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Comparison of a Classical Th1 Bacteria Versus a Th17 Bacteria as Adjuvant in the Induction of Experimental Autoimmune Encephalomyelitis

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Abstract

The relative contribution of myelin-specific Th1 and Th17 cells in the pathology of multiple sclerosis (MS) and its animal model, experimental autoimmune encephalomyelitis (EAE), is controversial. IL-12, the key cytokine necessary for the differentiation of Th1 cells, has been found to be dispensable for EAE induction; while the related cytokine associated with Th17 cells, IL-23, is a critical factor for inducing EAE. Since EAE is induced by immunization with myelin proteins in CFA which contains *M. tuberculosis* that generates a prototypical Th1-mediated immune response, we sought to determine if replacing the *M. tuberculosis* in the adjuvant with a bacterium that induces an IL-23-dependent Th17 cell response during infection would induce EAE with a different phenotype. *C. rodentium*, a bacterium that requires IL-23 for protective immunity, was used as the adjuvant in EAE and compared to CFA. Mice immunized with *C. rodentium* adjuvant (CRA) developed classical signs of EAE, similar to CFA-immunized mice, but disease was less severe with a later onset and slower progression than CFA. Surprisingly, the peripheral cytokine profile revealed similar numbers of Th1 and Th17 cells for both CFA and CRA-immunized mice; however, the number of Th1 and Th17 cells was significantly reduced in the CNS of CRA-immunized mice. The development of EAE in CRA-immunized mice was associated with epitope spreading. The unique clinical course of CRA immunizations helps serve as a useful alternative model for studying EAE pathogenesis and potential therapeutics for MS.

Keywords

experimental autoimmune encephalomyelitis; Th1 cells; Th17 cells; adjuvant

1. Introduction

Experimental autoimmune encephalomyelitis (EAE) has a long history as a model of Multiple Sclerosis (MS), and it has expanded our understanding of inflammatory processes that occur in demyelinating disease. EAE originated from observations over one hundred

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years ago when individuals immunized with Pasteur's rabies vaccine developed an encephalomyelitis caused by a reaction to the CNS tissue contaminating the inactivated virus. When scientists attempted to replicate the observed encephalomyelitis by injecting animals with homogenized CNS tissue, they had only limited success inducing disease (Baxter, 2007, Mackay and Anderson, 2010). It was not until Jules Freund introduced an adjuvant containing dried, killed *Mycobacterium tuberculosis* that disease could be induced consistently in animals, thus establishing the EAE model (Freund et al., 1947, Kabat et al., 1947, Morgan, 1947). Today EAE induced by immunization with myelin protein emulsified in Complete Freund's Adjuvant (CFA) is one of the most studied immune-mediated diseases and has enhanced our understanding of autoimmunity, as well as fundamental immunologic mechanisms.

Partially spurred by the use of *M. tuberculosis* in the adjuvant, EAE has long been considered a Th1 disease mediated by T cells secreting key cytokines, such as IFN- γ . This paradigm was called into question with observations that IFN- γ -deficient and IFN- γ receptor-deficient mice remained susceptible to EAE (Ferber et al., 1996, Willenborg et al., 1996). Since IL-12 is a critical cytokine for the differentiation of Th1 cells, Cua et al. (Cua et al., 2003) developed mice deficient for the subunits of IL-12 and a related cytokine, IL-23, and determined if these mice could develop EAE. Mice deficient in the p40 subunit shared by IL-12/IL-23 or the p19 subunit specific for IL-23 were protected from EAE, whereas mice deficient in the IL-12 specific p35 subunit were still susceptible to disease (Cua et al., 2003). Subsequent papers corroborated this finding demonstrating a critical role of IL-23 in the pathogenesis of EAE (Awasthi et al., 2009, Chen et al., 2006, Thakker et al., 2007). The mechanism by which IL-23 mediates EAE pathogenesis remains unclear. One mechanism that has gained the most attention has been the observation that IL-23 promotes the production of inflammatory Th17 cells (Langrish et al., 2005).

Although *M. tuberculosis* infections generate small amounts of Th17 cytokines, control of infection is dependent on a Th1 response (Cooper et al., 1993, Cooper et al., 2002, Khader et al., 2005). Immunizations with CFA also generate high levels of IFN- γ and other Th1 cytokines (Cua et al., 2003, Langrish et al., 2005). IL-23 plays a contributory role, but it is not required to limit *M. tuberculosis* growth (Khader et al., 2005). On the other hand, infections with other organisms are associated with dominant Th17 responses and are dependent on IL-23 for clearance. With the observed dependence of IL-23 in EAE pathogenesis, we hypothesized that the clinical course of EAE could be altered by replacing *M. tuberculosis* in the adjuvant with a Th17-inducing or more particularly an IL-23-dependent bacterium in the adjuvant. *Citrobacter rodentium* is a gram-negative bacterium that causes a distal colitis in mice that is typically cleared within 2-3 weeks. *C. rodentium* is commonly used as a model to study enteropathogenic and enterohemorrhagic *E. coli* infections. Infections in mice produce high levels of IL-17-producing lymphocytes in the lamina propria, and mice deficient in the IL-23-specific p19 subunit develop severe infections resulting in death by day 14 (Mangan et al., 2006, Zheng et al., 2008), indicating that IL-23 plays a critical role in protective immunity to *C. rodentium*. In the current study, we compare EAE induction in mice using the standard CFA adjuvant to EAE induced with *C. rodentium* emulsified in incomplete Freund's adjuvant to determine if bacterium that induce different T cell-mediated immune responses during infection generate different phenotypes of EAE.

2. Methods

2.1 Preparation of *C. rodentium*

C. rodentium (ATCC 51638) was grown to approximate late log phase growth in nutrient broth (Sigma-Aldrich), washed with PBS and heat killed at 65°C for one hour. *C. rodentium*

was lyophilized, crushed and stored for later use. Heat killing of bacteria was confirmed by plating on nutrient agar plates.

2.2 Induction of EAE

B10.PL V β 8.2 T cell receptor (TCR)-transgenic mice express a gene encoded a TCR V β 8.2 chain that when paired with the appropriate TCR V α chain recognize MBP Ac1-11 (Goverman et al., 1993). The frequency of T cells that recognize MBP Ac1-11 in V β 8.2 TCR transgenic mice is estimated to be 1:100-1:1000, making them highly susceptible to EAE. C57Bl/6 mice were purchased from Jackson Laboratory. All mice were bred and kept in a pathogen-free facility at The Ohio State University (OSU). EAE was induced by administering subcutaneous injections over four sites at the flanks of mice with an emulsion of Incomplete Freund's Adjuvant supplemented with heat-killed *C. Rodentium* (CRA) or *M. Tuberculosis* H37Ra (CRA) (Difco) and 200 μ g MOG35-55 or MBP Ac1-11. Pertussis toxin (Ptx; List Biological Laboratories) was given i.p. at a dose of 200 ng at the time of immunization and two days later. Mice were scored based on the following scale: 0-no clinical disease, 1-limp tail, 2-mild hind limb weakness, 3-severe hind limb weakness, 4-hind limb paralysis, 5-moribund, 6-death from EAE.

2.3 Flow cytometric analysis

Draining lymph nodes were isolated and crushed between microscope slides to generate single cell suspensions. Cells were suspended in EAE media containing 1X RPMI 1640 supplemented with 1 mM Sodium Pyruvate, 13 mM HEPES, 1X MEM Nonessential Amino Acids, 2 mM L-Glutamine, 100 I.U. Penicillin, 100 μ g/ml Streptomycin (Cellgro), 10% FBS (Invitrogen), and 50 μ M 2-ME (Sigma-Aldrich) at 5×10^6 cells/ml. Cells were cultured in 24-well plates at 2 ml/well for three days with 2 μ g/ml MOG35-55. Six hours before cytokine staining 50 ng/ml PMA and 1 μ g/ml ionomycin were added, and one hour later 1 μ l/ml Golgi Plug (BD Biosciences) was added to the culture. CNS mononuclear cells were isolated by crushing brains and spinal cords between microscope slides. CNS tissue was washed with 37% Percoll (GE Healthcare) solution and subjected to a 15 minute 2118 x g centrifugation over a 30/70% Percoll gradient. The interphase layer was collected, washed and suspended in EAE media at 2.5×10^6 cells/ml. Cells were cultured overnight in 24-well plates at 2 ml/well with 2 μ g/ml MOG35-55, and Golgi Plug was added five hours before staining. For flow cytometry staining, cells were washed and incubated with FC block (BD Biosciences) for 15 minutes. Cells were then incubated with surface staining antibodies for 30 minutes and washed. Cells were fixed in Cytofix/Cytoperm buffer (BD Biosciences) for 20 minutes and permeabilized in Perm/Wash buffer (BD Biosciences) for 30 minutes. Cells were suspended in Perm/Wash buffer and incubated with intracellular staining antibodies for 30 minutes. Cells were washed, suspended in stain buffer and 100,000 events were collected on a FACSCanto 2 (BD Biosciences). Data was analyzed using Flowjo software (Tree Star Inc.). Antibodies used included the following from BD Biosciences: PerCP-conjugated anti-mouse CD45, V500-conjugated anti-mouse CD4, APC-conjugated anti-mouse CD4, APC-conjugated anti-mouse IFN- γ , PE-Cy7-conjugated anti-mouse IFN- γ , and PE-conjugated anti-mouse IL-17. Additional antibodies used include FITC-conjugated anti-mouse T-bet (Santa Cruz Biotech) and eFluor® 450-conjugated anti-mouse CD44 (eBioscience).

2.4 ELISA

Supernatants were collected from same cultures used for flow cytometric analysis. Immulon 2 HB 96-well plates (Thermo Scientific) were coated overnight with 2 μ g/ml anti-mouse primary antibody at 50 μ l/well diluted in 0.1M NaHCO₃ pH 8.2. Plates were washed twice with PBS/tween and blocked with PBS + 1% BSA for 2 hours. Plates were washed twice and incubated overnight with samples and standards at 100 μ l/well. Cytokine standards were prepared in EAE media by diluting each individual cytokine in serial dilutions from 2 ng/ml

– 0.03 ng/ml. Plates were washed 4X with PBS/tween and incubated with 1 µg/ml biotinylated anti-mouse secondary antibody at 100 µl/well diluted in PBS + 1% BSA for one hour. Plates were washed 6X with PBS/tween and incubated with 2.5 µg/ml avidin-peroxidase at 100 µl/well diluted in PBS + 1% BSA for 30 minutes. Plates were washed 8X with PBS/tween followed by the addition of 100 µl ABTS substrate containing 0.03% H₂O₂ to each well. Following appropriate color development, plates were read at A405 on an Emax microplate reader (Molecular Devices).

2.5 Thymidine Incorporation Assay

Spleens were isolated and crushed between microscope slides to generate single cell suspensions. Red blood cells were lysed in buffer containing 155 mM NH₄Cl, 10 mM KHCO₃, and 0.1 mM EDTA for one minute at room temperature followed by two washes with PBS. Cells were cultured in 96-well round bottom plates at 2.5×10⁵ cells/well for 48-72 hours with MOG35-55 at 0.4, 2 or 10 µg/ml or bovine MBP (bMBP) at 4, 20 or 100 µg/ml in replicates of four. Cells were pulsed with 0.5 mCi/well [³H]thymidine for 18 or 36 hours. Cells were then harvested onto Unifilter-96 GF/C plates (PerkinElmer), and plates were dried. Micrisint 20 (PerkinElmer) was added to each well, and [³H]thymidine was measured using a TopCount NXT (PerkinElmer). The stimulation index was calculated determining the mean CPM for the no antigen and antigen replicates and determining the ratio of mean CPM of the antigen relative to the mean CPM of the no antigen. For the precursor frequency assays, cells were plated in 96-well plates at 2×10⁵, 1×10⁵ and 5×10⁴ cells per well with no antigen, 2 µg/ml MOG35-55 or 20 µg/ml bMBP in replicates of 32. Cells were pulsed with 0.5 mCi/well [³H]thymidine after 72 hr and harvested as described above. The mean and standard deviation of the no antigen wells was determined for each sample. For the antigen stimulated wells, a positive well was defined as having a CPM greater than the mean of the no antigen wells plus three standard deviations. After determining the number of positive wells for each condition, a chi square minimization analysis was performed to determine the estimated frequency of cells responding to a specific antigen for each sample.

2.6 Statistical analysis

All data were analyzed using GraphPad Prism software. Statistics on EAE clinical scores were performed using Mann-Whitney non-parametric analysis. Student t test was used for analysis of proliferation data. Significant differences represent analysis with a p<0.05.

3. Results

3.1 *Citrobacter rodentium* adjuvant (CRA) induces late onset EAE

C. rodentium was heat-inactivated, lyophilized, and mixed with Incomplete Freund's Adjuvant (IFA) to generate *C. rodentium* adjuvant (CRA). Similarly, heat-inactivated *M. tuberculosis* was emulsified in IFA at the same concentration. B10.PL mice, which express the Vβ8.2 T cell receptor (TCR) transgene specific for myelin basic protein (MBP) Ac1-11 and thus have an increased frequency of T cells specific for MBP, were used in our initial experiment to determine if CRA had the capacity to induce EAE in a highly susceptible strain of mice (Goverman et al., 1993, Ratts et al., 1999). Vβ8.2 TCR transgenic mice were immunized with MBP Ac1-11 in emulsion with CFA, CRA and IFA, with and without co-administration of pertussis toxin (Ptx). Mice that received CFA developed severe EAE with an early onset, whereas mice that received CRA developed less severe EAE with a later onset (Figure 1 A). As expected, mice that received IFA did not develop EAE. Ptx caused an earlier onset of disease in CRA-immunized mice, but it did not have any effect with any other adjuvant. To determine if *C. rodentium* could induce EAE in a more physiological-relevant model, C57Bl/6 mice were immunized with MOG35-55 in emulsion with CFA or

CRA, and Ptx was also given in this and all subsequent experiments. Mice that received the CFA developed a typical rapid-onset, chronic disease course followed by only a mild diminution of disease (Figure 1 B). Mice that received the CRA developed a disease course that resembled that of CFA, but there was more variation in disease onset. Most mice did not get EAE until after day 30, which accounts for the late rise in mean clinical score. In both strains of mice, the clinical signs of EAE were that of classical EAE, and they were similar between the CFA and CRA-immunized mice.

To determine if the differences in disease onset and severity in the CFA and CRA immunizations was due to the dose of bacterium, both *C. rodentium* and *M. tuberculosis* were titrated, and C57Bl/6 mice were immunized with decreasing doses of each. The concentration of *M. tuberculosis* and *C. rodentium* that generated the greatest severity and incidence of EAE was 2 mg/ml, and as the concentration of bacterium decreased there was a decrease in incidence and/or severity of disease (Figure 2 A,B). Again, CRA-immunized mice continued to develop a later onset and less severe disease course than CFA-immunized mice as disease onset was observed as late as 62 days after immunization, whereas the latest all mice in the CFA group developed disease was day 27. *C. rodentium* was also titrated using increasing doses, and the 2 mg/ml dose still generated the highest disease incidence and severity (data not shown), indicating the bacterium concentration was not responsible for the difference in disease incidence or severity observed relative to CFA.

3.2 CRA-immunization generates cytokine responses similar to CFA

C. rodentium infections generate an immune response that is dominated by high IL-17 and low IFN- γ production in lamina propria lymphocytes (Mangan et al., 2006), so we speculated that EAE induced with *C. rodentium* would generate myelin-specific Th17 cells. CD4 T cells from draining lymph nodes of C57Bl/6 mice immunized with MOG35-55 in emulsion with CFA or CRA were evaluated for IFN- γ and IL-17 production. Unexpectedly, the CRA-immunized mice did not produce the same pattern of cytokine expression as shown in the infection model. The pattern of IFN- γ and IL-17 expression was similar to CFA-immunized mice with a higher number of CD4 T cells expressing IFN- γ than IL-17 in both CFA and CRA groups (Figure 3A), and there was a modest increase in the amount of both cytokines being produced in the CRA-immunized mice compared to the CFA mice (Figure 3B). The production of IL-4 and IL-10 was also measured, but there was none detected. This data suggests that *C. rodentium* induces a similar immune response as *M. tuberculosis* when used as an adjuvant for subcutaneous immunization. In addition, neither the number of Th1 and Th17 cells, nor the amount of IFN- γ or IL-17 in the periphery appears to determine the incidence or severity of EAE.

The phenotype of infiltrating immune cells within the CNS provides a more accurate picture of the inflammatory response directly responsible for EAE. CNS mononuclear cells were isolated from CFA and CRA-immunized mice with clinical signs of EAE. Cells were isolated at early (day 16) and late (day 42) time points, corresponding with the time when CFA and CRA-immunized mice reach peak disease activity respectively. At both early and late time points, CFA-immunized mice had increased numbers of IFN- γ and IL-17-producing T cells relative to EAE-affected CRA mice (Figure 4 A,B). When comparing the number of CNS infiltrating cells that have an activated T cell phenotype (CD45⁺CD4⁺CD44^{hi}), the CFA-immunized mice had more activated T cells infiltrating the CNS at day 16. However, at day 42, although there was a decrease in the number of CNS infiltrating cells, there were more activated T cells within the CNS of CRA-immunized mice compared to CFA mice (Figure 4C), correlating with the differences in time of disease onset between CFA and CRA-immunized mice.

3.3 Epitope spreading correlates with development of EAE

Epitope spreading is believed to play a role in the development of relapses and/or the chronic phase of EAE, and possibly with the relapsing-remitting nature of MS. Although the pattern of epitope spread is well characterized in some models of EAE, such is not the case for MOG35-55 immunization in C57Bl/6 mice, now the most commonly used model of EAE. To determine if epitope spreading occurred in MOG35-55 induced EAE in C57Bl/6 mice, whole bovine myelin basic protein (bMBP), as opposed to specific peptides, was used as the antigen in lymphocyte proliferation assays to determine if additional myelin-specific T cells had been activated during the course of EAE in CFA and CRA-immunized mice that may contribute to disease. Splenocytes from CFA-immunized mice showed robust proliferation in response to MOG35-55, whereas the stimulation index from CRA-immunized mice was reduced by approximately 50%. Interestingly, the proliferative response to bMBP was equal between both CFA and CRA-immunized mice thus showing both adjuvants are equally capable of inducing epitope spreading (Figure 5).

Since all the mice used in the lymphocyte proliferation assay had EAE regardless of whether the mice were immunized with CFA or CRA and both developed similar T cell responses to bMBP, we wanted to determine if epitope spreading occurred in CRA-immunized mice that failed to develop EAE. To this end, precursor frequency analysis of MOG35-55 and bMBP-specific T cells in the spleen was determined in CRA mice that developed EAE and CRA immunized mice that remained free of disease. There was a correlation between mice that developed EAE and an increased frequency of bMBP-specific T cells, suggesting that epitope spreading may be a critical factor to develop EAE signs when *C. rodentium* is used as the adjuvant (Table 1). The same correlation was not observed with the frequency of MOG35-55-specific T cells in the CRA-immunized mice. The frequency of both MOG35-55 and bMBP-specific T cells in CFA-immunized mice was similar to that of CRA-immunized mice that developed EAE, suggesting that epitope spreading may be important in reaching a threshold of pathogenic T cells to generate signs of EAE, and CFA may do this more efficiently than CRA resulting in differences in disease incidence and severity.

4. Discussion

In light of the many recent studies in both EAE and MS that myelin-specific Th1 and Th17 cells both contribute to disease, but may yield distinct clinical phenotypes (Kroenke et al., 2010, Stromnes et al., 2008), we sought to determine if the bacterium in the adjuvant used in the induction of EAE influenced the phenotype of myelin-specific T cells and the clinical signs of disease. Since it is well established that *M. tuberculosis*, the bacteria in CFA, induces a strong Th1 cell response and IL-12 p35-deficiency results in an impaired capacity to control infection (Cooper et al., 2002, Khader et al., 2005), we performed a comparison study of EAE using a bacteria that is known to induce Th17 cells and is dependent on IL-23 p19 to establish protective immunity (Mangan et al., 2006). This study shows bacterium other than *M. tuberculosis* can serve as an effective adjuvant. The disease course of CRA immunizations is less severe and has a later onset in comparison to CFA immunization. When C57Bl/6 mice were immunized with CRA, 54% of the mice developed EAE within 25 days of immunization; however, 29% of the mice developed EAE at a later time point. The decreased incidence and severity of EAE in C57Bl/6 may make this a valuable model for studying certain aspects of MS. C57Bl/6 mice are readily available, and are the dominant genetic background for gene-deletion and gene-insertion studies; however, EAE in C57Bl/6 mice is one of the least representative of the clinical course observed in MS patients. MS is most frequently a relapsing-remitting disease with slow progression. In the CRA mice, there was more heterogeneity in the time of disease onset and slower progression of disease, more reminiscent of MS. These features are beneficial for studies that investigate novel therapies, because there is an increased window of time for the drug to mediate its effect before there

is severe neurological damage that would be resistant to most therapeutic intervention. This model would also be beneficial for studying molecules that contribute to disease severity. Since C57Bl/6 mice develop rapid-onset, severe EAE, it can be difficult to assess enhanced disease severity in these mice, which could be overcome by using CRA. Thus, C57Bl/6 mice immunized with CRA may serve as an alternative model of EAE in which the onset of disease is often delayed and severity reduced, yet most mice will develop EAE with the same peak clinical score as CFA induced EAE.

C. rodentium infections induce a strong IL-17 response within the lamina propria, so we hypothesized that we would see a similar response after immunization; nevertheless, we were surprised to observe a CD4 T cell immune response similar to immunization with CFA (Mangan et al., 2006). Lymph node cells showed similar ratios of IFN- γ and IL-17 expressing CD4 T cells in the CRA and CFA-immunized mice. There was a slight increase in the amount of IFN- γ and IL-17 observed in the CRA mice, but this did not seem to be biologically relevant, since the mice in both groups developed typical EAE characterized by ascending paralysis and not atypical EAE characterized by ataxia with minimal limb weakness often observed in Th17-mediated EAE (Stromnes et al., 2008). The observation that the peripheral CD4 T cell response to the immunogen was similar in both the CRA and CFA groups suggests that other factors were contributing to the time of disease onset and altered severity in the CRA immunized mice.

Three explanations can account for the difference between the cytokine profile seen in *C. rodentium* infection and the profile seen after CRA immunization. First, the nature of the bacteria may have changed in the preparation. The bacteria were heat-inactivated, and the high temperature may have broken down proteins and ligands critical for activating immune receptors. Immunizations with heat-killed bacteria or protein subunits commonly produced immune responses different from infection (Roberts et al., 1981, Zhan et al., 1993). *M. tuberculosis* is not an exception as infections are not dependent on IL-23 for protection (Khader et al., 2005), but EAE, which uses *M. tuberculosis* as an essential component of the adjuvant is dependent on IL-23 (Cua et al., 2003). The change in cytokine profile may also be due in part to the instability of the IL-17-producing phenotype as described by several groups (Hirota et al., 2011, Lee et al., 2009, Yang et al., 2009). The phenotype of the immune response may be dependent on the location. *C. rodentium* infects the colon of mice, and only in extreme infections are bacteria seen within other tissues (Simmons et al., 2003). Pepper et al. (Pepper et al., 2010) compared antigen-specific IFN- γ and IL-17 production in response to intravenous and intranasal infection with *Listeria monocytogenes*, and they found that intravenous infection resulted in a long-lasting memory population of IFN- γ -producing cells, whereas intranasal infection resulted in a short-lasting memory population of IL-17-producing cells. Therefore, use of *C. rodentium* as a mucosal adjuvant may produce a cytokine response more reminiscent of natural infection with enhanced production of IL-17 and reduced production of IFN- γ . Nonetheless, it appears that subcutaneous immunization of myelin peptides with heat-killed *C. rodentium* induces an immune response dominated by Th1 cells, in contrast to natural infection dominated by Th17 cells, resulting in EAE with classical ascending paralysis.

Toll like receptors (TLRs) play a critical role in driving the immune response necessary to develop EAE (Marta et al., 2008, Prinz et al., 2006). Different TLRs may affect the ratio of inflammatory and anti-inflammatory cytokines in different ways. Although there are limited studies on TLRs and *C. rodentium* in particular, it has been shown that both *M. tuberculosis* and *C. rodentium* stimulate TLR2 and TLR4 on immune cells (Abel et al., 2002, Gibson et al., 2008, Harding and Boom, 2010, Khan et al., 2006). We have previously shown that when C57Bl/6 mice are immunized with MOG35-55 using either a specific TLR2 or TLR4 agonists, instead of *M. tuberculosis* in the adjuvant, classical EAE is induced

(Hansen et al., 2006). To effectively induce EAE, the choice of bacterium in the adjuvant may not matter as long as it is capable of activating essential TLR signaling pathways. Differential expression of ligands for TLRs on individual bacteria and any disparity in bacterial numbers may account for differences in IFN- γ and IL-17 expression in lymph node cells and CNS of CRA-immunized mice.

The effects of adjuvants in the EAE model support the role of infections in the development and progression of MS. Although it is still not certain if infectious agents actually cause MS, evidence suggests that they play an important role in the development of disease (Milo and Kahana, 2010). Infections in patients with MS also cause an increased chance of relapse (Confavreux, 2002). Variation in the signs and symptoms of different MS patients may be influenced by environmental factors, infectious and noninfectious, encountered by individuals. The use of *C. rodentium* in the adjuvant shows that the use of different bacteria in the adjuvant can change the clinical course of EAE, illustrating that different pathogens may influence susceptibility and severity of autoimmune demyelinating disease.

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Figure 1.

C. rodentium adjuvant (CRA) induces a late onset of EAE. (A) B10.PL V β 8.2 TCR-transgenic mice were immunized with MBP Ac1-11 in emulsion with CFA (2mg/ml), CRA (2mg/ml), or IFA with or without co-injection of pertussis toxin (Ptx). Mice were monitored for EAE development. Disease incidence was CFA+Ptx (5/5), CFA (2/2), CRA+Ptx (3/5), CRA (2/5), IFA+Ptx (0/4), IFA (0/4). *Mice were euthanized due to EAE severity. (B) C57Bl/6 mice were immunized with MOG35-55 in emulsion with CFA (2mg/ml) and CRA (2mg/ml) with co-injection of pertussis toxin. Mice were monitored for EAE development. Disease incidence was CFA (5/6) and CRA (9/12).

Figure 2.

C. rodentium adjuvant (CRA) induces a milder EAE disease course. C57Bl/6 mice were immunized with MOG35-55. (A) MOG35-55 was given in emulsion with CFA (2mg/ml) or CRA at 2mg/ml, 1mg/ml, and 0.5mg/ml with co-injection of Ptx. Mice were monitored for EAE development. Disease incidence was CFA (7/7), CRA 2mg/ml (11/11), CRA 1mg/ml (6/11) and CRA 0.5mg/ml (2/11). (B) MOG35-55 was given in emulsion with CRA (2mg/ml) or CFA at 2mg/ml, 1mg/ml, and 0.5mg/ml with co-injection of Ptx. Mice were monitored for EAE development. Disease incidence was CRA (9/12), CFA 2mg/ml (12/12), CFA 1mg/ml (8/8) and CFA 0.5mg/ml (5/5). Representative of four independent experiments.

Figure 3.

CFA and CRA produce similar cytokine profiles dominated by IFN- γ . C57Bl/6 mice were immunized with MOG35-55 with CFA (2mg/ml; middle) or CRA (2mg/ml; right) with co-injection of Ptx. Draining lymph node cells were isolated 9 days after immunization and cultured with 2 μ g/ml MOG35-55 for 3 days. (A) Flow cytometric analysis was used to determine intracellular expression of IFN- γ and IL-17 on CD4⁺CD44^{hi} gated cells. (B) Supernatants were collected, and IFN- γ and IL-17 were measured by ELISA.

Figure 4.

Infiltration of activated T cells correlates with late incidence of EAE. C57Bl/6 mice were immunized with MOG35-55 with CFA (2mg/ml; middle) or CRA (2mg/ml; right) with co-injection of Ptx. CNS mononuclear cells were isolated from mice showing signs of EAE and were cultured overnight. Flow cytometric analysis was used to determine intracellular expression of IFN- γ and IL-17 on CD45⁺CD4⁺CD44^{hi} gated cells. (A) On day 16, CNS cells were isolated and cultured with 2 μ g/ml MOG35-55. Mean clinical score of mice was CFA=3 and CRA=2. (B) On day 42, CNS cells were isolated and cultured with 2 μ g/ml MOG35-55. Mean clinical score of mice was CFA=2.5 and CRA=3.5. (C) Represents number of gated CD45⁺ cells from A and B that were CD4⁺CD44^{hi}.

Figure 5.

Epitope spreading occurs in both CFA and CRA-immunized mice that develop EAE. C57Bl/6 mice were immunized with MOG35-55 with CFA (2mg/ml; middle) or CRA (2mg/ml; right) with co-injection of Ptx. Splenocytes were isolated from mice showing clinical signs of EAE 67 days after immunization and cells were cultured with MOG35-55 (10, 2, 0.4µg/ml) or bovine MBP (100, 20, 4µg/ml). Proliferation was measured using a [³H]-thymidine incorporation assay and the stimulation index (SI) was determined.

Table 1

Estimated frequency of autoreactive T cells

Adjuvant	EAE	Frequency	Frequency
		MOG35-55	bovine MBP
CRA	No	1/54,594	1/48,706
CRA	No	>1/12,000	1/58,497
CRA	Yes	>1/12,000	>1/12,000
CRA	Yes	>1/12,000	>1/12,000
CRA	Yes	1/29,904	1/14,308
CFA	Yes	1/14,308	>1/12,000