Early involvement of nitric oxide in mechanisms of pathogenesis of experimental autoimmune uveitis induced by interphotoreceptor retinoid-binding protein (IRBP)

A. Arroul-Lammali a, Z. Djeraba a, M. Belkhelfa a, H. Belguendouz a, D. Hartani b, O.S. Lahlou-Boukoffa c, C. Touil-Boukoffa a,∗

a Équipe cytokines et NO synthases/immunité et pathogénie, laboratoire de biologie cellulaire et moléculaire (LBCM), faculté des sciences biologiques, USTHB, BP 32, El Aliia, 16111 Alger, Algeria
b Service d’ophtalmologie, CHU Mustapha-Bacha, place du 1er mai, 16600 Sidi M’Hamed, Alger, Algeria
c Service d’ophtalmologie, CHU Ibn-Rochd, route de Bouhdid-Sidi Harb, 23000 Annaba, Algeria

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Summary
Introduction. — Autoimmune uveitis is a group of HLA-associated inflammatory diseases of the eye, prevalent worldwide, that may cause blindness. It can be limited to the eye, or associated with a systemic syndrome. Furthermore, patients suffering from uveitis exhibit high serum and local nitric oxide (NO) levels as a consequence of cellular responses to immunologically privileged antigens within the eye such as interphotoreceptor retinoid binding protein (IRBP). To investigate NO production kinetics in autoimmune uveitis and its implication in mechanisms of ocular pathogenesis, we first attempted to develop an experimental model of autoimmune uveitis (EAU) on the Wistar rat, using the whole bovine retinal interphotoreceptor matrix extract (IPMe) and isolated IRBP.

Material and methods. — Female Wistar rats (n = 24) were divided into three experimental groups: ‘‘control rats’’ (n = 3) consisting of non-immunized animals, ‘‘IRBP-immunized rats’’ (n = 12) and ‘‘IPMe-immunized rats’’ (n = 9), which received a subcutaneous injection, respectively, of 13 μg IRBP and 100 μg IPMe emulsified in complete Freund’s adjuvant. On days 7, 14

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and 21 post immunization, the rats were sacrificed. Nitrites were assessed in plasma and in homogenate of eyes using the Griess reaction. Meanwhile, eyes were collected for histological studies.

Results. – Our results show the sensitivity of the Wistar strain to both IPMe and IRBP-induced EAU. In fact, we observed histological disorders affecting the retinal tissue in both models of EAU. On the other hand, a significantly increased production of NO in plasma and homogenate of eyes was also observed in comparison to the control group. Moreover, we noted with interest that maximal production of NO occurs prior to the alteration of retinal tissue.

Conclusion. – In summary, our results suggest the early involvement of NO in the mechanisms of pathogenesis of EAU. NO can be considered as a key bio-marker of poor prognosis in ocular autoimmune inflammation.

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Introduction

Autoimmune uveitis is one of the most common causes of human visual disability and blindness with a prevalence of approximately 2% in developed countries [1]. It can be limited to the eye or associated with a systemic syndrome such as Behçet disease, sarcoidosis, Vogt-Koyanagi-Harada syndrome and Ankylosing spondylartharthritis [2]. The physiopathological process is characterised by a great infiltration of CD4+ Th1 cells recognizing retinal auto-antigens into the eyes. These cells recruit other leukocytes, where they cause an inflammatory reaction that destroys photoreceptors and neuronal cells and leads to decreased vision or even to blindness [1]. In fact, the eye is known to be an immuno-privileged site sequestrating resident antigens. This leads to inefficient peripheral tolerance to these antigens and to the persistence of autoreactive T lymphocytes in the circulation. The aetiological triggers leading to the priming of these autoreactive T cells are still unknown. However, it has been postulated to include antigenic mimicry by microorganisms or the breach of the blood ocular barrier and release of normally sequestered antigens [3].

Experimental autoimmune uveitis (EAU) in mice and rats constitutes an invaluable model for the study of basic mechanisms in autoimmune eye disease as well as a useful tool for testing new therapeutic strategies [4]. EAU is induced in susceptible animals by immunization with rather retina auto-antigens in complete Freund’s adjuvant or adoptive transfer of retina-antigen specific T lymphocyte [5–7]. Among the ocular antigens known to induce EAU in
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rodt models are interphotoreceptor retinoid-binding protein (IRBP) [8] and soluble retinal antigen (S-antigen) [9].

IRBP is a soluble glycoprotein of a high molecular mass (≈140 kDa in bovine) [10,11] found in the retinal interphotoreceptor matrix (IPM) located in the subretinal space, between the neural retina and the retinal pigment epithelium (RPE). It is also found in pinealocytes [12,13]. IRBP plays an important role in the visual cycle by binding free fatty acids and retinoids between the photoreceptor outer segments and the retinal pigment epithelium during the bleaching and regeneration of visual pigments [14,15]. Furthermore, it is also important in retinal development and its maintenance [16,17].

Nitric oxide (NO) is a free radical molecule formed from tissue L-arginine. This conversion is catalyzed by nitric oxide synthases (NOS). Inducible nitric oxide synthase (iNOS) is the main form of NOS in bone marrow-derived macrophages [18,19]. NO is characterized by a short lifetime. In fact, it is rapidly metabolized into nitrites (NO2−) and nitrates (NO3−), the mediators of its toxicity. However, NO can also bind to superoxide (O2−) to yield the potent oxidizing/nitrating agent, peroxynitrite [20,21], which has been widely implicated in photoreceptor damage observed in EAU [22–24].

Preliminary works elaborated by our team have revealed high serum levels of nitric oxide derivatives in patients suffering from uveitis of putative autoimmune origin “idiopathic and Behçet uveitis” [25,26]. Meanwhile, it has also been shown that NO derivatives have deleterious effects on cultured bovine ocular layers [27,28].

In the present work, we attempted to develop an experimental model of autoimmune uveitis on the Wistar rat using the whole bovine retinal interphotoreceptor matrix extract (IPMe) and isolated IRBP, in order to investigate nitric oxide production kinetics during this disease and its probable implication in mechanisms of ocular pathogenesis.

Material and methods

Animals

Female Wistar rats (n = 24) aged 6 to 8 weeks were used in this study. They were housed and maintained in the animal facility of the University of Algiers (USTHB). Treatment of the animals conformed to the ARVO statement for the use of animals in ophthalmic and vision research.

Preparation of retinal interphotoreceptor matrix extract and IRBP

Retinal interphotoreceptor matrix extract and isolated IRBP were prepared according to the protocol described by Adler and Evans [29]. All the procedures were carried out at middark and +4 C. Phenylmethanesulfonyl fluoride (PMSF) was also added during preparation of retinal extract and isolation of IRBP at a final concentration of 0.2 mM.

Fresh bovine eyes (n = 60) were used in the experiments. They were trimmed to remove connective tissue and then were cut just posterior to the ora serrata. The anterior portion was discarded. The vitreous was removed carefully, without causing retinal or choroidal detachment. Neural retinas were excised by cutting at the optic nerve. Then, they were pooled in PBS, pH 7.4 (1 mL per eye), stirred at a slow speed and put at +4 C for 1 hour. Meanwhile, material from the internal (apical) surface of the RPE, still in the eyecup, was extracted by rinsing (gently pipetting) with PBS (1 mL per eye for 30 seconds). The IPM preparations from retina and RPE were clarified by two centrifugations (15 minutes at 1000 rpm then 1 hour at 14000 rpm). Finally, they were combined and designated as IPMe.

IRBP is the major glycoprotein of the IPM [30], its isolation requires the method of affinity adsorption onto concanavalin A-Sepharose. Briefly, 1 mL of IPMe was applied to a con A-Sepharose column K9/15 (Pharmacia). The column was then washed with phosphate buffer 0.02 M pH 7.5. The “con A fraction” (IRBP) was finally liberated by washing the column with phosphate buffer 0.02 M-Sucrose 0.1 M pH 7.5.

The presence of IRBP in both IPMe and con A fraction was confirmed using polyacrylamide gel electrophoresis (SDS PAGE).

Induction of experimental autoimmune uveitis on the Wistar rat

Female Wistar rats (n = 24) were divided into three experimental groups:

• “IPMe-immunized rats” (n = 9). They were immunized subcutaneously with 100 µg of IPMe in 150 µL of emulsion with complete Freund’s adjuvant; CFA (Difco Laboratories) (v/v), that had been supplemented with Mycobacterium tuberculosis H37Ra to a final concentration of 1 mg/mL, without pertussis toxin (PTX);
• “control rats” (n = 3). They consisted of non-immunized animals;
• “IRBP-immunized rats” (n = 12). They were immunized subcutaneously with 13 µg of isolated IRBP emulsified with an equal volume of complete Freund’s adjuvant CFA containing 1 mg/mL of M. tuberculosis H37Ra without pertussis toxin, in a total volume of 200 µL.

All the rats were monitored daily. On day 7, 14 and 21 after each immunization, three rats chosen randomly from “IPMe-immunized” group and four rats from “IRBP-immunized” group were sacrificed. In parallel, all the rats from “control group” were also sacrificed.

Plasma collection

Blood was collected from each experimental group by cardiac puncture in ethylenediaminetetraacetic acid (EDTA) containing tubes. After centrifugation at 9000 rpm for 10 minutes, plasma was collected and stored at −45 °C until nitrates determination by Griess reaction.

Eyes collection for histological studies

The eyes of rats from each experimental group were enucleated and fixed in aqueous Bouin. Sections of 5 µm in thickness, realized in the posterior segment of the eyes, were stained with haematoxylin eosin and examined under a light microscope.

Eyes homogenization

For “IRBP-immunized” and “control” groups, one eye was fixed in aqueous Bouin for histological studies while the
other eye was homogenized in phosphate buffer 50 mM pH 7.2. After centrifugation at 9000 rpm for 10 minutes, the supernatant was collected and stored at −45 °C until Nitrites determination. Estimation of total proteins per eye was performed using Bradford method [31]. Nitrite contents of each eye were then expressed as µmol/mg of total protein.

Nitric oxide assay

NO production was assessed as nitrite accumulation as described by Touil-Boukoffa et al. [32]. In brief, 100 µL of each sample (plasma and supernatants of homogenized eyes) was mixed with 100 µL of Griess reagents [0.5% N-1-naphthyl ethylene diamine in 20% HCL, 5% sulphanilamide in 20% HCL (v/v)] and 800 µL of distilled water. After 20 minutes incubation at room temperature, the formation of the chromogenic derivative was detected by spectrophotometry at 543 nm. NaNO₂ standards were run along with the experimental samples for the calibration of the test.

Statistical analysis

All data were expressed as means ± standard deviation (SD). Statistical analyses were performed by one-way Anova with the Fisher’s LSD test modified by the Bonferroni and Sidak correction as post-hoc, or unpaired Student’s t test. Probability values of \( P \leq 0.05 \) were considered to be significant.

Results

Induction of experimental autoimmune uveitis on the Wistar rat by retinal interphotoreceptor matrix extract and IRBP

Experimental autoimmune uveitis was induced on Wistar female rats by the whole retinal IPMe and isolated IRBP. As shown in Fig. 1, rats of the Wistar strain were sensitive to both IPMe and IRBP-induced EAU. In fact, we have noticed on both EAU models the development of an intraocular inflammation affecting the posterior segment of the eyes and characterized by a great retina structure disruption (Fig. 1).

On IPMe-immunized group, a normal retina structure with a stratified appearance was noticed on day 7 post immunization (Fig. 1B), it was similar to that observed on control rats (Fig. 1A). On day 14 after immunization, retina histological disorders were noted. In fact, we have noticed retina folding and the presence of granuloma; indicating a great infiltration of immune cells into retina (Fig. 1C). On day 21 post immunization, retina architecture disruption is still noted and characterized by severe photoreceptor degeneration. However, neither granulomatous structures nor infiltrating immune cells were observed (Fig. 1D).

On IRBP-immunized group, no retina histological disorders were observed on day 7 and 14 after immunization (Fig. 1E et F). On day 21 after immunization, a great architecture disruption was noted characterized by photoreceptor degeneration and damage affecting all retina layers (Fig. 1G).

Systemic nitric oxide production during interphotoreceptor matrix extract and IRBP-induced experimental autoimmune uveitis

Nitric oxide was assessed in vivo (in plasma) of immunized and non-immunized Wistar rats by measuring its end product: nitrites. The results showed a significant high production of nitrites during the three stages of both IPMe and IRBP-induced EAU, when compared to control animals (Fig. 2).

During IPMe-induced EAU, the production of nitrites in vivo was significantly upregulated at day 7 post immunization (16.425 ± 2.38 µM, \( *P < 0.0001 \)) compared to control group (2.12 ± 0.54 µM). The production was then decreased on day 14 post immunization (12.06 ± 1.456 µM) and more on day 21 post immunization (7.3 ± 0.55 µM). It remained however significantly elevated when compared to control group (respectively \( * *P < 0.0001 \), \( *P < 0.001 \)).

During IRBP-induced EAU, the production of nitrites in vivo was also significantly upregulated at day 7 post immunization (12.5 ± 1 µM, \( *P < 0.001 \)) when compared to control group. However, on day 14 post immunization, unlike to what have been observed during IPMe-induced EAU the production of nitrites was further increased (23.5 ± 1.32 µM). In fact, it was significantly higher in comparison with control and IPMe-immunized groups (\( **P < 0.0001 \)). On day 21 post immunization we noticed a slight decrease of nitrites production (18.166 ± 1.527 µM), which remained significantly elevated when compared to control and IPMe-immunized groups (\( * *P < 0.0001 \)).

We also noticed that maximal nitrite production in vivo occurred on day 7 post immunization for IPMe-induced EAU and on day 14 post immunization for IRBP-induced EAU.

Furthermore, when we compare nitrite production kinetics in vivo in relation with histological disorders evolution for the two models of EAU, we observe that the highest levels of nitrites are found in the initial phase of EAU prior to the peak of the disease, characterized by severe histological disorders (Fig. 3).

Local nitric oxide production during IRBP-induced experimental autoimmune uveitis

Local nitrite production in eyes homogenates of IRBP-immunized and non-immunized Wistar rats revealed low levels on day 7 post immunization (0.032 ± 0.018 µmol/mg protein), not significantly different from those obtained on control group (0.01 ± 0.0034 µmol/mg protein). On days 14 and 21 post immunization, nitrite production significantly increased (respectively 0.0904 ± 0.015 µmol/mg protein, \( *P < 0.001 \) and 0.1136 ± 0.0136 µmol/mg protein, \( *P < 0.001 \)), in comparison to the control group (Fig. 4).

Moreover, on day 14 post immunization, we detected significant elevated amounts of nitrites, whereas no retina histological disorders were observed (Fig. 5).
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Figure 1. Histological disorders observed on retinas of interphotoreceptor matrix extract (IPMe) and interphotoreceptor retinoid binding protein (IRBP)-immunized Wistar rats. (A) Light microscopy image of histological section from a control retina showing the typical stratiform morphology and the ordered retina layers. (B), (C) & (D) Histological retinal sections of IPMe-immunized Wistar rats, respectively on day 7, 14 and 21 after immunization. Normal retina morphology is seen on day 7 post immunization, while on day 14, a complete retina structure disruption is noted with retina folding and the presence of granuloma (yellow arrow). On day 21 after immunization, retina architecture disruption is still noted with severe photoreceptor degeneration (red arrow). (E), (F) & (G) Histological retinal sections of IRBP-immunized Wistar rats, respectively on day 7, 14 and 21 after immunization. Normal retina morphology is seen on both days 7 and 14 post immunization. On day 21, we notice a great architecture disruption with photoreceptor degeneration and damage affecting all retina layers. All images were taken of 5 μm sections from the posterior segment of Wistar rat eyes stained with haematoxylin eosin, magnification ×400. V: vitreous; GCL: ganglion cell layer; IPL: inner plexiform layer; OPL: outer plexiform layer; INL: inner nuclear layer; ONL: outer nuclear layer; PIS: photoreceptor inner segments; POS: photoreceptor outer segments; G: granuloma. (Retinas: n = 3, control; n = 3, IPMe-immunized group at each time point; n = 4, IRBP-immunized group at each time point).

Discussion

Experimental autoimmune uveitis serves as a useful tool for studying basic disease mechanisms and for testing new therapeutic strategies. To this end, many works have described the protocols for inducing EAU on different animal models and especially on rodents: rats and mice [4,33,34]. The Lewis rat is indeed the most commonly used model so far because of its great sensitivity for EAU induction [33,34], whereas very few works have used the Wistar strain. Our study shows for the first time that the Wistar rat is sensitive to EAU induction by both isolated IRBP and the whole retinal IPMe. In fact, few days following ocular auto-antigen injection in complete Freund’s adjuvant, we have noticed in the posterior segment of rat eyes, a great disruption of retina structure characterized mainly by retina folding, the presence of granuloma and photoreceptor degeneration, which is due to locally exacerbate inflammatory response. Our observations are consistent with those made on the Lewis rat and the B10 RII mouse [33,35–37]. Furthermore, our team has recently developed an EAU model on the Wistar rat using S-antigen, which forms with IRBP the most uveitogenic ocular auto-antigens [38]. During S-Ag-induced EAU, retina histological disorders with severe photoreceptor degeneration similar to that observed during IRBP-induced EAU were noticed also after 21 days post immunization [38]. This observation indicates that both IRBP and S-antigen are uveitogenic when injected to the Wistar rat and validates this strain as an effective model of EAU.

According to our results, immunization of the Wistar rat by the whole interphotoreceptor matrix extract has led to retina histological disorders development visible from the 14th day post immunization whereas, it took much longer time for IRBP-immunized animals to develop EAU histological signs. This difference in uveitis onset kinetics may be attributed to auto-antigen quantity injected. In fact, we have injected 100 μg IPMe per rat and only 13 μg IRBP per rat. Early data using the Lewis rat have shown that the smallest is the IRBP quantity injected, the slowest is the intraocular inflammatory response kinetics [6,39].

Moreover, on day 21 post immunization, we noticed on IPMe-immunized rat eyes a great alteration of retina structure with severe photoreceptor degeneration but without any presence of granuloma or infiltrative immune cells. This
observation may indicate that there was a spontaneous resolution of the intraocular inflammatory response. In fact, rat models of EAU are characterized by an acute and self-limited disease, which does not recur [3,33,34,40]. This could be due to the elimination of the target antigen, or to a change in the phenotype of the cells present in the resolution phase, both T cells and macrophages, to a more anti-inflammatory phenotype [40].

Furthermore, systemic NO production pattern (in plasma) of both EAU models studied, reflected by its end derivative product; nitrites, indicated significant elevated amounts when compared to control rats. Our observations are consistent with those made on the Wistar rat immunized by S-antigen [38]. In fact, nitric oxide is a bio-molecule that expresses important immune-inflammatory properties. Produced by the iNOS enzyme in response to multiple pro-inflammatory stimuli such as cytokines: IFN-γ, TNF-α and IL-6, NO is involved during the effector phase as well as in the regulation of cellular immune responses [31,41]. High systemic production of nitric oxide derivatives were detected during human uveitis of putative autoimmune origin “idiopathic and Behçet uveitis” [25,26].

![Figure 2](image2.png)

**Figure 2.** Nitrite production in vivo (in plasma) during interphotoreceptor retinoid binding protein (IRBP) and interphotoreceptor matrix extract (IPMe)-induced experimental autoimmune uveitis. During IRBP-induced experimental model of autoimmune uveitis (EAU) (magenta histogram) and IPMe-induced EAU (yellow histogram), the in vivo nitrite production pattern shows significantly elevated amounts on the three stages of EAU (Day 7, 14 and 21 post immunization) when compared to control rats (non-immunized). Note that maximal nitrite production in vivo is observed on day 14 after immunization for IRBP-induced EAU and on day 7 post immunization for IPMe-immunized EAU.

All results are expressed as mean ± standard deviation. (Control, n = 3; IRBP-immunized group at each time point, n = 4; IPMe-immunized group at each time point, n = 3). *P* values from LSD post-hoc Anova test are indicated (NS: not significant, **P** ≤ 0.001, ***P*** ≤ 0.0001).

![Figure 3](image3.png)

**Figure 3.** Comparing nitrite production kinetics in vivo with histological disorders evolution during interphotoreceptor matrix extract (IPMe) and interphotoreceptor retinoid binding protein (IRBP)-induced experimental model of autoimmune uveitis (EAU). (A) During IRBP-induced EAU and (B) IPMe-induced EAU, maximal nitrite production in vivo (respectively on days 14 and 7 post immunization) is detected before the appearance of severe histological disorders (respectively on days 21 and 14 post immunization). For histological grades: grade 0 refers to normal retina morphology; grade 1 refers to a severe retina structure disruption.
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Moreover, comparing nitrite production pattern in plasma with histological disorders evolution for the two models of EAU led us to notice that highest levels of nitrites are found in the initial phase of EAU before reaching the peak of the disease, characterized by severe histological disorders. This observation indicates the probable implication of nitric oxide derivatives during the early phase of EAU onset. Indeed, immunization with uveitogenic peptide triggers in early events an immune response in the periphery characterized by the activation and clonal expansion of pathogenic autoreactive CD4+ T cells which differentiate mainly towards Th1 phenotype. This leads to an increased production of IFN-γ, which stimulates macrophages and other effector cells to produce elevated amounts of reactive oxygen and nitrogen species [33,39,42,43]. These activated T cells can afterward cross the blood–retina barrier and here, activation by local antigen presenting cells leads to the influx of a mixture of leukocytes, whose presence causes retinal disruption [40].

Local nitrite production pattern (in homogenate of eyes) of IRBP-immunized Wistar rats showed significant elevated amounts on day 14 and 21 post immunization when compared to control group. Our results are consistent with those obtained on the Lewis rat model. In fact, many works demonstrated high production of nitric oxide as well as an intense iNOS mRNA and protein expression by retina infiltrating macrophages during EAU [44–47]. The high production of nitric oxide derivative detected on day 21 post immunization correlates with the appearance of retina histological disorders, suggesting its probable implication in
retina disruption. In fact, many works elaborated by our team showed the multiple deleterious effects of nitric oxide derivatives (nitrites and nitrates) on cultured bovine ocular layers [27,28]. Moreover, it has been demonstrated that NO reacts with superoxide (O2−), also produced in large amounts during EAU by infiltrating neutrophils, and yields the potent oxidizing/nitrating agent, peroxynitrite (ONOO−). This latter has been widely implicated in the initiation of photoreceptor cell apoptosis and disintegration via peroxidation of cellular lipid components. Peroxynitrite seems also to mediate the amplification of the inflammatory process in uveitis [23,24,40].

Furthermore, the significant high production of nitrates detected on day 14 post immunization, before the appearance of retina histological disorders suggests a probable implication of nitric oxide, produced locally, during the early phase of EAU onset. Our results are consistent with those found by Rajendram et al. [48]. They reported an increased generation of oxidants in the photoreceptor mitochondria during the early phase of EAU, before infiltration of inflammatory cells. This oxidative stress in turn may lead to recruitment of inflammatory cells on subsequent days, resulting in amplification of the process [48].

In conclusion, autoimmune uveitis is a complex disorder. Our understanding of this pathology has considerably increased with the established in vivo models, among them: IRBP-immunized Wistar rat which seems to be a potent model of experimental autoimmune uveitis. Our study reveals a clear evidence for increased nitric oxide generation in plasma and eyes (target organ) during IRBP-induced experimental autoimmune uveitis. This high production mainly occurred prior to the appearance of histological ocular disorders, suggesting a probable implication of nitric oxide in the early phase of EAU onset and leading to consider this bio-molecule as an early key marker of poor prognosis in the pathophysiology of ocular autoimmune inflammation. Our study highlights the importance of targeting nitric oxide in elaborating new diagnostic methods and therapeutic strategies that would contribute for a better monitoring and future treatment of patients with autoimmune uveitis.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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