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Bowman-Birk Inhibitor attenuates experimental autoimmune encephalomyelitis by delaying infiltration of inflammatory cells into the CNS.

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Abstract

Bowman-Birk Inhibitor (BBI), a serine protease inhibitor derived from soybeans, has anti-inflammatory properties and is able to suppress the development of central nervous system (CNS) autoimmunity in animal models. Experimental autoimmune encephalomyelitis (EAE), a widely used animal model of multiple sclerosis (MS), is characterized by breakdown of the blood-brain barrier and infiltration of inflammatory cells into the CNS, resulting in pathology. In this study, we observed that BBI-treated mice showed delayed onset of EAE and reduced disease severity compared to control mice. BBI-treated mice had fewer inflammatory cells in the CNS including significantly reduced numbers of Th1 and Th17 cells. In the periphery, BBI treatment suppressed the development of encephalitogenic Th1 and Th17 responses early on [day 7 post-immunization (p.i.)], while after disease onset (day 14 p.i.) BBI-treated mice had stronger Th responses, as determined by antigen-specific proliferation and cytokine production. These results demonstrate that BBI treatment temporarily suppressed the development of encephalitogenic responses, but these responses eventually attained normal magnitude. Given that BBI-treated mice exhibited stronger encephalitogenic responses in the periphery during clinically manifesting EAE, delayed disease onset, and reduced numbers of CNS-infiltrating cells, it appears likely that BBI impedes the exit of pathogenic Th1 and Th17 cells from lymphoid organs, thereby delaying their migration into the CNS.

Keywords

Bowman-Birk inhibitor; BBI; EAE; CNS; multiple sclerosis

Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) that afflicts approximately 400,000 people in the United States and more than 2.5 million individuals worldwide. The etiology and pathogenesis of MS are unknown but it is now generally accepted that MS involves an autoimmune process against CNS myelin. Histopathological features of the disease include white matter lesions (MS plaques) in the CNS with inflammatory infiltrates, demyelination, and axonal loss [1, 2, 3].

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There is no cure for MS. FDA-approved medications for the treatment of MS rely on manipulation of the immune system [4]. Immunotherapeutic strategies have been in clinical use for over 15 years; however, the first-line treatments have only limited efficacy, and the safety of more recently developed treatments, such as Natalizumab, is still under clinical investigation [5]. Introduction of a novel treatment that can be administered orally, and acts synergistically with existing therapies, would be highly beneficial for MS therapy.

Experimental autoimmune encephalomyelitis (EAE) is the most commonly used animal model for studying the disease mechanisms of MS and for testing potential therapies [6]. Most therapies currently approved for MS were developed based on EAE studies. EAE can be induced in susceptible mouse strains by immunization with myelin peptide fragments, or it can be transferred to naïve recipient mice (adoptive transfer EAE) by transplantation of myelin-reactive T cells [7]. Immunization induces the development of myelin-specific CD4⁺ T cells, which traffic into the CNS where they initiate an inflammatory cascade through recruitment of inflammatory cells such as monocytes and neutrophils. Accumulation of immune cells within the CNS leads to myelin damage, axonal loss and clinical deficit in affected animals [8].

Bowman–Birk Inhibitor (BBI) was first described five decades ago [9] as a soybean-derived protein consisting of 71 amino acid residues and 7 disulfide bonds with a molecular weight of 7,975 Da [10]. Functionally, BBI is a double-headed inhibitor of both trypsin- and chymotrypsin-like serine proteases [9]. BBI can withstand boiling water temperature for 10 min, is resistant to a wide pH range and proteolytic enzymes of the gastrointestinal tract; it is bioavailable, and non-allergenic [11, 12, 13]. Thus far, several human serine proteases associated with the function of inflammatory cells have been shown to be highly sensitive to inhibition by BBI [14, 15, 16, 17]. It is likely that there are additional, yet unknown, serine proteases susceptible to inhibition by BBI.

BBI suppresses carcinogenesis [18] and has anti-inflammatory properties without affecting systemic immunity [19]. Our previous studies indicated that BBI reduces CNS inflammation in EAE and attenuates neuronal loss, making it a candidate therapeutic for MS [20]. However, the immunological mechanisms underlying the beneficial effects of BBI on EAE are poorly understood.

The goal of this study was to investigate the potential immunological mechanisms underlying the effects of BBI on EAE. Here, we show that BBI significantly delayed the onset of EAE and temporarily reduced its clinical severity. Consistent with the reduced clinical severity, BBI-treated mice had milder CNS inflammation than controls as demonstrated by reduced Th1 and Th17 cell infiltration into the CNS, in addition to lower total numbers of immune cells. Nonetheless, BBI-treated mice progressively accumulated clinical deficit and eventually became as sick as the control mice. In the periphery, BBI-treated mice showed delayed development of encephalitogenic Th responses that, post-disease onset, actually became stronger than responses in the control group. Our data strongly support the hypothesis that BBI over the course of EAE does not substantially suppress the development of encephalitogenic responses, but delays exit of effector Th cells

from peripheral organs and thereby slows down infiltration of inflammatory cells into the CNS and disease development.

Materials and methods

Mice

C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and were housed in the Thomas Jefferson University animal facility for at least 1 week before inclusion in experiments. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University.

Reagents

The Bowman–Birk inhibitor (BBI) was purchased from Aldrich Sigma (St. Louis, MO). The following antibodies for flow cytometry were from BD Biosciences: anti-CD4 (RM4–5), anti-IFN- γ (XMG1.2), anti-IL-17A (TC11–18H10) and anti-CD16/32 (2.4G2). DuoSet ELISA kits used to quantify IFN- γ , GM-CSF and IL-17A were from R&D Systems.

Induction of EAE and BBI treatment

EAE was induced in female C57BL/6 mice as previously described [21]. Briefly, female 8- to 10-week-old mice were immunized subcutaneously at two sites on the back with 180 μ g of myelin oligodendrocyte glycoprotein peptide (MOG35–55) in complete Freund's adjuvant containing Mycobacterium tuberculosis strain H37Ra (5 mg/ml; Difco). Mice were injected intraperitoneally on days 0 and 2 with 200 ng pertussis toxin (Difco) in 200 μ l PBS. The mice immunized with MOG35–55 were treated daily with BBI (1 mg/day) dissolved in PBS or PBS by oral gavage from day 0 post-immunization (p.i.).

Isolation of CNS-infiltrating cells

EAE mice were sacrificed, and brains and spinal cords were removed and pooled after transcardial perfusion with PBS. Tissues were mechanically dissociated through a 100- μ m strainer and washed with PBS. The resultant single cell suspension was fractionated on a 70–30% Percoll step gradient by centrifugation at 300 g for 20 min. Infiltrating mononuclear cells were collected from the interface and washed, then were counted and analyzed by flow cytometry as described below.

Flow cytometry

For intracellular staining, cells were stimulated for 4 h with PMA (50 ng/ml; Sigma) and ionomycin (500 ng/ml; Sigma) and were treated with GolgiPlug (1 μ g per 1×10^6 cells; BD Pharmingen). In the staining procedure, Fc receptors on cells were first blocked with anti-CD16/CD32 (2.4G2; BD Pharmingen) and surface and intracellular staining was done according to manufacturer's instructions, with Fix and Perm reagents (Caltag Laboratories). Data were acquired on a FACSARIA (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Proliferation assay

Splenocytes and LN cells were stimulated with MOG35–55 for 3 days and were pulsed for the last 18 h of culture with 1 μ Ci of [3H]thymidine. Thymidine incorporation was measured using a scintillation counter.

ELISA

The splenocytes and lymph nodes cells were cultured with MOG35–55 for 3 days; thereafter, supernatants were harvested and analyzed for IFN- γ , GM-CSF and IL-17A by ELISA kits according to the manufacturer's instructions.

Statistical analysis

The two-tailed Student's t test and the χ^2 test were used to analyze the significance of results. Additionally, analysis of variance (ANOVA) was used for the comparison of average clinical scores. P values of less than 0.05 were considered significant.

Results

BBI delays development of EAE

To investigate the role of BBI in the development of EAE, C57BL/6 mice were immunized with the encephalitogenic MOG35–55 peptide and treated with 1 mg/mouse/day of BBI from day 0 post-immunization (p.i.) until mice were sacrificed. BBI treatment delayed disease onset for several days and significantly reduced clinical EAE severity until day 25 p.i., as compared to the PBS control group (Fig. 1a). Control mice reached disease peak at day 18 p.i., while BBI-treated mice continued to accumulate clinical deficit until day 28 p.i. when their disease became similarly severe as in PBS-treated mice. Moreover, the incidence of EAE in BBI-treated mice was initially markedly lower compared with that in PBS-treated control animals but eventually all mice also developed disease (Fig. 1b). These data indicate that BBI does not permanently suppress EAE, but rather substantially delays disease development.

BBI treatment reduces CNS inflammation

We next sought to investigate the effect of BBI on the infiltration of inflammatory cells into the CNS during EAE. To this end, we isolated mononuclear cells from the CNS on days 7 (asymptomatic phase), 14 (onset), 21 (peak) and 30 p.i. BBI-treated mice had fewer numbers of inflammatory cells infiltrated into the CNS at all time points analyzed except day 30 p.i. (Fig. 2), suggesting a reduced migration of inflammatory cells to the CNS. Flow cytometric analysis showed also reduced total numbers of CD4+IFN γ + (Th1), CD4+IL-17+ (Th17), and CD4+IFN γ +IL-17+ (Th1/Th17) cells in the CNS of BBI-treated mice compared with PBS-treated control animals. However, the proportion of CD4+ T cells among CNS-infiltrated mononuclear cells was not affected by BBI treatment. Similarly, the proportions of IFN- γ - and IL-17-producing cells among CD4+ T cells was similar among PBS- and BBI-treated animals, indicating that BBI does not cause immune deviation, at least in the case of Th1 and Th17 cells. These data demonstrate that BBI substantially delays infiltration

of immune cells into the CNS without pronounced alterations in composition of the cellular infiltrate.

BBI delays development of Th1 and Th17 cells

We next asked whether BBI treatment suppresses the development of MOG-specific T cells in the periphery. Therefore, we measured the proportions of IFN- γ +CD4+ (Th1) and IL-17+CD4+ (Th17) cells in spleen and lymph nodes (LNs) from both BBI-treated and PBS-treated mice. On day 7 p.i., BBI treatment resulted in a small but reproducible decrease in the proportion of Th1 and Th17 cells in LNs and spleen compared with control mice (Fig. 3). However, on days 14 and 21 p.i., BBI-treated mice showed somewhat higher proportions of Th1 and Th17 cells in both spleens and LNs compared with PBS-treated mice (Fig. 3). These data demonstrate that BBI treatment does not substantially alter proportions of IL-17- and IFN- γ -producing Th cells among CD4+ T cells in the periphery. However, there is a tendency toward somewhat reduced proportions of cytokine-producing Th cells early on in BBI-treated mice, whereas post-onset of clinical disease the opposite is true.

BBI suppresses the early development of encephalitogenic Th responses

To determine the effect of BBI treatment on development of MOG35–55-specific Th responses, we immunized mice with the peptide and analyzed MOG-specific proliferative responses in several time points p.i., Splenocytes and LN cells from BBI- or PBS-treated mice harvested on days 7, 14, 21 and 30 p.i., were stimulated with MOG35–55 for 3 days. Both splenic and LN cells of BBI-treated mice harvested 7 days p.i., proliferated significantly less than cells of PBS-treated mice. However, the opposite was true when cells were harvested on days 14 and 21 p.i.; cells of BBI-treated mice displayed significantly higher proliferation rates in response to MOG35–55 (Fig. 4). On day 30 p.i., the two groups of mice exhibited similar proliferation in response to MOG35–55.

We next analyzed cytokine production in cultures of spleen and LN cells harvested at different time points p.i. and stimulated with MOG35–55 for 3 days. In contrast to the suppression of proliferation seen above, BBI treatment did not affect production of IFN- γ , GM-CSF or IL-17A by cells harvested 7 days p.i. However, consistent with both the proliferation and flow cytometric data, IL-17A and IFN- γ production were both upregulated on days 14 and 21 p.i. in the BBI-treated mice (Fig. 5). Taken together, these results clearly indicate that the antigen-specific response *ex vivo* was weaker at the early stage of autoimmunity and became stronger after the early stage in BBI-treated mice compared with control mice. This was true even on day 14 p.i., when they lacked apparent EAE clinical symptoms.

Discussion

We show here that BBI treatment delayed the onset of EAE and slowed the pace of clinical deficit accumulation, but ultimately did not stop disease development, with incidence and severity eventually reaching levels similar to the control mice. Consistent with the delay in clinical disease development, infiltration of inflammatory cells into the CNS was also

delayed and reduced, although without marked perturbation in cellular composition of CNS infiltrate.

A possible reason for protracted EAE development in BBI-treated mice was the suppressed development of encephalitogenic responses in the periphery. Indeed, we have found suppressed MOG35–55-specific responses in BBI-treated mice, but only before onset of clinical disease. After disease was manifested, BBI-treated mice had stronger myelin-specific responses in the periphery compared with control mice. In later phases of disease (day 30 p.i.), all measured parameters in the periphery and in the CNS were similar between BBI- and PBS-treated mice. We interpret these data as indicating that BBI delays EAE development primarily by retarding CNS infiltration by inflammatory cells, and not by the suppression of encephalitogenic responses in the periphery. If BBI strongly suppresses the development of MOG35–55-specific responses in the periphery, these responses would have been less than those of the control mice at all time points. This does not exclude the possibility that BBI has some suppressive effect on development of immune responses, as shown by reduction in MOG35–55-specific proliferation on day 7 p.i. However, given that at later time points, the suppression was lost it seems that BBI only temporarily exerts its suppressive effect, which can be viewed as a delay in the development of responses that eventually reach normal magnitude despite continuous treatment with BBI. We and others have shown previously that BBI enhances IL-10 production [20, 22] and that IL-10 is required for the suppression of EAE development by BBI (data submitted for publication). Given that IL-10 has well established immunosuppressive effects [23, 24], it is possible that the observed delay in development of MOG35–55-specific responses is dependent on IL-10. However, it is also possible that IL-10 mediates the suppressive effect of BBI through a pathway or in a stage of disease development that is distinct from the priming of encephalitogenic responses (i.e., infiltration into the CNS). To distinguish between these possibilities, development of immune responses during early stage (i.e., day 7 p.i.) would need to be compared between WT and IL-10^{−/−} mice.

We have observed that, during the post-initial priming phase (day 14, 21 p.i.), BBI-treated mice exhibit stronger MOG35–55-specific responses in the periphery as compared to PBS-treated mice. We believe that these stronger responses are not caused by enhanced priming of the immune response by BBI, but rather by retention of more MOG35–55-specific CD4⁺ T cells in peripheral lymphoid organs of BBI-treated mice. In this scenario, BBI- and PBS-treated mice have developed MOG35–55-specific responses of similar magnitude by day 14 p.i.; however, the proportions of CD4⁺ T cells specific for MOG35–55 that have left lymphoid organs and infiltrated the CNS substantially differ, with greater numbers of these cells migrating into the CNS of control mice. Hence, smaller cell numbers remained in the periphery than in the BBI-treated group. The mechanistic reason for delayed exit of CD4⁺ T cells from lymphoid organs in the presence of BBI is not known, but it is likely that enhanced production of IL-10 plays a role.

Both splenic and LN cells of BBI-treated mice harvested 7 days p.i. proliferated significantly less in response to MOG35–55 than cells of control mice, indicating that BBI suppressed the development of the immune response against immunizing peptide. However, secretion of cytokines in response to both MOG35–55, and non-antigen-specific stimulation

with PMA and ionomycin, resulted in similar levels of INF- γ , GM-CSF and IL-17A, indicating a similar magnitude of immune responses in both BBI- and PBS-treated groups. The most likely explanation for the discrepancy between proliferation and cytokine production data is that BBI, directly or indirectly, selectively affects some, but not all aspects of an immune response.

While BBI treatment reduces CNS inflammation and ameliorates EAE, its exact mechanism of action remains unclear. Since numerous serine proteases participate in a multitude of processes, BBI can potentially impact the development of autoimmune responses, diminish invasiveness of immune cells through the blood–brain barrier, dampen effector mechanisms of immune cells within the CNS, or curtail recruitment of additional inflammatory cells into the CNS, or a combination of these. One known anti-inflammatory mechanism of BBI, similar to other inhibitors of chymotrypsin proteolytic activity [25], is the ability to prevent the production of reactive oxygen species (superoxide and hydrogen peroxide) [26, 27]. In addition, BBI inhibits production of nitric oxide and suppresses the prostaglandin E2 pathway in LPS-induced macrophages [28]. Furthermore, BBI suppresses expression of proinflammatory cytokines (IL-1 β , IL-6 and TNF) by LPS-activated macrophages [22]. It is likely that BBI exerts its effect in EAE through more than one mechanism, by simultaneously inhibiting several serine proteases and affecting multiple pathways. Since different processes dominate distinct phases of EAE (i.e., early priming phase, followed by infiltration of the CNS, etc.), BBI can potentially affect various disease phases by interfering with unrelated pathways.

A notable finding of this study, that BBI alters the distribution of MOG-specific T cells between the peripheral compartment and the CNS, is reminiscent of fingolimod, a recently approved oral therapy for MS. It is thought that fingolimod exerts its beneficial effect by blocking lymphocyte egress from secondary lymphoid organs to the peripheral blood circulation and thus reduces lymphocyte trafficking into the CNS [29].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

BBI delays development of EAE. C57BL/6 mice were immunized with MOG₃₅₋₅₅ and treated daily with 1 mg/day BBI ($n = 13$) or PBS ($n = 14$) by oral gavage from the day of immunization (p.i.). Mice were scored daily for clinical disease. Data are representative of three independent experiments.

Figure 2.

BBI reduces infiltration of the CNS by inflammatory cells. C57BL/6 mice were immunized with MOG_{35–55} and treated daily with 1 mg/mouse BBI or PBS starting on the day of immunization. On day 7, 14, 21, and 30 p.i., mice were sacrificed, their spinal cords pooled and cells from the CNS isolated. **a** Total numbers of cells isolated from the CNS, and total numbers of CD4⁺, CD4⁺IFN- γ ⁺, and CD4⁺IL-17A⁺ cells. **b** Mononuclear cells isolated from the CNS were analyzed by flow cytometry for proportion of CD4⁺IFN- γ ⁺IL-17A⁺ cells. Data shown are of three independent experiments.

Figure 3.

BBI-treated mice initially have reduced numbers of Th1 and Th17 cells, followed by an increase. C57BL/6 mice were immunized with MOG₃₅₋₅₅ and treated daily by oral gavage with 1 mg/mouse BBI or PBS starting on the day of immunization. On days 7, 14, and 21 p.i., mice were sacrificed, and splenocytes and LN cells were analyzed by flow cytometry for the proportions of IL-17A⁺ and IFN- γ ⁺ cells among CD4⁺ T cells. Data are representative of three independent experiments.

Figure 4.

BBI-treated mice have initially weaker but then stronger MOG-specific T-cell responses in the periphery. C57BL/6 mice were immunized with MOG₃₅₋₅₅ and treated daily by oral gavage with 1 mg/mouse BBI or PBS starting on the day of immunization. On days 7, 14, 21, and 30 p.i., mice were sacrificed, splenocytes, and LN cells were stimulated with MOG₃₅₋₅₅ for 3 days, and proliferation was measured by ³[H]-thymidine incorporation. Data are representative of three independent experiments.

Figure 5.

BBI treatment results in enhanced cytokine production by peripheral cells at later stage of EAE development. C57BL/6 mice were immunized with MOG₃₅₋₅₅ and treated daily by oral gavage with 1 mg/mouse BBI or PBS starting on the day of immunization. On days 7, 14, and 21 p.i., mice were sacrificed, splenocytes, and LN cells were stimulated with MOG₃₅₋₅₅ for 3 days, and GM-CSF, IL-17A, and IFN- γ concentrations in the supernatants measured by ELISA. Data are representative of three independent experiments.