Modulating phenotype and cytokine production of leucocytic retinal infiltrate in experimental autoimmune uveoretinitis following intranasal tolerance induction with retinal antigens

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Abstract

***Background/aim*—Nasal administration of retinal antigens induces systemic tolerance which results in suppression of experimental autoimmune uveoretinitis (EAU) when subsequently exposed to antigen. The aim was to establish if tolerance induction alters retinal infiltrating leucocyte phenotype and cytokine profile in tolerised animals when there is significantly reduced tissue destruction despite immunisation with retinal antigen.**

***Methods*—Female Lewis rats were tolerised by intranasal administration with retinal extract (RE) before immunisation with RE to induce EAU. Control animals were administered phosphate buffered saline (PBS) intranasally. Post immunisation, daily clinical responses were recorded and at the height of disease, retinas were removed and either infiltrating leucocytes isolated for flow cytometric phenotype assessment and intracellular cytokine production, or chorioretina processed for immunohistochemistry. Fellow eyes were assessed for cytokine mRNA by semiquantitative RT-PCR.**

***Results*—Flow cytometric analysis showed that before clinical onset of EAU there is no evidence of macrophage infiltration and no significant difference in circulating T cell populations within the retina. By day 14 a reduced retinal infiltrate in tolerised animals was observed and in particular a reduction in numbers of "activated" (with respect to CD4 and MHC class II expression) macrophages. Immunohistochemistry confirmed these findings and additionally minimal rod outer segment destruction was observed histologically. Cytokine analysis revealed that both IL-10 mRNA and intracellular IL-10 production was increased in tolerised eyes 7 days post immunisation. Although by day 14 post immunisation, IL-10 production was equivalent in both groups, a reduced percentage of IFN-γ^+ macrophages and IFN-γ^+ CD4$^+$ T cells with increased percentage of IL-4$^+$ CD4$^+$ T cells were observed in tolerised animals.**

***Conclusions*—Leucocytic infiltrate is not only reduced in number but its distinct phenotype compared with controls implies a reduced activation status of infiltrating monocytes to accompany increased IL-10 and reduced IFN-γ production in tolerised animals. This modulation may in turn contribute towards protection against target organ destruction in EAU.**

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Diminishing tissue destruction in organ specific autoimmune diseases by mucosal administration of autoantigen is potentially a powerful method of immunosuppression.[1] Experimental autoimmune uveoretinitis (EAU) is a CD4$^+$ T cell mediated animal model for organ specific autoimmune posterior uveitis[2 3] which experimentally can be induced with a variety of retinal antigens.[4] Suppression of EAU may be achieved by administering retinal antigens via mucosal surfaces, such as the gastrointestinal tract[5] and nasorespiratory tract.[6] The experimental success of such therapy has led to phase I/II clinical trials of oral tolerance in a variety of autoimmune diseases, but with inconclusive results.[7] However, the potential of such therapy remains if a greater understanding of the mechanisms of tolerance induction and effector mechanisms of such suppression can be attained. Improved efficacy of such therapy may be achieved with administration of antigen via the nasorespiratory tract rather than orally, because of the smaller quantities of antigen or antigenic peptide required to induce tolerance and the fact that antigen/peptide will not be so readily degraded by the enzymatic environment more prevalent in the gut. Mechanisms of tolerance induction are dependent upon the dosage and route of antigen administration[8 9] and include T cell anergy/deletion in high dose oral tolerance[10] and active suppression via generation of regulatory cells in low dose tolerance.[11]

We have previously established a model of tolerance suppressing EAU by repetitive intra-

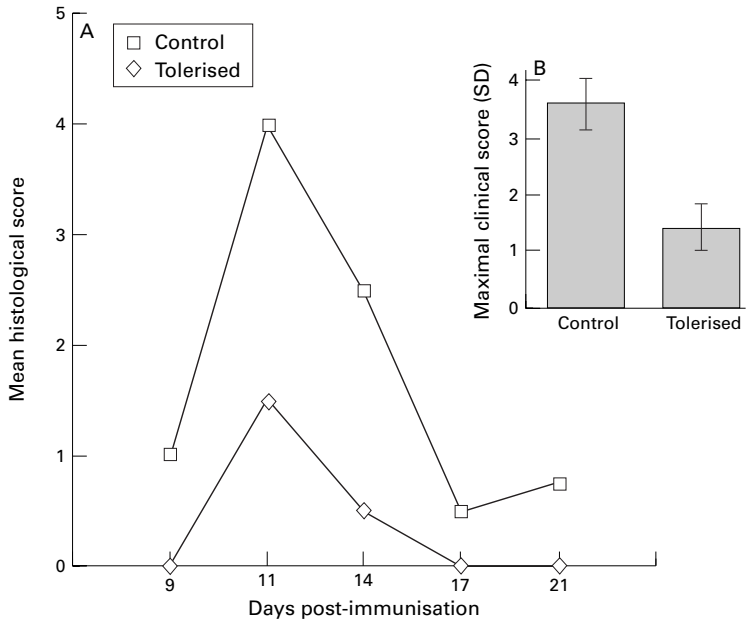

Figure 1 Suppression of clinicopathological features of experimental autoimmune uveoretinitis (EAU) by intranasal tolerance induction. (A) Histological grading[12] of EAU shows extent of histopathological damage is reduced in tolerised animals. (B) Clinical inflammatory scores[12] are significantly suppressed in tolerised animals.

nasal administration of retinal antigens[6 12] similar to suppression observed in other animal models of autoimmune disease.[13–16] Suppression of EAU via intranasal retinal antigen administration is antigen specific and inhibits Th1 reactivity (DTH and T cell proliferation), while maintaining T dependent antibody responses.[17 18] As with other models of low dose tolerance induction, regulatory cells are generated, as tolerance can be transferred by splenocytes[12 17] and also systemic Th2 cytokine production is increased (Laliotou, unpublished data). Although there is a reliable and consistently significant reduction in target organ destruction (rod photoreceptor outer segment (ROS) loss), secondary neuronal destruction and retinal atrophy in tolerised animals, infiltrating cells are still observed within the vitreous and retina, in particular around the inner retinal vessels.[6 12 18] One of the conflicts regarding the safety and reliability of such tolerance therapy is whether mucosal administration of antigen can suppress ongoing active disease.[19] Animal models can be adapted to mimic more of a lower grade chronic relapsing disease in which tolerance therapy has had unconfirmed reports of success of disease suppression.[13 20 21] However, to date we have unreliably been able to suppress active disease unless combined with other immunosuppressants.[22 23] In EAU, retinal infiltrating T cells and macrophages during the height of inflammation are predominantly activated Th1 $CD4^+$ T cells along with "activated" macrophages expressing high levels of MHC class II and CD4 antigen, with predominantly proinflammatory (Th1) cytokine synthesis and production.[24 25] Modulating T cell function (that is, deviating Th1 response towards Th2) while downregulating macrophage activation reduces retinal destruction in EAU.[25 26] We wished therefore to assess if via tolerance induction, suppression of antigen specific Th1 responses, and deviation towards Th2 responses systemically[16 27] could also modulate the retinal leucocytic infiltrate and thus contribute to suppression of tissue damage.

Methods

ANIMALS, TOLERANCE INDUCTION, AND INDUCTION OF EAU

Inbred adult female Lewis rats (8–10 weeks of age) were obtained from the animal facility, medical school, University of Aberdeen. Animals were used in all experiments according to the ARVO statement for the use of animals in ophthalmic and vision research. EAU was induced by 0.1 ml intradermal footpad injection of 100 μl of 6 mg/ml of retinal extract (RE) v/v in complete Freund's adjuvant containing 5 mg H37RA *Mycobacterium tuberculosis*. RE was prepared as described[6] by hypotonic lysis of freshly dissected bovine retinas in the dark. RE contains uveitogenic proteins (S-Ag and interphotoreceptor binding protein, IRBP) as confirmed by SDS-PAGE electrophoresis (Pharmacia, Sweden) and western blot analysis. S-Ag accounts for 4–6% and IRBP 5–10% of the total protein in RE preparations as measured by competitive ELISA estimations.[18] At least four animals per experimental group were used. Intranasal tolerance induction was induced by a previously described successful regime.[6] Thirty μl of RE or control phosphate buffered saline (PBS) were directly administered intranasally using an Oxford micropipette. The concentration of tolerising antigen, RE, was 6 mg/ml (total protein). Nasal inoculations were given on week days for 2 weeks (10 inoculations), followed by a 1 week break before immunisation with RE. Total inoculum dose was 3.2 mg (total protein) of RE.

FLOW CYTOMETRIC ANALYSIS, IMMUNOHISTOCHEMISTRY, AND MONOCLONAL ANTIBODIES

Retinas were dissected from perfused animals. Leucocytes were isolated and viable cell counts were determined by trypan blue exclusion as described previously.[22 25] Cell surface molecules to characterise different cell populations were identified by specific monoclonal antibodies. Mouse mAb specific for rat cell surface markers used were obtained from Serotec (UK) unless otherwise stated and included OX1 (anti-CD45), OX6 (anti-MHC class II; I-A), W3/25 (anti-CD4), R73 (anti-αβTCR), ED7 (CD11b/c; macrophage/monocyte markers), and OX22 (anti-CD45RB, high molecular weight form of LCA). OX21 (anti-human C3bi and not rat cells) was used as an isotype control for flow cytometry. mAb used were either unconjugated or conjugated to biotin, PE, or FITC for two colour immunofluorescence. Unconjugated mAb was detected with rat absorbed reagent, FITC conjugated sheep $F(ab')_2$ anti-mouse Ig (Sigma, USA), and biotinylated antibodies were detected with streptavidin-PE (SA-PE; Caltag, USA) for two colour. Phenotyping by flow cytometry (FACSCalibur, Becton Dickinson, USA) was

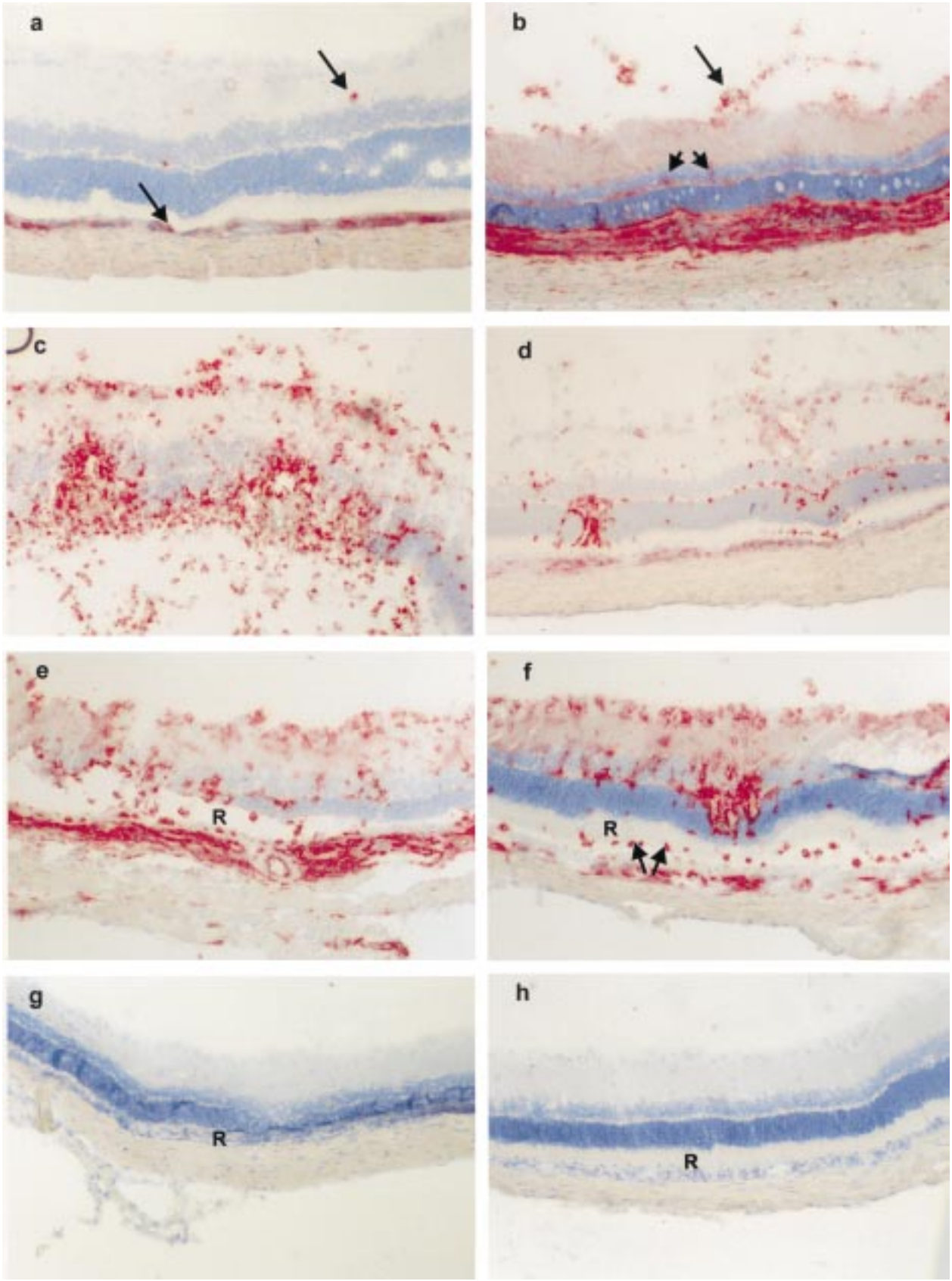

Figure 2 Composite of immunohistochemical analysis of chorioretina from tolerised and control animals. By day 9 post immunisation ED1⁺ macrophages are found infiltrating both the choroid and retina (arrows) (a), concomitant with an increase in MHC class II staining (b) particularly at the choroid/RPE, inner retinal vessels (arrow), and retinal parenchyma (arrowhead representing parenchymal microglia), both panels are from control eyes. During active inflammation (day 11 post immunisation) ED1⁺ cell infiltrate increases in number in both control (c) and tolerised animals (d), although staining is more intense in control animals (c). By day 14 post immunisation with persistent ED1⁺ infiltrate, retinal architecture (particularly ROS (R)) is markedly damaged in control animals (e) whereas tolerised animals display preserved ROS (R) despite persistent ED1⁺ infiltrate (f). This is further confirmed by day 21 post immunisation. Although leucocytic infiltrate has largely resolved total ROS loss (R) can be observed in retinas of control (g) and not tolerised animals (h). Original magnifications: a–f ×350 and g, h ×300.

Table 1 Absolute cell numbers (×10⁴)/animal (two retinas) *

Cell populations/days post immunisation	*Controls*		*Tolerised*	
	7	*14*	*7*	*14*
$CD4^+\alpha\beta TCR^+$	1.62	109 (130)	1.44	69.7 (58.3)
$CD8^+\alpha\beta TCR^+$	1.17	42 (47.1)	1.86	28.7 (16.8)
$ED7^+CD4^+$†‡	24.3	67.1 (54.8)	12.3	24.6 (37.3)
$ED7^+$MHC class II⁺§	nd	109.8	nd	98.4
$OX22^+CD4^+$	nd	51	nd	41

*Figures are a representative experiment and calculated from the mean recovery from two animals in each group (four retinas). (Cell numbers for a second experiment in parenthesis.)
†Cells were backgated from $CD45(OX1)^+$ positive cells (see Methods) and only $ED7^{high}$ cells were included in analysis of macrophage infiltrate thereby excluding resident microglia ($ED7^{low}$ expressing; see Dick *et al* [22]) which did not change in number between groups in any of the experiments.
‡See Figure 3 and legend.
§Athough MHC class II expression was increased on majority of $CD45(OX1)^+$ cells greater than negative control, increased numbers of $ED7^+$ cells expressed MHC class II in control retinas (see Fig 3).

performed as previously described.[26] Flow cytometric cytokine analysis on fixed, permeabilised cell suspensions was performed.[28] Specific mAb against rat IL-2, IL-10, IL-4, and IFN-γ (Pharmingen, USA) were used and positive controls (RiCK-2 cell line (Pharmingen, USA)) as well as blocking with recombinant cytokine to ensure specificity of cytokine stain were run in parallel with retinal samples. A total of 10 000 events were collected and analysed using CellQuest acquisition and analysis software. Appropriate liberal leucocyte gates and instrument variables were set according to forward and side scatter characteristics and analysis of fluorescence was performed after further backgating to exclude dead cells and aggregates. In other experiments (12 animals in each group) enucleated eyes were processed for routine single APAAP immunohistochemistry as previously described.[29]

RT-PCR

Cytokine mRNA was measured in eyes from tolerised and control animals 7 and 14 days post immunisation by RT-PCR. Total RNA was extracted from whole tissue and mRNA present within the sample was selectively reverse transcribed to cDNA as previously described. Published rat specific oligonucleotide primers for β actin, IL-2, IL-10, TGF-β, IFN-γ, and TNF-α were used for PCR amplification.[30] The RNA yield was calculated spectrophotometrically and the quality of the RNA was determined by the ratio of OD260:OD280 and integrity of the 18s and 24s ribosomal bands on electrophoresis of 1 μg of each RNA sample on a 1.5% agarose gel; 5 μl of cDNA was used as a template in each polymerase chain reaction (PCR). PCR was carried our under standard reaction conditions in a volume of 25 μl using purified Taq DNA polymerase (Boehringer-Mannheim, UK).

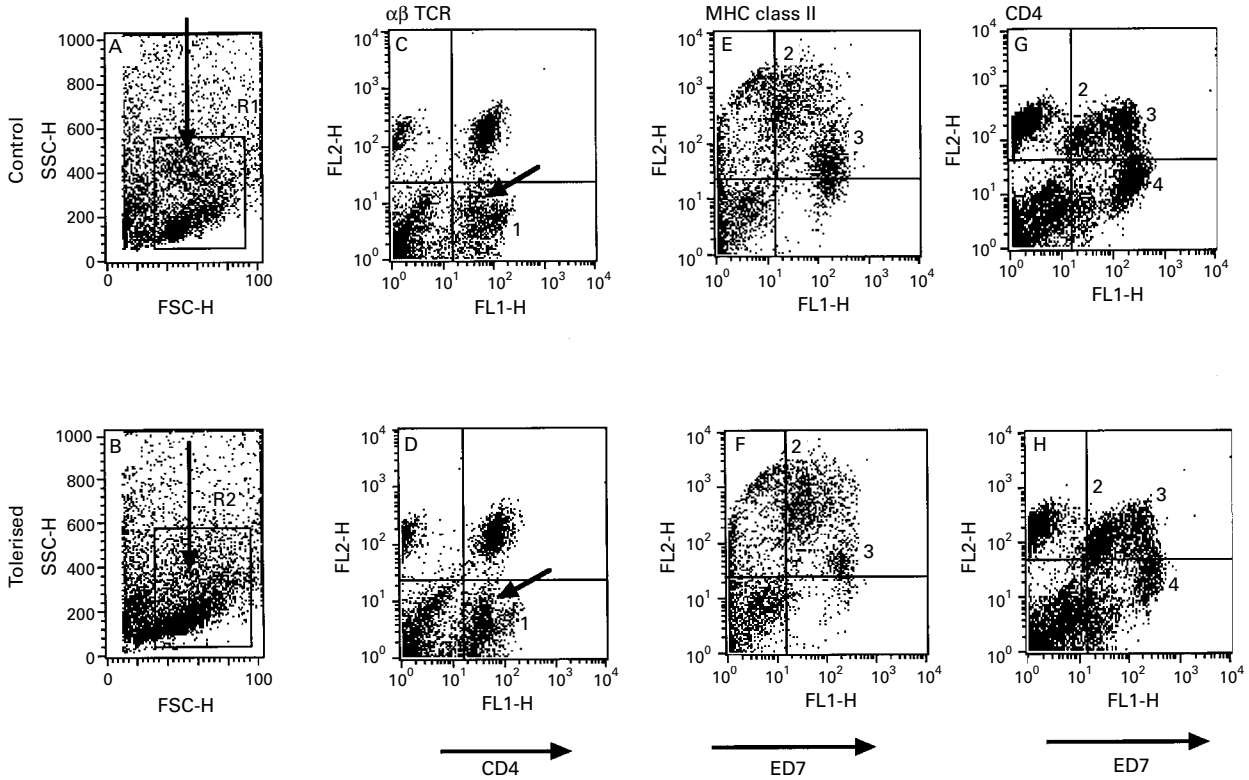

Figure 3 Flow cytometric analysis of retinal leucocytes in tolerised and control animals during EAU. Retinal leucocytes were isolated day 14 post immunisation The cells were derived from a pool of four eyes per experimental group, but comparable data were obtained in another identical experiment. Populations identified were based on two colour flow cytometric analysis including CD4 v αβTCR (CA and DC) and ED7 vMHC class II (BE and FD) and ED7 v CD4 (G and H)); see text. Plots A and B show scatter profile of retinal cell isolates demonstrating an increased population of granulocytes (arrow) in control animals (see text). Population 1 (plots C and D) identifies a $CD4^{high}\alpha\beta$ TCR^- population in control animals (CD4 MFI of 110), distinct from $CD4^{low}\alpha\beta TCR^-$ (arrow) population (CD4 MFI of 43), representing microglia (MG) and non-activated infiltrating macrophages.[22 25 26] MG, further shown (population 2) on plots E–H and characterised by $ED7^{low}$ expression (see text), are equal in number (data not shown) and express similar MHC class II and CD4 between the two groups. Population 3 represent $ED7^{high}$ cells (granulocytes are excluded for calculation of macrophage numbers by appropriate backgating to scatter plot). Increased numbers of macrophages express MHC class II (plots E and F) and high levels of CD4 (plots G and H) in control animals (see text). E and F do not show granulocyte population whereas plots G and H include granulocytes (population 4) to show that they do not express CD4 and are markedly reduced in number in tolerised animals.

The primer sequences and predicted product sizes are as previously specified.[30] All primers were used at a final concentration of 1 mM in the PCR reaction, which consisted of 35 cycles of 94°C for 50 seconds, 50°C for 60 seconds, and 72°C for 90 seconds. PCR products were analysed by electrophoresis through 1.5% agarose gels containing 0.4 μg/ml ethidium bromide and visualised under ultraviolet light. Semiquantitative analysis was performed by an image enhancement package (Imagestore 5000; UVP) and Gelbase analysis software. To control for discrepancies in the initial concentration of cDNA used, all the levels of cytokine expression are presented as a ratio of the value of β actin.

Results

INTRANASAL ADMINISTRATION OF RETINAL ANTIGENS REDUCES THE NUMBER OF INFILTRATING CELLS AND "ACTIVATION" PHENOTYPE OF INFILTRATING MONOCYTES DURING SUPPRESSION OF EAU

Intranasal administration of retinal antigens consistently and significantly ($p<0.05$) suppressed clinical disease (Fig 1B). Figure 1A documents the extent of histological changes[12] representing both the significant reduction in leucocytic infiltration and retinal damage in tolerised animals. Further immunohistochemical analysis shows that before clinical onset of disease, small numbers of $ED1^+$ macrophages infiltrate the choroid and retina (Fig 2a, arrows) and MHC class II expression by resident retinal cells is upregulated (Fig 2b, arrowhead). By day 11 in controls and day 14 in tolerised animals there was maximal inflammatory infiltrate within the retina. Immunohistochemistry demonstrated, in addition to $CD4^+$ and $CD8^+$ T cell infiltrate, $ED1^+$ macrophages within the retinas of both groups of animals (Fig 2c–f) concomitant with an increase in MHC class II expression in both groups of animals (not shown). Histological changes at this stage were typical of those previously described showing severe retinal destruction and retinal detachment in control eyes and, despite a retinal infiltrate, only minimal photoreceptor outer segment loss was observed in tolerised animals (Fig 2e, f). This was confirmed by histological assessment at day 21 post immunisation at the time leucocytic infiltrate had largely resolved, which showed minimal rod photoreceptor outer segment (ROS) destruction in tolerised animals (Fig 2g, h). Flow cytometric analysis confirmed the presence of a significant intraocular infiltrate in tolerised animals particularly during height of disease. Analysis on day 7 post immunisation, which preceded disease onset, documented minimal evidence of inflammatory cells within the retina in either group of animals (Table 1). Although there were equal numbers of $CD4^+$ T cells within the retina of each group, $CD8^+$ T cell infiltrate although increased was not significant in tolerised animals (1.86×10^4 tolerised and 1.17×10^4 controls). During the height of inflammation (day 14 post immunisation) leucocytic ($OX1^+$ [CD45] cell) retinal infiltrate was reduced in tolerised animals ($OX1^+$ cell numbers of 4.1×10^6 tolerised and 6.1×10^6 controls). In particular, granulocytes (as defined by their characteristic high scatter profile) were markedly reduced in tolerised animals (110×10^4 controls and 28.7×10^4 tolerised animals; Fig 3). One advantage of flow cytometry is that it not only allows evaluation of both numbers of positively stained cells but also analyses differences in extent of cell surface expression per cell. We used ED7 as a macrophage marker as ED1 is intracellular and assessment of extent of staining per cell is less interpretable. Although $ED7^+$ cell numbers (excluding granulocytes) were equivalent between groups (Table 1) in these series of experiments, macrophages isolated from the retinas of tolerised animals exhibited a reduction in activation phenotype. $ED7^{high}$ cells expressed high or low levels of CD4 antigen as determined by mean fluorescent intensity (MFI) on flow cytometry. CD4 expression exhibited MFI values of 131 in high expressing cells compared with values of 45 in low expressers. Using these criteria to differentiate extent of CD4 expression (after backgating to exclude granulocytes and $ED7^{low}$ microglia (MG)), 24.6×10^4 $ED7^+$ cells in tolerised animals compared to 67.1×10^4 cells in controls expressed high values of CD4 antigen (population 4, Fig 3 and Table 1). Corroborating a reduction in activation of $ED7^+$ cell numbers in tolerised animals was the reduced proportion of $ED7^+$ cells expressing MHC class II (39% controls and 24% tolerised). In parallel with the overall decrease in $OX1^+$ infiltrate, T cell numbers were also proportionally decreased in tolerised animals (1.09×10^6 in controls and 6.9×10^5 in tolerised animals), including both $CD4^+$ and $CD8^+$ T cell populations. Protection from target organ (ROS) destruction was not accounted for by any increase in $CD8^+$ or $\gamma\delta$ TCR^+ T cell numbers in tolerised animals at any stage of EAU.

INTRAOCULAR EXPRESSION OF IL-10 MRNA OCCURS EARLY AND IS MAINTAINED THROUGHOUT INFLAMMATORY RESPONSE IN EAU OF TOLERISED ANIMALS

On day 7 post immunisation, eyes from control animals displayed minimal but detectable expression of IL-2, TNF-α, and TGF-β. In tolerised animals, however, there was an increased expression of IL-10 mRNA in all eyes tested (Figs 4A and B). In a separate experiment intracellular cytokine analysis showed that retinal leucocytes produced increased IL-10, confirming the increase in IL-10 mRNA in tolerised animals 7 days post immunisation (0.71% of cells in controls and 2.22% of cells in tolerised animals were $IL\text{-}10^+$, Fig 4C).

By day 14 post immunisation, comparable proinflammatory cytokine mRNA expression was documented (IL-2, TNF-α, and IFN-γ) in both groups of animals, and although ocular IL-10 mRNA expression was still increased in tolerised animals, substantial differences were not apparent (arbitrary values of 0.215 and 0.16 in tolerised and control animals respectively). Intracellular cytokine analysis con-

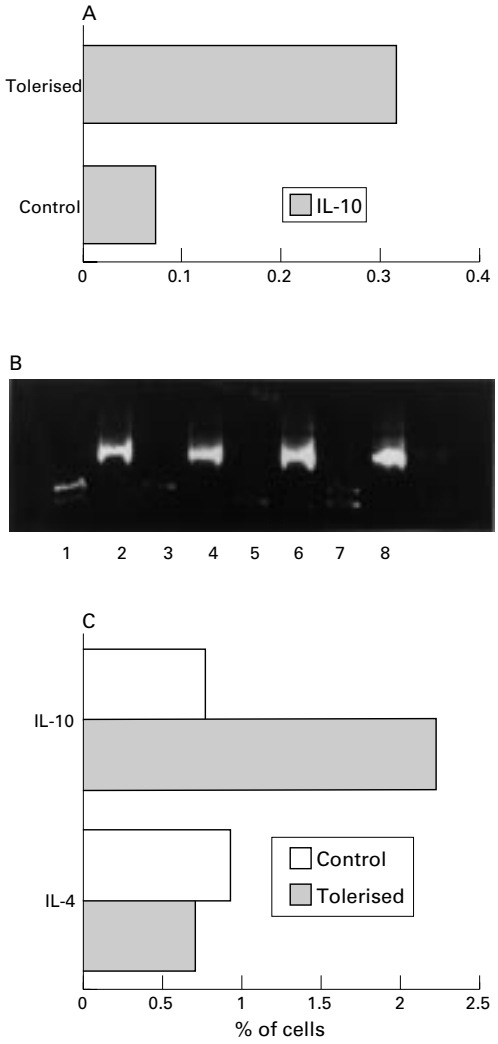

Figure 4 (A) RT-PCR cytokine analysis from eyes of control and tolerised animals during EAU. Values are from a representative experiment calculated from means of two animals/group at day 7 post immunisation each time point. (B) PCR IL-10 blots at day 7 post immunisation. Lanes 1 and 3, tolerised IL-10; lanes 2 and 4, tolerised β actin; lanes 5 and 7, control IL-10; lanes 6 and 8 control β actin. (C) Flow cytometric intracellular cytokine analysis day 7 post immunisation in control and tolerised animals. IL-10 expression represented as percentage of OX1⁺ cells. Values are mean of two animals/group.

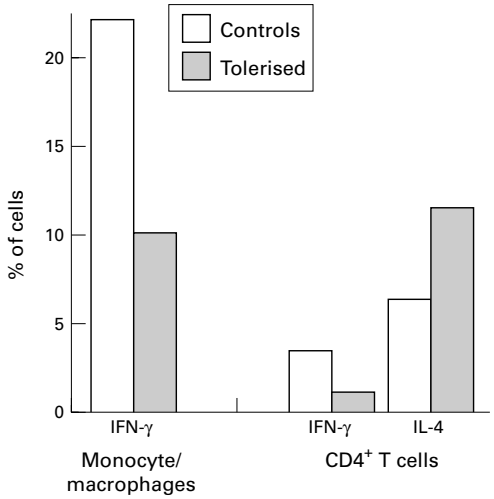

Figure 5 Flow cytometric intracellular cytokine analysis day 14 post immunisation in control and tolerised animals. Cytokine expression represented as percentage of either monocyte/macrophage gate or CD4⁺ T cells. Values are mean of two animals/group.

firmed that there were equivalent numbers of IL-10⁺ cells 14 days post immunisation (data not shown) but, furthermore, tolerised animals displayed a decrease in percentage of IFN-γ⁺ monocytes and CD4⁺ T cells, while IL-4⁺ CD4⁺ T cell percentage was increased (Fig 5).

Discussion

Despite the mechanisms of intranasal tolerance induction not being understood fully, intranasal administration of retinal antigens successfully prevents target organ (ROS) destruction.[6 12 18] Systemically, regulatory cells are generated within the spleen, capable of transferring suppression and thus cell mediated and delayed hypersensitivity responses are inhibited.[17] However, as in previous experiments, in all tolerised animals leucocytic retinal infiltration occurred in reduced number although with minimal signs of an effector response (ROS destruction) even after the infiltrate has resolved. It would appear, therefore, that at tolerising doses we use either (a) suppression of tissue damage occurs because the chorioretinal infiltrate is significantly less; or that (b) tolerance therapy, while suppressing cell mediated Th1 responses systemically, modulates T cell and consequently monocyte/macrophage function within infiltrating cells and that despite tolerance therapy, it would appear that "primed" cells are still capable of "trafficking" and infiltrating the eye. Against the former explanation is that we have previously shown that there was no dose response in relation to tolerising dose and suppression, so that reducing the tolerising dose of antigen still maintained suppression until finally at a lower doses protection against tissue destruction was lost completely.[6] Furthermore, data[12] have documented that the degree of ROS loss in RE induced EAU does not correlate with the extent of ocular infiltrate, but depends upon the dose of immunising antigen. Consequently, we wished to examine further any characterisitcs which may help define the "non-destructive" ocular infiltrate in tolerised animals, such as changes in cell phenotype and cytokine production.

Flow cytometric analysis of the retinal infiltrate in these experiments confirmed previous histological evidence for a reduced retinal cell infiltrate in tolerised animals (Table 1) and, in addition, showed that there was proportionally an equal reduction in both CD4⁺ and CD8⁺ T cell numbers at the height of disease. However, earlier at day 7 post immunisation it was noted that tolerised animals had increased, albeit small, CD8⁺ T cell numbers (as well as percentage of T cell infiltrate). These cells may have a role in suppression of target organ damage, as observed during the late stages of EAU, where unconfirmed reports of TGF-β Th2 CD8⁺ T cells have been implicated as suppressor cells during resolution phase.[24] Whether specific regulatory cells—that is, antigen specific Th2

cells, are generated via tolerance induction remains unclear. These data have shown that before disease onset and in later stages of EAU there was increased IL-10 mRNA expression maintained in later stages of EAU, a Th2 cytokine normally associated with recovery and suppression in experimental models of autoimmune disease. During the course of EAU differences in OX22 expression,[31] which distinguishes Th1 and Th2 phenotype in rats, was not observed between controls and tolerised animals (data not shown). OX22 expression, however, is lost from activated Th1 T cells (normally $OX22^+$), and therefore during a Th1 mediated inflammatory response without additional cytokine data distinguishing between activated Th1 and Th2 cells (both now $OX22^-$) is not possible using OX22 phenotype expression alone.

In tolerised animals, infiltrating $ED1^+$ monocyte/macrophages were noted immunohistochemically. Although increased $ED1^+$ expression confers increased phagocytic ability of the macrophage, activation of macrophages was further assessed by flow cytometric analysis as previously described.[25 26] These results showed a reduced activation phenotype (lower levels of CD4 and MHC class II antigen expression). Early monocyte infiltration before signs of clinical disease, also showed reduced $CD4^+$ expression (data not shown). Intranasal tolerisation, therefore, not only reduces the ocular cellular infiltrate but also modulates predominantly IL-10 cytokine production. Although the present data cannot exclude fully the possibility that these differences reflect a mere delay in macrophage recruitment, this explanation alone remains unlikely as immunohistochemical analysis on day 17 post immunisation showed a reduced leucocytic infiltrate and in addition reduced ED1 expression in both groups of animals. Moreover, the documentation that tolerised animals preserve retinal architecture despite leucocytic infiltration would indicate remarkable differences in effector cells between the two groups. Intracellular cytokine analysis confirmed this opinion. By day 14 post immunisation tolerised animals displayed reduced percentage of IFN-γ^+ cells while the percentage of IL-4^+ cells was increased (Fig 5). This is despite recording equivalent proinflammatory cytokine mRNA levels in both groups of animals on day 14 post immunisation, suggesting as previously described that increased mRNA expression does not always correlate with production of bioactive cytokines.[24]

The relative effector cell role for individual infiltrating cell populations in EAU remains undefined. For example, when the function of a major proinflammatory cytokine, such as TNF is neutralised, target organ damage is suppressed despite ongoing tissue leucocytic infiltration, particularly T cells.[26] T cell function is however modulated and in addition there is a reduction in monocyte activation,[25] similar to data described here. Supporting evidence for effector role for bone marrow derived macrophages has been described previously in EAE[32] and EAU.[29 33] Macrophages are not integral to tissue destruction and the role for T cells as potent effectors in EAU is supported by previous data which showed that intranasal tolerance therapy combined with mycophenolate mofetil immunosuppression did not protect against marked tissue destruction and ROS loss despite an absent granulocyte and macrophage infiltrate.[22] It has to be noted, however, that tolerance induction was administered after immunisation and in these experiments tolerance was induced before immunisation. The mechanisms therefore, which are presently not clearly defined, may be fundamentally different.

These results stress previous experimental findings which document suppression of systemic Th1 responses and tissue damage via generation of regulatory cells during low dose tolerance therapy, although in this model tissue infiltration still occurs. How, therefore, does tolerance therapy suppress tissue damage if infiltrating leucocytes are present within the target organ? Recent unpublished data show that in addition to generation of regulatory cells, Th2 responses (IL-10 and IL-4 production) in regional drainage lymph nodes and spleen predominate probably as a response to Th1 T cell suppression rather than generation of regulatory Th2 T cells directly inhibiting Th1 cells.[34] Although we do not know if these cells are antigen specific or not, the non-destructive IL-10^+/IL-4^+ retinal T cell infiltrate may represent trafficking of Th2 cell population from mucosal drainage lymph nodes and spleen to the eye. One proposal, therefore, is as a consequence of downregulating Th1 cytokine production, suppression of both macrophage activation and tissue damage occurs, akin to effects of neutralising TNF-α production.[25 26] We have been unable to demonstrate TGF-β secreting cells in our tolerance model to date, but our data similar to other models of tolerance show that increased production of one or more of IL-10, IL-4, and TGF-β are produced by or at least concomitant with generating regulatory cells during tolerance induction and subsequent suppression of disease.[35] Both TGF-β and IL-10, independently or synergistically, are capable of directing the immune response towards a Th2 response.[36]

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1 Rizzo LV, Caspi RR. Immunotolerance and prevention of ocular autoimmune disease. *Curr Eye Res* 1995;**14**:854–64.
2 Caspi RR, Roberge FG, McAllister CG, *et al*. T cell lines mediating experimental autoimmune uveoretinitis (EAU) in the rat. *J Immunol* 1986;**136**:928–33.
3 Forrester JV, Liversidge J, Dua HS, *et al.* Comparison of clinical and experimental uveitis. *Curr Eye Res* 1990;**9**:75–84.
4 Gery I, Mochizuki M, Nussenblatt RB. Retinal specific antigens and immunopathogenic processes they provoke. *Progr Retinal Res* 1986;**5**:75–109.
5 Nussenblatt RB, Caspi RR, Mahdi R, *et al.* Inhibition of S-Ag induced experimental autoimmune uveoretinitis by oral induction of tolerance with S-Ag. *J Immunol* 1990;**144**:1689–95.
6 Dick AD, Cheng YF, McKinnon A, *et al.* Nasal administration of retinal antigens suppresses the inflammatory response in experimental allergic uveoretinitis. *Br J Ophthalmol* 1993;77:171–5.

7 Nussenblatt RB, Gery I, Weiner HL, *et al*. Treatment of uveitis by oral administration of retinal antigens: results of a phase I/II randomised masked trial. *Am J Ophthalmol* 1997;**123**:583–92.

8 Gregerson DS, Obstrisch WF, Donoso LA. Oral tolerance in experimental autoimmune uveoretinitis: distinct mechanisms of resistance are induced by low zone versus high zone feeding protocols. *J Immunol* 1993;**151**:5751–61.

9 Metzler B, Wraith DC. Inhibition of experimental autoimmune encephalomyelitis by inhalation but not oral administration of the encephalitogenic peptide: influence of MHC binding affinity. *Int Immunol* 1993;**5**:1159–65.

10 Melamed DAF. Direct evidence for anergy in T lymphocytes tolerised by oral administration of ovalbumin. *Eur J Immunol* 1993;**23**:935–42.

11 Khoury SJ, Hancock WW, Weiner HL. Oral tolerance to myelin basic protein and and natural recovery from experimental autoimmune encephalomyelitis are associated with down regulation of inflammatory cytokines and differential upregulation of trnasforming growth factor beta, interleukin 4 and prostaglandin E expression in the brain. *J Exp Med* 1992;**176**:1355–64.

12 Dick AD, Cheng YF, Liversidge J, *et al*. Immunomodulation of experimental autoimmune uveoretinitis: a model of tolerance induction with retinal antigens. *Eye* 1994;**8**:52–9.

13 Bai XF, Shi FD, Xiao BG, *et al*. Nasal administration of myelin basic protein prevents relapsing experimental autoimmune encephalomyelitis in DA rats by activating regulatory cells expressing IL-4 and TGF-beta mRNA. *J Neuroimmunol* 1997;**80**:65–75.

14 Myers LK, Seyer JM, Stuart JM, *et al*. Suppression of murine collagen-induced arthritis by nasal adminiatration of collagen. *Immunology* 1997;**90**:161–4.

15 Shi FD, Bai XF, Li HL, *et al*. Nasal tolerance in experimental autoimmune myasthenia gravis (EAMG): induction of protective tolerance in primed animals. *Clin Exp Immunol* 1998;**111**:506–12.

16 Tian J, Atkinson MA, Clare-Salzer M, *et al*. Nasal administration of glutamate decarboxylase (GAD65) peptides induces Th2 responses and prevents murine insulin-dependent diabetes. *J Exp Med* 1996;**183**:1561–7.

17 Dick AD, Cheng YF, Liversidge J, *et al*. Intranasal administration of retinal antigens suppresses retinal antigen-induced experimental autoimmune uveoretinitis. *Immunology* 1994;**82**:625–31.

18 Laliotou B, Liversidge J, Forrester JV, *et al*. Interphotoreceptor retinoid binding protein is a potent tolerogen in Lewis rat: suppression of experimental autoimmune uveoretinitis is retinal antigen specific. *Br J Ophthalmol* 1997;**81**:1–7.

19 Chen Y, Inobe JI, Kuchroo VK, *et al*. Oral tolerance in myelin basic protein T cell receptor transgenic mice: suppression of autoimmune encephalomyelitis and dose-dependent induction of regulatory cells. *Proc Natl Acad Sci USA* 1996;**93**:388–91.

20 Bai XF, Li HL, Liu JQ, *et al*. Complexities of applying nasal tolerance induction as a therapy for ongoing relapsing experimental autoimmune encephalomyelitis (EAE) in DA rats. *Clin Exp Immunol* 1998;**111**:205–10.

21 Thurau SR, Chan CC, Nussenblatt RB, *et al*. Oral tolerance in a murine model of relapsing experimental autoimmune uveoretinitis (EAU): induction of protective tolerance in primed animals. *Clin Exp Immunol* 1997;**109**:370–6.

22 Dick AD, Kreutzer B, Laliotou B, *et al*. Effects of mycophenolate mofetil on nasal mucosal tolerance induction. *Invest Ophthalmol Vis Sci* 1998;**3**: 835–40.

23 Kreutzer B, Laliotou B, Cheng Y, *et al*. Nasal antigen administration of retinal antigens maintains immuosuppression of uveoretinitis in cyclosporin A treated Lewis rats: future treatment of endogenous posterior uveoretinitis. *Eye* 1997;**11**:445–5.

24 Barton K, Lightman S. T lymphocyte effector mechanisms in the retina in posterior uveitis. *Eye* 1994;**8**:60–5.

25 Dick AD, Duncan L, Hale G, *et al*. Neutralizing TNF-alpha activity modulates T cell phenotype and function in experimental autoimmune uveoretinitis. *J Autoimmun* 1998;**11**: 255–64.

26 Dick AD, McMenamin PG, Korner H, *et al*. Inhibition of tumour necrosis factor activity minimises target organ damage in experimental autoimmune uveoretinitis despite quantitatively normal activated T cell traffic to the retina. *Eur J Immnol* 1996;**26**:1018–25.

27 Wolvers DA, van der Cammen MJ, Kraal G. Mucosal tolerance is associated with, but independent of, up-regulation Th2 responses. *Immunology* 1997;**92**:328–33.

28 Prussin C, Metcalfe D. Detection of intracytoplasmic cytokine using flow cytometry and directly conjugated anti-cytokine antibodies. *J Immunol Meth* 1995;**188**:117–28.

29 Forrester JV, Huitinga I, Lumsden L, *et al*. Marrow-derived activated macrophages are required during the effector phase of experimental autoimmune uveoretinitis in rats. *Curr Eye Res* 1998;**17**:426–37.

30 Hoey S, Grabowski PS, Ralston S, *et al*. Nitric oxide accelerates the onset and increases the severity of experimental autoimmune uveoretinitis through an IFN-γ dependent mechanism. *J Immunol* 1997;**159**:5132–42.

31 Powrie FDM. Ox22high CD4+ T cells induce wasting disease with multiple organ pathology: prevention of the OX-22low subset. *J Exp Med* 1990;**172**:1701–8.

32 Huitinga I, van Rooijen N, de Groot CJA, *et al*. Suppression of experimental allergic encephalomyelitis in Lewis rats after elimination of macrophages. *J Exp Med* 1990;**172**: 1025–33.

33 Caspi RR, Chan CC, Fujino Y, *et al*. Recruitment of antigen-nonspecific cells plays a pivotal role in the pathogenesis of a T cell-mediated organ-specific autoimmune disease, experimental autoimmune uveoretinitis. *J Neuroimmunol* 1993;**47**:177–88.

34 Laliotou B, Duncan L, Dick AD. Intranasal administration of retinal antigens induces transient T cell activation and apoptosis within drainage lymph nodes but not spleen. *J Autoimmun* 1999 (in press).

35 Yotsukura J, Huang H, Singh AK, *et al*. Regulatory cells generated by testicular tolerization to retinal S-Antigen: possible involvement of IL-4, IL-10 and TGF-beta in the suppression of experimental autoimmune uveoretinitis. *Cell Immunol* 1997;**182**:89–98.

36 D'Orazio TJ, Niederkorn JY. A novel role for TGF-beta and IL-10 in the induction of Immune privilege. *J Immunol* 1998;**160**:2089–98.