally, all eyes were surveyed for histopathology as follows. The posterior pole was excised using a 7 mm trephine, producing a disc centered about the macula with the neural retina separated from the retinal pigment epithelium (RPE) and choroid. Samples of the RPE-choroid portion of the trephined samples were embedded in Epon-Araldite resin and examined with light and electron microscopy. Specimens showing signs of retinal pathology, such as the presence of absence of drusen, pigmentary disturbance, abnormal collagen deposition, neovascular or atrophic lesions, were excluded from the study. Normal eyes (n = 25) were divided into three age groups: 0-29 years (mean age 21.7 ± 4.6 years), 30-59 years (43.4 ± 7.2 years) and 60-90 years (74.3 ± 9.6 years).

Histology

Immunohistochemistry: Paramacular and peripheral regions of normal and AD neural retinae were excised using a 3 mm trephine. Specimens were rinsed, then soaked in 0.2% Triton X-100 (BDH Chemicals, Australia) in 0.1 M tris-buffered saline (TBS) (pH 7.6) for 12 h at 4°C prior to incubation in primary antibody for 24 h at 4°C. The primary antibody used was HLA-DR, CR3/43, which reacts with the beta-chains of all products of the gene subregions DP, DQ and DR (1:50 dilution; DAKO, Carpinteria, CA, Cat. no. M775); additional samples from three retinæ were incubated with anti-human LCA (CD45) (prediluted; DAKO, Cat. no. L1826). After several washes specimens were incubated in biotinylated secondary antibody (1:50 dilution; Amersham, Australia, Cat. no. RPM1001) for a further 24 h at 4°C. Primary and secondary antibodies were diluted in 2% fetal calf serum (FCS) in 0.1 M TBS. Bound antibody was detected using the avidin-biotin peroxidase labeling technique (Vector, Vector Laboratories, Burlingame, CA). Peroxidase was visualized using a nickel-enhanced, 3,3'-diaminobenzidine tetrahydrochloride (DAB) solution (34). Following immunohistochemical procedures specimens were placed onto gelatinised slides, nerve fiber layer uppermost, air-dried in a humidified chamber, dehydrated in graded alcohols and xylene and mounted in DePeX (BDH Chemicals).

Retinal Sections: Trephined samples of neural AD retinae (n = 10) were resin-embedded (Epon-Araldite) and toluidine blue-stained semi-thin sections were examined for the presence of leukocytic infiltrates.

Image Analysis

In order to measure the intensity of MHC class II expression, each labeled flat mount was viewed under a Zeiss Axiophan microscope using a ×10 objective lens. A Sony color video camera was used to select fields and the image was acquired by a Tracor Northern image analysis system (IAS). The size of each field was 0.7 mm², three different fields being selected from each specimen. A binary image was created to enable the computer to distinguish areas positively stained with the peroxidase-labeled anti-MHC class II antibody. Computer calculations were made of the optical density of positive areas (MHC class II-positive cells) in each field from which the average optical density of positive cells was calculated. In addition, the percentage of retinal area labeled with anti-MHC class II antibody in each field was measured by the computer. The values obtained from normal and pathological retinae were compared statistically using the Tukey-Kramer test (35).

Cell Numbers

In order to measure the density (number of cells/mm²) of labeled cells in the different layers of the retina, flat mounts from three normal and three AD retinae were viewed with the Tracor Northern IAS using a ×40 objective lens. The size of each field was 0.03 mm², with each field taken from a similar retinal location in the different retinæ. The number of labeled cells in each field viewed on the television monitor was manually counted. This process was repeated for fifty selected fields in each retina.

Visual Assessment of MHC Class II Activity

The intensity of individual labeled cells was evaluated at the light microscopy level by two independent observers, without knowledge of specimen pathology, using a grading system: no staining (−), weak (+), moderate (+++), strong (+++) and very strong (++++)+. The values obtained from normal and pathological retinae were compared statistically using the Wilcoxon/Kruskal-Wallis test.

Controls

MHC Class II Expression and Age: In order to test for the possibility that MHC class II expression varies with age, optical density measurements (see Image Analysis) of the intensity of MHC class II reactivity in all normal specimens were plotted as a function of age. Optical density data were divided into three groups based on donor age: 0-29 years, 30-59 years and 60-90 years. The Tukey-Kramer test was used to statistically compare intensity of MHC class II expression between groups.

Immunohistochemistry: Nonspecific binding was excluded by substituting a non-immune monoclonal IgG isotype of the same species (DAKO, Cat. no. X931) for the primary antibody. In addition, the primary and secondary antibodies were excluded in control experiments to exclude the expression of endogenous
peroxidase. An anti-gial fibrillary acidic protein antibody (1:1,000 dilution, GFAP, DAKO, Cat. no. Z334) was used to distinguish astrocytes from microglia.

RESULTS

Controls

Age-Related Changes: Levels of MHC class II antigen expression were similar in all age ranges of normal retinae studied. The Tukey-Kramer test showed no significant variation in optical density measurements of MHC class II intensity between specimens of 0-29, 30-59, 60-90 years of age (p > 0.05).

Immunohistochemistry: In control experiments where non-immune monoclonal IgG isotype of the same species was substituted for the primary antibody, and where preincubation with primary and secondary antibodies was excluded, only endogenous reactivity of red blood cells (RBC) was detected. Retinal cells labeled with anti-GFAP antibody had the morphological and distributional features of astrocytes (13), distinguishing them from the populations labeled using the other markers. In both normal and AD retinae the vascular endothelium was unlabeled following incubation with anti-GFAP.

MHC Class II-Positive Cells in Normal Retinae

Microglia: Microglia expressing MHC class II immunoreactivity were detected in all 25 normal adult human retinae studied. MHC class II expression was evident on ramified microglia and perivascular macrophages, although perivascular cells appeared to express slightly higher levels than ramified forms (Figs. 1, 2).

Labeled microglia had a laminar topography in normal retinae, being distributed in the nerve fiber layer (NFL), inner plexiform layer (IPL) and inner nuclear layer/outer plexiform layer (INL/OPL) border. Different morphological forms were apparent in each layer (Fig. 2A, C, E). Parenchymal microglia in the NFL appeared elongated, apparently constrained by nerve fiber bundles (Fig. 2A). Perivascular macrophages were evident in association with the larger retinal vessels (36); they had a rounded morphology and a reduced number of cell processes which conformed to the vessel profiles and did not extend into the retinal parenchyma (14) (Fig. 1E). In the IPL, parenchymal microglia displayed typical dendritic morphology with multiple smooth processes extending from a small, oval cell body (Figs. 1C, 2C). Perivascular macrophages on the smaller vessels were less ramified but had cell processes extending into the parenchyma (Fig. 2C). At the INL/OPL border, parenchymal microglia were predominantly of dendritic morphology (Fig. 2E).

Retinal Vasculature: Visual examination by light microscopy of trephined retinal samples taken from central, equatorial and peripheral regions of 25 normal specimens indicated that immunoreactivity was confined to perivascular macrophages and endogenous peroxidase reactivity to intravascular RBC (Figs. 1A, C, E, 2A). There was no evidence of MHC class II reactivity on EC, pericytes or astrocytic processes.

MHC Class II-Positive Cells in AD Retinae

Two distinctive features of MHC class II expression were evident in AD retinae compared with normals; firstly, the induction of MHC class II expression on EC and, secondly, the hypertrophy of microglia and perivascular macrophages.

Microglia: The laminar topographical arrangement of microglia expressing MHC class II evident in normal retinae was also apparent in AD retinae (Fig. 2B, D, F). Labeled microglia in the NFL had a similar elongated appearance to those seen in normal retinae (Fig. 2B). Perivascular macrophages appeared hypertrophied against a background of increased levels of immunoreactivity of the retinal vasculature in the NFL (Figs. 1B, 2B). In the IPL, labeled microglia within the retinal parenchyma displayed morphological changes, including hypertrophy of the cell body and increased numbers of vacuolated cell processes, but appeared, using visual assessment (see Methods), to have unchanged levels of MHC class II immunoreactivity compared to ramified microglia in corresponding locations in normal retina (p > 0.05, Wilcoxon/Kruskal-Wallis test) (Figs. 1D, 2D). At the INL/OPL border immunoreactive cells appeared to be predominantly perivascular macrophages which were more numerous than in normal retinae; ramified forms were less evident in this layer than in normal retinae (Figs. 1F, 2F). There was no evidence of CD45- or MHC class II-positive cellular infiltrates of lymphoid morphology present in AD retinal flat mounts (Figs. 1, 2) (13). Similarly, there was no evidence of parenchymal leukocytic infiltrates in semi-thin retinal sections. Of ten specimens examined in semi-thin sections in the paramacular region, six showed apparent reduction in the thickness of the ganglion cell layer, however, the tissue cross-section was too small for statistically significant conclusions to be drawn.

Retinal Vasculature: Visual examination by light microscopy of trephined retinal samples taken from central, equatorial and peripheral regions of 35 AD specimens indicated that there was a generalized induction of MHC class II antigen expression throughout the retinal vasculature. Diffuse immunoreactivity was apparent on retinal blood vessels of all calibers including arterioles, venules and capillaries (Figs. 1B, 2B, D, F), consistent with the distribution of EC.

Image Analysis

The mean intensity of MHC class II antigen expression, as measured by optical densitometry, in AD retinae was moderately but significantly increased relative to normal
Fig. 1. MHC class II expression in flat mounted normal (A, C, E) and AD (B, D, F) retinæ. Low magnification views of normal retinæ reveal endogenous peroxidase reactivity of intravascular RBC (small arrows) but absence of reactivity on blood vessel walls (A), while in AD retinæ a diffuse expression of MHC class II antigens on blood vessels is evident (B). Higher magnification views of MHC class II-positive parenchymal microglia reveal the typical dendritic morphology in normal retinæ (C); arrowheads indicate endogenous peroxidase reactivity in intravascular RBC in the absence of evident staining of the vessel walls. Hyper trophy and vacuolation of parenchymal microglia is evident in AD retinæ (D). On large vessels in normal retinæ, labeled perivascular macrophages (arrows) have a rounded profile with a small number of cell processes which appear to be constrained by the vessel wall (E). In AD retinæ, perivascular macrophages with processes extending into the parenchyma were most evident associated with small blood vessels (F). Note that the ramified form (arrow) is not associated with the vasculature. Visualization was performed using the avidin-biotin labeling technique with nickel-enhanced DAB as a peroxidase substrate. A, B × 80; C, D × 480; E × 320; F × 200.
Fig. 2. Class II-immunoreactive cells are shown in three planes of focus in normal (A, C, E) and AD (B, D, F) flat mounted retinae. In the NFL of both normal (A) and AD (B) retinae, MHC class II-positive microglia have an elongated appearance; increased staining on blood vessel walls in AD retinae is apparent (B). Arrowheads indicate endogenous peroxidase reactivity in RBC. In the IPL, two populations of MHC class II-reactive microglia are evident in normal retinae (C); ramified forms (arrows) with moderate levels of immunoreactivity and perivascular macrophages (arrowheads) which are less ramified and more intensely immunoreactive. Two populations of MHC class II-reactive microglia are also evident in AD retinae (D); ramified forms (arrows) which are hypertrophied and reveal a moderate level of immunoreactivity and perivascular forms (arrowhead) which are difficult to distinguish against the upmodulated immunoreactivity of the vasculature. In outer retina (INL/OPL border), MHC class II-reactive cells in normal retinae (E) were predominantly parenchymal microglia of dendritic morphology (arrows), while labeled cells in AD retinae (F) appeared to be predominantly perivascular macrophages (arrows). Visualization was performed using the avidin–biotin labeling technique with nickel-enhanced DAB as a peroxidase substrate. A–F ×200.
Optical Density (OD) of MHC Class II-positive Cells in AD and Normal Retinae

![Graph showing optical density (OD) of MHC Class II-positive cells in AD and Normal Retinae]

**Fig. 3.** Computerized optical densitometry measurements indicated that the mean intensity of MHC class II antigen expression in AD retinae was significantly increased compared to normal retinae (p < 0.05, Tukey-Kramer test). The percentage of retinal area reactive for MHC class II was 17.8 ± 12.4% of AD retina reactive compared to 6.2 ± 4.3% in normal retina (Fig. 4; p < 0.05, Tukey-Kramer test). These measurements of both area and intensity include all reactive retinal constituents.

**Cell Numbers**

Analyses of normal retinae reveal significant differences in the mean cell density of microglia (including perivascular and ramified forms) in the three distinct laminae (p < 0.05, Tukey-Kramer test). However, the mean total cell density of microglia in AD retinae compared to normal retinae was not significantly different (317 ± 126 cells/mm² [mean ± SD] vs 319 ± 141 cells/mm²; p > 0.05, Tukey-Kramer test). In addition, the mean cell density in each lamina was not significantly different between the two groups (p > 0.05, Tukey-Kramer test).

**DISCUSSION**

The expression of AD-related degeneration in the retina appears to reflect the close anatomical relationship of the brain and retina. However, the histopathological features of AD in the cerebral cortex (neurofibrillary tangles and neuritic plaques) have not been observed in the retina, supporting the observation that the pathogenesis of AD is not uniformly expressed in all regions of the brain (1).

Percentage of Retinal Area Positive For MHC Class II

![Graph showing percentage of retinal area positive for MHC Class II in AD and Normal Retinae]

**Fig. 4.** The percentage area reactive for MHC class II in both normal and AD retinae was assessed; a three-fold increase in area immunoreactivity was detected in AD retinae.

It has been previously suggested that ganglion cell loss results from retrograde degeneration associated with a primary lesion in the visual cortex (2, 4). The present results indicate that modulation of MHC class II antigen expression and hypertrophy of microglia is related to the pathogenesis in the retina, possibly associated with loss of ganglion cells.

**Retinal Microglia**

Consistent with our previous observations, MHC class II antigens were detected on both ramified microglia and perivascular macrophages in normal retinae (14). We have identified distinctive responses of ramified microglia and perivascular macrophages associated with the pathogenesis of AD in the retina. Both ramified and perivascular forms were hypertrophied apparently without significant upmodulation of MHC class II reactivity.

A number of studies have observed that microglia in the neural tissue of animals and humans comprise a heterogeneous population of leukocyte lineage cells (13, 14, 37–40). We have shown previously that ramified microglia express phenotypic characteristics in common with dendritic antigen-presenting cells (14) and that perivascular cells express macrophage antigens (13). Together with the present study, these observations challenge the traditional view that microglia are a homogeneous population of macrophages (41). Other groups have demonstrated that in skin, gut and lung populations of both...
MPS cells coexist, and that MPS cells can suppress the antigen-presenting functions of resident dendritic cells (31, 32). The hypertrophy of the perivascular macrophages observed in AD retinae in the present study may, therefore, indicate increased suppressor activity of this population. Changes apparent in ramified microglia, observed in AD retinae, are open to a number of interpretations, one possibility being that vacuolation and hypertrophy of ramified microglia are related to the synthesis of complement proteins, recently reported to be present in AD brains (7).

It has been suggested that perivascular macrophages can be distinguished from ramified microglia by their relationship to the glia limitans, perivascular macrophages lying between the glia limitans and the endothelium, microglia being distributed within the parenchyma (40). In an earlier study we showed that ramified microglia and perivascular macrophages express distinct phenotypic characteristics (13). The present study further describes two populations of MHC class II-positive perivascular macrophages in both normal and AD retinae, one associated with larger blood vessels with cell processes apparently constrained by the glia limitans (see Fig. 1E) and a second form in which cell processes extend into the parenchyma. Similar morphological forms were evident in normal retinae in a previous study (14).

MHC Antigen Expression in Neural Tissue

Upregulation of MHC class II antigen expression by microglia has previously been observed in AD brains (16, 21–23, 42), and it has been suggested that these cells may participate in immune reactions in AD tissue including antigen presentation and the activation of lymphocytes (6, 41, 43). Increased numbers of T lymphocytes have been reported in both the parenchyma and blood vessels of AD-affected brain (5, 6, 22); however, in the present study no evidence of increased numbers of parenchymal or intravascular cells of lymphoid morphology was detected. Furthermore, upmodulation of MHC class II expression on retinal vascular or microglial elements was not associated with increased numbers of retinal microglia.

In the normal retina vascular elements showed no detectable expression of MHC class II antigens, and in normal brain EC appear to be unreactive with anti-MHC class II antibodies (24, 25). In the present study we have observed increased levels of MHC class II immunoreactivity, apparently due to a uniform induction of MHC class II antigen expression on EC of the vasculature of AD retinae (Fig. 1A, B). Optical density measurements indicate that the level of expression is marginally higher in AD than in normal retinae, while the percentage of retinal area positive for MHC class II is significantly greater. These findings, together with morphological observations, indicate that the modulation of MHC class II antigens, which is detected in AD retinae, is a result of an induced expression of these antigens on EC. The distribution is not consistent with the arrangement of other cellular constituents of the vasculature, including astrocytes and pericytes. Although patchy expression of MHC class II antigens on AD brain EC has been reported (23), the present results suggest that higher levels and a more even distribution of expression of these antigens occurs in AD retinae. In addition, while MHC class I immunoreactivity is present on EC of normal retinal vessels (10), it has also been reported that class I immunoreactivity is upregulated in vascular endothelium in AD brain (23).

Expression of MHC antigens is conventionally associated with antigen presentation and promotion of immune responses, where MHC class II antigens are thought to be responsible for the presentation of exogenously derived peptides (44) and class I antigens for the presentation of endogenously derived peptides (45). However, it has been suggested that aberrant MHC class II expression may inactivate T cells and downregulate immune responses in some circumstances (30). Upregulation of MHC class II antigens on microglia may also be related to other functional properties of these molecules (15, 46), such as the trapping of antigenic peptides and the limitation of release of antigens arising from neuronal degeneration. One study has interpreted activation and upmodulation of MHC class II on microglia as an early indicator of patterns of neuronal damage in rat brain (47). In the absence of leukocytic infiltrates, the present results are consistent with suggestions that both aberrant MHC class II expression and suppressor activity of perivascular macrophages may restrict immune responses against the neural retina.

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