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Rat retinal pigment epithelial cells express an inducible form of nitric oxide synthase and produce nitric oxide in response to inflammatory cytokines and activated T cells

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SUMMARY

In this report we show that rat retinal pigment epithelial (RPE) cells express an inducible form of nitric oxide synthase (iNOS) and secrete high levels of nitric oxide (NO·) when co-cultured with activated lymphocytes. We have previously shown that cultured rat RPE cells suppress syngeneic lymphocyte proliferation, an effect attributed to prostaglandin E_2 (PGE_2) secretion by the RPE cells. However supernatants from such co-cultures were also found to contain high levels of nitrite (NO_2^-), the stable end-product of NO· synthesis. RPE cell secretion of NO· was stimulated by the cytokines interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α), an effect enhanced by endotoxin [lipopolysaccharide (LPS)], reduced by the competitive inhibitor of L-arginine metabolism, N^G-monomethyl-L-arginine (L-NMMA) and inhibited by cycloheximide. These effects were dose dependent. Using reverse transcription (RT)/PCR a product of 1398 bp was amplified which showed sequence identity with iNOS cloned from rat vascular smooth muscle. Northern blot analysis of total RNA extracted from rat RPE before and after cytokine stimulation showed induction of a 4·5 kb (kilobase) transcript which hybridized with a 1398 bp (base pair) polymerase chain reaction (PCR)-generated cDNA probe derived from the sequence of rat RPE cell iNOS. These results indicate RPE cells express an inducible form of nitric oxide synthase (NOS) and that high levels of NO· may be produced locally in the eye by the RPE in the presence of activated lymphocytes. Given the cytostatic and cytotoxic properties of this molecule, NO· may play an important role as an inducible mediator of immunosuppressive mechanisms within the microenvironment of the eye at the site of lymphocyte activation.

INTRODUCTION

The retinal pigment epithelium (RPE) which phagocytoses and recycles components of the rod outer segments (ROS) during physiological photoreceptor recycling, occupies a critical position at the blood–retina interface and has been implicated in the pathogenesis of autoimmune uveoretinitis.[1] Our previous work has focused on the possible role of RPE cells as inducers of retinal autoimmunity as T cells must be activated before they can cross Bruch's membrane and enter the retina.[2] However, despite the ability of RPE cells to express major histocompatibility complex (MHC) class II molecules *in vitro*, and to possess many of the adhesion and accessory molecules characteristic of antigen-presenting cells (APC),[3] we and others[4,5] have found that although able to process antigen, RPE were only poor presenters of antigen to sensitized T lymphocytes under normal culture conditions. Indeed lymph node cell (LNC) proliferation to antigen, mitogen or interleukin-2 (IL-2) was profoundly suppressed by RPE cells, even in the presence of exogenous APC, an effect mainly attributed to high prostaglandin E_2 (PGE_2) secretion by the RPE cells.[5]

Similar immunosuppressive effects on lymphocyte proliferation by macrophages added to cultures of T cells and APC have been described which have been attributed to reactive nitrogen intermediates, in particular, nitric oxide (NO·).[6–8] Nitric oxide is a highly soluble free radical whose diverse roles include the regulation of vascular resistance, signal transduction and cytostatic and cytotoxic effector roles in tumour cell killing, antimicrobial activity and in the killing of a variety of intracellular parasites.[9–11] Recently, in addition to these actions as an immune defence molecule, a role for NO· as an immunoregulator in inflammation has been proposed. In particular, the cytostatic effects of L-arginine metabolism by macrophages have been shown to regulate the immune system

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Abbreviations: iNOS, inducible nitric oxide synthase; L-NAME, N^G-nitro-L-arginine methyl ester; L-NMMA, N^G-monomethyl-L-arginine; NO·, nitric oxide; NO_2^-, nitrite; PGE_2, prostaglandin E_2; RPE, retinal pigment epithelial.

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by reducing or suppressing allogeneic activation[11] and mitogen-induced proliferation of lymphocytes.

This wide range of activity has been attributed to the existence of different isoforms of the nitric oxide synthase (NOS) family of enzymes,[12] at least two of which are known to be products of separate genes.[13,14] Bovine RPE are known to secrete NO_2^- in response to stimulation with exogenous cytokines,[15] but the form of the enzyme involved has not been characterized. In this study we analyse rat RPE mRNA for NOS species and identify an inducible NOS product identical to rat inducible (i)NOS. In addition rat RPE cells co-cultured with syngeneic T cells were found to secrete very high levels of NO· in the absence of exogenous stimulation indicating the potential for cytostatic or cytotoxic NO· production in the eye in response to local inflammation.

MATERIALS AND METHODS

Rats

Adult PVG R1 rats from an inbred line maintained in the Aberdeen Medical School animal house were used as sources of lymphoid tissue and RPE cell lines. Groups of male or female rats weighing 200–250 g were used for immunization; 8–16-day-old rats were used as a source of RPE cells.

Antigens and other reagents

Retinal extract was prepared from bovine eyes as previously described.[16] Indomethacin (Sigma, Poole, UK) was dissolved in ethanol and diluted in phosphate-buffered saline (PBS) to a stock solution of 1 mg/ml, aliquoted and used at a final dilution of 1 μg/ml. Concanavalin A (Con A), lipopolysaccharide (LPS) (*Escherichia coli*) cycloheximide and N^G-monomethyl-L-arginine (L-NMMA) were purchased from Sigma. Human cytokines (cross-reactive with rat) IL-1β and tumour necrosis factor-α (TNF-α) were obtained from Genzyme (Kings Hill, West Malling, UK) and rat recombinant interferon-γ (IFN-γ) was from Holland Biotechnology (Leiden, the Netherlands).

Culture medium

RPE cells were grown in Eagle's minimum essential medium (Gibco, Paisley, UK) supplemented with 20% fetal calf serum (FCS) (Advanced Protein Products, West Midlands, UK), 1 mM sodium pyruvate and non-essential amino acids (Flow Laboratories, Irvine, UK). Lymphocytes were isolated and cultured in RPMI-1640 medium (Gibco) with 2 mM glutamine, 1 mM sodium pyruvate, non-essential amino acids, 100 μg/ml each penicillin G and streptomycin sulphate, 5×10^{-5} M β-mercaptoethanol (all Gibco) and 5% heat-activated FCS or 2% normal serum as required.

RPE cell culture

Rat RPE cells were isolated from the eyes of 8-day-old rats, cultured as described previously,[5] and lines established. Flat-bottomed 96-well plates were seeded with 10^4 cells/well in 0·2 ml growth medium (GMEM) and grown to confluence for use in the proliferation assays. For the tests the monolayers were washed twice with Hanks' balanced salt solution (HBSS) and the medium was replaced by RPMI-1640 5% FCS. For supernatant sampling cells were cultured in flasks or dishes at 10^5 cells/ml.

Responder lymphocytes

Rats were immunized in one hind footpad with soluble retinal extract emulsified with an equal volume of complete Freund's adjuvant (CFA) containing 1 mg/ml *Mycobacterium tuberculosis* (H37a, Difco, East Molesey, UK). As additional adjuvant 1 μg pertussis toxin (Wellcome Strain 28, Porton Products Ltd, Maidenhead, UK) was injected i.p. Animals were killed on days 9 or 10 and single-cell suspensions of cells from popliteal lymph nodes (LNC) or spleens (SPL) prepared. Adherent cells were removed by incubation (5×10^5 cells/ml) on tissue culture plastic for 2 hr at 37°, 5% CO_2 in air. Non-adherent cells were washed off with warm RPMI and further purified on nylon wool columns to remove all residual macrophages. T cells prepared in this manner were 85–90% $CD5^+$ by flow cytometric or cytospin analysis, and retained dendritic cells and residual B cells as accessory cells. Proliferation assays were set up with 2×10^5 T cells/well in 96-well plates with optimum stimulating concentrations of antigen or mitogen in a final volume of 0·2 ml, with or without confluent monolayers of syngeneic RPE cells. In all experiments using RPE co-culture, parallel cultures were set up (using the same pool of responder cells) to measure responses of T cells alone. In preliminary experiments the optimum challenge of antigen was found to be 10 μg/ml for S-antigen (SAg) and 2·5 μg/ml Con A. Wells were pulsed 16 hr prior to harvesting with 0·5 μCi/well [^{3}H]thymidine (Amersham International, Amersham, UK) and incorporated radioactivity measured using the Packard Matrix 96 system. Results are expressed as mean 6 min counts $\times 10^{-3} \pm 1$ SD of triplicate or quadruplicate cultures, and are representative of at least two experiments.

Quantification of NO·

Cell-free supernatants were prepared from RPE cells cultured at 10^5 cells/ml and T cells cultured at 1×10^6/ml. Culture medium contained 240 mg/l L-arginine available as free base and was taken directly from cytokine-stimulated cultures or from parallel cultures set up with the proliferation assays. Nitric oxide production was quantified by accumulation of nitrite in the supernatants measured spectrophotometrically using the Greiss reaction with sodium nitrite as the standard.[17] Results are expressed as means of duplicate cultures and are representative of at least three experiments (Tables 1 and 2). Figure 1 data are expressed as means ± 1 SD of triplicate samples and significance of results tested using the Student's *t*-test.

Measurement of PGE_2

Cell-free supernatants were prepared and PGE_2 levels measured by competition enzyme-linked immunosorbent assay (ELISA) (Cascade Biochem Ltd, Reading, UK).

RNA isolation, Northern blotting and hybridization

Total RNA was extracted from cultures of RPE cells, T-cell-enriched SPL and from RPE cells by the method of Chomczynski & Sacchi,[18] quantified spectrophotometrically and stored in a solution of 75% ethanol at − 80°. Immediately before use, aliquots of the RNA were recovered by precipitation with 0·3 M sodium acetate, washed twice in 75% ethanol and dissolved in an appropriate volume of diethylpyrocarbonate (DEPC; Sigma) treated water. Degenerate oligonucleotide

Table 1. Suppression of antigen-specific and mitogen-driven T-cell proliferation in co-culture with rat RPE cell monolayers co-relates with accumulation of NO_2^- and PGE_2 in the culture supernatants

	Nitrite* (μM NO_2^-)	PGE_2* (ng/ml)	DNA replication by lymphocytes [^{3}H]TdR ($\times 10^{-3}$)
RPE + medium alone	1·6	0·9	1·1 ± 0·5
RPE + T cells	6·1	1·5	6·8 ± 1·6
RPE + T cells + antigen	34·9	> 90·0	11·0 ± 0·8
T cells + antigen	6·0	0·8	34·6 ± 3.8
RPE + T cells + Con A	72·7	> 120·0	18·0 ± 1·9
T cells + Con A	9·6	0·8	107·3 ± 2·2

* Results expressed as means of duplicate measurements, SD were within 10% of the mean. Data are representative of two separate experiments.

Table 2. Effect of indomethacin (indo. 1 μg/ml) on nitric oxide production by RPE cells co-cultured with cytokines or T cells and mitogen (Con A, 2·5 μg/ml)

	Nitrite in supernatant (μM NO_2^-)*	DNA replication by lymphocytes [^{3}H]TdR ($\times 10^{-3}$)
RPE cells + T cells	16	4·3 ± 1·6
RPE cells + T cells + indo	10	7·1 ± 1·5
RPE + T cells + Con A	138	11·9 ± 6·0
RPE + T cells + Con A + indo	127	102·9 ± 6·3
RPE + cytokines†	81	NT
RPE + cytokines + indo	46	NT

* See footnote to Table 1.
† 100 U/ml IFN-γ, 500 U/ml TNF-α and 1 μg/ml LPS.
NT, not tested.

primers were designed based upon conserved motifs in published NOS nucleotide sequences in the GenBank database.[19] All gave products of predicted size: DGN1 = GG[C/T]-TGGTACATG[A/G]GCAC[C/T]GAGAT[C/T]GG; DGN5 = GG[A/T]GCGT[A/G]ATGTCCAGGAAG[A/T]AGGTGAG; 5NOS = CC[C/T]GT[CTG]TTCCA[C/T]CAGCAGATG; 3NOS = [A/G]AAGGC[A/G]CA[A/G]AA[CG]TG[A/G]GG-GTA and were synthesized by Oswel DNA services (Edinburgh, UK). A 1·4 kbp cDNA product was generated by reverse transcription/polymerase chain reaction (RT/PCR) from cytokine-stimulated RPE total RNA, and cloned into the pCRII vector (In Vitrogen, R & D Systems, Abingdon, UK). RT/PCR was performed as previously described;[20] briefly, 5 μg of total RNA was reverse transcribed with a genetically engineered MuMLV reverse transcriptase (Superscript; Gibco/BRL) using oligo $d(T)_{18}$ primer. Reverse transcriptase was inactivated by heating the same to 95° for 5 min. The reaction volume was brought up to 100 μg with sterile double-distilled water and the sample stored at 4° prior to use. PCR was performed on 5 μl reverse-transcribed total RNA template in 50 μl final reaction volume using purified Taq polymerase (Boehringer-Mannheim, Lewes, UK) under the reaction conditions specified by the manufacturer; 35 cycles of amplification were performed as standard, each cycle comprising a denaturation step of 94° for 1 min; an annealing step at 50° for 1 min and a denaturing step at 72° for 1 min 30 seconds. During the initial cycle the denaturation was extended to 3 min and during the final cycle the extension step was extended to 5 min. Primers were used at a final concentration of 0·5 μM each. Sequencing and restriction analysis demonstrated that the cDNA product corresponded to bases 1122–2519 (1398 bp) of the published sequence for rat smooth muscle iNOS.[21] Total RNA (30 μg/lane) from control and cytokine-stimulated RPE was separated in a denaturing agarose gel (1% agarose/6% formaldehyde) run in 1 × MOPS buffer for Northern blot analysis. After transfer to a nylon membrane (Hybond N; Amersham) the blot was probed with ^{32}P-dCTP-labelled 1·4 kbp cDNA iNOS probe, and with a 1·4 kb probe for 18 S rDNA[22] (Dr R. Fulton, Beatson Institute, Glasgow, UK). Blots were quantified by densitometry of exposed X-ray film and the iNOS signal corrected for the 18 S rDNA signal.

RESULTS

Nitrite in rat RPE/activated T-cell co-cultures

Lymphoid cell (LNC or SPL) proliferation to specific antigen or mitogen is profoundly suppressed when co-cultured on a monolayer of rat RPE cells.[5] As NO· is reported to inhibit lymphocyte proliferation we examined supernatants from such co-cultures for the presence of nitrite (NO_2^-), as a stable end-product of L-arginine metabolism. Table 1 shows the results from an experiment in which adherent cell-depleted lymph node T cells from retinal extract-immunized rats were challenged with retinal extract or Con A in a proliferation assay alone or in co-culture with monolayers of RPE cells as described in the Materials and Methods. Suppression of proliferation in the challenged co-cultures co-related with accumulation of nitrite and PGE_2 in the supernatants. In comparison with the levels found in the suppressed cultures (34·9 or 72·7 μM/10^5 RPE cells/ml) levels of nitrite were low in control RPE and T-cell cultures and in unstimulated co-cultures (1·6–9·6 μM/ml). In another experiment, however, proliferation to mitogen was restored in RPE co-cultures treated with 1 μg/ml indomethacin (a specific inhibitor of PGE_2 synthesis) despite continued high levels of NO_2^- in the supernatant (Table 2). In cytokine-stimulated RPE cells, however, indomethacin treatment reduced NO_2^- levels by nearly 50%.

Nitrite production by RPE cells in response to cytokine stimulation

Experiments were conducted to identify the cytokines required for the maximal induction of NO synthesis by RPE cells. IL-1β in combination with IFN-γ, which has been reported to be effective for other non-haematopoietic cells,[9] did not increase supernatant NO_2^- levels (data not shown). However, when challenged for 72 hr with a cytokine cocktail (100 U/ml IFN-γ, 10 μg/ml LPS and 1000 U/ml TNF-α) reported to be optimum for macrophage NO· synthesis,[8] rat RPE cell supernatant levels of 131·1 μM nitrite were found. Of the three components included in the stimulatory cocktail, only IFN-γ was found to

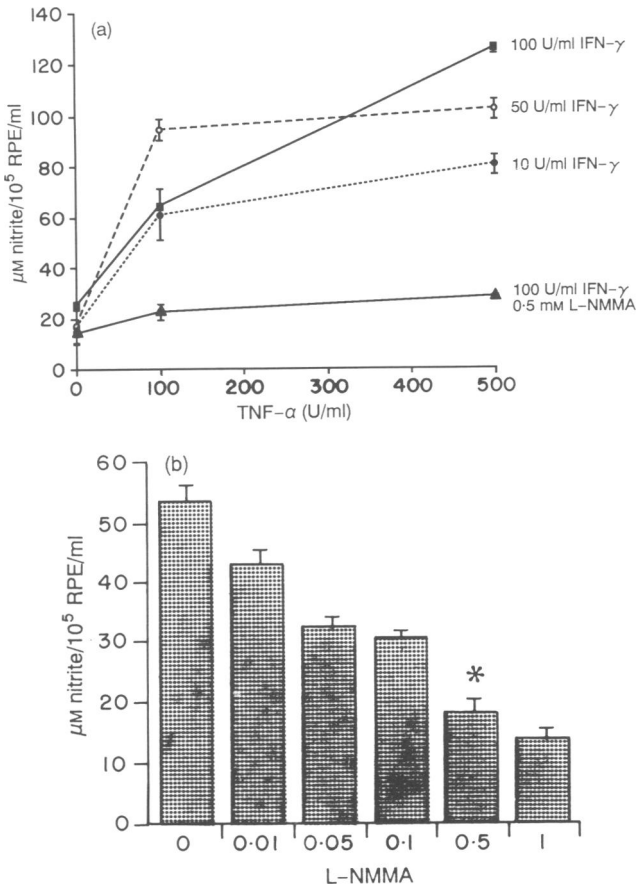

Figure 1. (a) Dose-dependent effects of IFN-γ and TNF-α on NO· production by rat RPE cells. Rat RPE cells were cultured with 1 μg/ml LPS and various concentrations of IFN-γ and TNF-α. Duplicate cultures stimulated with 100 U/ml IFN-γ and TNF-α were also set up with 0.5 mM L-NMMA. (b) Dose-dependent effect of L-NMMA on NO_2^- production by RPE cells stimulated with 1 μg/ml LPS, 100 U/ml IFN-γ and 500 U/ml TNF-α. After 72 hr supernatants were collected and NO_2^- measured by Greiss reaction. Results are expressed as means ± SD of two to four samples. Data in (b) are derived from three separate experiments, 0·5 mM L-NMMA reduced NO_2^- production by 66%; $P = 0{\cdot}019$.

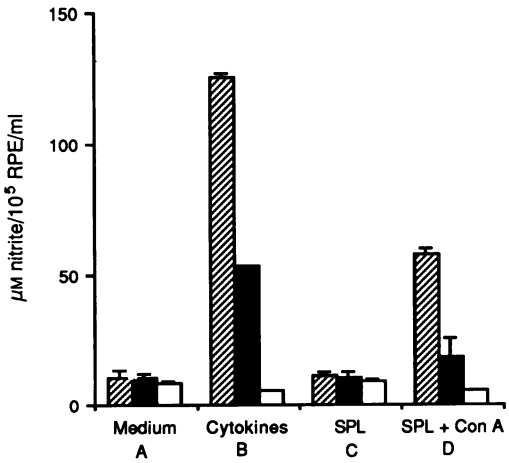

Figure 2. Inhibition of rat RPE NO· production by L-NMMA or cycloheximide. Rat RPE cells were cultured with: (A) 1:3000 ethanol in medium (inhibitor vehicle control); (B) 100 U/ml IFN-γ, 500 U/ml TNF-α and 1 μg/ml LPS; (C) splenocytes (SPL), 2 × 10^6/ml; (D) SPL, 2 × 10^6/ml + 5 μg/ml Con A. Additions: (▨), no inhibitor; (■) 0·5 mM L-NMMA; (□) 10 μg/ml cyclohexamide. Supernatants were harvested after 72 hr and NO_2^- levels measured by Greiss reaction. Results are expressed as means ± SEM of two similar samples. NO_2^- values for SPL cultured without RPE cells were: SPL, 8·8 μM/10^6 cells/ml; SPL + Con A, 8·0 μM/10^6 cells/ml; SPL + Con A + 0·5 mM L-NMMA 7·27 μM/10^6 cells/ml.

be essential, and was optimal at 100 U/ml in combination with 500 U/ml TNF-α and 1 μg/ml of the endotoxin LPS (Fig. 1a), increased NO_2^- was present in the cultures by 24 hr which accumulated to give maximum values by 72 hr. The competitive inhibitor of L-arginine metabolism via the NOS pathway, L-NMMA,[9] inhibited cytokine-induced rat RPE cell NO· production in a dose-dependent manner, with optimal inhibition (66%) at 0·5 mM L-NMMA ($P = 0{\cdot}019$, Fig. 1b). Both cytokine-induced and activated lymphocyte-induced NO· secretion were completely inhibited when cycloheximide was included in the cultures at 10 μg/ml and reduced by 0·5 mM L-NMMA (Fig. 2) indicating that increased NO_2^- in the culture supernatants was owing to NOS metabolism of L-arginine present in the culture medium.

Analysis of mRNA levels for NOS species in rat RPE cells

By using degenerate oligonucleotide primers we amplified and cloned a cDNA product corresponding to RPE iNOS from cytokine-induced RPE RNA. Sequencing demonstrated that the cDNA product corresponded to bases 1122–2519 (1398 bp) of the published sequence for rat vascular smooth muscle iNOS.[21] Primers used for these experiments were designed to recognize nucleotide motifs conserved between the published sequences for both constitutive (cNOS) and inducible forms of NOS and will distinguish between cNOS and iNOS species by generating different-sized PCR products. Northern blot analysis of total RNA extracted from rat RPE before and 24 hr after cytokine treatment showed a fivefold induction of a 4·5 kb mRNA transcript which hybridized with the 1·4 kbp cDNA probe (Fig. 3). This transcript size is consistent with the mRNA transcript for iNOS expressed in rat vascular smooth muscle. Culture supernatants from these 18-hr cytokine-stimulated RPE cells contained 37·28 μM NO_2^-.

DISCUSSION

Numerous investigations have demonstrated that exogenously applied T-cell-derived cytokines in various combinations and concentrations are potential inducers of the NOS gene[9,15,23] and proposed a cytostatic or cytotoxic role for NO· in inflammatory disease. However T-cell-derived cytokines are also known to be inhibitors of iNOS[10,24] and the *in vivo* significance of NO· in T-cell-mediated autoimmune disease was unclear. We have shown that in co-culture with RPE cells, activated, but not resting, T cells generate an endogenous cytokine profile which permits macrophage-type iNOS gene expression in the RPE cells and secretion of NO·. As the RPE is located immediately adjacent to the rod outer segments of the regina which are the target for activated, autoreactive T cells it

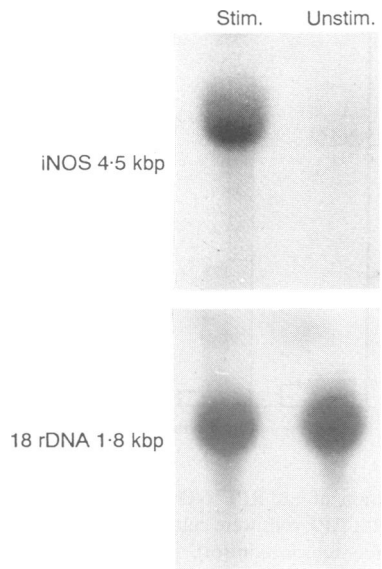

Figure 3. Northern blot of iNOS in rat RPE cells. Rat RPE cells were grown in 75-cm^2 flasks at 10^5 cells/ml in: growth medium alone (Unstim.), or growth medium plus cytokines, 100 U/ml IFN-γ, 500 U/ml TNF-α and 1 μg/ml LPS (Stim.). Cells were incubated for 24 hr, the medium decanted and RNA extracted as described in the Materials and Methods. Total RNA (30 μg) was electrophoresed through a denaturing agarose gel and blotted onto hybond N membrane. The blot was probed with the cloned iNOS PCR product (upper blot) and with a probe for 18 S rDNA (lower blot). iNOS mRNA was analysed by densitometry and showed a fivefold increase upon stimulation when corrected for sample loading using the 18 S ribosomal DNA probe.

seems likely therefore that NO· will be produced locally during uveoretinitis.

Nitric oxide clearly has a profound immunosuppressive effect on immune responses in many systems, mediating macrophage suppression of T-cell proliferation to antigen[25] as well as mitogen,[8] demonstrated by the effects of NO· inhibitors such as L-NMMA or N^G-nitro-L-arginine methyl ester (L-NAME) on experimental systems. The failure of indomethacin-treated RPE co-cultures to suppress lymphocyte proliferation, despite continued NO· production, was therefore surprising. Indomethacin-treated rat RPE cells cultured with T cells do not appear to inhibit T-cell proliferation. Indeed we have previously observed enhanced responses over SPL values alone,[5] which would imply that in our *in vitro* model RPE NO· secretion on its own is not directly involved in inhibiting lymphocyte proliferation. Albina *et al.*,[8] using indomethacin to inhibit PGE_2 production, also observed an increased proliferation of mitogen-stimulated murine T cells in the presence of macrophages despite an unchanged rate of NO_2 production. These authors suggested that the iNOS pathway was necessary, but not sufficient alone, for suppression to occur. In support of this theory, Warren *et al.*[26] have shown that vasodilatation *in vivo*, induced by arachidonic acid, was inhibited by either L-NAME or indomethacin suggesting that either a synergy between prostaglandins and NO· exists or that their release is linked. Our observation of a marked reduction of NO· secretion by cytokine-stimulated RPE cultures in which PGE_2 synthesis was inhibited concurs with this hypothesis. Gaillard *et al.*[27] found that increases in intracellular cAMP as a result of endogenous PGE_2 production increased NO· synthesis in rat Kuppfer cells. We are currently investigating the relationship between eicosanoids and the L-arginine pathway on RPE suppression of antigen-driven lymphocyte proliferation.

The contribution of RPE cell iNOS to the pathogenesis of autoimmune uveoretinitis remains to be elucidated. The role of induced NO· as an effector molecule may be cytotoxic or cytostatic and may participate in inflammatory and autoimmune-mediated tissue destruction.[28] As high levels of cGMP cause destruction of photoreceptor cells in the retina,[9] induction of NO· at high levels as a non-specific defence mechanism would result in bystander damage to healthy retinal tissue. Nitric oxide-mediated lysis of pancreatic islet cells leading to development of insulin-dependent diabetes has been demonstrated,[29] and tissue damage by locally deposited immune complexes is also mediated by locally released excess NO·.[30] The ability of macrophages to express iNOS and secrete high levels of NO· in response to inflammatory mediators is now well documented, and this may contribute to the role of macrophages in disease pathogenesis. For instance, a higher activation state of macrophages has been reported in various autoimmune conditions[33,34] and macrophage participation in the induction and early stages of experimental autoimmune encephalitis (EAE)[33] and EAU has also been described.[1] If this indicates enhanced secretion of NO· in the induction phase of disease, modulation of the iNOS pathway in macrophages may be therapeutically beneficial. Conversely, if RPE iNOS is involved in suppression of lymphocyte proliferation in the retina, therapy to inhibit NO· may exacerbate disease. It is of note that the Brown Norway rat, which secretes very high levels of NO·, is also highly resistant to the induction of T-cell-mediated autoimmune diseases, while susceptible Lewis rats are low NO· producers.[34]

Although the eye has been described as a site of immune privilege, circulating autoreactive T cells can be isolated from normal individuals,[35] indicating that peripheral mechanisms are in force which prevent activation of these cells and their migration into the retina. The ability of RPE cells to secrete cytostatic NO· in response to contact with an activated lymphocyte would therefore constitute an integral component of the immunosuppressive mechanisms postulated to exist within the eye.[36]

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