Nasal administration of retinal antigens suppresses the inflammatory response in experimental allergic uveoretinitis

A preliminary report of intranasal induction of tolerance with retinal antigens

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Abstract

Current immunotherapy of posterior uveitis is non-specific and limited by drug toxicity and unpredictable relapses on therapy. Alternative modes of therapy being investigated using the rat model of experimental autoimmune uveoretinitis (EAU) have included the induction of tolerance with oral administration of milligram quantities of retinal antigens. In this preliminary report we demonstrate that tolerance to retinal antigens can be induced via the upper respiratory tract with microgram doses of antigen, preventing subsequent induction of EAU.

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Current immunosuppressive agents act through relatively non-specific mechanisms on the immune system. Despite the advent of the newer immunosuppressive drugs with more selective modes of action, such as cyclosporin A, treatment of endogenous posterior uveitis requires prolonged drug usage and is limited by the development of drug resistance, unpredictable relapse on dose reduction and significant side effects.

Experimental autoimmune uveoretinitis (EAU)^{1,2} is an established model for human endogenous posterior uveitides, the study of which has led to a greater understanding of the underlying immunopathogenesis of this group of conditions.³⁻⁵ EAU is a CD4+ T-lymphocyte mediated disease which can be induced by at least two soluble retinal antigens (S-Ag) and interphotoreceptor retinol binding protein (IRBP), both of which have several uveitogenic epitopes.⁶⁻⁸ The S-Ag induced EAU model is of particular interest as patients demonstrate abnormal in vitro T-cell proliferative responses to S-Ag and its peptide fragments,⁹ although humoral antibody titres to S-Ag occur in both patients and normal subjects.¹⁰

EAU has proved to be an extremely valuable model for preclinical trials of immunosuppressive therapy as has been shown with cyclosporin A¹¹ and with several antigen-specific and immunospecific targeted therapies such as anti-S Ag antibody therapy,¹²⁻¹⁴ anti-Ia antibodies,¹⁵ and antigen coupled splenocyte transfer.¹⁶ Recently, tolerance (immune unresponsiveness) to S-Ag induced EAU has been achieved in susceptible animals by oral administration of

milligram quantities of S-Ag.¹⁷ Oral feeding of specific immunopathogenic antigens suppresses successfully the inflammatory response in many other models of autoimmune diseases¹⁸ and the mechanism of 'oral tolerance' is mediated by CD8+ T-lymphocytes.¹⁹ In the case of experimental autoimmune encephalomyelitis, suppression of the disease could be induced by a heterogeneous mixture of neuroantigens.²⁰

Tolerance may also be induced via the respiratory tract.^{21,22} Aeroallergens, when first encountered, are capable of conferring protection to subsequent allergic sensitisation. The proposed mechanism of inhalational tolerance is analogous to oral tolerance, described above, but requires much smaller (physiological) quantities of antigen to induce the suppression.²³ In addition to the antigen-specific mechanisms of tolerance induction which are influenced by genetic factors under control of local antigen presenting cells, alveolar macrophages induce non-specific immunological unresponsiveness, resulting in suppression of T-cell activation.²⁴

We have found that intranasal inoculation of a heterologous mixture of retinal antigens (retinal extract) induces tolerance to the multiple auto-antigens present in retinal extract, inhibiting subsequent induction of EAU to both retinal extract and S-Ag. The mechanism of tolerance induction by this regime of antigen inhalation would appear to be antigen specific since tolerisation to S-Ag did not protect against EAU induced by the other uveitogenic proteins (for example IRBP) present in the retinal extract.

Materials and methods

ANTIGENS

Soluble bovine retinal extract (RE) was prepared by hypotonic lysis of the retina in the dark into 0.02% TRIS-HCl pH 8.0, followed by ultracentrifugation at 25 000 rpm for 15 hours at 4°C and was dialysed against phosphate buffered saline (PBS) over 24 hours. Bovine S-Ag was prepared by HPLC on a TSK-DEAE column as previously described¹⁴ or by affinity column extraction. The S-Ag by both methods was homogeneous by silver staining on SDS polyacrylamide gel using Pharmacia Phast System, according to the manufacturer's instructions. The protein concentration of RE used in the experiments ranged from 5-7 mg/ml and S-Ag

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Table 1 Summary of the dose of retinal extract (RE) and S-Ag for both induction of EAU (immunisation) and tolerance

Antigen	Concentration of antigen	
	Immunisation (100 µl of antigen)	Tolerisation (60 µl of antigen/day)
RE (mg/ml)	5–7	5–7
S-Ag (µg/ml)	600	20
RE (dose response)	7 (mg/ml)	7 mg–700 ng/ml

Table 2 Clinical grading of EAU in Lewis rats

Grade	Clinical signs (biomicroscopy)
0	No disease
1	Iris vessel engorgement
2	Anterior chamber cells
3	Early fibrinous exudate at pupillary margin
4	Fibrin plugging pupil. Retroiridal hypopyon

Table 3 Summary of the histological grading of inflammatory infiltrate in EAU

Anterior segment		Posterior segment	
Iris infiltrate	0–4	Ciliary body infiltrate	0–4
Cells	0–4	Vitreous cells	0–5
Cornea	0–2	Vasculitis	
		Extent	0–5
		Severity	0–4
		ROS loss	0–5
		Choroidal infiltrate	0–4
		Choroidal granulomas	0–3

Grading
1 <10
2 10–15
3 15–20
4 20–30
5 30–35
6 >35

accounted for between 4 and 6% of the total protein concentration as determined by competitive ELISA estimations. The doses of both RE and S-Ag used for the induction of EAU and the induction of tolerance in the following experiments are shown in Table 1.

INDUCTION OF EAU

EAU was induced in 175–250 g Lewis rats by a single hind footpad injection of 100 µl of retinal antigen (retinal extract or S-Ag) emulsified in 100 µl of complete Freund's adjuvant (CFA). Immunisation was performed in animals 1 week after the last dose of intranasal inoculation for tolerance induction (see below).

CLINICAL EVALUATION

Ocular examination was carried out daily using slit-lamp biomicroscopy. The day of onset, severity, and course of uveitis were noted. The severity of inflammation was graded 0–4 according to the degree of cellular activity in the anterior chamber (Table 2).

HISTOLOGICAL EXAMINATION

Day 21 post-immunisation, animals were sacrificed and eyes were enucleated and prepared for haematoxylin and eosin staining. Histological inflammatory response was graded 0–6 using a system which graded the inflammatory infiltrate (cells and tissue thickening) of the iris, ciliary body, retina, and choroid. Both anterior seg-

Figure 1 Clinical response of retinal extract (RE) induced EAU in both control and RE-inoculated Lewis rats.

ment and vitreous cavity cellular activity were graded and more specifically, the extent and severity of retinal vasculitis and rod outer segment (ROS) loss, and the number of choroidal granulomas were also determined (Table 3).

TOLERANCE INDUCTION

Tolerance was induced under light ether anaesthesia by inoculation of 30 µl of antigen of PBS (for controls) into each nostril using an Oxford micropipette. This was performed daily for 10 days before induction of EAU by immunisation with retinal antigens in CFA.

Results

TOLERANCE INDUCTION WITH RETINAL EXTRACT
All control (non-tolerised) animals developed clinical signs of EAU by day 10 post immunisation (Fig 1 and Table 4), with a mean clinical score of 3 (SD 0.5) and mean histological grade of 4. Five of eight eyes in rats that were tolerised intranasally with RE (7 mg/ml; total inoculum

Table 4 Clinical and histological response in re-induced EAU in both controls and re-inoculated Lewis rats

Inoculum (7 mg/ml)	Immunisation (7 mg/ml)	Incidence (No eyes)	Day of onset	Severity	Histology grading
PBS	RE	10/10	10	3	4
RE	RE	3/8	12	0.7	1

Fig 2A

Fig 2B

Figure 2 Histological preparations (haematoxylin and eosin) of retinal extract induced EAU in both control and intranasal antigen inoculated Lewis rats. (A) Normal rat retina. (R=rod outer segments). (B) Retinal extract induced EAU. There is a marked inflammatory infiltrate in all retinal layers, with necrosis (N) loss of rod outer segments (R) and associated vitritis (V). (C) vasculitis (V) in tolerised rats as demonstrated by the early leucocytic infiltration of the vessel lumen and wall (arrows). (D) Thickening of the iris (I) and inflammatory cell infiltrate (arrows) in tolerised rats. (E) Ciliary body (C) inflammatory cell infiltrate (arrows) in tolerised rats.

dose of 4.2 mg) failed to develop EAU after immunisation with RE in CFA. Overall there was a significant reduction in clinical score of EAU from 3 (SD 0.5) to 0.7 (SD 0.31) ($p < 0.001$, Student's *t* test). Histological examination of the control group demonstrated a severe posterior uveitis, with a marked vitritis, loss of rod outer segments (ROS), leucocytic infiltration of the retinal layers and ultimately necrosis when compared with normal rat retina (Fig 2A and 2B). Histological findings within tolerised animals, where little or no clinical activity was present, demonstrated minimal or no signs of inflammation with low grade retinal vasculitis, intraluminal and intramural leucocytic infiltration (Fig 2C), and inflammatory cell infiltrate in the iris and ciliary body (Fig 2D and 2E) in four out of eight eyes. None of these animals demonstrated ROS loss, which is a primary feature of the EAU model.⁵

DOSE RESPONSE OF INTRANASAL TOLERANCE INDUCTION WITH RETINAL EXTRACT

All controls developed clinical EAU with a maximal mean severity of 3 at day 12, and onset of the disease at day 10. Suppression of RE-induced EAU was dependent on the tolerising dose of RE inoculated intranasally, with significant suppression ($p < 0.001$, Student's *t* test) being obtained over a dose range of 7 mg/ml to 7 µg/ml of RE (total inoculum dose of 42 µg). At the lowest dose 2/8 eyes had a delayed onset of disease (day 19) with a mean clinical score of 1. Partial clinical suppression with a mean score of 2.5 in 6/8 eyes and delayed onset of the disease (day 12) was achieved with a dose of 700 ng/ml equivalent to a total inoculum dose of 4.2 µg or 420 ng/day of RE. Histological findings within the tolerised group again showed minimal evidence of ROS loss, as described above.

SUPPRESSION OF EAU BY INTRANASAL TOLERANCE INDUCTION WITH DIFFERENT RETINAL ANTIGENS

All control animals that were immunised with S-Ag and CFA developed clinical EAU with a mean clinical score of 3 and the onset of EAU at day 12. Intranasal inoculation with S-Ag at a concentration of 20 µg/ml successfully suppressed S-Ag induced EAU (Table 5). Although two eyes developed low grade EAU, the onset of the disease was delayed to day 19 post immunisation with a mean clinical score of 0.6. However, S-Ag intranasal inoculation failed to suppress significantly RE-induced EAU, where 6/8 eyes developed the disease with a mean clinical score of 3 ($p > 0.1$, Student's *t* test). Conversely, RE intranasal inoculation conferred protection against S-Ag induced EAU, with a significant reduction of clinical score to grade 1.5 in 2/8 eyes, and a delay in onset of EAU to day 19 post-immunisation (Table 5).

Discussion

The immune system receives many external antigenic stimuli through its mucosal surfaces, which may either sensitise or tolerise the individual to subsequent antigenic exposure. We

Fig 2D

can be induced with retinal extract (RE) and S-Ag, where rats were inoculated intranasally with either RE or S-Ag before immunisation with these antigens, which resulted in a suppression of the inflammatory response. We found that inhalation of S-Ag protects only against subsequent challenge with S-Ag and not other autoantigens present in RE, whereas inhalation of whole RE will protect against S-Ag-induced EAU.

Tolerance is a natural form of immune suppression that has been extensively studied with particular reference to oral tolerance, and to the role of the respiratory tract in the protection against sensitisation to aeroallergens. As described in the introduction, the mechanism of 'inhalational tolerance' is analogous to oral tolerance, and it is proposed as the last line of defence against sensitisation to aeroallergens.²³ Previous work on inhalational tolerance with ovalbumin has demonstrated that both aerosol and direct intranasal inoculation confer equal protection against further sensitisation to ovalbumin in susceptible animals.²⁵ The question of oral absorption of the antigen by the nasal route has been addressed by radiolabelled studies of ovalbumin distribution²⁶ and with radiolabelled S-Ag

Fig 2E

Table 5 The effect of intranasal antigen inoculation on RE or S-Ag induced EAU

Inoculum	Immunisation	Incidence of disease (eyes)	Day of onset	Severity (mean)	Histology score
S-Ag	S-Ag	2/8	19	0.67	1
S-Ag	RE	6/8	12	3.0	3.5
RE	S-Ag	2/8	19	1.5	2
PBS	S-Ag	4/4	12	3.0	4

Concentration of antigens: S-Ag inoculum=20 µg/ml; S-Ag immunisation=60 µg/ml; RE inoculum=5 mg/ml; RE immunisation=5 mg/ml.

distribution (work in progress). These studies have demonstrated that although a significant proportion of antigen is presented via the gastrointestinal tract, up to 25% of the antigen is taken up via the trachea and major bronchi. With both ovalbumin and RE or S-Ag, the amount of antigen that is required to induce tolerance via the gastrointestinal tract is several log units greater than that required to induce tolerance via inhalation. In the current experiments the dose of the antigen taken up via the oral route was considerably less than that required to induce a state of tolerance in EAU,¹⁷ indicating that tolerance in this study was achieved via the nasal route.

Tolerance induction by inhalation with ovalbumin is mediated by CD8+ T-lymphocytes²⁷ in the regional drainage lymph nodes of the respiratory tract.²⁸ Before the effector cell activation, antigen processing is presumed to occur in the contiguous network of dendritic cells that line the epithelium of the respiratory tract.²⁹ S-Ag requires minimal processing³⁰ and dendritic cells are highly efficient antigen presenting cells with an endosomal system capable of processing IRBP for presentation (B Chain, personal communication). There therefore appear to be all the facilities required to deal with retinal antigenic stimuli by the intranasal route.

The histological changes that occur in EAU in the Lewis rat and other animal models have been well described.²³⁻³³ Inflammatory cells are first detected in the ciliary body and choroid, before the major inflammatory infiltration of the retina, with concomitant vasculitis by day 12-14. The result of the acute inflammatory reaction is retinal necrosis and loss of rod outer segments. In the late stages, evidence of chronic inflammation with choroidal granulomas and subretinal neovascular membranes occurs up to 3 to 4 weeks post-immunisation. Intranasal tolerance did not confer complete protection in all cases. Histological examination of tolerised animals revealed a mild iridocyclitis with little or no evidence of retinal inflammation, except minimal retinal vasculitis and vitritis. Thus 'tolerised' animals, that were subsequently immunised, at worst appeared only to develop a minor inflammatory response, with minimal retinal damage, indicative of an ongoing immunomodulation and subsequent alteration in both anatomical distribution and severity of the intraocular inflammation. This, however, was not the case with animals that were tolerised with S-Ag and immunised with RE, who developed the typical histological changes described in EAU. This is perhaps not surprising, as many recognised uveitogenic proteins are

Figure 3 Dose response of retinal extract (RE) intranasal inoculation on the suppression of RE induced EAU.

present in the RE, and protection against these will not have been conferred.

At present we are studying the underlying cellular and humoral immune responses in this model of tolerance against EAU to increase our understanding of this process as a possible future immunotherapy. Tolerance is an attractive method of therapy because of its natural and powerful immunosuppression. Unlike oral tolerance, inhalational tolerance requires only small doses of antigen. Future studies are also aimed at studying the effect of intranasal tolerisation after immunisation and also during established intraocular inflammation. This is of paramount importance because in humans 'tolerance therapy' must avoid sensitisation or exacerbation of established disease in already sensitised individuals.

In summary we have established a method of successfully suppressing EAU. The model will allow further study of the underlying immune mechanisms of tolerance and of any future potential for this form of immunomodulation.

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