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Tolerance induction by molecular mimicry: prevention and suppression of experimental autoimmune encephalomyelitis with the milk protein butyrophilin

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Abstract

Multiple sclerosis (MS) is a common inflammatory disease of the central nervous system. Although the etiology of MS remains unknown, studies in experimental autoimmune encephalomyelitis (EAE) have suggested that foreign molecules, which show molecular mimicry with myelin antigens, may play an important role as causative agents of the human disease. In this study, we investigate the molecular mimicry between the extracellular Ig-like domain of the cow's milk protein butyrophilin (BTN) and the extracellular domain of myelin oligodendrocyte glycoprotein (MOG), a candidate autoantigen in MS. Interestingly, we found that as a result of a non-pathogenic cross-reactivity that is localized to a subdominant region of MOG, treatment of C57BL/6 mice with BTN either before or after immunization with MOG was shown to prevent and also suppress the clinical manifestations of EAE. BTN treatment resulted in a significant reduction in both proliferation and production of T_h1-related cytokines (IFN-γ, IL-2, IL-12 and granulocyte macrophage colony stimulating factor) in response to MOG. This specific inhibition was consistently associated with an up-regulation in IL-10 secretion. Furthermore, adoptive transfer of BTN-specific T cells prior to active immunization with MOG resulted in a transitory reduction of the clinical symptoms. Our results suggest that the clinical improvement associated with BTN treatment involved the combination of both anergy and regulatory cells secreting high levels of IL-10. In conclusion, we show that despite the traditional link between molecular mimicry and pathogenic immune response, environmental agents that share homology with autoantigens may also represent a source of cells with a protective phenotype.

Introduction

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS) that causes demyelination and axonal degeneration resulting in the disruption of neurological functions (1). MS is considered to be an organ-specific autoimmune disorder that involves selective activation of T and B lymphocytes reactive against myelin autoantigens (2,3). Although the mechanisms underlying the initiation and progression of MS remain poorly understood, converging lines of evidence suggest that both genetic predisposition and exposure to undefined environmental agents proceed synergistically to trigger an autoimmune reaction against CNS myelin antigens (4,5). Evidence in favor of this model comes largely from epidemiological family studies that illustrate the lack of complete concordance among monozygotic twins (25–

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30%), stressing the role of additional non-genetic variables in the genesis of MS (6). Infectious organisms, particularly viruses, have been commonly associated with the occurrence of autoimmune disorders (7–9). The pathogenic mechanism by which this potential threat may instigate an autoimmune response remains incompletely defined (9). Nevertheless, due to the high degeneracy of T cell recognition (10–12), the concept of molecular mimicry in which structural similarity is shared by foreign antigens and self-proteins has offered a viable hypothesis to explain the activation of self-reactive specific T cells (13,14).

In MS, the molecular mimicry hypothesis is supported by the finding that antigenic determinants of certain viruses are able to stimulate myelin basic protein (MBP)-specific $CD4^+$ T cell clones, presumably based on their structural homology to MBP (15). Moreover, studies undertaken in experimental autoimmune encephalomyelitis (EAE), an autoimmune CNS disorder broadly studied as an animal model of MS, have also shown that virus sequences that share similarity with myelin autoantigens can trigger a cross-reactivity *in vivo* that ultimately results in the development of clinical signs of the disease (16,17). In contrast, recent studies have shown that certain MBP-mimicking peptides derived from human papilloma virus and *Bacillus subtilis* were able to protect against EAE by means of cross-reactivity with self-peptide $MBP_{87–99}$ (18). Therefore, the notion that foreign antigens specifically interplay with autoaggressive T cells brings forward a new scenario wherein molecular mimicry may also assume a protective role in autoimmune diseases.

Epidemiological and recent experimental studies have raised the possibility that dietary compounds containing proteins that cross-react with myelin antigens may also have a role in the pathogenesis of the disease (19–21). Enhanced T cell reactivity against several cow's milk proteins, such as BSA, has been reported in MS patients (21). Furthermore, $BSA_{193–204}$ was demonstrated to be highly immunogenic in SJL/J mice and probably, as a result of its homology with the exon 2 of MBP, was able to induce clinical signs of EAE in some of these mice (21). Similarly, based on the homology shared by the extracellular IgV-like domain of butyrophilin (BTN), a major protein of the milk fat globule membrane (22,23), and the myelin oligodendrocyte glycoprotein (MOG) (24), a candidate target for the pathogenic autoimmune response in MS (25,26), immunization of Dark Agouti rats with BTN resulted in pathological damage in the CNS (27). Since the response to MOG in humans appears to be highly heterogeneous, characterized by different immuno-dominances among individuals (28), it is the purpose of this paper to further evaluate the deleterious and/or protective role of the milk protein BTN in C57BL/6 mice, a strain that is susceptible to MOG-induced EAE. This may, therefore, further characterize the relevance of this structural homology with respect to human disease. Here, we present evidence that the cross-reactivity between the two proteins entails a preventive and protective response against the disease, rather than representing an encephalitogenic menace. Thus, BTN treatment was able to not only prevent EAE by subsequent immunization with MOG, but also ameliorate the clinical manifestations of a pre-existing disease. Furthermore, the underlying mechanisms of this protection appear to be associated with an inhibition of the T_h1 encephalitogenic response to MOG that seems to be mediated by the generation of anergy and/or regulatory T cells.

Methods

Mice

Female C57BL/6 (B6; H-2^b) and NOD/Lt (H-2^{g7}) mice aged 12–15 weeks, were obtained from the Central Animal House at La Trobe University. All experiments involving animals and their care were performed under an approved protocol in accordance with the Australian code of practice for the care and use of animals for scientific purposes (NHMRC, 1997).

Antigens

Recombinant proteins consisting of the N-terminal Ig-like domain of human MOG (amino acids 1–120) and the first Ig-like domain of bovine BTN (amino acids 1–115) both containing an N-terminal six histidine tag, were expressed in *Escherichia coli* M15(pREP4) and purified as described by Ni-NTA superflow (Qiagen) chromatography. An alternating organic wash with 60% isopropanol was included for better removal of contaminants as well as endotoxins from the Ni-NTA beads or recombinant protein, as previously described (29). Eluted proteins were filter sterilized (0.2 μm) before stored at – 20°C until further use. Purity was verified by SDS–PAGE and protein concentration was determined via the BCA protein assay (Pierce). Bacterial endotoxin levels were <0.1 EU/ml as determined by the Limulus amebocyte lysate assay (Sigma, St Louis, MO). The his-tagged ubiquitin glutathione S-transferase (His-Ub-GST) was kindly provided by Dr Rohan Baker and purified as above. Keyhole limpet hemocyanin (KLH) was purchased from Sigma. $MOG_{35–55}$ (MEVGWYRSP-FSRVVHLYRNGK) was synthesized by Auspep (Melbourne, Australia). A set of overlapping (by three residues) 15mer MOG peptides covering the extracellular domain molecule of rat MOG (amino acids 1–132) was obtained from Chiron Technologies (Melbourne, Australia).

Induction of EAE and clinical evaluation

EAE was induced by immunizing animals s.c. over two sites in the thigh with either 100 μg of MOG or 150 μg of $MOG_{35–55}$ emulsified in an equal volume of complete Freund's adjuvant (CFA; Gibco/BRL) supplemented with *Mycobacterium tuberculosis* H37 RA (400 μg/mouse; Difco). Pertussis toxin (300 ng; List Biological, Campbell, CA) was injected i.v. at the time of immunization and repeated 48 h later. Animals receiving adjuvant alone served as controls. Mice were assessed daily beginning at day 5 for clinical signs of EAE and were graded according to the following scale: 0, no disease; 1, loss of tail tone; 2; hind limb weakness or partial paralysis; 3 complete hind limb paralysis and body paresis; 4 hind and front limb paralysis; 5 death. Gradations of 0.5 for intermediate scores were assigned to animals exhibiting signs of a lesser severity than any of these stages.

Pretreatment and treatment with BTN

B6 mice to be evaluated for BTN-mediated protection were injected s.c. into the hind flanks 7 days before EAE induction

Table 1. Splenic CD4+ T cell proliferation, and cytokine production of MOG- and BTN-primed B6 mice[a]

Immunization	*In vitro* stimulation	Proliferation (SI)[c]		Cytokine concentration (ng/ml)			
		CD4+ T cells[b]	Total	IFN-γ	IL-2	GM-CSF	IL-10
MOG	MOG	7.04 ± 0.32	12.7 ± 2.5	4.9 ± 0.6	0.41 ± 0.09	0.29 ± 0.08	0.18 ± 0.2
MOG	BTN	5.39 ± 0.35	5.9 ± 0.47	0.75 ± 0.08	0.11 ± 0.06	0.19 ± 0.03	1.72 ± 0.7
BTN	MOG	5.8 ± 0.84	8.7 ± 0.75	–	–	–	–
BTN	BTN	7.57 ± 1.47	11.4 ± 2	–	–	–	–

[a]B6 mice were immunized s.c. with 100 μg of either MOG or BTN. Spleen cells were collected 13 days after immunization and stimulated with 20 μg/ml of the relevant antigen for T cell proliferation or 30 μg/ml for cytokine determination. Spontaneous secretion of cytokines in the absence of antigen was subtracted.
[b]CD4+ T cells (4×10^4/well) were cultured with irradiated syngeneic splenocytes (2×10^5/well) in the presence or absence of antigen.
[c]Stimulation indexes ± SD of triplicate cultures (c.p.m. + antigen)/(c.p.m. – antigen) were calculated from individual mice ($n = 6$).

with either 200 μg of BTN or KLH emulsified in Freund's incomplete adjuvant (IFA; Difco). A second control group received adjuvant in PBS at the same time. For the treatment experiments, when mice started to show signs of paralysis they were injected i.p. with PBS or with 200 μg of either BTN or KLH emulsified in IFA.

Adoptive transfer experiments

B6 donors mice were immunized s.c. with 200 μg of either BTN or KLH emulsified in IFA. Twelve days later, their spleens were collected and stimulated *in vitro* with BTN or KLH (20 μg/ml) in the presence of IL-2 (50 U/ml) for 3 days. Cells were separated on a Ficoll gradient (Amersham Biosciences), resuspended in PBS and 3×10^7 splenocytes were injected i.v. via the tail vein of B6 recipient mice. Twenty-four hours after transfer, animals were immunized s.c. with 100 μg of MOG emulsified in CFA followed by 300 μg of pertussis toxin i.v. on days 0 and 2. EAE was scored daily as described above.

Antigen specific proliferation assay and cytokine production

To quantify the *in vitro* proliferative response to antigen, lymph nodes (LN; inguinal, popliteal, axillary and mesenteric) and spleens were obtained 13, 30 and 50 days after EAE induction. Tissues were mechanically homogenized to make a single-cell suspension and cells were seeded in triplicate in 96-well flat-bottomed plates at a concentration of 1×10^6 cells/ml in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, 10 mM HEPES, 1 mM sodium pyruvate, 2×10^{-5} M 2-mercaptoethanol and 5% heat-inactivated FCS. Cells were cultured with MOG or BTN (20 μg/ml), His-Ub-GST (40 μg/ml), concanavalin A (Con A; 2 μg/ml), heat-inactivated *Mycobacterium tuberculosis* (10 μg/ml) or medium alone in the presence or absence of rIL-2 (200 U/ml). After 48 h of incubation at 37°C with 5% CO_2 and humidified atmosphere, plates were pulsed with [^{3}H]thymidine (1.0 μCi/well; Amersham Pharmacia Biotech) for an additional 16 h before harvesting the cells. Results are expressed as mean thymidine uptake (c.p.m.) of triplicate cultures. CD4+ T cells were purified by positive selection using CD4 (L3T4) MicroBeads on an autoMACS magnetic separator (Miltenyi Biotech). CD4+ purity was verified by flow cytometry and showed >95%. Cytokine production was determined by quantitative capture ELISA according to the supplier's guidelines from 2-ml cultures containing 2×10^6 spleen or LN cells. Plates were stimulated with no antigen, MOG (30 μg/ml) and Con A (5 μg/ml). Culture supernatants were collected at 24 h to measure IL-2 and IL-4 production or at 48 h for levels of IL-12, IFN-γ, granulocyte macrophage colony stimulating factor (GM-CSF), transforming growth factor (TGF)-β and IL-10. All paired mAb used in the assay were from BD PharMingen and cytokine concentrations were calculated using standard curves generated with known amounts of recombinant proteins (Peprotech, Rocky Hills, NJ). Cytokine IL-4 mRNA expression in the spleen and LN cells was detected by RT-PCR using total cellular RNA isolated from 2×10^6 stimulated cells in the presence or absence of the relevant antigen. Total RNA was extracted using TRIzol Reagent (Invitrogen) according to the manufacturer's protocol. RNA samples were reverse transcribed into cDNA with oligo$(dT)_{20}$ primer and Superscript II reverse transcriptase (Gibco/BRL). For PCR analysis, equivalent amounts of cDNA were amplified using 2.5 U of Taq DNA polymerase (Gibco/BRL), 1 mM of each dNTP, 50 pmol of each primer, and a PCR buffer mixture containing 50 mM KCl, 10 mM Tris (pH8.3), 1.5 mM $MgCl_2$ and 0.01% gelatine. The primers used were: β-actin sense 5′-GGA CTC CTA TGT GGG TGA CGA GG-3′; antisense 5′-GGG AGA GCA TAG CCC TCG TAG AT-3; IL-4 sense 5′-GAG CCA TAT CCA CGG ATG CGA CAA-3′; antisense 5′-CAT GGT GGC TCA GTA CTA CGA GTA-3′. PCR reactions were carried out in a Perkin-Elmer Cetus 9600 Gene amp thermal cycler for 35 cycles (denaturation, 30 s at 94°C, annealing, 30 s at 55°C and elongation for 30 s 72°C). PCR products were resolved on a 1.5% agarose gel containing 0.5 μg ethidium bromide/ml and visualized under UV light.

Assay of antigen-specific IgG subclasses

Serum MOG-specific IgG subclasses were measured by ELISA. Briefly, 96-well Maxisorp microtiter plates were pretreated with 0.2% glutaraldehyde and were coated with 5 μg/ml of MOG in 10 mM carbonate buffer (pH 9.6) for 3 h at 37°C. The plates were washed 5 times with PBS/Tween 20 and then blocked with 2% BSA in 10 mM carbonate buffer (pH 9.6) overnight at 4°C in a humidified chamber. After washing, 100 μl of 1:500 dilution sera, in triplicate, was added to the wells and incubated at 37°C for 1 h. Peroxidase-conjugated anti-rat IgG1, IgG2b and IgG3 (Clonotyping System-HRP; Southern Biotechnology Associates, Birmingham, AL) were added at a final dilution of 1:800 for 1 h at 37°C. The mAb biotinylated anti-

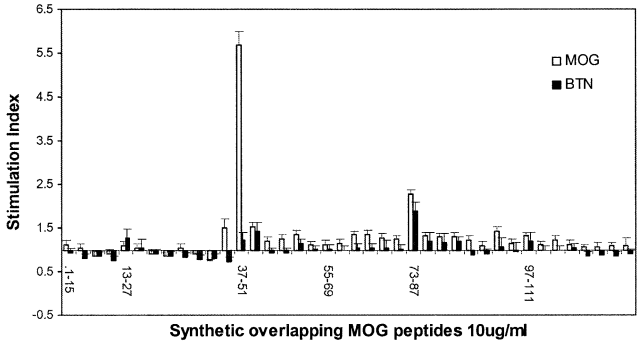

Fig. 1. T cell responses to synthetic MOG peptides encompassing the extracellular domain of MOG in B6 mice immunized with 100 μg of MOG (solid bar) or 200 μg of BTN (open bar) in CFA. Spleen cells were collected 13 days post-immunization and were cultured *in vitro* with 10 μg/ml of the indicated peptide. Data is expressed as the mean stimulation index ± SD obtained from five mice per group.

mouse IgG2a^b (Igh-1^b) (PharMingen) was used to detect anti-MOG IgG2c in the mouse sera followed by avidin–peroxidase (Sigma) for 30 min at 1:1000 dilution. ABTS was used for the development of the colorimetric reaction and optical density was read in a microplate reader using a 405-nm filter. Results were expressed as OD units.

Results

T cell cross-reactivity between MOG and BTN did not result in neurological damage.

In our initial studies, two strains of mice NOD/Lt (H-2^{g7}) and B6 (H-2^b) highly susceptible to EAE induced by MOG (25) were evaluated for susceptibility to the disease by immunization with BTN. Sensitization of animals with dosages ranging from 50 to 200 μg of BTN in CFA did not result in any clinical signs of EAE. Histopathological examinations of paraffin-embedded sections from the brain (cerebellum, pons and medulla) and spinal cord (cervical, thoracic and lumbrosacral) of mice (n = 7–9 per group) sacrificed at day 30 and 55 post-immunization demonstrated no signs of inflammation or demyelination as assessed by Hematoxylin & eosin and Luxol fast blue staining (data not shown). Although BTN was not found be encephalitogenic in the strains of mice tested, lymphocytes from BTN-immunized animals responded vigorously to the priming antigen and, more remarkably, also mounted a significant proliferative response to MOG (Table 1). Concordantly, a marked lymphoproliferative response was elicited by MOG-immunized animals in recall response to BTN, further indicating the mutual cross-reactivity between the two proteins. Splenocytes isolated from MOG and BTN immunized mice did not proliferate in response to the control proteins His-Ub-GST and KLH (SI < 1.1 and SI < 1.15 respectively). As shown in Table 1, the nature of this cross-reactivity appears to be MHC class II restricted since CD4$^+$ T cells from MOG-immunized animals showed a significant response to BTN in the presence of irradiated antigen-presenting cells. Interestingly, the cytokine profile of spleen cells from MOG-primed animals upon stimulation with BTN *in vitro* strongly differed from the T_h1-type pattern characteristic upon recall response to MOG. Thus, response to BTN significantly inhibited the production of IFN-γ, GM-CSF and IL-2, whereas the production of IL-10 was up-regulated.

We next analyzed the peptide-specific proliferation of spleen cells from MOG- and BTN-primed mice using a panel of overlapping synthetic 15mer MOG peptides encompassing the whole extracellular domain of MOG. As seen in Fig. 1, lymphocytes from MOG-primed mice showed a dominant response to the MOG sequence 37–51, whereas the dominant response of lymphocytes derived from BTN-primed mice was restricted to the MOG sequence 73–87, suggesting that the primary cross-reactivity between the two antigens did not comprise encephalitogenic regions of MOG in H-2^b mice (16,30). However, residual cross-reactivity was also found in the MOG sequence 40–54.

BTN protects B6 mice against MOG-induced EAE

Having observed that BTN was recognized by MOG-specific T cells *in vitro*, we next sought to considered whether treatment with the milk protein could possibly modulate the natural course of EAE by interfering with the encephalitogenic MOG-specific T cells. Therefore, to investigate whether BTN could confer protection to MOG-induced EAE, groups of eight to 11 mice were treated s.c. with BTN in IFA 1 week prior to challenge with MOG in CFA. Control animals received the unrelated antigen KLH, the vehicle of administration (IFA in saline) or were left untreated before MOG immunization. As shown in Fig. 2 in B6 mice, active immunization with MOG in CFA induces severe paralysis in all of the animals. As opposed to the recently described protective effect of IFA/saline in EAE (31), the clinical symptoms in the vehicle-treated animals or KLH control group were virtually indistinguishable from those exhibited by the untreated group. In contrast, administration of BTN in IFA 1 week before the encephalitogenic challenge resulted in a dramatic reduction of the clinical symptoms with a lower incidence and delayed onset than those of the control groups. Thus, only 33.3% of BTN-pretreated animals developed the disease, while the incidence of EAE in the control groups was 100%. The mean maximal clinical score in the three BTN-pretreated mice that exhibited EAE was 1 ± 1.08 as compared to 3.1 ± 0.01 in the KLH-pretreated controls, 3.57 ± 0.49 in the PBS group and 3.41 ± 0.44 in the untreated group. The lower disease severity observed in BTN affected mice was accompanied by a reduction in the number of days of illness that could be attributed to an atypical relapsing-remitting character displayed by these animals, which differed from the single acute episode that follows MOG immunization in B6 mice. The mean day of disease onset of the BTN-pretreated group was also delayed by 3 days in comparison with that of the control groups.

BTN reduces MOG-induced lymphocyte proliferation and alters cytokine responses

To ascertain whether the inhibition of the clinical signs seen in BTN-pretreated mice was associated with an altered response to MOG in the periphery, the ability of LN cells and splenocytes from all of the groups to respond to antigen *in vitro* was assayed 13 days after the encephalitogenic challenge. As seen in Fig. 3, LN cells from control animals that received KLH or PBS in IFA proliferated vigorously and to a

Table 2. BTN pretreatment depresses MOG-specific T_h1 cytokine secretion

Pretreatment	MOG-specific cytokine secretion (ng/ml)[a]						
	IFN-γ	IL-2	IL-12	GM-CSF	TGF-β	IL-10	IL-10 (day 50)
None[b]	5.1 ± 0.1	0.8 ± 0.09	0.9 ± 0.5	0.5 ± 0.09	0.19 ± 0.09	2.1±1	15 ± 1.6
PBS	4.9 ± 0.5	0.9 ± 0.1	0.9 ± 0.1	0.6 ± 0.2	0.17 ± 0.07	2.6 ± 0.5	16.9 ± 1.2
KLH	5.3 ± 0.3	0.8 ± 0.05	0.7 ± 0.2	0.8 ± 0.1	0.22 ± 0.1	2.4 ± 0.5	17.1 ± 2
BTN	0.076 ± 0.05	0.2 ± 0.06	0.09 ± 0.09	0.06 ± 0.05	0.26 ± 0.1	16.3 ± 1.6	26.5 ± 1

[a]Spleen cells were collected at day 13 and 50 (as indicated) after MOG immunization. Cytokine concentration was determined by ELISA in supernatants stimulated with 30 μg/ml MOG. Mean ± SD for eight individually mice are given. Values shown correspond to one independent experiment performed in triplicate.
[b]A control group that was not pretreated, but challenged with MOG in CFA at day 0 was also included.

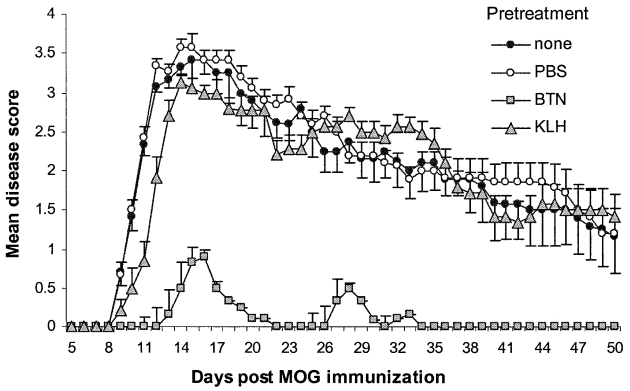

Fig. 2. Pretreatment with BTN protects mice against MOG-induced EAE. B6 mice were either left untreated (n = 10) or were pretreated with a single s.c. injection of BTN (200 μg per mouse, n = 11), KLH (200 μg per mouse, n = 8) or PBS (n = 11) emulsified in IFA. EAE was induced 7 days later by immunization with 100 μg MOG in CFA. Results are given as mean disease score ± SD. Data shown is a representative of three independent experiments.

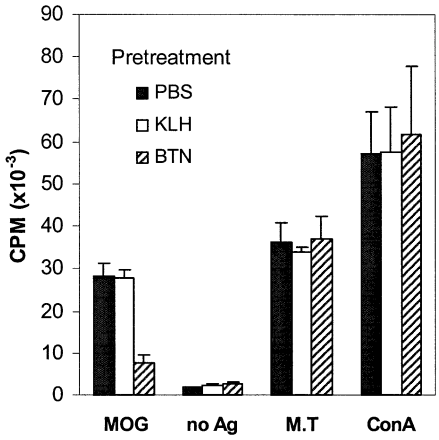

Fig. 3. Decreased LN cells proliferative response in mice pretreated with BTN. LN cells from BTN-pretreated and control groups (1×10^6 cells/ml) were harvested 13 days after MOG immunization and incubated with 20 μg/ml MOG, 2 μg/ml Con A, 10 μg/ml heat-inactivated *M. tuberculosis* or medium alone. Proliferation was determined by measuring [^{3}H]thymidine uptake. The indicated c.p.m. represents the mean ± SD of eight individually tested mice.

similar degree in response to MOG. In contrast, lymphocytes isolated from BTN-pretreated animals were significantly less responsive to *in vitro* re-stimulation with MOG. The lymphoproliferative response to Con A or *M. tuberculosis* was not affected by the BTN treatment, which confirms the competence of the T cells populations from BTN-injected mice to respond to non-specific stimulation. Comparable results were obtained when the proliferative responses of spleen cells were studied (data not shown).

In order to examine the possibility that a deviation of the characteristic T_h1 phenotype of the pathogenic MOG-specific T cells may account for the protection achieved by BTN, cytokine production of stimulated splenic cells from BTN-pretreated and control animals was studied in supernatants from cultures set up in parallel with those described above. Spleen cells from mice immunized with BTN, but not with KLH or adjuvant alone, secreted reduced amounts of T_h1-type cytokines (IFN-γ, IL-2, IL-12 and GM-CSF) in response to MOG, whereas very high levels of the T_h2 type cytokine IL-10 were readily detected in the same culture supernatants (Table 2). IL-4 has been reported to be a requisite for the generation and regulation of the T_h2-type immune response (32). Since levels of this cytokine were undetectable by the ELISA system and in order to further clarify the involvement of the T_h2 subset in the depressed T_h1 response associated with the BTN administration, transcription of IL-4 mRNA was evaluated by semiquantitative RT-PCR. No IL-4 mRNA expression in spleen or LN cells was found in any of the groups (data not shown). There were no significant differences in TGF-β production in all three groups, suggesting that the development of T cell unresponsiveness to MOG in the protected animals was not likely due to T_h3 regulatory cells secreting TGF-β. An analogous pattern of cytokine production was seen 50 days after EAE induction; however, at this time very high levels of IL-10 were also detected in culture supernatants of control mice (Table 2). This correlates with the time when the resolution of the acute phase of the disease was seen in the majority of the animals. The lack of IL-10 secretion at the height of clinical disease and the increased levels detected late during the immune response is consistent with the reported regulatory role of IL-10 during clinical recovery (33,34).

BTN alters the anti-MOG IgG antibody isotype repertoire

Cytokines are major factors for B cell differentiation (35) as they influence which class or subclass of antibody is produced. Therefore, to address the question of whether the functional change observed in the cytokine profile of mice

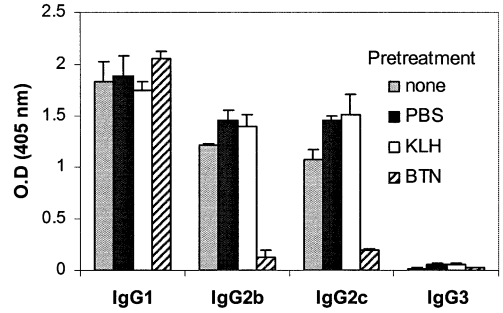

Fig. 4. Isotype profile of anti-MOG IgG antibody measured by ELISA. Sera were collected on day 50 post-EAE induction. Results are expressed as mean OD ± SD of 1/500 sera dilution of individual serum samples from six mice per group. A control group that was not pretreated, but challenged with MOG in CFA, was also included. Results are representative of one independent experiment out of three performed.

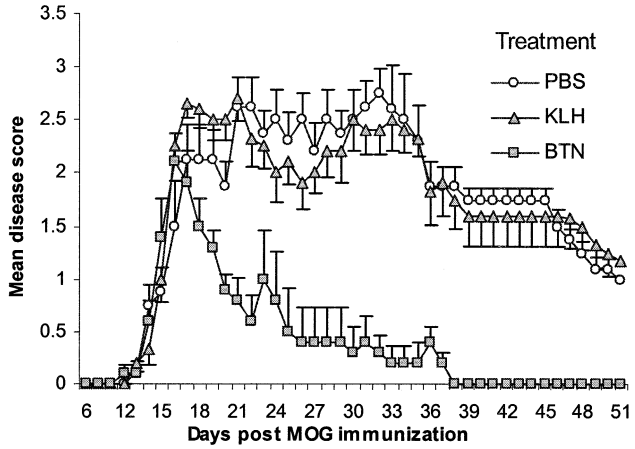

Fig. 5. BTN treatment suppressed ongoing EAE. MOG was injected to induce EAE and at the first signs of paralysis, mice were injected i.p. with either BTN, KLH or PBS emulsified in IFA. All of the animals exhibit signs of disease when the injections were performed. Results are given as mean disease score ± SD, each group consisting of eight mice.

treated with BTN was further accompanied by an altered MOG-specific humoral immune response, serum levels of MOG-specific IgG subclasses were analyzed by ELISA 50 days after EAE induction with MOG. It can be seen in Fig. 4 that mice pretreated with BTN express anti-MOG IgG1 serum antibodies to a similar level to those of the control groups, whereas their anti-MOG IgG2b and IgG2c levels were markedly reduced. In mice, switching from IgM to IgG2, in particular IgG2a (36,37), is thought to be promoted by IFN-γ. Interestingly, it has been recently shown that in B6 mice the IgG2a gene is absent, they express instead the closely associated isotype IgG2c (38). The variation in the amino acid sequences between the two subtypes may therefore reveal dissimilarity of their functional activity. To this end, there is no previous information regarding the role of cytokines in regulating murine IgG2c development. However, the low synthesis of anti-MOG IgG2c antibody exhibited by the protected animals well matched the decreased T_h1 cytokine response

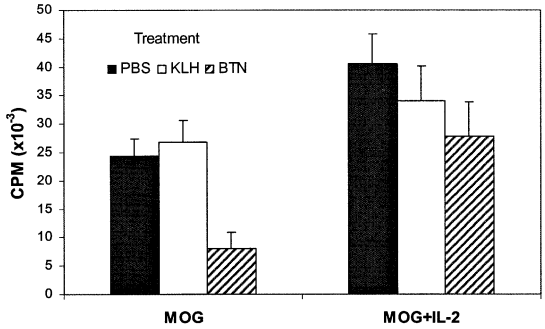

Fig. 6. Exogenous IL-2 restores response of LN cells from BTN-treated mice. MOG-specific proliferation was measured 30 days after EAE induction. LN cells were cultured at 1×10^6 cells/ml in the presence or absence of 200 U/ml of rIL-2 for 48 h. The cultures were then pulsed with 1 μCi [^{3}H]thymidine for 16 h before harvesting. Results are expressed as the mean c.p.m. ± SD of triplicate cultures for six individual mice. Background counts in absence of antigen were 1846 ± 126, 2507 ± 349 and 2796 ± 579 for PBS, KLH and BTN groups respectively; in the presence of IL-2 the resultant background counts were 28,551 ± 3479, 29,430 ± 5049 and 23,341 ± 1926.

described before, supporting the view that IgG2c may also be IFN-γ dependent.

Effect of BTN treatment in ongoing EAE

Having seen that pretreatment with BTN was highly efficient in preventing mice from development of EAE, we next sought to determine whether treatment with BTN could also ameliorate the clinical manifestations of an established disease in B6 mice. For this purpose, groups of eight mice were immunized with MOG/CFA to develop active EAE. Starting at the time of paralysis (i.e. when animals reached a clinical score ⩾2) mice were treated with a single i.p. injection of BTN, KLH or PBS emulsified in IFA. As can be seen in Fig. 5, while both control groups (PBS and KLH treated) progressed to developed severe disease, BTN-treated mice promptly recuperated and by the end of the experiment none of the mice showed clinical signs of disease.

Up-regulation of IL-10 and down-regulation of the T_h1 response by BTN treatment

In order to analyze the mechanisms that contribute to the clinical improvement observed in BTN-treated mice, we determined the antigen-specific proliferation and the pattern of cytokine secretion in response to MOG in BTN-treated and control groups. As shown in Fig. 6, there was a significant suppression of the proliferative response to MOG in LN cells of BTN-treated animals as compared to KLH or PBS control groups (similar results were obtained when spleen cells were studied). Treatment with BTN also reduced the production of the T_h1 cytokines IFN-γ, GM-CSF, IL-2 and IL-12 in recall response to MOG, whereas the secretion of IL-10 was up-regulated (Fig. 7). Once again, we observed no difference in TGF-β production among groups and there were no detectable levels of mRNA IL-4 expression in spleen or LN from any of the animals. Anti-MOG IgG antibody isotype repertoire was

Table 3. Protection transferred by BTN-specific splenocytes

Cell transfer	EAE incidence (MDS)[a]	Duration of EAE (days)	T cell proliferation (c.p.m.)[b]	
			MOG	Con A
None	5/5 (3.5, 3, 3, 3, 2)	19–30	23047 ± 870	133154 ± 5575
KLH	7/7 (5, 5, 5, 4, 4, 3, 2)	21–34	37327 ± 1407	144715 ± 9935
BTN	3/5 (3.5, 3, 1.5, 0, 0)	5–26	13139 ± 1174	132856 ± 9865

[a]MDS = maximal disease score obtained per individual mouse in each group.
[b]Proliferative responses of draining LN cells were determined 45 days after MOG immunization. Background counts were 4300 ± 735, 5246 ± 660 and 2634 ± 220 for BTN, KLH and no transfer group respectively.

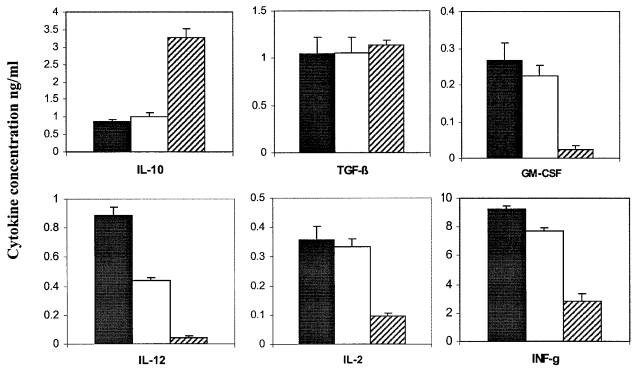

Fig. 7. Cytokine production of spleen cells from mice treated at the disease onset. EAE was induced by immunizing animals with MOG/CFA; at the disease onset mice were treated with a single i.p. injection of BTN (cross-hatched bar), KLH (open bar) or PBS (solid bar). Cytokine concentration in the supernatants was determined by ELISA 30 days after EAE induction. Mean ± SD for six individual mice are given.

comparable to that attained in the pretreatment experiments (data not shown).

Treatment with BTN induces both anergy and regulatory cells

Because LN and splenic cells from mice that received BTN had a stunted proliferative response and secreted low levels of IL-2 upon stimulation with MOG, we next evaluated whether clonal anergy was induced by BTN administration. To address this issue, we assessed the ability of exogenous IL-2 to rescue anergic T cell responses *in vitro*. As can be seen in Fig. 6, addition of 200 U/ml of rIL-2 to the cultures restored the capacity of the LN cells from BTN-treated mice to proliferate in response to MOG (Fig. 6). The impaired proliferative response was also restored by rIL-2 when LN cells from BTN-pretreated animals were studied (not shown).

In order to further investigate whether the immune regulation that has originated in the periphery by BTN administration could be adoptively transferred to naive hosts, groups of 20 animals were immunized with BTN, or the control protein KLH, in IFA. On day 12, their spleens were taken and activated *in vitro* with the priming antigen. Three days later, 3×10^7 splenocytes were transferred into naive animals that were subsequently challenged for EAE induction with MOG. A group of mice that received no cells was also included in this experiment as control for the development of disease. The results in Fig. 8 show that BTN/IFA splenocytes, but not KLH/IFA cells, transferred protection to actively induced EAE. Protection was limited to 75% of the mice and lasted up to 4 weeks. After that period, two out of the remaining four recipients started to develop signs of the disease with a severity that was almost the same as the no transfer control group, whereas the other two mice remained completely protected throughout the period of observation (Table 3). Interestingly, recipient animals that received KLH cells exhibited a significant earlier disease onset than the control group that received no cells; there was also an aggravation in the severity of EAE since three of seven mice died within 4 weeks, whereas none of the mice died in the other two groups. As seen in Table 3, transfer of BTN splenocytes also specifically compromises, the ability of MOG-specific LN cells to proliferate *in vitro*. This data supports the hypothesis that specific regulatory mechanisms acting on the pathogenic MOG-specific population of the recipients may account for the clinical improvement observed.

BTN fails to protect against $MOG_{35–55}$-induced EAE

To obtain further insights into the protective mechanisms induced by BTN, we next determined whether the subtle cross-reactivity identified at the T cell immunodominant level of MOG might also account for the protective role of BTN. For this propose, mice were pre-immunized with BTN in IFA or vehicle alone and 7 days later EAE was induced by immunization with the encephalitogenic peptide $MOG_{35–55}$. In contrast to what we observed when MOG was used as immunogen, pre-immunization with BTN had no effect on the incidence or severity of EAE actively induced by $MOG_{35–55}$ (Fig. 9), pointing out the importance of the cross-reactivity at the T cell subdominant level of the autoantigen in the generation of the regulatory mechanisms.

Discussion

Although the existence of self-reactive T cells in the normal repertoire of healthy individuals has been extensively described (39–41), the incidence of MS is relatively rare. This clearly indicates that complimentary events may occur to compromise the maintenance of self-tolerance (42). In this regard, the mimicry hypothesis has long been proposed as a means to explain the instigation and exacerbations of autoimmune disorders (43). However, our results prompt a holistic interpretation that brings together the protective and the detrimental aspect that the molecular mimicry concept entails. The cow's milk protein BTN, consistent with its shared

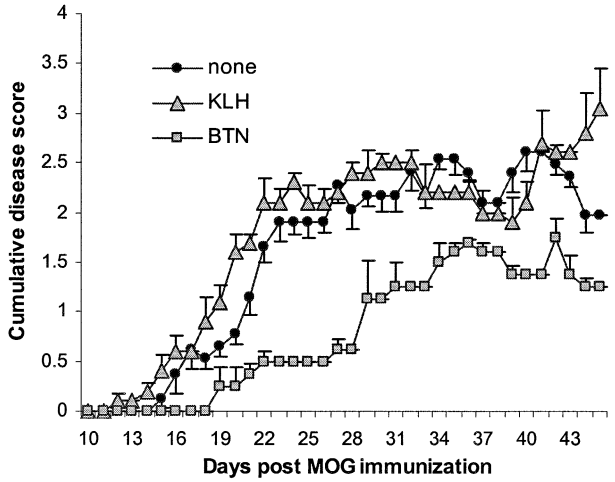

Fig. 8. Transfer of splenocytes from mice primed with BTN provides significant protection to actively induced EAE. Donor B6 mice were injected with 200 μg of either BTN or KLH emulsified in IFA. On day 12, spleens were removed and stimulated *in vitro* with 20 μg/ml of BTN or KLH for 3 days. Each recipient received 30×10^6 cells i.v. and EAE was actively induced 24 h later with 100 μg MOG. An additional group of mice that received no cells was also included as a control for disease development. Results are given as group mean of the sum of daily disease score changes for individual mice (± SD).

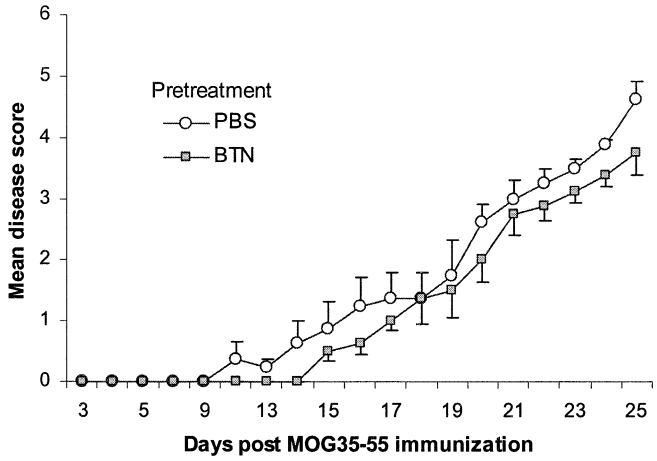

Fig. 9. Pretreatment with BTN fails to protect mice against $MOG_{35–55}$-induced EAE. B6 mice were pre-immunized with a single s.c. injection of BTN (200 μg per mouse, $n = 5$) or PBS ($n = 5$) emulsified in IFA. EAE was induced 7 days later by immunization with 150 μg $MOG_{35–55}$ in CFA. Results are given as mean disease score ± SD.

structural similarity to MOG, has been previously described as pathogenic for Dark Agouti rats (27). However, as we show here in B6 mice, BTN treatment leads to the production of a protective source of regulatory cells that not only prevents, but also suppresses, the autoimmune process induced by MOG. Interestingly, our results appear to indicate that the protective condition associated with BTN treatment could not be explained by means of cross-reactive events at the encephalitogenic core of the autoantigen. Our data shows that the T cell response to BTN cross-reacts primarily with a subdominant region of MOG in B6 mice and this cross-reactivity appears to dictate a subsequent cascade of events that represses the encephalitogenic response to MOG. Interestingly, we observed that although the $CD4^+$ T cell response between BTN and MOG was reciprocally cross-reactive, immunization with BTN was unable to induce any clinical or histological signs of EAE in the strains of mice studied. Recent work by Stefferl *et al.* has shown that BTN immunization induced an inflammatory response in the CNS of Dark Agouti rats. Further examination of the cross-reactivity between MOG and BTN unveiled the involvement of an encephalitogenic T cell epitope of MOG ($MOG_{74–90}$) in the Dark Agouti strain (27). Our studies undertaken on a B6 background reveal that the cross-reactivity at the T cell level between the two proteins is mostly restricted to the sequence $MOG_{73–87}$ and only a minimal response is found within the immunodominant region $MOG_{35–55}$ (Fig. 1). $MOG_{35–55}$ (44) and more recently $MOG_{18–32}$ (16) have been the only two encephalitogenic epitopes identified in B6 mice. Therefore, it is conceivable that molecular mimicry events not involving the encephalitogenic core of the autoantigen would not initially represent a source of auto-aggressive cells. Thus, these findings provide one line of support for the failure of BTN to induce disease. In addition, immunization with BTN was unable to induce an antibody response that cross-reacts with MOG (Mañá, unpublished results). To this end, data from previous studies have highlighted the critical role that MOG-specific antibodies play in the priming phase of MOG-induced EAE. B cell-deficient mice are not susceptible to EAE by immunization with MOG (45) and transfer of MOG-specific antibodies reverse susceptibility for the disease (46).

The striking shift in the pattern of cytokines released by MOG-primed mouse lymphocytes in response to BTN compared to the characteristic T_h1-type pattern released in recall response to MOG encouraged us to speculate that the cross-reactivity between the two proteins could also provide a basis for the suppression of EAE. In fact, this hypothesis has been previously sustained by others, by showing that prevention of EAE could be achieved by pretreatment with certain microbial sequences bearing structural resemblance with an encephalitogenic MBP peptide (18). The protective effect appeared to rest in the ability of such microbial determinants to behave as altered peptide ligands. Accordingly, pretreatment with BTN and treatment at the disease onset was carried out in B6 mice immunized with MOG. In both settings, protection against the disease was readily obtained and was correlated with a decrease in MOG-specific T cell proliferation and a depressed T_h1 response, as evidenced by reduced antigen-specific secretion of IFN-γ, IL-2, IL-12 and GM-CSF. Previous studies have shown that injection of autoantigens or cross-reactive peptides in IFA prevents EAE (18,47). Several mechanisms including suppressor cells (48), anergy (49), exhaustion (47) and, particularly, T_h2 immune deviation (50) have been associated with such protective effect. Interestingly, the protection imparted by BTN was not associated with an up-regulation in the expression of IL-4 at any time point studied, therefore arguing against T_h2 immune deviation as the protective mechanism that would explain the clinical improvement observed. However, the reduced proliferation and decreased IL-2 secretion in response to antigen and the finding that addition of exogenous IL-2 could revert the

unresponsiveness state of MOG-specific population appears to indicate that T cell anergy was at least one of the mechanisms accountable for the protection. One of the major effects associated with the BTN administration was the consistent up-regulation of IL-10 secretion. Recent investigations have made a connection between anergy and IL-10. IL-10 prevents dendritic cell maturation and thus the stimulation of $CD4^+$ T cells by immature dendritic cells favors the development of anergy (51). Moreover, high levels of this cytokine promote the expansion of a T cell population that secretes IL-10, but exhibits low proliferative response (52). It is, therefore, likely that the protective effect provided by BTN may account for the generation of regulatory cells that upon stimulation with MOG display high levels of IL-10, thereby influencing the remaining MOG-specific population, which probably harbors precursor and/or effector pathogenic MOG-specific T cells. In support of this hypothesis, IL-10 is believed to function as a regulatory cytokine in EAE. IL-10-deficient mice develop a more severe form of EAE than wild-type mice, and this enhanced response has been shown to be associated with an up-regulation of IFN-γ and tumor necrosis factor-α secretion (53). The regulatory role of IL-10 in our setting was further evidenced by the enhanced IL-10 production exhibited by the untreated mice during the remission phase of the disease (Table 2). The latter observation is compatible with the finding that IL-10-deficient mice also lose the spontaneous recovery from EAE (34). Taken together, these data suggest that the increased levels of IL-10 during remission appear to be an inherent property of MOG-induced EAE.

To further characterize the regulatory phenotype of BTN-specific cells, splenocytes from BTN-primed mice were expanded *in vitro* and injected into naive recipients prior to MOG immunization. Interestingly, transfer of a syngeneic BTN-specific population into naive animals provides significant protection against actively induced EAE. It should be noted that although the protective state was regarded as transient when compared with the protection achieved by the active administration of the protein, the immunological studies revealed that transfer of such regulatory cells induced a considerable effect in the MOG-specific repertoire of recipients mice, thus supporting the regulatory role that BTN-specific cells play in MOG-induced EAE.

The striking observation that BTN was also efficient at suppressing an established disease suggests that the maintenance of the encephalitogenic response was also affected by the milk protein administration. Although it is reasonable to suggest that the regulatory mechanisms that originated in the periphery by BTN treatment may compromise the continuous influx of active T cells into the CNS, we cannot rule out the possibility that regulatory mechanisms may also arise at the site of inflammation, as it has been previously described (54). Thus, the protection achieved by BTN in the setting of an established disease may reflect the ability of these regulatory cells to home to sites of inflammation where, by revealing its immunomodulatory functions, it may assist to arrest the local inflammatory environment. In this context, it has been shown that delivery of IL-10 within the CNS induces down-regulation of the microglia/macrophage functions (55), which are believed to play a critical role in the effector phase of EAE (56–58). In addition, the ability of the regulatory cells to enter the CNS would be facilitated by the chemokine milieu (59,60) and by the disruption of the blood–brain barrier during EAE (61,62).

Since the cross-reactivity between BTN and MOG involved primarily a subdominant region of MOG in B6 mice (Fig. 1), we set out to evaluate whether the occurrence of such cross-reactivity denotes a mandatory phenomenon for the instigation of regulatory mechanisms associated with the BTN treatment. Under these circumstances, one would presume that protection provided by BTN could, in principle, reflect a collateral suppression of the response to the encephalitogenic determinants of MOG in our model. In fact, our results appear to support that hypothesis, as by using $MOG_{35–55}$ to induce EAE the administration of BTN/IFA prior challenge had no effect upon disease incidence or severity. This observation suggests that immunization with BTN probably engenders the expansion of regulatory clones that cross-react with the sequence $MOG_{73–87}$ and these in essence are primarily responsible for the inhibition of the disease induced by MOG.

Taken collectively, our results highlight the importance of the cross-reactivity between environmental agents and determinants of endogenous proteins that, although not included in the immunodominant core, can generate regulatory cells capable of modulating an autoimmune process. For this, we believed that molecular mimicry is a framework that entails more than autoimmunogenic dominant peptides, and the analysis of another possible cross-reactivity could be useful for achieving a better and comprehensive understanding of the complex interplay between molecular mimicry and autoimmunity.

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Abbreviations

BTN	butyrophilin
CFA	complete Freund's adjuvant
CNS	central nervous system
Con A	concanavalin A
EAE	experimental autoimmune encephalomyelitis
GM-CSF	granulocyte macrophage colony stimulating factor
IFA	incomplete Freund's adjuvant
KLH	keyhole limpet hemocyanin
LN	lymph nodes
MBP	myelin basic protein
MOG	myelin oligodendrocyte glycoprotein
MS	multiple sclerosis
TGF	transforming growth factor

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