EXTENDED REPORT

Photoreceptor mitochondrial oxidative stress in early experimental autoimmune uveoretinitis

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Aims: In early S-antigen induced experimental uveitis (EAU), photoreceptor mitochondrial proteins are nitrated prior to macrophage infiltration of the retina, suggesting that oxidative stress is an initial event in the development of EAU. We attempted to detect the oxidative stress and localise it in the EAU retina.

Methods: Lewis rats were immunised with S-antigen in complete Freund's adjuvant (CFA). Animals were injected with CFA alone and non-immunised animals served as controls. Immunised and non-immunised animals were killed on day 5 and subsequent days. Isolated retinas were processed for inducible nitric oxide synthase (iNOS), tumour necrosis factor (TNF) α , interferon (IFN) γ , interleukin (IL) α and CD28 expression by real time polymerase chain reaction. In addition, iNOS was colocalised with cytochrome c oxidase on day 5 of EAU. Oxidative stress was detected by 2', 7'-dichlorodihydrofluorescein diacetate and localised by a mitochondrial specific marker. Leucocyte and T cell infiltration in the retina/choroid was evaluated by immunohistochemistry.

Results: The iNOS, TNF α , IFN γ , IL α and CD28 transcripts were significantly upregulated on day 5 in EAU, and iNOS was colocalised with cytochrome c oxidase in the photoreceptor mitochondria. Oxidative stress was seen primarily in the photoreceptor mitochondria. Occasional T cells were present in the retina at this stage.

Conclusions: During early EAU, mitochondrial oxidative stress is selectively noted in the photoreceptor inner segments. The oxidative stress appears to result from iNOS upregulation in the photoreceptor mitochondria and cytokine generation in the retina by a few antigen specific infiltrating T cells.

Experimental autoimmune uveitis (EAU) is an established animal model used to study the mechanism of uveitis development in humans.^{1–3} Blood borne, activated macrophages and neutrophils are the major effectors of the observed tissue damage in EAU.⁴ These phagocytes cause damage to the retina through the production and release of cytokines and proteolytic enzymes and by the production of nitric oxide and superoxide. These oxidants react rapidly to form the highly cytotoxic peroxynitrite.^{5–6} The macrophages in the outer retina have been identified as a major source of inducible nitric oxide synthase (iNOS).⁵

The iNOS isoform was originally described in macrophages; however, it has since been detected in numerous other cell types, including neurones, and in many regions of the brain.^{7–9} The activity of iNOS is regulated mainly at the transcriptional level and is strongly induced by various cytokines and other immunological stimuli.^{10–14} iNOS expression in the retina is likely to cause tissue damage by interfering with the beneficial activities of the constitutive neuronal NOS and endothelial NOS.^{11–15–23}

Our recent studies of EAU²⁴ revealed nitration of photoreceptor mitochondria related proteins on day 5 after S-antigen injection, before the macrophage and neutrophil infiltration of the retina (early EAU) that occurs on days 9–11 post immunisation.^{23–24} These observations suggest that oxidative stress is an early event in the development of uveitis. The purpose of this study was twofold: to detect oxidative stress and to localise the stress during early EAU.

MATERIALS AND METHODS

Induction of experimental uveitis and detection of infiltrating leucocytes in the retina

Male Lewis rats weighing 150–175 g were used in all studies. Animals were treated in accordance with the ARVO Statement

for the Use of Animals in Ophthalmic and Vision Research. EAU was induced in 58 Lewis rats by immunising with a hind foot pad injection of bovine S-antigen (60 μ g) in complete Freund's adjuvant (CFA) containing 4 mg/ml of heat killed *Mycobacterium tuberculosis*.²⁵ Controls consisted of two groups: 40 Lewis rats injected with CFA alone and 28 non-immunised animals.

Groups of six antigen immunised animals each were killed on days 5, 11 and 14 post immunisation and the eyes used for haematoxylin–eosin staining to evaluate the inflammatory infiltrates and morphological changes. They were compared with groups of six CFA injected animals each, killed on days 5 and 14 post immunisation and six non-immunised rats. Sections were examined for clinical signs of uveitis, including conjunctival hyperaemia, cells in the anterior chamber and vitreous opacity.

Another group of six antigen immunised animals each from day 5 and day 11 were used for immunohistochemical detection of CD45 and CD3 positive cells and the results compared with those of groups of six CFA injected animals each from day 5 and day 11 and six non-immunised controls. In each period, experiments were performed in triplicate. Cryostat sections of the retina (10 μ m) obtained from these animals were incubated with rat anti-mouse CD45 (1:50; BD Biosciences, San Jose, California, USA) Secondary antibody was biotin labelled mouse anti-rat IgG (1:50; BD Biosciences). The signal was enhanced with avidin–biotin complex and visualised with 3-amino-9-ethylcarbazole. For CD3 immunostaining, 10 μ m cryostat

Abbreviations: CFA, complete Freund's adjuvant; DCF, dichlorofluorescein; EAU, experimental autoimmune uveitis; GAPDH, glyceraldehyde phosphate dehydrogenase; H2DCFDA, 2,7-dichlorodihydrofluorescein diacetate; IFN, interferon; iNOS, inducible nitric oxide synthase; PBS, phosphate buffered saline; PCR, polymerase chain reaction; TNF, tumour necrosis factor

Figure 1 Histological examination (A–D) of eyes from non-immunised control and complete Freund's adjuvant (CFA) injected animals and S-antigen immunised animals on day 5 after immunisation shows intact retina with no inflammatory cell infiltration (haematoxylin-eosin; $\times 240$). On day 14 after immunisation with S-antigen (E), the eyes reveal infiltration of mononuclear inflammatory cells in the retina. There is disruption of the retinal architecture and disruption of photoreceptors (haematoxylin-eosin; $\times 240$).

sections of the retina were incubated with mouse anti-rat monoclonal antibody (1:50; BD Biosciences) and subsequently with FITC labelled goat anti-mouse IgG (1:100; Molecular Probes, Eugene, Oregon, USA). The sections were then viewed with a Zeiss LSM-510 laser scanning confocal microscope.

Rat spleen sections were used as positive controls, and the primary antibody replaced by phosphate buffered saline (PBS) was used as a negative control.

Detection of iNOS, TNF α , IFN γ , IL1 α and CD28 transcripts during EAU development by real time polymerase chain reaction

The iCycler optical system (Bio-Rad Laboratories, Hercules, California, USA) was used to perform real time polymerase chain reaction (PCR) for sequential study of the gene expression changes of iNOS, tumour necrosis factor (TNF) α , interferon (IFN) γ , interleukin (IL)1 α and CD28 during EAU

Figure 2 (A–F) Immunolocalisation of CD45 in the retina on days 5 and 11 post immunisation with S-antigen, compared with complete Freund's adjuvant (CFA) injected and non-immunised controls. A few CD45-positive cells were detected in the choroid of non-immunised controls (A) and day 5 (B) and 11 CFA injected animals (C). A few positive cells were also noted in the choroid of day 5 S-antigen immunised animals (D). Large numbers of CD45 positive cells were observed in the retina and choroid on day 11 after immunisation with antigen (E). (F) Sections from normal rat spleen. All sections were incubated with rat anti-mouse CD45 as the primary antibody and with biotin labelled mouse anti-rat IgG as the secondary antibody. Sections were treated with avidin-biotin complex and visualised with 3-amino-9-ethylcarbazole (AEC). (G–H) Immunofluorescent detection of CD3 positive cells in the retina and choroid obtained from days 5 and 11 after immunisation in experimental autoimmune uveitis (EAU) animals compared with non-immunised and CFA injected animals from days 5 and 11. The stain reveals positive cells in the inner nuclear layer near the outer plexiform layer and choroid in day 5 EAU (J). CD3 positive cells were not detected in the retina of day 5 CFA injected animals (H) and non-immunised controls (G). However, occasional positive cells were noted in the outer nuclear layer of the retina and choroid of day 11 CFA injected animals (I). CD3 positive cells were detected in the retina and choroid of day 11 of experimental autoimmune uveitis (K). (L) Sections from normal rat spleen. Sections were treated with mouse monoclonal anti-CD3 and the secondary antibody used was FITC labelled goat anti-mouse IgG. The sections were then visualised with confocal microscope. Magnification $\times 400$.

Figure 3 Changes in gene expression of inducible nitric oxide synthase (iNOS), tumour necrosis factor (TNF) α , interferon (IFN) γ and interleukin (IL)1 α during experimental autoimmune uveitis (EAU). iNOS, TNF α , IFN γ and IL1 α mRNA were elevated during the initial phase of EAU (day 5 after immunisation) compared with non-immunised controls (A). There was no significant change in gene expression of iNOS, TNF α , IFN γ , IL-1 α and CD28 in the retina of complete Freund's adjuvant (CFA) injected and non-immunised controls (B). Gene expression of CD28 was upregulated 1.98-fold on day 5 after immunisation compared with controls (C). Total RNA was extracted from the retinas using Trizol and reverse transcribed. Real time polymerase chain reaction was performed using gene specific primers and normalised with glyceraldehyde phosphate dehydrogenase (GAPDH). The fold increase compared with the control mRNA expression was determined using the $2^{-\Delta\Delta Ct}$ method. Results expressed are mean (SEM) of three independent experiments. * $p < 0.05$. CN, control (non-immunised); ** $p < 0.001$.

development. Changes in mRNA expression of these genes in CFA injected animals compared with changes in non-immunised controls were also studied. Retinal samples from groups of four antigen immunised and four CFA injected animals each from days 5, 8, 11 and 14 post immunisation and four non-immunised controls were subjected to RNA extraction using Trizol reagent (Invitrogen, Carlsbad, California, USA) and DNase 1 treatment (Ambion, Austin, Texas, USA). RNA (1 μ g) was reverse transcribed and used as a template for Quantitative PCR reactions.

Specific primers were then used to amplify glyceraldehyde phosphate dehydrogenase (GAPDH) (forward 5'-TGC ACC ACC

ACC AGT GCT TA-3; reverse 5'-GGA TGC AGG GAT GAT GTT C-3), TNF α (forward 5'-AGC AGA TGG GCG TAC CTT-3'; reverse 5'-CTG GAA GAC TCC TCC CAG GT-3') and CD28 (forward 5'-AGA GAC TTG CAG CGT ACC G-3'; reverse 5'-ATT GGT GGC CCA GTA GAG GT-3'). The primers used to detect iNOS, IFN γ and IL1 α transcripts were obtained from SuperArray Bioscience Corporation (Frederick, Maryland, USA). GAPDH was used to normalise cDNA input levels. Reactions were performed in a 25 μ l volume containing 2 μ l cDNA, 0.5 μ M each of forward and reverse primers, and the buffer included in the SYBR Green 1 supermix (Qiagen, Valencia, California, USA). Triplicate reactions were performed for each gene to minimise individual tube variability. The cycling parameters were as follows: denaturation 95°C, 10 s; annealing 57°C, 30 s; and extension 72°C, 30 s. The cycle threshold (Ct) difference was used to calculate the amount (x-fold) of change in gene expression as $x = 2^{-\Delta\Delta Ct}$. Statistical analysis of $\Delta\Delta Ct$ was performed with a Student's *t* test for three independent samples, with significance set as $p < 0.05$, and compared between EAU and non-immunised control groups. CFA injected animals were also compared with non-immunised controls. The experiments were performed in triplicate.

Localisation of iNOS in the retina

Frozen sections (6 μ m) from 12 EAU eyes on day 5 after immunisation and 12 non-immunised rat eyes were used to immunolocalise iNOS and cytochrome c oxidase in the retina. Gene expression of iNOS by PCR clearly showed no significant difference between the CFA injected and non-immunised animals; hence in all further experiments we used non-immunised animals as control animals. Sections were fixed in 4% paraformaldehyde, washed with PBS and blocked with 5% bovine serum albumin. Sections were then incubated at 4°C overnight with the mouse anti-rat monoclonal antibody and anti-cytochrome c oxidase subunit IV (Molecular Probes) 1:200, in combination with rabbit polyclonal anti-iNOS, 1:100 (BD Bioscience). Sections were incubated with fluorescein labelled goat anti-rabbit IgG (1:100; American Qualex, San Clemente, California, USA) and Rhodamine Red goat anti-mouse IgG (1:100; Molecular Probes) at room temperature for 1 h. Isotype controls and primary antibody replaced by PBS were used as control procedures to ascertain the specificity of the primary antibody binding. Experiments were performed in triplicate. All sections were viewed with a Zeiss LSM-510 laser scanning confocal microscope.

Localisation of oxidative stress in the retina

Oxidative stress in the retina was localised within the mitochondria using 2,7 dichlorodihydrofluorescein diacetate (H2DCFDA; Molecular Probes) in combination with a marker for mitochondria, MitoTracker Deep Red 633.²⁶ Six control non-immunised animals and six EAU animals were killed on day 5 after immunisation. One retina from each animal was removed and incubated at 37°C for 30 min in Dulbecco's modified Eagle's medium containing 300 nM MitoTracker Deep Red 633 and 20 μ M H2DCFDA (Molecular Probes). The other retina from each animal was incubated for 40 min at 37°C with 300 nM of a reduced form of MitoTracker Red (MitoTracker Red CM-H2Xros) that fluoresces at 599 nm when oxidised by reactive oxygen species. Each retina was then washed twice with Dulbecco's modified Eagle's medium and 10 μ m cryostat sections were viewed with a confocal microscope (Carl Zeiss, Thornwood, New York, USA). Control and day 5 post immunisation EAU retinas were evaluated qualitatively (side by side and superimposed images) and quantitatively by measuring relative fluorescence profiles.

Figure 4 Immunolocalisation of inducible nitric oxide synthase (iNOS) in the retina in day 5 post immunisation animals. Note punctate positive staining in the inner segments (IS) and along the cell membranes of the outer nuclear layer (ONL). Sections from day 5 post immunisation rat eyes were incubated with polyclonal anti-iNOS as the primary antibody and with fluorescein labelled goat anti-rabbit IgG as the secondary antibody. Sections were then visualised with confocal microscopy. Magnification $\times 400$.

RESULTS

Induction of EAU and detection of infiltrating leucocytes in the retina

On day 11 after immunisation, animals showed clinical evidence of uveitis, characterised by conjunctival injection, presence of cells in the anterior chamber and vitreous haze (data not shown). Histological examination of the enucleated globes confirmed the presence of uveoretinitis on day 11 after immunisation, characterised by infiltration of mononuclear cells in the uvea and retina, and the presence of cells in the anterior chamber and vitreous. Similar changes were also noted in eyes enucleated from day 14 after immunisation animals; however, these animals also had extensive photoreceptor damage (fig 1E). The S-antigen injected animals killed on day 5 showed no clinical signs or histological evidence of uveitis (fig 1D). Similarly, the retina of non-immunised and CFA injected animals on day 5 and day 14 after immunisation showed no signs of uveitis (fig 1A–C).

No CD45 positive inflammatory cells were seen in the day 5 post immunisation retina. The retinal architecture was well preserved and the photoreceptors were intact. A few CD45 positive cells were seen in the inner choroid on day 5 after immunisation (fig 2D) and large numbers of CD45 positive cells were noted on day 11 in the retina and choroid (fig 2E). However, a few CD45 positive cells were also seen in the choroid of non-immunised controls, and day 5 and 11 CFA injected animals (fig 2A–C).

Anti-CD3 immunostaining showed positive cells in the inner nuclear and outer plexiform retinal layers on day 5 after immunisation (fig 2J). Immunopositive cells were also detected in the inner choroid. We could not detect any CD3 positive cells in the retina and choroids of day 5 CFA injected animals (fig 2H) whereas occasional CD3 positive cells were detected in the retina and choroid on day 11 CFA injected animals (fig 2K).

Detection of iNOS, TNF α , IFN γ , IL1 α and CD 28 transcripts during EAU development

Changes in retinal gene expression levels of iNOS, TNF α , IFN γ , IL1 α and CD28 in EAU and CFA injected animals at different time periods compared with non-immunised controls, as determined by real time PCR, are shown in fig 3. No significant change was seen in mRNA expression of any genes studied in the adjuvant injected animals compared with the non-immunised animals (fig 3B). Expression of iNOS and TNF α were upregulated in the initial phase of EAU at day 5 after immunisation compared with controls. Expression was further increased, with strong signals on days 11 and 14 after immunisation at the peak of the disease. Levels of IFN γ and IL1 α mRNA were also elevated on day 5 after immunisation, although to a lesser degree, and a high level of expression was detected from day 11 to day 14 after immunisation (fig 3A). Expression of CD28 also increased significantly in the retina on day 5 after immunisation compared with controls (fig 3C).

Localisation of iNOS in the retina

In the retinas of animals killed on day 5 after immunisation, immunoreactive iNOS was found in the inner segments of the photoreceptors and in the outer plexiform layer. The staining was punctate in nature (fig 4). Dual staining for cytochrome c oxidase subunit IV and iNOS revealed positive staining in the inner segments of the photoreceptors. iNOS colocalised with cytochrome c oxidase subunit IV, which is a marker of the inner mitochondrial membrane (fig 5). In control retinas, immunoreactivity for iNOS was minimal compared with the EAU retina. The isotype and control without primary antibody were negative for iNOS and cytochrome c oxidase staining.

Localisation of oxidative stress in the retina

The EAU retinas exposed to 2',7'-H₂DCFDA revealed fluorescence at 520 nm at the site of the photoreceptor inner segments by conversion of H₂DCFDA to fluorescent dichlorofluorescein (DCF) by reactive nitrogen species. This fluorescence colocalised with the mitochondrial marker MitoTracker Deep Red 633 at the inner segments (fig 6). A marked coincidence of the fluorescence of DCF with the site of MitoTracker Deep Red was noted. The day 5 post immunisation EAU retina showed increased fluorescent intensity of DCF compared with control retinas while the level of MitoTracker Deep Red fluorescence was similar in both retinas. A similar positive reaction was noted at 599 nm at the photoreceptor inner segments of the day 5 post immunisation retinas with the reduced form of MitoTracker Red (fig 7). The reaction was negative in the control retinas.

DISCUSSION

Uniquely, by virtue of the retinal architecture, oxidative stress and increased iNOS could be demonstrated in early EAU, primarily in the inner segments of the retina. Moreover, at this site in these areas, iNOS protein colocalised with the cytochrome c oxidase, and the punctate quality of the iNOS immunofluorescence (figs 4, 5) further supports its photoreceptor mitochondrial location.¹⁷ Once induced, iNOS may translocate from the cytosol to the mitochondria where it exhibits a characteristic, high, Ca²⁺ independent activity and nitric oxide generation.^{27–30}

The localisation of iNOS primarily in the photoreceptor mitochondria, along with the localisation of reactive oxidants by DCF and the reduced form of MitoTracker (figs 6, 7) suggests that the observed mitochondrial oxidative stress in the mitochondria could be primarily due to peroxynitrite formation. As peroxynitrite formation requires nitric oxide and superoxide generation in proximity, and as both of these oxidants are increased markedly in the photoreceptor mitochondria on day 5

Figure 5 Immunolocalisation of inducible nitric oxide synthase (iNOS) (green) in the photoreceptor mitochondria using a dual immunostain for iNOS and cytochrome c oxidase subunit IV (red). The retinal sections obtained from the control and day 5 post immunisation animals were exposed to anti-iNOS and anti-cytochrome c oxidase antibodies and examined under the confocal microscope. The control retina examined for iNOS shows an absence of positive fluorescence (A). The control retina examined for cytochrome c oxidase (B) and the combination of (A) and (B) overlapping image (C) reveals distribution of cytochrome c oxidase primarily in the photoreceptor inner segments (IS), outer plexiform layer (OPL) and nerve fibre layer (NFL). Note that the overlapping image (C) shows negative staining for iNOS. In contrast, the day 5 post immunisation retina reveals positive staining for iNOS in the inner segments, along with the inner nuclear cell membranes, outer plexiform layer and vessel walls of the nerve fibre layer (D). The same section reveals distribution of cytochrome c oxidase (E) as noted in the above controls. The combination image of (D) and (E) shows colocalisation of iNOS at the site of the cytochrome c oxidase in the inner segments (IS), outer plexiform layer (OPL) and to some extent the outer nuclear layer (F). Original magnification $\times 400$.

after immunisation, it appears that oxidative stress in early EAU takes place primarily in the photoreceptor mitochondria and that the stress is probably mediated by peroxynitrite. Such a conclusion is supported by the previous report by Wu *et al.*²⁴ which showed peroxynitrite mediated selective nitration of mitochondrial related proteins in early EAU.

Both DCF and the reduced form of MitoTracker are excellent indicators for reactive oxidants and peroxynitrite accumulation, particularly in cells that produce nitric oxide.^{31–34} The colocalisation of fluorescent DCF with the MitoTracker Deep Red probe, which labels mitochondria, demonstrates that the main source of the reactive oxidants is in the photoreceptor inner segment mitochondria (fig 6). The reduced form of the MitoTracker used in our study showed the same result (fig 7), thus confirming generation of the oxidant species within the photoreceptor mitochondria. The nitric oxide generated by iNOS reacts with superoxide by a third order reaction that is up to 300-fold greater in the membrane under hydrophobic conditions compared with its reactivity in the cytosol or in an aqueous medium. This increased reaction is due to the concentrated oxygen present in the membrane.¹⁰ The increased level of iNOS

on day 5 after immunisation and its colocalisation with the cytochrome c oxidase located in the mitochondrial membrane also indicates peroxynitrite formation in the mitochondria, as well as the vulnerability of cytochrome c, other mitochondrial proteins and mitochondrial membrane lipids to oxidative stress.

The above histochemical findings and our previous report²⁴ show evidence of mitochondrial oxidative stress before macrophage and other inflammatory cell infiltration. In our study, CD45 positive cells were absent from the retina on days 5 and 8 after immunisation. Lack of staining of these cells in the retina might be due to the small number of infiltrating cells during these time periods. However, the infiltrating cells were noted in the choroid of day 5 EAU, CFA injected and non-immunised control animals.

The mechanism that induces such stress is not clear. However, a few CD3 positive cells were found in the retina on day 5 after immunisation (fig 2B) and real time quantitative PCR showed a 1.98-fold increase in CD28 transcripts, suggesting that small numbers of activated T cells migrate to the retina soon after priming in the periphery with S-antigen. Such homing of a few T cells during early EAU was previously reported by Prendergast *et al.*³⁵ However, we could not detect

Figure 6 Colocalisation of reactive oxidants by dichlorofluorescein (DCF) and mitochondrial marker MitoTracker Deep Red 633. The retinas from the control non-immunised animals and day 5 post immunisation animals (experimental autoimmune uveitis (EAU)) were exposed to 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) and MitoTracker Deep Red 633 and viewed using a Zeiss LSM 510 confocal microscope. EAU day 5 after immunisation retina (A) showing fluorescence of the mitochondrial specific probe, MitoTracker Deep Red, primarily localised to the inner segments of the photoreceptors. EAU day 5 after immunisation retina (B) exhibits increased DCF fluorescence localised to the photoreceptor inner segments ($\times 400$). Simultaneous visualisation of the MitoTracker Deep Red and DCF (C) reveals colocalisation of these fluorescent agents primarily in the photoreceptor inner segments. The illustration depicts a phase contrast confocal image and shows photoreceptor nuclei, with intense fluorescence of inner segments (yellow). (D) Control retina showing minimal DCF fluorescence. The graph shows increased intensity of DCF in EAU retina (E), with a similar level of MitoTracker Deep Red fluorescence as in the control retina (F). The graph shows relative fluorescent intensity (Y axis) of DCF (channel 2, green line) and MitoTracker Deep Red (channel 1, red line) against distance across section (X-axis/ μm).

any T cells in the retina of day 5 CFA injected animals and, moreover, there was no significant change in mRNA levels of CD28 compared with non-immunised controls.

Real time PCR of the retina on day 5 after immunisation also revealed significant upregulation of $\text{TNF}\alpha$, along with iNOS, $\text{IFN}\gamma$ and $\text{IL1}\alpha$ (fig 3). Similar expression of various cytokines at

Figure 7 Localisation of the oxidants in the photoreceptor inner segments using the reduced form of MitoTracker (CM-H₂Xros) alone with confocal microscopy. Retinas were obtained from day 5 post immunisation animals and exposed to the reduced form of MitoTracker. After examination under confocal microscopy, the same section was stained with haematoxylin-eosin. The confocal image (A) reveals fluorescence of the MitoTracker at 599 nm, localised to the photoreceptor inner segments. Histology of the same frozen section (B) reveals intact retinal architecture and absence of inflammatory cell infiltration (haematoxylin-eosin; $\times 400$).

different periods in the mouse EAU model was reported by others.³⁶ However, there was no significant change in the gene expression levels of these cytokines in the CFA injected animals, indicating that the changes are purely antigen driven and not due to CFA. These observations suggest that during early EAU, before inflammatory cell infiltration, a few activated T cells could infiltrate the retina and that these cells might generate the cytokines, including TNF α .

TNF α expression is known to upregulate iNOS and the subsequent production of nitric oxide.^{37–40} IFN- γ , which is also increased in the retina on day 5 after immunisation, has been shown to be involved in the upregulation of iNOS expression in many cell types.⁷ It is likely that the increase in TNF α and IFN γ on day 5 after immunisation leads to increased iNOS expression. Elevation of IL1 α can lead to expression of proinflammatory genes and to the subsequent upregulation of iNOS.⁴¹ The combination of IL1 α and IFN γ leads to a time dependent expression of iNOS.⁴² These observations indicate that inflammatory signals begin to appear in the early EAU retina on day 5 after immunisation or earlier, even though there is no histological or clinical evidence for uveitis. Although PCR revealed significant upregulation of the several proinflammatory genes at the mRNA level, further studies are required to confirm increased levels of transcripts of these genes.

In summary, there is clear evidence for increased generation of oxidants in the photoreceptor mitochondria during the early phase of EAU, before infiltration of inflammatory cells. Based on histological, immunohistochemical and PCR results, the oxidative stress changes are from EAU and not from CFA. The data suggest that a low level of antigen specific T cells infiltrate the retina on day 5 in EAU leading to cytokine upregulation and causing mitochondrial oxidative stress. This stress in turn may lead to recruitment of inflammatory cells on subsequent days, resulting in amplification of the process. Agents that could prevent mitochondrial oxidative stress and the subsequent amplification of uveitis may be useful for prevention of retinal degeneration caused by oxidative stress.

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