Brief Definitive Report

IL-12 Unmasks Latent Autoimmune Disease in Resistant Mice

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Summary

Inbred mice exhibit a spectrum of susceptibility to induction of experimental allergic encephalomyelitis (EAE). We have compared the immune responses of the susceptible SJL ($H\text{-}2^s$) and resistant B10.S ($H\text{-}2^s$) strains to determine factors other than the MHC background which control resistance/susceptibility to EAE. The resistance of the B10.S strain was found to be secondary to an antigen-specific defect in the generation of Th1 cells that produce IFNγ. This defect in IFNγ production could be restored by exposure of the myelin basic protein (MBP)-reactive T cells to IL-12 with the subsequent induction of the ability to transfer EAE to naive recipients. These findings have important implications for the therapeutic use of IL-12 and IL-12 antagonists and may explain the association between relapses/exacerbation of autoimmune disease and infectious diseases.

EAE is a demyelinating disease of the central nervous system induced by an autoimmune response against myelin proteins, particularly myelin basic protein (MBP) and its derivative peptides (1–3). EAE is mediated by CD4+ T cells and the T cells responsible for disease induction produce Th1 type cytokines. Thus, MBP-reactive T cell clones and lines that are encephalitogenic have been found to produce either IFNγ, TNFα, or TNFβ or a combination of these cytokines, while T cell clones or lines that produce IL-4 or IL-10 are non-encephalitogenic (4–8). Exposure of MBP-reactive LN cells to IL-12, a powerful inducer of IFNγ, enhances their encephalitogenicity. Conversely, treatment of animals that have received MBP-reactive cells with neutralizing antibodies to IL-12 attenuates the symptomatology of EAE (9). Examination of spinal cords from diseased animals has revealed the presence of mRNA for IFNγ and TNF during clinical episodes, whereas mRNA for IL-10 appeared at the time of clinical remissions (10). This suggests that remissions may be induced by downregulation of the Th1 response by the production of Th2-type cytokines. Furthermore, EAE can be ameliorated by the infusion of either IL-4 or IL-10 (11–12).

Inbred strains of mice exhibit a spectrum of susceptibility to induction of EAE. The MHC plays a critical role in disease susceptibility and strains bearing $H2^s$ or $H2^u$ haplotypes have been found to be susceptible, while those bearing $H2^d$ or $H2^k$ are resistant. One important exception is the B10.S strain, which is resistant to both active and passive EAE despite bearing the same MHC ($H2^s$) as mice of the prototypical susceptible strain, SJL (13). In this paper, we compare the immune responses of the SJL and B10.S strains to MBP in an attempt to uncover factors that may control resistance/susceptibility to EAE. As the differential induction of a Th1 vs a Th2 response has been shown to critically influence both the immune responses of different mouse strains to infection by pathogens and the immune responses needed for induction of autoimmune diseases (14–16), we have focused our efforts on a comparison of cytokine production by the two strains. We demonstrate that the resistance of the B10.S strain to the induction of EAE after immunization with MBP is secondary to an antigen-specific defect in the generation of Th1 cells that produce IFNγ. However, the defect in IFNγ production can be corrected by exposure of the MBP-reactive T cells to IL-12 in vitro with the subsequent induction of encephalitogenic T cells. These results have important implications for furthering our understanding of the role of cytokines in the susceptibility to, and pathogenesis of, autoimmune disease.

Materials and Methods

Mice. Female B10.S and SJL/J mice were obtained from McLaughlin Research Institute (Great Falls, MT) and Jackson Laboratory (Bar Harbor, ME), respectively, and kept in a pathogen free facility. All mice were 8–12 wk of age when experiments were initiated.

Antigens. MBP was prepared from guinea pig spinal cords, as previously described (17). Peptides corresponding to residues 260–283 of Influenza A nucleoprotein ($NP_{260\text{-}283}$, ARSALILRGSVAHKSCLPACVYGP), and residues 87–106 of MBP ($MBP_{87\text{-}106}$, VVHFFKNIVTPRTPPPSQGK) were synthesized and purified by HPLC by the Laboratory of Molecular Structure, Peptide Synthesis Laboratory (NIAID, NIH, Bethesda, MD).

Immunization. Mice were immunized subcutaneously at four sites over the flanks with an emulsion containing equal volumes

of CFA (DIFCO Laboratories, Detroit, MI) and antigen dissolved in PBS. Each mouse received a total of 0.1 ml of emulsion (0.025 ml/site) containing 30 μg of Mycobacterium tuberculosis and an optimal dose of the relevant antigen. Optimal doses were determined in pilot experiments as follows: MBP, 400 μg; $NP_{260-283}$, 5 μg; MBP_{87-106}, 100 μg.

Generation of Ag-Specific LN Cells for Cell Transfer and Analysis of Cytokine Production. 10–11 d after immunization draining LN cells (inguinal and axillary) were removed and processed as previously described. Cells were cultured in RPMI 1640 containing 10% FCS and standard supplements (18) with either MBP (25 μg/ml), MBP_{87-106} (50 μg/ml), or $NP_{260-283}$ (12.5 μg/ml). Supernatants were removed at 24 h intervals for quantification of cytokine levels. After four days of culture, cells were harvested and washed extensively. Some of these cells were injected into naive recipients (35×10^6 cells/mouse intraperitoneal) to test their encephalitogenicity. Other cells were recultured in fresh medium with or without soluble antigen and supernatants were removed at 24 and 48 h for cytokine analysis.

Where specified, cytokines or neutralizing antibodies were added to the primary cultures as follows: IL-12, 20 ng/ml (gift of S. Wolf, Genetics Institute, Cambridge, MA); anti–IL-4 mAb, 10 μg/ml (clone 11B11; gift of W.E. Paul, NIAID, NIH); rat anti–mouse IL-10 mAb, 0.5 μg/ml (clone JES5-2A5; PharMingen, San Diego, CA); recombinant murine IFNγ, 30 ng/ml (PharMingen); and anti-mouse IFNγ, 20 μcg/ml (clone XMG 1.2; gift of R. Seder, NIAID, NIH).

Purification of T Cells. T cells were purified to levels of ~90% purity from LN cell preparations by passage through nylon wool columns followed by "mouse T cell enrichment" columns (R & D Systems, Minneapolis, MN). Recovered T cells were washed twice, resuspended in complete medium at 1×10^6 cells/well, and then cocultured with irradiated (3,000 rads) splenocytes added at a 1:4 ratio. Before being added, splenocytes were depleted of T cells by c-mediated lysis using anti-Thy 1.2 (present in supernatants of cell line HO13.4-9).

Proliferation Assays. LN cells (5×10^5/0.2 ml) were cultured with various concentrations of antigen or with media alone for 4 d in 96-well round-bottom plates (Costar, Cambridge, MA) at 37°C in 3% CO_2-air. Wells were pulsed for the final 16 h of culture with 1 μCi of [^{3}H]TdR (Amersham Corp., Arlington Heights, IL) and counted as previously described (18).

Cytokine ELISA. Cytokines were quantified using a sandwich ELISA technique based on noncompeting pairs of antibodies. The capture and detection mAbs used for each cytokine were obtained from PharMingen except for rabbit anti–mouse IFNγ (Spring Valley Laboratories, Woodbine, MD). Goat anti–rabbit IgG-HRP (Southern Biotechnology Associates, Inc., Birmingham, AL) was used as a tertiary Ab in the IFNγ ELISA. Peroxidase labeled streptavidin (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) was applied as a last step in the ELISAs using biotinylated detection antibodies. Plates were developed with TMB peroxidase substrate (Kirkegaard & Perry) and stopped with 10% sulfuric acid.

Clinical Evaluation. After injection of MBP-reactive cells, mice were examined daily for signs of neurologic impairment and rated for severity of symptomatology as previously described (18).

Results

B10.S Mice Have A Specific Defect in IFNγ Production in Response to MBP. As an initial approach to determine the basis of the resistance of B10.S mice to EAE, we immunized B10.S and SJL mice with guinea pig MBP in CFA and compared the proliferative responses and cytokine production of draining LN cells to antigen stimulation 10 d later. LN cells from the two strains mounted comparable proliferative responses to MBP and to PPD (Fig. 1 *A*) and produced comparable quantities of IL-2 in an antigen-specific manner (data not shown). However, LN cells from SJL mice produced significant quantities of IFNγ which accumulated over time, while LN cells from B10.S mice failed to secrete detectable quantities of that cytokine (Fig. 1 *B*). Furthermore, LN cells from B10.S mice failed to produce significant amounts of IFNγ upon secondary in vitro stimulation, while LN cells from SJL produced heightened amounts of IFNγ with accelerated kinetics (Fig. 1 *C*). TNFα production was not detectable in LN cells of either strain (data not shown).

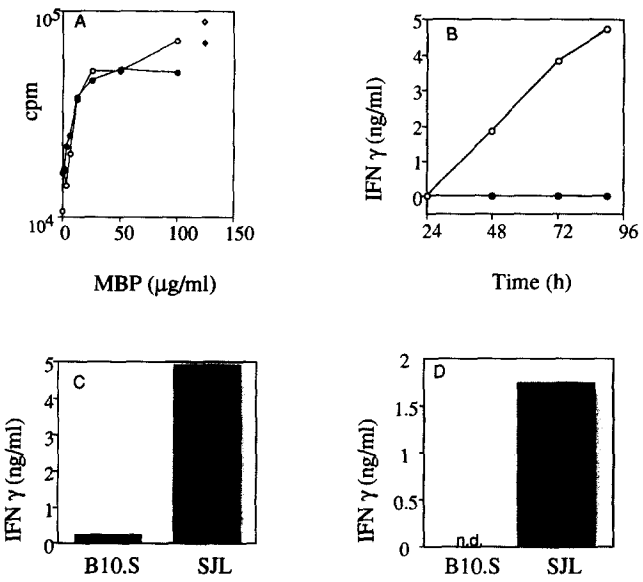

Figure 1. MBP-reactive LN cells from B10.S mice proliferate, but do not produce IFNγ on antigenic challenge in vitro. Draining LN cells from MBP-primed B10.S (*closed symbols*) or SJL (*open symbols*) mice were stimulated in vitro with MBP (*circles*) or PPD (*diamonds*). Proliferative responses (*A*) were measured after 96 h and IFNγ production assayed over a 96-h period (*B*). Draining LN cells from B10.S and SJL mice primed with MBP (*C*) or with the peptide, MBP_{87-106}, (*D*) were cultured for 96 h with MBP or with MBP_{87-106} respectively, washed, restimulated for 48 h with antigen, and IFNγ production measured. Results are representative of 10 experiments each using LN cells pooled from five or more mice. *nd*, none detected.

The failure of the B10.S mice to produce IFNγ was not related to the overproduction of suppressive Th2 cytokines as LN cells from neither strain produced detectable amounts of IL-4 or IL-10 (data not shown), nor did the addition of anti–IL-4 or anti–IL-10 alone or in combination to primary cultures restore the capacity of B10.S T cells to produce IFNγ on secondary in vitro stimulation with MBP (see Fig. 3). Furthermore, treatment of B10.S animals during the course of priming with MBP with large quantities of a neutralizing anti–IL-4 mAb (0.5 mg of 11B11 intraperitoneal on day 0, 3, and 7) failed to result in IFNγ production upon stimulation of the LNC in vitro (data not shown). The defect was also not related to a failure of pro-

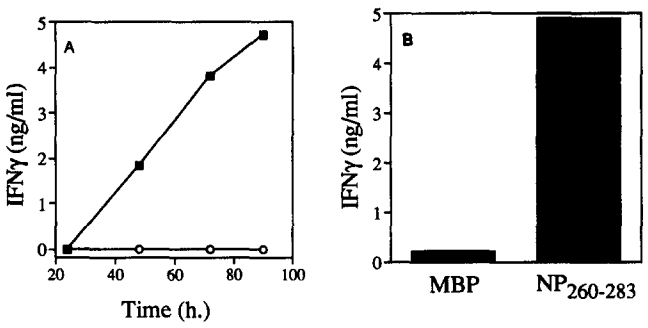

Figure 2. The failure of MBP-primed B10.S T cells to produce IFNγ in vitro is antigen-specific. LNCs from $NP_{260-283}$-primed (*closed squares*) or MBP-primed (*open circles*) B10.S mice were stimulated for 96 h in vitro with antigen and IFNγ levels were measured at 24-h intervals (*A*). The cultures were then washed and restimulated for 48 h and IFNγ production assayed (*B*). Results are representative of seven experiments each using LN cells pooled from five or more mice.

cessing or presentation of the encephalitogenic epitope of MBP as similar results were obtained after immunization of the two strains with the encephalitogenic peptide, MBP_{87-106} (Fig. 1 *D*). In addition, the inability of MBP-reactive B10.S T cells to produce IFNγ was not secondary to a defect in cytokine production by B10.S APC. The capacity of purified MBP-specific B10.S T cells to produce IFNγ was not restored when they were cultured with T-depleted spleen cells from SJL mice, nor was the capacity of MBP-specific SJL T cells to produce IFNγ diminished when they were cultured with B10.S T-depleted spleen cells (data not shown). Although the studies shown above were performed with unseparated LNC or purified T cells, identical results were observed when highly purified CD4+ T cells were stimulated with MBP in the presence of T-depleted spleen cells from normal animals.

The inability of B10.S LNC to produce IFNγ in response to MBP was antigen-specific as primed B10.S LNCs produced significant quantities of IFNγ on both primary and secondary in vitro stimulation with a peptide of Influenza A virus nucleoprotein, $NP_{260-283}$, previously shown to be highly immunogenic in this strain (19) (Fig. 2, *A* and *B*). Primed B10.S LNC also produced as much IFNγ as primed SJL LNC when stimulated in vitro with PPD (data not shown).

Reconstitution of the Capacity of MBP-primed B10.S LNCs to Produce IFNγ by Treatment with IL-12. As IL-12 has been shown to play a critical role in the induction of IFNγ production in response to several pathogens, we attempted to induce IFNγ production by MBP-primed B10.S T cells by adding IL-12 to the primary in vitro cultures. Surprisingly, the addition of IL-12 resulted in substantial production of IFNγ in the secondary cultures that was equal to or greater than that produced by LN cells from SJL mice on secondary in vitro challenge. The presence of neutralizing mAb to IFNγ did not compromise the ability of IL-12 to promote IFNγ production on subsequent challenge, suggesting that the effect of IL-12 is direct and not mediated by the IFNγ it induces. Furthermore, the addition of IFNγ to the primary cultures failed to prime for its own synthesis (Fig. 3). No enhancement of IFNγ production in secondary cultures was seen when either anti–IL-4 or anti–IL-10 were used together with IL-12 in the primary cultures (Fig. 3).

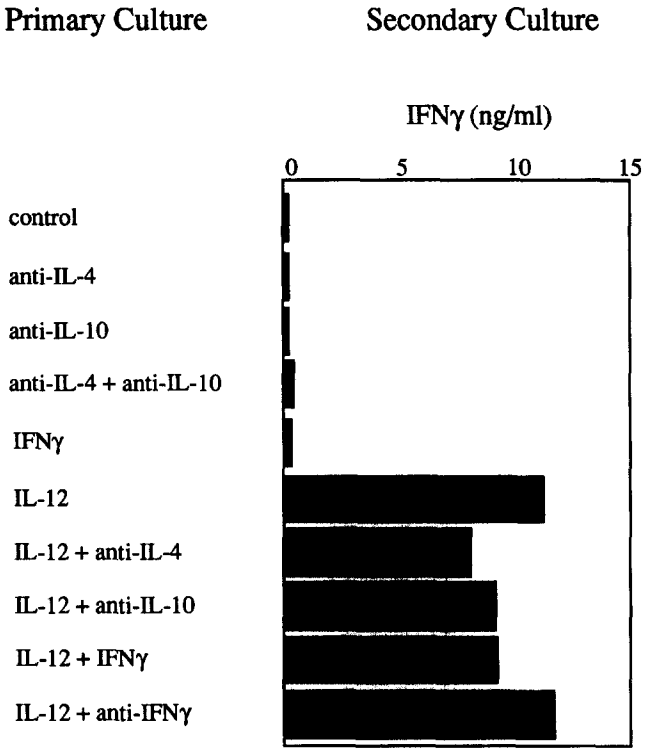

Figure 3. Exposure of MBP-reactive LNCs from B10.S mice to IL-12 promotes IFNγ production on subsequent antigenic challenge. LN cells from MBP-primed B10.S mice were cultured for 4 d in the presence of MBP and the cultures were supplemented with the reagents listed in the figure. All cultures were then harvested, washed, and restimulated with MBP alone and IFNγ production measured after 48 h. Results are representative of four experiments each using LNCs pooled from five or more mice.

Encephalitogenicity of IL-12–treated B10.S and SJL LNC. The capacity of LN cells from MBP-primed mice to transfer EAE to normal adoptive recipients is dependent on restimulation of the cells in culture. However, the critical parameters that permit in vitro stimulated cells to induce disease have not been fully elucidated. Although the induction of IFNγ production by MBP-specific B10.S T cells with IL-12 is of interest, it is not clear if the production of IFNγ per se relates to the capacity of MBP-specific effector cells to induce EAE in vivo. We therefore transferred SJL and B10.S T cells that had been cultured with MBP alone or B10.S T cells which had been cultured with MBP in the presence of IL-12 to naive recipients and monitored them for the induction of EAE. MBP-reactive cells from SJL mice transferred moderate to severe relapsing-remitting EAE with 100% incidence, while no disease was observed in recipients of MBP-reactive T cells from B10.S mice during a 40 d follow-up period. More importantly, B10.S T cells that had been cultured with MBP and IL-12 acquired the ability to induce EAE upon adoptive transfer. The resultant disease occurred at 100% incidence, but was not as severe as that induced by SJL LNC. The induction of EAE

Table 1. *IL-12–treated MBP-primed B10.S LN Cells Induce EAE*

Strain*	Primary culture conditions	Disease incidence	Mean peak severity	Disease course‡
SJL	MBP	100%	3.5	relapsing
B10.S	MBP	0%	N/A	N/A
B10.S	MBP + IL-12	100%	2.25	monophasic
B10.S	$NP_{260-283}$ + IL-12	0%	0	N/A

*LN cells from antigen-primed B10.S or SJL mice were cultured with the indicated antigen in the presence or absence of IL-12 (20 ng/ml) for 4 d. The cells were then washed and injected (3.5×10^7 cells intraperitoneal per mouse) into naive syngeneic recipients. Animals were monitored daily for neurological signs. Results shown are representative of four experiments performed with five mice in each group.
‡Average day of disease onset was 8.2 d for B10.S mice and 7.6 d for SJL mice.

was absolutely dependent on the presence of MBP-specific T cells as B10.S LNCs sensitized to $NP_{260-283}$ and exposed to IL-12 do not induce EAE (Table 1).

Discussion

Multiple genetic factors control the susceptibility of experimental animals and man to autoimmune diseases. Although the MHC plays a major role in determining the susceptibility of rodents to EAE, our comparison of the factors responsible for the induction of EAE in the B10.S and SJL strains which both bear $H\text{-}2^s$ has defined a critical, non-MHC related, step in the activation cascade of autoreactive T cells that is defective in the B10.S mouse. We initially reasoned that resistance of B10.S mouse to MBP-induced EAE was mediated at the level of the T cell and APC and was not due to factors peripheral to the immune reaction, such as the impermeability of the blood–brain barrier or resilience of the oligodendrocyte to autoimmune attack, as B10.PL ($H\text{-}2^u$) mice are readily susceptible to induction of EAE (4, 20). Hence, there is no characteristic of the B10 background which prevents manifestations of EAE if functional encephalitogenic T cells are present and properly primed. We found that the distinguishing characteristic of the B10.S immune response to intact MBP, as well as to MBP_{87-106}, was the failure of primed T cells to produce IFNγ in vitro in the presence of a vigorous IL-2–driven antigen-specific proliferative response. This failure to produce IFNγ in response to MBP was antigen-specific and was not secondary to an ongoing Th2 response.

MBP-reactive B10.S LN cells acquired the ability to produce IFNγ upon exposure to IL-12 during primary ex vivo challenge with antigen. Furthermore, this ability was retained upon subsequent antigenic challenge in the absence of IL-12. Concomitantly, exposure to IL-12 unmasked the latent potential of these T cells to induce EAE on adoptive transfer. It is not clear whether the acquisition of the capacity to produce IFNγ was directly responsible for encephalitogenicity of the IL-12–treated T cells. It is possible that IL-12 has an effect on T cells in addition to its well characterized ability to prime for IFNγ production that is critical in conferring encephalitogenicity. Indeed, previous studies have raised the possibility that endogenous IL-12 might play a role in the pathogenesis of EAE by inducing the upregulation of adhesion molecules (9). The role of IFNγ in the pathogenesis of EAE is not well understood as attempts to assess the effect of neutralizing antibodies to IFNγ on the disease have yielded contradictory results (21–23) and mice of a susceptible strain with a disrupted IFNγ gene still manifest clinical signs of EAE after transfer of MBP-reactive cells (24). Hence, although IFNγ might play an integral role in establishing or perpetuating the CNS inflammation characteristic of EAE, its presence is not essential and probably can be compensated by production of other proinflammatory cytokines. The induction of IFNγ production by IL-12 may thus simply serve as a surrogate marker of encephalitogenicity. We cannot as yet conclude that reconstitution of encephalitogenicity by treatment of primed B10.S T cells with IL-12 is indicative of a critical role for this cytokine in the induction of IFNγ production in vivo as studies with neutralizing anti–IL-12 reagents in vivo are still in progress.

In conclusion, we have found that exposure to IL-12 unmasks a latent capacity of MBP-reactive T cells from a resistant strain to transfer EAE. These findings have important implications for the use of IL-12 or antagonists to IL-12 for therapeutic purposes. Use of IL-12 as an adjunct in anti-tumor therapy or as a component of a vaccine could trigger an autoimmune syndrome in genetically predisposed individuals. Our results also suggest that the association between intercurrent infections and presentation or relapse of autoimmune diseases, including multiple sclerosis (25, 26), may be secondary to IL-12 production in response to pathogens. On the other hand, our data support the view that antagonists to IL-12 may have therapeutic value in the treatment of inflammatory autoimmune diseases by inhibiting the activation and/or action of Th1 T cells.

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