Single dose intranasal administration of retinal autoantigen generates a rapid accumulation and cell activation in draining lymph node and spleen: implications for tolerance therapy

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Abstract

Background/claims—A single intranasal delivery of retinal autoantigen suppresses effectively experimental autoimmune uveoretinitis (EAU). To further unravel underlying mechanisms the authors wished to determine, firstly, the kinetics of antigen delivery and, secondly, the early cellular responses involved in the initial stages of nasal mucosal tolerance induction.

Methods—Flow cytometry, cell proliferation assays, and microscopy were used to track antigen following a single, intranasal dose of Alexa-488 labelled retinal antigen.

Results—A rapid accumulation of antigen within both superficial cervical lymph nodes (SCLN) and spleen was observed after 30 minutes. Significant proliferative responses to IRBP were elicited by 48 hours indicating that systemic priming of naive T cells to retinal antigen had occurred. Cell activation was further confirmed by immunoprecipitation studies, which demonstrated phosphorylation of STAT4 but not STAT6 in both lymph nodes and spleen. However, at 24 hours, STAT4 heterodimerisation with STAT 3 was only observed in spleen.

Conclusions—The results provide novel evidence that following a single intranasal application rapid transfer of antigen occurs. Resulting T cell proliferation develops consequent to differential cell signalling in SCLN and spleen. Further understanding of these underlying cellular mechanisms, in particular as is inferred by the results the contribution of local versus systemic tolerance induction, may assist in strategies to clinically apply mucosal tolerance therapy successfully.

(Br J Ophthalmol 2001;85:1001–1006)

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Accepted for publication
2 April 2001

resulting in destruction of photoreceptors that clinicopathologically closely resembles uveitis in humans.⁴ Antigen specific suppression of EAU is obtained consistently when retinal autoantigen is delivered before immunisation via a variety of mucosal surfaces^{5–8} and with appropriate immunosuppression, some protective immunity can be also be induced in primed animals.^{9–11} However, clinical trials in multiple sclerosis, rheumatoid arthritis, and uveitis have underlined the need to fully define the parameters involved in mucosal tolerance to avoid worsening ongoing autoimmune disease.¹² Recent research has focused on the antigen used and its presentation to the immune system, including both peptide affinity for MHC class II binding¹³ as well as inherent hierarchical ability of T cell epitopes to induce tolerance.¹⁴ Together with data on the dosage and timing of treatment, these important observations indicate that routes of administration such as nasal delivery and intravenous injection¹⁵ that avoid proteolytic degradation and therefore deliver tolerogenic doses of antigen accurately will be important therapeutically.

With respect to nasal tolerance induction, evidence in support of intranasal antigen administration regulating CD4+ T cell function is accumulating. Recent data in allergy, and autoimmune models including EAU, have shown that mucosal administration of autoantigen results in early activation followed by apoptosis of T cell populations and invoke a role for IL-10 in the generation of immunological non-responsiveness.^{1–3 16–19} Notably, in the EAU model, we demonstrated that although regulatory cells were generated, tolerance could only be transferred from spleen, and cells that did infiltrate the retina in treated animals produced IL-10.¹⁹ The data imply an important role for antigen presenting cells in the process, and a pivotal role for the regional drainage lymph nodes in the induction of tolerance has been shown.²⁰ However, the cellular mechanisms involved remain undefined. If antigen specific T cell activation and proliferation is an essential first step in the process, then presentation of antigen on functionally mature dendritic cells (DC) must be involved.²¹

The respiratory tract contains a contiguous network of dendritic cells (RTDC) that have a rapid turnover particularly in response to local inflammatory stimuli.^{22 23} In both rats and mice data have shown that freshly isolated RTDC

Models of tolerance induction via the mucosa have been shown to be effective for the prevention of several autoimmune diseases. The effectiveness of the treatment is dependent on parameters such as the antigen used, its dose and route of delivery, and various mechanisms have been identified, including T cell anergy, generation of regulatory Th3 (TGFβ producing) cells or CD8+ T cells and γδ T cells.^{1–3} Models used include experimental autoimmune uveoretinitis (EAU), an organ specific CD4+ T cell mediated ocular inflammation

are functionally immature and following an OVA pulse are capable of presenting antigen to naive T cells.^{24,25} Functional immaturity in DC is linked to their capacity to induce peripheral tolerance rather than immunity^{21,26}; however, the ability of RTDC to provide a “default” tolerogenic response *in vivo* may also rely on the microenvironment provided by the cervical regional drainage lymph nodes.²⁰ Soluble antigen not processed by RTDC but entering the lymphatics and blood stream may also be important as systemic presentation of soluble antigen (involving both lymph nodes and spleen), in the absence of inflammatory stimuli, has also been linked to the induction of peripheral tolerance (reviewed by Liblau *et al*²⁵). Tolerance induction is rapid, as we have recently shown following a single intranasal dose of retinal antigen, which suppressed EAU.²⁷ In this preliminary study, therefore, we wished to document the early events during induction of nasal mucosal tolerance induction by observing, firstly, the kinetics of antigen distribution to superficial cervical lymph nodes (SCLN) and spleen and, secondly, cellular activation via cell proliferation and immunohistochemistry of signalling proteins—for example, signal transducer and activator of transcription (STAT) expression.

Methods

ANIMALS, ANTIGEN, AND ANTIGEN ADMINISTRATION

Adult female Lewis rats (Charles Rivers, UK), 6–9 week old, were housed in the Biological Services Unit, University of Aberdeen under non-specific pathogen-free conditions. Animals used in all experiments were treated according to the ARVO statement for the use of animals in ophthalmic and vision research. S-antigen (S-Ag), interphotoreceptor retinoid binding protein (IRBP), or retinal extract (RE; containing a mixture of these and other soluble uveitogenic antigens) induce tolerance to S-Ag, IRBP, or RE induced EAU.^{5,9,28} RE was prepared as described⁹ by hypotonic lysis of freshly dissected bovine retinas in the dark. S-Ag and IRBP were purified from retinal extract under aseptic conditions and filter sterilised as previously described.²⁹ For kinetic experiments, antigen (maximum of 2 mg/ml) was fluorescently labelled with Alexa-488 labelling kit (Molecular Probes Inc, Leiden, Netherlands), following the manufacturer's instructions. Free excess dye was removed with extensive dialysis against phosphate buffered saline (PBS). Labelled antigen was detected on western blots with rabbit polyclonal anti-Alexa-488 antibody (Molecular Probes Inc) and was functional in proliferation assays (data not shown). Single doses of intranasal antigen in PBS (30 µl; either total S-Ag dose of 60 µg or RE dose of 150 µg) or PBS alone as control were administered directly via an Oxford pipette.

T CELL PROLIFERATION ASSAY

Proliferation assays were performed on both lymph node and spleen preparations following intranasal administration of antigen. Cells were

seeded in quadruplicate at 2×10^5 /well into round bottomed 96 well plates in either compete media alone, media containing 20 µg/ml IRBP, or control stimulation with PPD (20 µg/ml) or Con A (2.5 µg/ml). After 72 hours of incubation at 37°C, 5% carbon dioxide cells were pulsed with 0.5 µCi of 3[H]-thymidine for a further 16 hours. Plates were then harvested and counts/min (CPM) recorded as mean (SD) (Packard matrix system). Stimulation index (mean CPM of quadruplicate cultures with stimulant divided by mean CPM of quadruplicate control cultures) was used to present proliferation data, as experiments were carried out on separate days.

MICROSCOPIC EXAMINATION OF CELLS AND TISSUE AND IMMUNOHISTOCHEMISTRY

Lymph nodes and spleen were dissected at given time points after intranasal administration of retinal antigen. Tissue was analysed for detection of (a) Alexa-488 labelled antigen by scanning laser confocal microscopy (Bio-Rad MRC 1024), and (b) STAT 4 and STAT 6 by immunohistochemistry (alkaline phosphatase antialkaline phosphatase; APAAP). Fresh frozen tissue in OCT was used for sectioning. Serial 5–10 µm sections were cut, air dried and, for STAT immunostaining, fixed in 100% ice cold acetone. Following rehydration in TRIS buffered saline (TBS), sections were blocked with TBS/1% normal rabbit serum and then avidin D block solution (Vector Laboratories, USA). STAT staining was performed using rabbit polyclonal antibodies (STAT4, C20; STAT6, M20, Santa Cruz Biotechnology, CA, USA) using appropriate blocking peptides as negative control and detected with rat serum absorbed antirabbit IgG-AP conjugate and APAAP substrate (Vector Labs). Sections were lightly counterstained with haematoxylin.

WESTERN BLOT ANALYSIS OF STAT PROTEINS

STAT expression was further analysed by immunoblotting of immunoprecipitates prepared from nuclear and cytosolic cellular extracts from spleen and pooled lymph node lymphocyte populations. Briefly, cytosolic extracts were prepared from 10^7 cells in 20 µl lysis buffer A (10 mM HEPES, 1.5 mM MgCl₂, 10 mM KCl 0.1%, nonidet P40, 0.2 mM PMSF, 0.5 mM DTT) pH 7.9. Nuclei were removed by centrifugation and supernatants collected as the cytoplasmic fractions. Nuclei were then incubated in 20 µl lysis buffer B (20 mM HEPES, 0.42M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.1% nonidet P40, 2 mM PMSF, 0.5 mM DTT) pH 7.9, centrifuged, and supernatants collected as nucleic fractions. Protein estimates were made on all fractions and then adjusted to contain equivalent protein concentrations, thus permitting equal loading (protein amount) of wells. STAT 4 or STAT 6 associated proteins were immunoprecipitated by anti-STAT 4 or anti-STAT 6 antibodies (see above), collected on Protein-A-sepharose beads (Amersham-Pharmacia) and resolved by SDS-PAGE on gradient (7–14%) minigels (Pharmacia, Sweden). Protein was transferred onto Hybond PVDF membrane

(Amersham Life Science) by electroblotting. Initial probing was performed for phosphotyrosine (PTY) (mouse anti-phosphotyrosine mAb 4G10; Upstate biotechnology, USA) detected by enhanced chemiluminescence (ECL) with rat absorbed anti-rabbit IgG-HRP conjugate. After stripping (stripping buffer: TRIS-HCl pH 6.7, 2% SDS, 100 mM 2-mercaptoethanol), membranes were re-exposed to confirm removal of signal, and re-probed for further analysis of STAT 4, 6 or STAT3 (C20 Santa Cruz). Preincubation of STAT antibodies with blocking peptides was used to control for specificity of the signal.

Results

SINGLE DOSE INTRANASAL APPLICATION OF ALEXA-488 LABELLED ANTIGEN RAPIDLY ACCUMULATES WITHIN REGIONAL DRAINAGE LYMPH NODE (SCLN) AND SPLEEN ALLOWING NAIVE T CELL PRIMING

Following direct intranasal administration of 30 μ l Alexa-488 labelled RE, animals were sacrificed at varying time points and spleen and SCLN dissected and snap frozen for subsequent scanning laser confocal microscopy. Figure 1 shows that by 30 minutes Alexa-488 fluorescence was detected within both SCLN and spleen, which had markedly diminished by 24 hours. Examination of other lymph nodes (submandibular, internal jugular, parathyroid, mediastinal, and mesenteric) indicated the antigen also reached these sites with similar kinetics (data not shown). By 24 hours Alexa-488 RE could still be detected within cells of SCLN (Fig 1C, arrow), but not spleen or other non-draining nodes, although systemic presentation of antigen was evident as proliferative T cell responses could be elicited from both SCLN and spleen. Naive T cell priming was assessed by lymph node and splenocyte proliferative responses to IRBP. Although IRBP is a known immunogen that induces EAU, when administered nasally either alone or within RE (that contains 4–8% of IRBP⁵), it is a potent

Figure 1 Alexa-488 labelled antigen is present in both draining LN and spleen by 30 minutes post-tolerisation. Figure represents merged (red and green fluorescent) confocal images of Alexa-488 labelled S-Ag accumulation within superficial cervical lymph node (SCLN), top panels (A–C); and spleen, bottom panels (D–F), following intranasal antigen administration. By 30 minutes green fluorescing antigen was detected in both SCLN and spleen and was maximal by 4 hours (not shown, FITC 488 nm channel saturated). At 24 hours there was residual detection of labelled antigen in SCLN (arrow) present in green channel only, distinct from autofluorescent tissue resident cells detected in both red and green channels, which appear yellow/orange.

Figure 2 Antigen specific proliferation in both SCLN and spleen following a single nasal delivery of retinal extract. Splenocyte (solid bar) and SCLN (open bar) stimulation by IRBP, a potent tolerogen in RE. Histogram represents stimulation index (SI; mean of quadruplicate cultures with stimulant divided by mean of quadruplicate control cultures). Data representative of three similar experiments. Maximum Con A responses were 21 745.8 (SD 5420.7) in lymph node and 110 750 (21 745) in spleen. Maximal background levels were 468.3 (226.8) in SCLN to 3991.5 (188.5) in spleen. By 48 hours both SCLN and spleen generated significant proliferative responses to IRBP stimulation (SI of 2.4 and 6.4, respectively).

inducer of tolerance, protecting against RE induced EAU.²⁸ Figure 2 shows that both spleen and SCLN show significant proliferative responses to IRBP (SI of 2.4 rising to 3.5 at 96 hours for SCLN and an SI of 6.4 for spleen at 48 hours); data representative of three separate experiments) by 48 hours after intranasal antigen administration.

ADMINISTRATION OF RETINAL ANTIGEN INTRANASALLY GENERATES CHANGES IN STAT TRANSCRIPTION FACTORS

We have previously observed that repeated intranasal doses of retinal antigen induce an IFN- γ burst with increased CD4⁺ T cells numbers within SCLN but not spleen, and that on subsequent antigen challenge there is increased T cell apoptosis in SCLN.¹⁹ We wished to ascertain the overall effect of cytokine signalling in the tissues following a single intranasal application of antigen which might be associated with our observed previous changes and the T cell proliferation responses we had currently noted. STAT 6 and STAT4 mediate specific IL-4 and IL-12 signalling respectively, and are important regulators of T cell function.³⁰ Experiments were therefore repeated and SCLN and spleen were analysed for evidence of Th1 (IL-12 mediated STAT 4 activation) or Th2 type cell activation (IL-4 mediated STAT 6 activation) at varying times following a single intranasal application of retinal antigen. Figure 3 shows kinetics in STAT expression in tissue. In SCLN, STAT 6 expression appeared to diminish over first 24 hours after intranasal administration, whereas STAT 4 expression (low in normal SCLN) peaked at 24 hours. In spleen both STAT 4 and 6 expression increased over 96 hours. Upregulation of STAT 4 at 24 hours after treatment was not observed in the submandibular or mesenteric nodes.

The change in STAT patterns of expression were further investigated for evidence of activation (nuclear translocation) by western

Figure 3 STAT 4 expression transiently increases over 24 hours in SCLN. APAAP immunohistochemical analysis of STAT 4 and 6 expression in SCLN and spleen following antigen nasal delivery. A transient increase in expression of STAT 4 with decreased STAT 6 expression occurred at 24 hours in cervical lymph nodes (SCLN) (all $\times 125$), followed by a reduction in STAT 4 by 96 hours ($\times 62.5$). In contrast, the spleen showed weak constitutive expression of both STAT 4 and STAT 6 (0 hours) ($\times 62.5$) although this was increased at later time points, with strong, focal expression of both at 96 hours after treatment ($\times 125$). Controls with appropriate blocking peptides did not demonstrate any STAT expression or background colour. Data representative of three separate experiments.

blotting of STAT 4 and STAT 6 immunoprecipitates from cytoplasmic and nuclear extracts of SCLN and spleen. Because tyrosine phosphorylation of STAT proteins is associated with, and is required, for the binding of STATs to conserved promoter elements within the nucleus anti-phosphotyrosine (PTY), immunoblotting was also carried out. Figure 4 shows that STAT 6 protein expression in SCLN was generally low and fluctuated in cytoplasmic extracts, and although higher expression was found in spleen nuclear extracts, this appeared to be constitutive and was not phosphorylated. In contrast, STAT 4 and coprecipitated STAT 3 expression appeared to fall in the SCLN cytoplasm, suggesting dissociation of heterodimers with time, although notably, STAT4/STAT3 heterodimers increased in cytoplasmic extracts from spleen at 24 hours. STAT 4 expression in the nuclear extracts remained strong and was not associated with STAT 3 after treatment. STAT 4 immunoprecipitates were tyrosine phosphorylated.

Discussion

Evidence to date supports distinct mechanistic differences in tolerance induction between nasal and systemic antigen delivery, primarily because without drainage lymph nodes, tolerance is abrogated. Such inference is supported by the many reports that have shown the nasal compartment as an extremely efficient route for tolerance induction to both non-pathogenic and autoantigens. The unique microenvironment that includes not only the regional drainage lymph nodes²⁰ but also the nasorespiratory mucosa and its resident cells (for example, RTDC)^{24, 25} are therefore highly selected for tolerance induction. Until recently the kinetics of such responses have been poorly understood.³¹ Following intranasal delivery of titrated tolerogenic peptide of myelin basic protein (MBP), [3H]Ac1-9[4y], concentrations (as well as presence of intact peptide) were high in draining lymph nodes and spleen between 2.5–4 hours of peptide delivery and significant levels were observed in blood and

Figure 4 Western blot analysis of phosphorylation and heterodimer formation by STAT4 and STAT6 in both SCLN and spleen following a single nasal delivery of retinal extract. STAT6 or STAT4 and associated proteins (STAT3) were immunoprecipitated (IP) from cytoplasmic and nuclear extracts from SCLN and spleens from rats at 0, 4, and 24 hours after intranasal delivery of RE antigen. Equivalent quantities of protein were run on SDS gel (lanes 1–6), blotted onto nitrocellulose, and probed with antibody 4G10 to detect phosphorylated tyrosine residues (PTY). Blots were the stripped and reprobed for STAT proteins as indicated. Immunoblot analysis of resolved proteins indicated STAT4 phosphorylation (PTY) in both cytoplasmic and nuclear extracts at all time points. Phosphorylation of STAT6 proteins was very weak or absent by comparison. Some STAT4/STAT3 heterodimers were constitutively present but these appeared to dissociate over time, with the exception of spleen cytoplasm at 24 hours after treatment which stained strongly for co-precipitated STAT3. STAT4/STAT3 heterodimers were detectable in the SCLN nuclei of normal but not treated rats. IP denotes antibody used to immunoprecipitate protein, Ab denotes antibody used to stain blot.

lymphatics, peaking at around 4 hours. Our present data also support rapid traffic of antigen from the nasal mucosa to drainage lymph nodes and spleen, as early as 30 minutes after delivery (Fig 1).

One of the major mechanisms of active suppression in mucosal tolerance highlighted to date is the generation regulatory T cells.^{32–33} However, the cellular mechanisms preceding either active suppressor mechanisms or T cell anergy/deletion in EAU are not fully identified.

Application of soluble antigens via mucosal surfaces leads to a transient activation of T cells in the drainage lymph nodes and Peyer's patches of the gut and apoptosis¹⁷ (reviewed by Lowrey *et al*² and Wardrop and Whitacre³). Such activation induced cell deletion is not complete and the resulting T cell populations are unresponsive to further stimulation³⁴ but may still produce IL-10.^{18–19} Further to our previous reports of T cell activation and apoptosis within SCLN following repeated intranasal antigen administration, we now show that even a single dose (able to actively suppress EAU²⁸) of retinal extract (a mixture of soluble retinal antigens), results in a predominance of phosphorylated STAT4 in both SCLN and spleen and T cell priming and proliferation to IRBP, a major tolerogen²⁷ within retinal extract. As STAT4 is highly phosphorylated within 5 minutes of IL-12 receptor engagement, constitutive expression of the activated form of this STAT within the lymphoid tissues of normally housed rats (non-SPF condition) is perhaps not surprising.³⁵ Co-precipitation of STAT3 with STAT4 heterodimers as well as formation of STAT4 homodimers is specific to IL-12 signalling and results in augmented IFN- γ secretion from Th1 cells.³⁶ The reduction in co-precipitated STAT3 protein over 24 hours in the SCLN and absence of STAT3

from the nuclear fraction of treated lymphoid tissues is consistent with a reduction or dissociation of heterodimer formation, and may represent an association of STAT4 with another component. One notion is that our observed early proliferative responses and STAT activation may represent inductive events that lead to antigen stimulated Th1 cell anergy. Antigen stimulated Th1 anergy has been related to increased levels of p27/Kip1 cyclin dependent kinase inhibitors³⁷ and we are presently investigating such possibilities in mucosal tolerance induction. The significance of the increase in STAT3/STAT4 heterodimers at 24 hours in spleen cytoplasmic fractions is currently unexplained. It may represent additional signalling input to the spleen as several cytokines including IL-10 are known to activate STAT3. Increased availability of phosphorylated STAT3 may enable increased heterodimer formation. A mechanism of a potentially stable Th2 commitment remains obscure, but non-phosphorylation of STAT6 in our model would suggest that IL-4 dependent signalling is not involved in the initial 24 hours following intranasal exposure to antigen. STAT6 is expressed in a wide range of cell types and the patterns of expression revealed by immunostaining of lymphoid tissue staining reflect this.

The data generated following a single intranasal application of autoantigen does not support the notion of a Th1 to Th2 CD4+ T cell switch mediated via default "Th2 polarity" of RTDC function during T cell priming as previously proposed during allergic responses.²⁴ Whether IL-4 activated STAT6 or other associated molecules are involved during tolerance induction by repetitive low dose antigen administration remains to be elucidated. What is well appreciated is the involvement of cytokine activated STAT molecules in the regulation of T helper cell differentiation and apoptosis,³⁸ and the ability of regulatory cytokines such as TGF- β to modulate this process.³⁹ Despite previously demonstrating, after tolerance induction and immunisation, Th2 responses (IL-2 and IL-10 producing) from infiltrating T cells within retina,¹⁹ there remains little evidence to support a bias toward Th2 responses as a direct result of tolerance mechanisms within the lymph node.⁴⁰ Here we have shown increased STAT4 signalling, concomitant with antigen specific proliferation, signifying Th1 reactivity and is in keeping with our previous data of a transient IFN- γ burst within SCLN¹⁷ and supports an initial T cell activating event. Together with the observation by Burkhart *et al*¹⁸ that IFN- γ and IL-4 cytokine production followed different kinetics in cervical lymph nodes and spleen, our data support the possibility of separate roles for SCLN and spleen in the induction of nasal tolerance.

This work was supported by a grant from WH Ross Foundation for the Prevention of Blindness, Scotland. We would like to thank Zoe Fletcher, Linda Duncan, Rosemary Dawson, and Neil Taylor for expert technical assistance.

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