IL-12 Unmasks Latent Autoimmune Disease in Resistant Mice

By Benjamin M. Segal and Ethan M. Shevach

From the Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892

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-2b) and resistant B10.S (H-2b) 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 H2b or H2d haplotypes have been found to be susceptible, while those bearing H2a or H2k 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) 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 (NP260_283, ARSALLRGSVAKHKSCLPACVYGP), and residues 87–106 of MBP (MBP87_106, VVHFFKNIIVTPRTTPPSQGK) 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 MBP and complete Freund's adjuvant (CFA) on days 0 and 21. Control mice were immunized with CFA alone on day 0. Upon challenge with MBP and CFA on day 21, animals were monitored for paralysis and encephalitis (EAE) scores as previously described (18).
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, 200-250, 5 μg; MBP, 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 (50 μg/ml), or NP, 25-250 (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 X 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, 1 μg/ml (clone JES-2A5; PharMingen, San Diego, CA); recombinant murine IFNγ, 30 ng/ml (PharMingen); and anti-mouse IFNγ, 20 μg/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 X 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 X 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% CO₂-air. Wells were pulsed for the final 16 h of culture with 1 μCi of [³H]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). TNFa production was not detectable in LN cells of either strain (data not shown).

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-
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<sub>87-106</sub> (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<sup>+</sup> 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<sub>260-283</sub>, 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).

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 re-stimulation 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   | NP260-283 + 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 × 10⁷ 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.

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-2b has defined a critical, non-MHC related, step in the activation cascade of autoreactive T cells. Antigen presentation (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-2b) 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.

We wish to thank Dr. Sidney Wolf and the Genetic Institute for the generous gift of recombinant IL-12.

Address correspondence to Ethan M. Shevach, Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bldg. 10, Rm 11N311, 10 Center Drive-MSC 1892, Bethesda, MD 202892-1892.

Received for publication 9 April 1996 and in revised form 3 June 1996.

774  IL-12 Unmasks Latent Autoimmune Disease in Resistant Mice
References

1. Mohkhatarin, F., D.E. McFarlin, and C.S. Raine. 1984. Adaptive transfer of myelin basic protein-sensitized T cells produces chronic relapsing demyelinating disease in mice. Nature (Lond.) 309:356–358.

2. Kono, D.H., J.L. Urban, S.J. Horvath, D.G. Ando, R.A. Sazaveda, and L. Hood. 1988. Two minor determinants of myelin basic protein induce experimental allergic encephalomyelitis in SJL/J mice. J. Exp. Med. 168:213–227.

3. Sakai, K., S.S. Zamvil, D.J. Mitchell, M. Lim, J.B. Rothbard, and L. Steinman. 1988. Characterization of a major encephalitogenic T cell epitope in SJL/J mice with synthetic oligopeptides of myelin basic protein. J. Neuroimmunol. 19:21–32.

4. Ando, D., J. Clayton, D. Kono, J. Urban, and E.E. Sercarz. 1989. Encephalitogenic T cells in the B10.PL model of experimental allergic encephalomyelitis (EAE) of the Th-1 lymphokine subtype. Cell. Immunol. 124:132–143.

5. Powell, M.B., D. Mitchell, J. Lederman, J. Buckmeier, S.S. Zamvil, M. Graham, N.H. Ruddle, and L. Steinman. 1990. Lymphotoxin and tumor necrosis factor-alpha production by myelin basic protein-specific T cell clones correlates with encephalitogenicity. Int. Immunol. 2:539–544.

6. van der Veen, R.C., and S.A. Stohlman. 1993. Encephalitogenic Th1 cells are inhibited by Th2 cells with related peptide specificity: relative roles of interleukin (IL)-4 and IL-10. J. Neuroimmunol. 48:213–220.

7. Baron, J.L., J.A. Madri, N.H. Ruddle, G. Hashim, and C.A. Janeway, Jr. 1993. Surface expression of e4 integrin by CD4 T cells is required for their entry into brain parenchyma. J. Exp. Med. 177:57–68.

8. Khoruts, A., S.D. Miller, and M.K. Jenkins. 1995. Neuroantigen-specific Th2 cells are inefficient suppressors of experimental autoimmune encephalomyelitis induced by effector Th1 cells. J. Immunol. 155:5011–5017.

9. Leonard, J.P., K.E. Waldburger, and S.J. Goldman. 1995. Prevention of experimental autoimmune encephalomyelitis by antibodies against interleukin. J. Exp. Med. 181:381–386.

10. Kennedy, M.K., D.S. Torrance, K.S. Picha, and K.M. Mohler. 1992. Analysis of cytokine mRNA expression in the central nervous system of mice with experimental autoimmune encephalomyelitis reveals that IL-10 mRNA expression correlates with recovery. J. Immunol. 149:2496–2505.

11. Racke, M.L., A. Bonomo, D.E. Scott, B. Canella, A. Levine, C.S. Raine, E.M. Shevach, and M. Rocken. 1994. Cytokine-induced immune deviation as a therapy for inflammatory autoimmune disease. J. Exp. Med. 180:1961–1966.

12. Rott, O., B. Fleischer, and E. Cash. 1994. Interleukin-10 prevents experimental allergic encephalomyelitis in rats. Eur. J. Immunol. 24:1434–1440.

13. Arnon, R. 1981. Experimental allergic encephalomyelitis—susceptibility and suppression. Immunol. Rev. 55:5–50.

14. Reiner, S.L., and R.M. Locksley. 1995. The regulation of immunity to leishmania major. Annu. Rev. Immunol. 13:151–177.

15. Sher, A., and R.L. Coffman. 1992. Regulation of immunity to parasites by T cells and T cell-derived cytokines. Annu. Rev. Immunol. 10:385–409.

16. Libau, R.S., S.M. Singer, and H.O. McDevitt. 1995. Th1 and Th2 CD4+ T cells in the pathogenesis of organ-specific autoimmune diseases. Immunol. Today. 16:34–38.

17. Deibler, G.E., R.E. Martenson, and M.W. Kies. 1972. Large scale preparation of myelin basic protein from central nervous tissues of several mammalian species. Prep. Biochem. 2:139–165.

18. Segal, B.M., C.S. Raine, D.E. McFarlin, R.R. Voskuhl, and H.F. McFarland. 1994. Experimental allergic encephalomyelitis induced by the peptide encoded by exon 2 of the MBP gene, a peptide implicated in remyelination. J. Neuroimmunol. 51:7–19.

19. Gao, X., F.Y. Liew, and J.P. Tito. 1998. Identification and characterization of T helper epitopes in the nucleoprotein of influenza A virus. J. Immunol. 143:3007–3014.

20. Merrill, J.E., D.H. Kono, J. Clayton, D.G. Ando, D.R. Hinton, and F.M. Hofman. 1992. Inflammatory leukocytes and cytokines in the peptide-induced disease of experimental allergic encephalomyelitis in SJL and B10.PL mice. Proc. Natl. Acad. Sci. USA. 89:574–578.

21. Billiau, A., H. Heremans, F. Vandekerckhove, R. Dijkmans, H. Sobis, E. Meulepas, and H. Carton. 1988. Enhancement of experimental allergic encephalomyelitis in mice by antibodies against IFN-γ. J. Immunol. 151:1506–1510.

22. Vooitihuis, J.A., B.M. Uitdehaag, C.J. De Groot, P.H. Goede, P.H. van der Meide, and C.D. Dijkstra. 1990. Suppression of experimental allergic encephalomyelitis by intraventricular administration of interferon-γ in Lewis rats. Clin. Exp. Immunol. 81:183–188.

23. Duong, T.T., J. St. Louis, J.J. Gilbert, F.D. Finkelman, and G.H. Strejan. 1992. Effect of anti-interferon-γ and anti-interleukin-2 monoclonal antibody treatment on the development of actively and passively induced experimental allergic encephalomyelitis in the SJL/J mouse. J. Neuroimmunol. 36:105–115.

24. Ferber, I.A., S. Brocke, C. Taylor-Edwards, W. Ridgway, C. Dinisco, L. Steinman, D. Dalton, and C.G. Fathman. 1996. Mice with a disrupted IFN-γ gene are susceptible to the induction of experimental autoimmune encephalomyelitis (EAE). J. Immunol. 156:5–7.

25. Gay, D., G. Dick, and G. Upton. 1986. Multiple sclerosis associated with sinusitis: case-controlled study in general practice. Lancet. 1:815–819.

26. Sibley, W.A., C.W. Bamford, and K. Clark. 1985. Clinical viral infections and multiple sclerosis. Lancet. 1:1313–1315.