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Initiation and Exacerbation of Autoimmune Demyelination of the Central Nervous System via Virus-Induced Molecular Mimicry: Implications for the Pathogenesis of Multiple Sclerosis

J. Ludovic Croxford,† Julie K. Olson,† Holly A. Anger, and Stephen D. Miller*

Department of Microbiology and Immunology and Interdepartmental Immunobiology Center, Northwestern University Feinberg School of Medicine, 303 E. Chicago Ave., Chicago, Illinois 60611

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Epidemiological studies indicate that infectious agents are important in the pathogenesis of multiple sclerosis (MS). Our previous reports showed that the infection of SJL mice with a nonpathogenic variant of Theiler's murine encephalomyelitis virus (TMEV) engineered to express a naturally occurring *Haemophilus influenzae*-encoded molecular mimic ($HI_{574-586}$) of an immunodominant self-myelin proteolipid protein epitope ($PLP_{139-151}$) induced a rapid-onset demyelinating disease associated with the activation of $PLP_{139-151}$-specific Th1 responses. The current results extend our previous findings in four critical respects. We show that disease initiation by the *H. influenzae* mimic is prevented by tolerance to the self $PLP_{139-151}$ epitope, definitively proving the occurrence of infection-induced molecular mimicry. We demonstrate that the *H. influenzae* mimic epitope can be processed from the flanking sequences within the native mimic protein. We show that the *H. influenzae* mimic epitope only induces an immunopathologic self-reactive Th1 response and subsequent clinical disease in the context of the TMEV infection and not when administered in complete Freund's adjuvant, indicating that molecular mimicry-induced disease initiation requires virus-activated innate immune signals. Lastly, we show that the infection of SJL mice with TMEV expressing the *H. influenzae* mimic can exacerbate a previously established nonprogressive autoimmune disease of the central nervous system. Collectively, these findings illustrate the evolving mechanisms by which virus infections may contribute to both the initiation and exacerbation of autoimmune diseases, and they have important implications for MS pathogenesis.

Multiple sclerosis (MS) is a human autoimmune demyelinating disease of the central nervous system (CNS) which is thought to be mediated by autoreactive T cells. Both genetic and environmental factors, such as infections, have been implicated in MS susceptibility (8, 14, 15, 29), and virus infections can induce demyelinating diseases (7, 26, 32, 34). Interestingly, both healthy individuals and MS patients possess peripheral $CD4^+$ T cells specific for myelin antigens (25, 30). Nonetheless, the mechanism of autoreactive T-cell activation in MS remains unknown. One proposed mechanism of activation is molecular mimicry, whereby autoreactive T cells can be activated by infectious agents encoding epitopes with similar structures or sequence homology to self-tissue epitopes (10, 21). T-cell mimicry between myelin and viral peptides has recently been described for MS patients (33, 36). Molecular mimicry has also been implicated in the pathogenesis of diabetes, systemic lupus erythematosis, and human T-lymphotropic virus type 1-associated myelopathy/tropical spastic paraparesis (1, 11, 16, 19). MS patients present with multiple clinical forms, including relapsing-remitting and chronic-progressive forms of disease. Although the mechanism(s) involved in the initiation and in the relapse phase of relapsing-remitting MS is currently not fully understood, several factors, including viral infection, may be important (2, 27, 28).

We previously demonstrated (20) that a nonpathogenic variant of Theiler's murine encephalomyelitis virus (TMEV) expressing either the mouse myelin proteolipid protein (PLP) epitope, $PLP_{139-151}$, or a core mimic sequence of $PLP_{139-151}$ derived from *Haemophilus influenzae* ($HI_{574-586}$) (3) induced a mild, slowly progressing form of CNS demyelinating disease. Clinical disease was associated with the cross-activation and Th1 differentiation of myelin epitope $PLP_{139-151}$-specific $CD4^+$ T cells, and the disease induced by the virus encoding the self $PLP_{139-151}$ epitope could be prevented by tolerance to the homologous self peptide (22).

The present studies were designed to further understand the mechanisms of infection-induced molecular mimicry in both the initiation and progression of CNS autoimmunity. The present results extend our previous studies by illustrating four additional critical observations regarding the role of infection-induced molecular mimicry in activating CNS demyelinating disease. We demonstrate (i) that the CNS disease induced by infection with the core *H. influenzae* mimic-expressing virus is due to the cross-activation of autoreactive T cells, as peripheral tolerance induced to the self myelin $PLP_{139-151}$ epitope specifically inhibited disease induction; (ii) that the mimic *H. influenzae* epitope can be naturally processed from the flanking sequences in the native *H. influenzae* protein and presented to cross-activate $PLP_{139-151}$-specific autoreactive $CD4^+$ Th1 cells; (iii) that molecular mimicry-induced disease initiation requires virus-activated innate immune signals, since infection with TMEV expressing the *H. influenzae* mimic induced disease, while the core *H. influenzae* mimic epitope administered in complete Freund's adjuvant (CFA) did not; and (iv) that a

* Corresponding author. Mailing address: Dept. of Microbiology-Immunology, Northwestern University, 303 E. Chicago Ave., Chicago, IL 60611. Phone: (312) 503-1449. Fax: (312) 503-1154. E-mail: s-d-miller@northwestern.edu.

† These authors contributed equally to this work.

slowly progressing CNS autoimmune disease can be significantly exacerbated upon reinfection with the *H. influenzae* mimic-expressing virus. Collectively, these studies demonstrate that autoreactive T cells triggered by infection-induced molecular mimicry can both initiate and exacerbate clinical autoimmune demyelination and have important implications for the initiation and relapse phases of MS pathogenesis.

MATERIALS AND METHODS

Mice. Five- to 6-week-old female SJL mice were obtained from Harlan Sprague-Dawley (Indianapolis, Ind.). Mice were housed under barrier conditions at the National Institutes of Health-approved Northwestern University Medical School animal facilities. All protocols were approved by the Northwestern University Animal Care and Use Committee. Paralyzed mice were afforded easier access to food and water.

Construction of mimic-expressing TMEV. The cDNA encoding the BeAn strain of TMEV was modified by inserting ClaI sites and deleting 23 amino acids, as previously described (20). Genomic DNA containing the sequence coding for *Haemophilus influenzae* serine protease IV was obtained from the American Type Culture Collection (Manassas, Va.). ClaI sites were introduced by PCR to the *H. influenzae* DNA at either end of a coding sequence for 39 amino acids, $HI_{566-604}$, which contained the $PLP_{139-151}$ mimic sequence, $HI_{574-586}$, by using the following primers: HI39A, 5′ GCA CCT TAA TCG ATT TGA GCG 3′; and HI39AS, 5′ CTG ACC ATC GAT TTT AGG ATC 3′. Following an enzyme restriction cut with ClaI, the 39-amino-acid-encoding piece containing the $HI_{566-604}$ sequence was inserted into the ClaI site in the ΔCla-BeAn virus cDNA. This was designated HI39-BeAn cDNA. Infectious virus was produced as previously described (20). The PLP-BeAn, OVA-BeAn, and HI-BeAn viruses were produced as previously described (20).

Infection of SJL mice with TMEV. Mice ($n = 8$) were infected by the intracerebral injection of 3×10^6 PFU of either wild-type TMEV (strain BeAn 8386), HI-BeAn, PLP139-BeAn, OVA-BeAn, or ΔCla-BeAn and were scored at weekly intervals on a clinical scale of 0 to 5, as follows: 0, asymptomatic; 1, mild waddling gait; 2, severe waddling gait; 3, paralysis of one hindlimb; 4, total hindlimb paralysis, severe dehydration, and/or malnutrition; and 5, death. The data were plotted as mean clinical scores for each group of animals.

Peptides. The proteolipid protein (PLP) peptide $PLP_{139-151}$ (HSLGKWLGH PDKF), the TMEV capsid peptide $VP2_{70-86}$ (WTTSQEAFSHIRIPLPH), the ovalbumin peptide $OVA_{323-339}$ (ISQAVHAAHAEINEAGR), the *H. influenzae* peptide $HI_{574-586}$ (EQLVKWLGLPAPI), the *H. influenzae* 30-mer peptide $HI_{566-595}$ (SDTKKGAQEQLVKWLGLPAPIQKLQKELNI), and the PLP 30-mer peptide $PLP_{130-159}$ (QAHSLERVCHCLGKWLGHPDKFVGITYALT) (Fig. 1a) were purchased from Peptides International (Louisville, Ky.). Their amino acid compositions were verified by mass spectrometry, and their purity was assessed by high-performance liquid chromatography.

Induction of peripheral tolerance. Tolerance was induced by the intravenous injection of 5×10^7 peptide-pulsed, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (ECDI)-fixed syngeneic splenocytes, as described previously (13, 18, 22).

Delayed-type hypersensitivity responses. Delayed-type hypersensitivity (DTH) responses were elicited by injecting mice subcutaneously in alternate ears with 10 μg of the challenge peptides after measurements of ear thickness with a Mitutoyo model 7326 engineer's micrometer (Schlesinger's Tools, Brooklyn, N.Y.). Twenty-four hours following the peptide challenge, the ears were measured again, and differences in ear thickness (swelling) from the prechallenge thickness were expressed in units of 10^{-4} inches (mean ± standard errors of the means), as previously described (20).

T-cell proliferation and cytokine analysis. Spleens were removed from infected mice that had not been previously used for DTH responses ($n = 2$) at the indicated times following infection or immunization. T-cell proliferation and cytokine analyses were performed as described previously (20). Proliferation was determined for triplicate wells for each peptide concentration and then expressed in counts per minute. For gamma interferon (IFN-γ) cytokine analysis, a duplicate set of proliferation wells was used to collect supernatants at 48 and 72 h, and cytokine concentrations were determined by an enzyme-linked immunosorbent assay.

Immunohistochemistry. Mice ($n = 3$ per group) were anesthetized and perfused with phosphate-buffered saline (PBS) on the indicated days postinfection. Spinal cords and brains were removed by dissection, and 2- to 3-mm spinal cord blocks and brains were immediately frozen in OCT (Miles Laboratories, Elkhart, Ind.) in liquid nitrogen. Multiple 6-μm-thick cross sections from the spinal cord or brain were cut and mounted on Superfrost Plus electrostatically charged slides (Fisher, Pittsburgh, Pa.), air dried, and stored at −80°C. CNS immunohistochemistry was performed as previously described (12). The results shown are representative of the cellular infiltrates from multiple sections cut from three individual mice per group. Photomicrographs of immunostained sections from a spinal cord, cerebellum, and brain stem representative of the cellular infiltrates in the different groups were used to quantify the numbers of positive inflammatory cells per area of each tissue section. Data from photomicrographs were stored as 8-bit binary images in a grayscale format. Quantification was determined with ImageJ software v1.32j (http://rsb.info.nih.gov/ij/). Threshold values of brightness and contrast were determined for the photomicrographs and were kept constant for each sample. Prior to analysis, the minimum and maximum sizes of pixels to be counted were determined whereby sections with no positive staining gave a measurement of 0.0 pixels. The data are presented as percentages of positive pixels per area of photomicrograph.

Statistics. Clinical severity results are presented as mean group clinical scores, and the statistical difference was calculated by the Mann-Whitney nonparametric ranking test. Statistical analyses of DTH and proliferative responses and IFN-γ secretion were performed by using the two-tailed Student *t* test.

RESULTS

Peripheral tolerance to $PLP_{139-151}$ inhibits HI-BeAn virus-induced autoimmune demyelinating disease. As we have previously reported (20), mice infected intracerebrally with a nonpathogenic variant of the BeAn strain of TMEV (ΔClaI-BeAn, which carries a 23-amino-acid deletion in the viral leader sequence) engineered to express a 30-mer peptide encompassing the encephalitogenic myelin epitope $PLP_{139-151}$ (PLP-BeAn) develop a severe, rapid-onset, progressive demyelinating disease, while infection with BeAn containing the $PLP_{139-151}$ epitope mimic, consisting of amino acids 574 to 586 of the *Haemophilus influenzae* serine protease IV protein (HI-BeAn), develop a less severe nonprogressive clinical disease (Fig. 1b). HI-BeAn induces a cross-reactive $PLP_{139-151}$-specific $CD4^+$ T-cell response, as determined by in vivo DTH and in vitro T-cell proliferative assays (Fig. 1c and d), and results in the infiltration of $CD4^+$ T cells and activated $F4/80^+$ macrophages/microglia into the CNS (see Fig. 5d and l) and associated focal areas of demyelination, as assessed by staining with an anti-PLP antibody (manuscript in preparation), similar to the lesions found in PLP-BeAn-infected SJL mice (20). To definitively determine whether cross-reactivity (mimicry) between $PLP_{139-151}$ and $HI_{574-586}$ is critical for the induction of demyelinating disease, we exposed HI-BeAn- or PLP-BeAn-infected mice (day 0) to the myelin epitope, $PLP_{139-151,}$ or the irrelevant $OVA_{323-339}$ epitope (on days −7 and +3) to induce tolerance. As we previously reported (22), $PLP_{139-151}$ tolerance inhibited clinical disease in mice infected with PLP-BeAn. Significantly, the present results show that $PLP_{139-151}$ tolerance also specifically inhibits the CNS disease, as assessed by the TMEV scoring system, characterized by a mild to moderate waddling gait induced by infection with the mimic-expressing HI-BeAn virus compared to the case for mice exposed to $OVA_{323-339}$ ($P < 0.05$) (Fig. 1b). $PLP_{139-151}$-tolerant mice infected with either HI-BeAn or PLP-BeAn displayed significantly reduced $HI_{574-586}$- and $PLP_{139-151}$-specific DTH and $CD4^+$ T-cell proliferative responses ($P < 0.05$) than those of the controls (Fig. 1c and d). The tolerance was specific to the $PLP_{139-151}$ epitope, as TMEV-specific $VP2_{70-86}$-specific T-cell responses were unaffected in $PLP_{139-151}$-tolerant mice, and the responses in $OVA_{323-339}$-tolerant mice were similar to those in untreated mice. Interestingly, tolerance induced with $HI_{574-586}$,

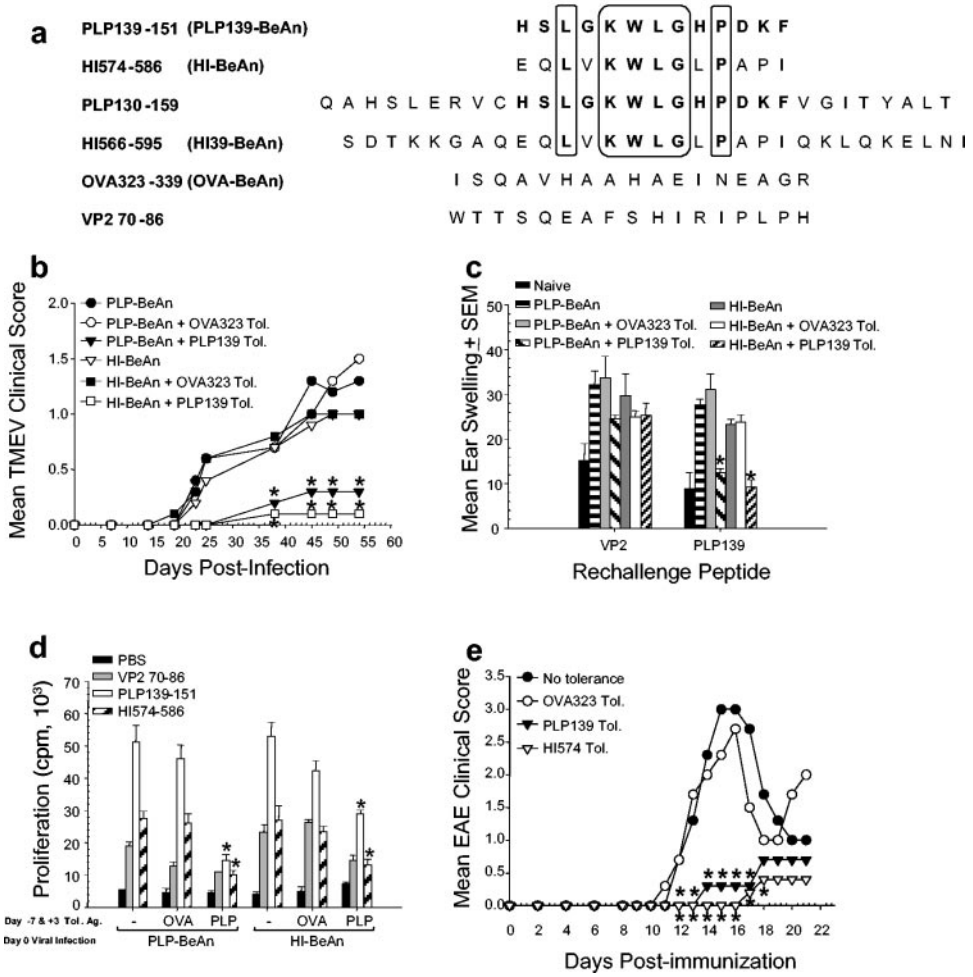

FIG. 1. Peripheral tolerance to $PLP_{139-151}$ inhibits HI-BeAn virus-induced autoimmune demyelinating disease. (a) Sequences of peptides used for this study, with shared residues outlined by boxes. Recombinant viruses expressing the peptide sequences used for the present study are shown in parentheses. (b) Mice ($n = 8$) were exposed to $PLP_{139-151}$ or $OVA_{323-339}$ at 7 days preinfection and 3 days postinfection with the HI-BeAn or PLP-BeAn virus. The mice were monitored for clinical disease by using the TMEV grading scale (see Materials and Methods) for 55 days. Mean clinical scores are plotted versus the time (days) postinfection. (c) DTH responses to the immunodominant virus epitope $VP2_{70-86}$ and the myelin epitope $PLP_{139-151}$ were measured at 14 days postinfection. (d) Splenic T-cell proliferative responses to $VP2_{70-86}$, $PLP_{139-151}$, and $HI_{574-586}$ were determined at 21 days postinfection. The clinical disease scores differed significantly from those for $OVA_{323-339}$-tolerant mice. *, $P < 0.05$. The results in each panel are representative of three separate experiments. (e) Mice ($n = 5$) were exposed to $PLP_{139-151}$, $HI_{574-586}$, or $OVA_{323-339}$ at 7 days preimmunization and 3 days postimmunization with 50 μg of $PLP_{139-151}$/CFA. Clinical scores were assessed by using an EAE grading scale as follows: 1, lack of tail tone; 2, impaired righting reflex; 3, partial hindlimb paralysis; 4, total hindlimb paralysis; and 5, moribund.

as well as that induced with $PLP_{139-151}$, was shown to inhibit experimental autoimmune encephalomyelitis (EAE) induced by priming with $PLP_{139-151}$/CFA (Fig. 1e), illustrating the bidirectional cross-reactivity of these epitopes. These results thus provide definitive evidence that the early-onset CNS disease induced by infection with the core *H. influenzae* mimic-expressing virus is due to the cross-activation of $PLP_{139-151}$-specific autoreactive $CD4^+$ T cells, i.e., the data prove the occurrence of infection-induced molecular mimicry.

The $HI_{574-586}$ mimic can be processed from its native protein sequence and induces demyelinating disease only in the context of virus infection. The data in Fig. 1 demonstrate that TMEV engineered to express the $HI_{574-586}$ myelin mimic within the core of a 30-amino-acid sequence spanning $PLP_{130-159}$ (HI-BeAn) induces the in vivo activation of cross-reactive $PLP_{139-151}$-specific $CD4^+$ T cells. To determine if the $HI_{574-586}$ mimic epitope can be processed from its native flanking sequences within the mimic protein, we constructed a TMEV virus (HI39-BeAn) expressing a 39-amino-acid ($HI_{566-604}$) piece of the *H. influenzae* protease IV protein. SJL mice infected with HI39-BeAn (Fig. 2a) developed an early-onset demyelinating disease which was less severe than that induced by PLP-BeAn but equivalent to that induced by HI-BeAn, while mice infected with TMEV expressing a 30-mer peptide encompassing the nonself $OVA_{323-339}$ sequence developed a late-onset disease typical of infections with the wild-type BeAn TMEV strain (20, 22). We have previously described that the insertion of base pairs encoding the $OVA_{317-346}$ 30-mer peptide in either the forward or reverse direction restores the ability of the parental ΔCla-BeAn virus to persist in the CNS and to induce late-onset clinical disease (18). Although the molecular basis for this is unknown, it is possible that the

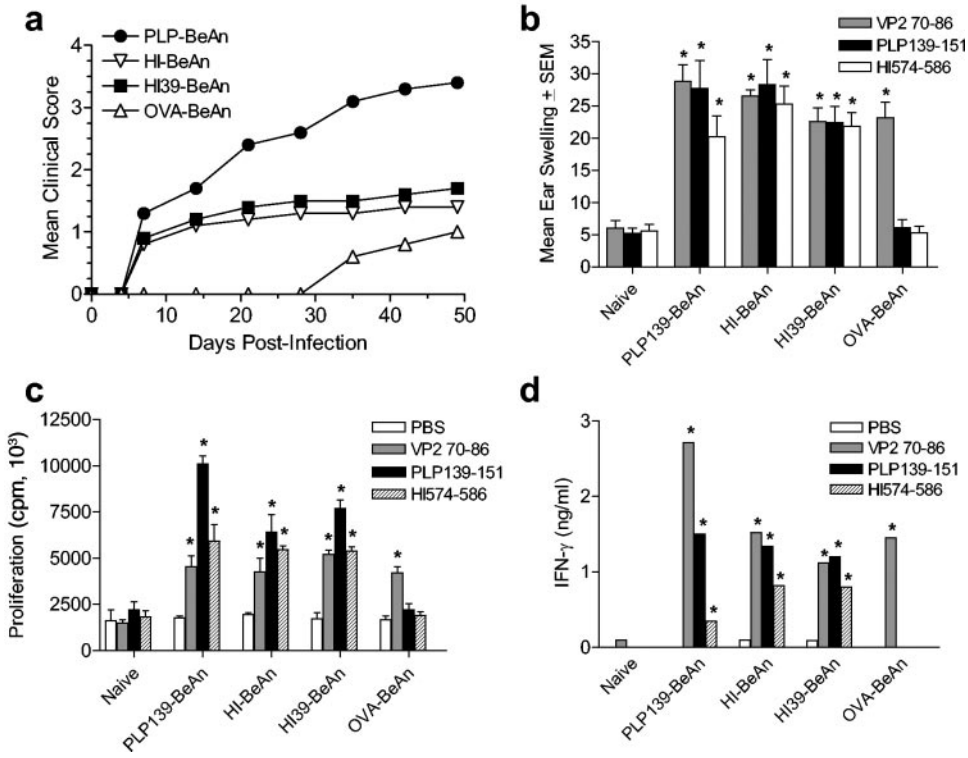

FIG. 2. HI39-BeAn virus infection activates cross-reactive $PLP_{139-151}$-specific $CD4^+$ T cells and induces autoimmune demyelinating disease. (a) Mice ($n = 8$) were infected with PLP-BeAn, HI-BeAn, HI39-BeAn, or OVA-BeAn and monitored for clinical signs of disease for 50 days. (b) DTH responses to the virus $VP2_{70-86}$, myelin $PLP_{139-151}$, and mimic $HI_{574-586}$ epitopes were measured at 14 days postinfection. (c) Splenic $CD4^+$ T-cell responses to $VP2_{70-86}$, $PLP_{139-151}$, and $HI_{574-586}$ were measured by proliferation assays at 21 days postinfection. (d) IFN-γ-specific secretion in response to $VP2_{70-86}$, $PLP_{139-151}$, and $HI_{574-586}$ was determined from proliferation culture supernatants. *, values were significantly above control levels ($P < 0.05$). The results in each panel are representative of three separate experiments.

addition of the OVA peptide coding sequence to the leader restores its original length and induces a conformational change in ΔCla-BeAn, which allows more efficient in vivo replication and virus persistence in mouse CNS cells. HI-BeAn- and HI39-BeAn-infected mice displayed strong $CD4^+$ DTH and proliferative T-cell responses associated with potent IFN-γ secretion, which is typical of Th1 responses, upon recall with either $HI_{574-586}$ or the $PLP_{139-151}$ self epitope (Fig. 2b to d). The level of $VP2_{70-86}$-induced IFN-γ secretion seen in PLP139-BeAn-infected mice compared to that in mice infected with the other recombinant viruses was less than twofold higher and not consistently seen and thus was not considered significant. Thus, $HI_{574-586}$ is arguably a natural $PLP_{139-151}$ mimic capable of being processed from its native sequence following virus infection to activate cross-reactive $PLP_{139-151}$-specific autoreactive $CD4^+$ T cells.

To determine if the HI mimic epitope could also induce clinical disease in the context of innate immune signals induced by complete Freund's adjuvant (CFA), we immunized SJL mice with either the minimal $HI_{574-586}$ mimic epitope or a 30-amino-acid peptide ($HI_{566-595}$) encompassing the core $HI_{574-586}$ mimic epitope in CFA. Immunization with either peptide activated both $HI_{574-586}$ mimic-specific and cross-reactive self-myelin $PLP_{139-151}$-specific $CD4^+$ T cells (Fig. 3c). However, unlike the case for SJL mice infected with TMEV engineered to express either the core $HI_{574-586}$ mimic epitope flanked by the native PLP sequences (HI-BeAn) or the long $HI_{566-604}$ epitope (HI39-BeAn) (Fig. 2a), priming with either $HI_{574-586}$ or $HI_{566-595}$ in CFA failed to induce a clinical demyelinating disease (Fig. 3a). This was also true if pertussis toxin and/or lipopolysaccharide was used in conjunction with CFA as an additional innate immune stimulus (data not shown). Disease induction following HI-BeAn infection appeared to induce more potent innate immune signals than priming with the mimic peptide/CFA, as exemplified by the failure of the self $PLP_{139-151}$ peptide to recall either in vivo DTH responses or in vitro IFN-γ production (Fig. 3b and d). In contrast, the self $PLP_{139-151}$ peptide recalled potent DTH and IFN-γ production in mice infected with either HI-BeAn or HI39-BeAn (Fig. 2b and d). Thus, the induction of autoimmunity via molecular mimicry requires critical innate immune signals specific to the infectious agent encoding the molecular mimic to induce an efficient differentiation of cross-reactive autoreactive Th1 cells with pathological potential.

Secondary virus infection in the CNS exacerbates clinical disease in mice previously infected with HI-BeAn. Mice infected with the HI-BeAn mimic virus consistently developed a less severe clinical demyelinating disease than mice infected with PLP-BeAn (Fig. 2a) (20). To determine if subsequent virus infections could exacerbate the nonprogressive demyelinating disease induced by HI-BeAn infection, we infected SJL mice intracerebrally with HI-BeAn (day 0) and then reinfected

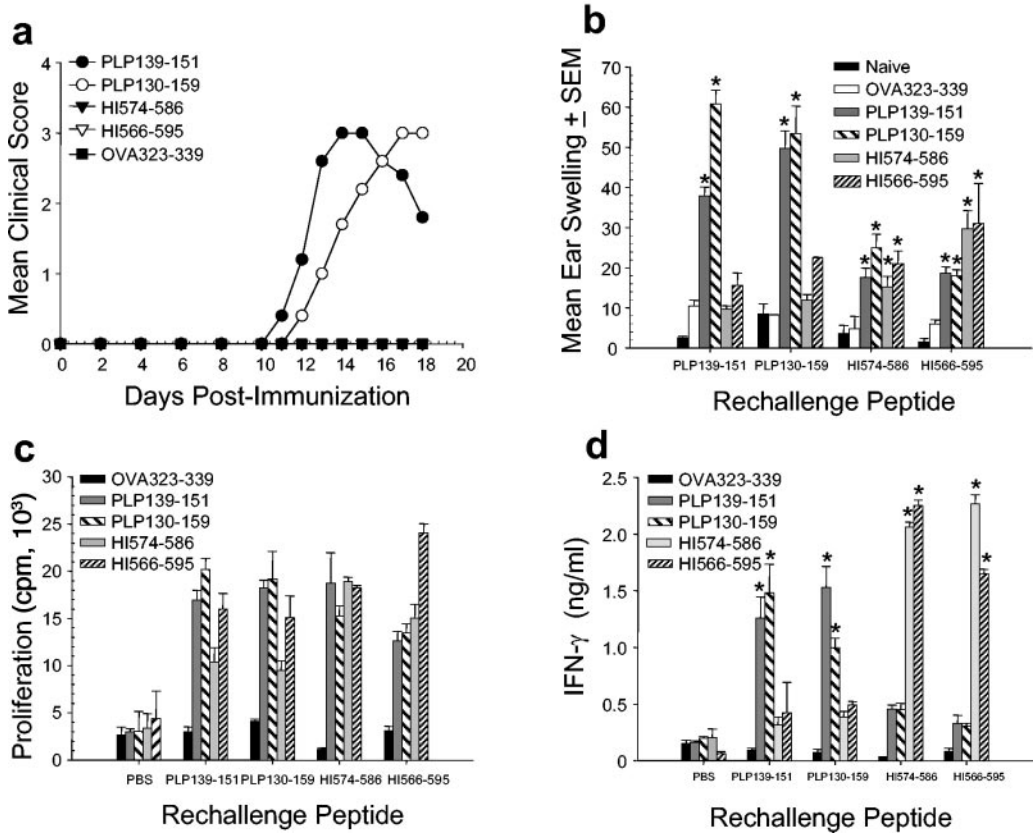

FIG. 3. *H. influenzae* mimic sequences fail to induce EAE upon immunization with complete Freund's adjuvant. (a) SJL mice ($n = 8$) were immunized subcutaneously with 100 μl of a CFA emulsion containing 400 μg of *Mycobacterium tuberculosis* H37Ra (Difco, Detroit, Mich.) and 50 μg of the indicated peptides distributed over three sites on the lateral hind flanks and dorsally. Clinical scores were assessed by using an EAE grading scale as follows: 1, lack of tail tone; 2, impaired righting reflex; 3, partial hindlimb paralysis; 4, total hindlimb paralysis; and 5, moribund. Immunization with *H. influenzae* mimic peptides failed to induce clinical disease in SJL mice, in contrast to immunization with PLP peptides, which induced characteristic EAE symptoms. (b) DTH responses to short ($PLP_{139-151}$) and long ($PLP_{130-159}$) versions of the self myelin PLP epitope and short ($HI_{574-586}$) and long ($HI_{566-595}$) versions of the *H. influenzae* mimic epitope were measured at 17 days postimmunization. (c) Splenic $CD4^+$ T-cell proliferative responses to the PLP and *H. influenzae* peptides were measured at 18 days postimmunization. (d) IFN-γ levels were determined from proliferation culture supernatants. *H. influenzae* peptide-immunized mice exhibited significant cross-reactive T-cell proliferative responses but poor in vivo DTH and in vitro IFN-γ responses after a rechallenge with the self $PLP_{139-151}$ epitope. In all experiments, the 30-mer peptides induced responses similar to those induced by the 13-mer peptides. OVA-immunized mice (negative controls) did not respond to rechallenge with either the *H. influenzae* or PLP peptides. *, values were significantly above control levels ($P < 0.05$).

them on day +14 with HI-BeAn, OVA-BeAn, the nonpathogenic parental virus (ΔCla-BeAn), or an equivalent volume of PBS. A primary infection with HI-BeAn and subsequent reinfection with HI-BeAn induced a significantly more severe clinical disease ($P < 0.05$) than that in mice given PBS on day +14 (Fig. 4a). Interestingly, mice infected with HI-BeAn that received a second infection with OVA-BeAn also developed an exacerbated clinical disease compared with that in PBS-injected controls ($P < 0.05$) (Fig. 4a). The reinfection of HI-BeAn virus-infected mice with the parental, nonpersisting ΔCla-BeAn virus resulted in a delayed onset of clinical disease compared with that in controls, and these mice developed a delayed exacerbated disease as well (Fig. 4a). However, as observed in multiple experiments, the clinical disease induced upon reinfection with the $PLP_{139-151}$ mimic-expressing HI-BeAn virus always had an earlier onset and was significantly more severe than the disease in mice reinfected with OVA-BeAn or ΔCla-BeAn. Mice infected with the HI-BeAn virus followed by a second infection with OVA-BeAn, ΔCla-BeAn, or HI-BeAn developed $HI_{574-586}$-specific $CD4^+$ T-cell proliferative responses as well as cross-reactive $PLP_{139-151}$-specific $CD4^+$ T-cell responses (Fig. 4d and e) ($P < 0.05$). All mice initially infected with HI-BeAn developed virus-specific $CD4^+$ T-cell responses which increased slightly upon secondary infection (Fig. 4c). The $CD4^+$ T-cell responses to the virus, myelin, and the mimic peptide in these infected mice were associated with IFN-γ-specific secretion, which is indicative of Th1-type $CD4^+$ T-cell responses (Fig. 4f). Thus, a primary infection with a mimic-encoding virus may result in a suboptimal activation of autoreactive T cells, resulting in a mild initial clinical disease, but secondary infections with neurotropic viruses expressing the $PLP_{139-151}$ mimic epitope more efficiently enhance the autoimmune response, leading to a severe clinical disease compared to that induced by non-mimic-expressing neurotropic viruses.

To further determine whether a prior infection of the CNS with a neurotropic virus (OVA-BeAn) that induces a late-onset, slowly progressing disease (Fig. 2a) could predispose

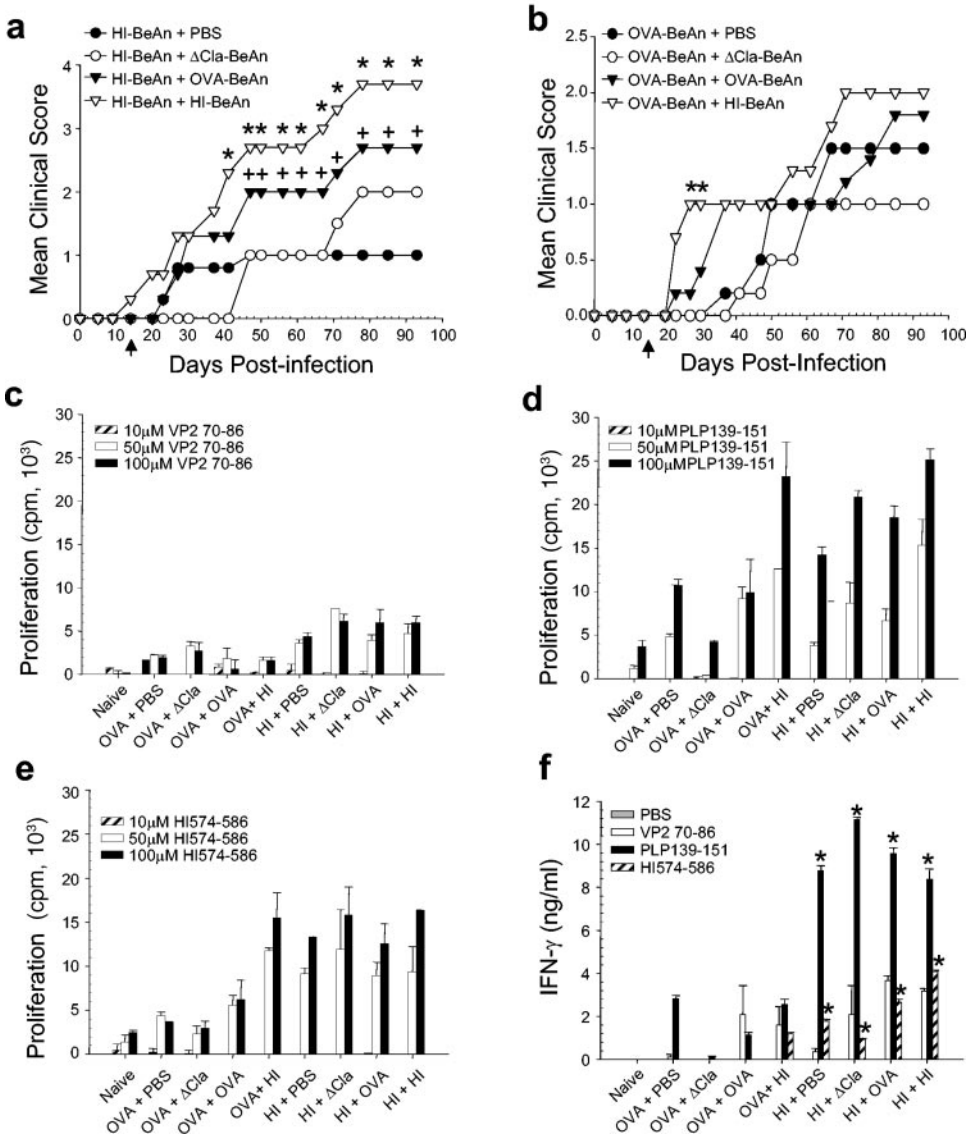

FIG. 4. Secondary CNS infection exacerbates clinical disease in mice previously infected with HI-BeAn. (a) Mice ($n = 8$) were infected with HI-BeAn (day 0) and then received either PBS or a secondary infection with HI-BeAn, OVA-BeAn, or ΔCla-BeAn at 14 days postinfection. Mice were observed for up to 94 days postinfection, and clinical disease was assessed with the TMEV disease scoring system. (b) Mice ($n = 8$) were infected with OVA-BeAn (day 0) and then received either PBS or a secondary infection with HI-BeAn, OVA-BeAn, or ΔCla-BeAn at 14 days postinfection. The mice were monitored for clinical disease for 94 days. (c to e) Splenic proliferation assays were conducted to determine T-cell reactivities to $VP2_{70-86}$ (c), $PLP_{139-151}$ (d), and $HI_{574-586}$ (e) at 60 days postinfection. (f) IFN-γ secretion in response to $VP2_{70-86}$, $PLP_{139-151}$, and $HI_{574-586}$ was determined from culture supernatants from proliferation assays. *, clinical disease scores were significantly higher than those for mice infected with HI-BeAn + PBS ($P < 0.05$). The results in each panel are representative of three separate experiments.

mice to a more severe autoimmune disease upon secondary infection with a mimic-encoding virus, we infected mice with OVA-BeAn (day 0) and then reinfected them with HI-BeAn, OVA-BeAn, ΔCla-BeAn, or an equivalent volume of PBS on day +14. Mice infected with OVA-BeAn that received a second infection with HI-BeAn developed a disease with a significantly earlier onset than that in mice that received a second infection with OVA-BeAn, ΔCla-BeAn, or PBS ($P < 0.05$) (Fig. 4b). OVA-BeAn virus-infected mice that received a second infection with HI-BeAn or OVA-BeAn developed a slightly more severe disease than control mice throughout the disease course. Mice infected with the OVA-BeAn virus and reinfected with the HI-BeAn virus developed $HI_{574-586}$-specific $CD4^+$ T-cell responses and had increased $PLP_{139-151}$-specific $CD4^+$ T-cell responses compared to those of control mice (Fig. 4d and e). All OVA-BeAn virus-infected mice had virus-specific $CD4^+$ T-cell responses, although interestingly, mice reinfected with the non-CNS-persisting ΔCla-BeAn virus exhibited reduced reactivities to all challenge peptides (Fig. 4c to f).

Brains and spinal cords were removed from mice ($n = 3$) at 60 days postinfection, and frozen sections were analyzed for their levels of $CD4^+$ T-cell and macrophage infiltrates to assess the effects of secondary virus infections on the levels of CNS inflammatory cell infiltration. Lumbar spinal cords from HI-

BeAn virus-infected mice that were reinfected with HI-BeAn displayed significantly more $CD4^+$ T cells and $F4/80^+$ microglia/macrophages (Fig. 5a and i) than did PBS-treated control mice (Fig. 5d and l). Mice initially infected with HI-BeAn and reinfected with either OVA-BeAn or ΔCla-BeAn had more $CD4^+$ T cells (Fig. 5b and c) and macrophages (Fig. 5j and k) than did PBS-treated controls (Fig. 5d and l) but fewer of these cells than did mice reinfected with HI-BeAn (Fig. 5a and i). Lumbar spinal cords from mice infected with the OVA-BeAn virus and subsequently reinfected with HI-BeAn had increased $CD4^+$ T cells and macrophages (Fig. 5e and m) compared to control mice (Fig. 5h and p). Cerebellum and brain stem sections from mice initially infected with HI-BeAn and reinfected with HI-BeAn displayed large numbers of infiltrating $CD4^+$ T cells and $F4/80^+$ cells (Fig. 5q to t). However, OVA-BeAn-infected mice that were reinfected with HI-BeAn displayed limited immune cell infiltrates in the brain (Fig. 5u to x). Overall, the levels of immune cell infiltration in the CNS correlated well with the severity of clinical disease observed for mice that received secondary infections.

DISCUSSION

Molecular mimicry is a putative mechanism for the activation of autoreactive T cells in MS, a human autoimmune demyelinating disease. However, little direct evidence exists for mimicry-induced T-cell autoimmunity using infectious agents. Previously, we described a model of molecular mimicry whereby the viral delivery of a $PLP_{139-151}$ myelin-mimic epitope naturally encoded in the protease IV protein of *H. influenzae*, $HI_{574-586}$, could induce an early-onset autoimmune demyelinating disease associated with the activation of autoreactive $PLP_{139-151}$-specific $CD4^+$ Th1 cells (20). The present experiments build upon this initial observation and illustrate four critically important conditions required for the induction and/or exacerbation of autoimmune disease by infection-induced molecular mimicry.

Firstly, the current demonstration that $PLP_{139-151}$ tolerance (13, 18) significantly inhibited the incidence and severity of disease following HI-BeAn infection and specifically inhibited both $HI_{574-587}$- and $PLP_{139-151}$-specific $CD4^+$ T-cell responses definitively shows the functional activation of pathological cross-reactive $HI_{574-586}$ and $PLP_{139-151}$ $CD4^+$ T cells following HI-BeAn infection. Secondly, the demonstration that infection with the HI39-BeAn virus induced a similar early-onset demyelinating disease and similar activation of $PLP_{139-151}$-specific $CD4^+$ Th1 cells, as observed following infection with the HI-BeAn virus, shows that the *H. influenzae* mimic sequence is a natural epitope which can be processed and presented in vivo by SJL antigen-presenting cells to activate $PLP_{139-151}$-specific autoreactive Th1 cells. We feel that this is a significant advance over past descriptions of pathological mimic epitopes, which uniformly employed minimal mimic sequences identified in T-cell hybridoma screens for their disease-inducing abilities and did not take into account the possibility that the mimic peptide may not be capable of being processed from its surrounding amino acid residues in the native mimic protein.

Thirdly, our data showing an induction of clinical CNS disease and autoreactive $PLP_{139-151}$-specific DTH and IFN-γ Th1 responses following infection with a TMEV engineered to express either the core $HI_{574-586}$ mimic epitope flanked by the native PLP sequences (HI-BeAn) or the long $HI_{566-604}$ epitope (HI39-BeAn) (Fig. 2), but not upon priming with HI-BeAn or HI39-BeAn in CFA (Fig. 3), demonstrate that an equally important component required for the infection-induced initiation of autoimmune disease via molecular mimicry is the ability of the pathogen to trigger an appropriate innate immune response leading to an efficient expansion and differentiation of pathological autoreactive Th1 responses. This finding is consistent with our demonstration that a TMEV infection of quiescent microglial cells leads to the induction of a functional antigen presentation function concomitant with a significant upregulation of major histocompatibility complex and costimulatory molecules and an increased expression of cytokines (interleukin-12 and interleukin-23) required for Th1 differentiation (23). Furthermore, we have more recently shown that TMEV infection is a more potent stimulus to resting microglia than is the ligation of other Toll-like receptors (TLRs). Compared to the activation of TLR2 via peptidoglycan, TLR3 alone via poly(I-C), TLR4 via bacterial lipopolysaccharide, or TLR9 via CpG DNA, the infection of microglia with viable TMEV caused a more potent upregulation of innate and effector immune cytokines, chemokines, and major histocompatibility complex class II and costimulatory molecules, enabling the microglia to more efficiently present myelin antigens to $CD4^+$ Th1 cells (24). Preliminary unpublished data indicate that TMEV is also tropic for dendritic cells, and thus it is possible that TMEV expressing the *Haemophilus*-encoded $PLP_{139-151}$ mimic causes a more efficient activation/maturation of dendritic cells than does CFA, resulting in an enhanced activation of autoreactive Th1 cells. In addition to the more efficient induction of peripheral cross-reactive $PLP_{139-151}$-specific Th1 responses by HI-BeAn infection than by $HI_{574-586}$/CFA priming, the fact that TMEV can persist and replicate in the CNS, leading to the local release of inflammatory cytokines and to possible deleterious effects on blood-brain barrier integrity, may also contribute to the efficient disease induction observed following infection with the mimic-expressing virus versus that with peptide priming.

Lastly, our results demonstrate that a secondary infection with a myelin mimic-expressing neurotropic virus can exacerbate a mild preexisting CNS disease, indicating that mimicry may also trigger disease relapses in autoimmune disease. Various environmental factors have been associated with initiating MS relapses, including viral and bacterial infections (2, 9, 27, 28, 31). The infection of SJL mice with the HI-BeAn mimic virus induces a mild, slowly progressive form of clinical disease. The nonprogressive disease induced by HI-BeAn may be due to the failure of the virus to fully activate the self-reactive $PLP_{139-151}$-specific T-cell population ($HI_{574-586}$ has a 50% infective concentration of 385 nM for $I\text{-}A^s$, while that of $PLP_{139-151}$ is 87 nM) (3), or the mimic peptide may only activate a subset of the self-reactive population (6). Significantly, mice infected with a second dose of HI-BeAn developed a severe, progressive clinical disease, consistent with the repriming of $HI_{574-586}$/$PLP_{139-151}$-specific $CD4^+$ T cells. We have also recently shown that the reinfection of prediabetic mice with a virus expressing an $H\text{-}2K^b$-restricted mimic ligand to a lymphocytic choriomeningitis virus (self) epitope transgenically expressed on pancreatic β-cells can accelerate the development of diabetes, but this

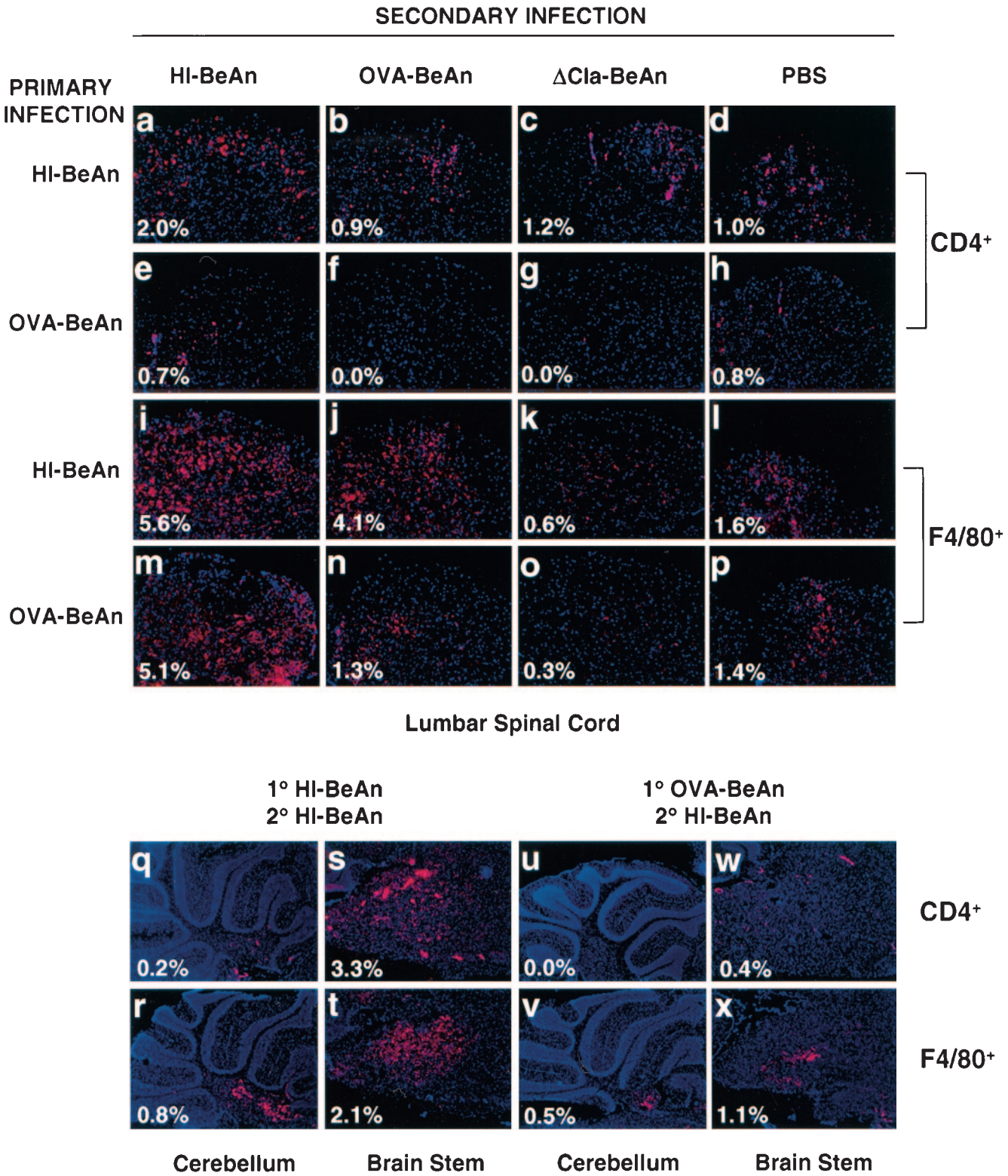

FIG. 5. Increased CNS infiltration of inflammatory cells in mice following secondary infection with HI-BeAn. HI-BeAn or OVA-BeAn virus-infected mice received a secondary infection with HI-BeAn, OVA-BeAn, or ΔCla-BeAn at 14 days postinfection or were administered PBS. At 60 days postinfection, mice ($n = 3$) from all groups were anesthetized and perfused. The lumbar spinal cords, brain stems, and cerebellums of mice were removed and sectioned into 5-μm-thick slices. The sections were stained for the presence of $CD4^+$ T cells and $F4/80^+$ monocytes/macrophages in the lumbar spinal cord (a to p) and in the cerebellum and brain stem (q to x). Quantification of the immunohistochemistry was determined for representative sections by using ImageJ software. The number in the lower left corner of each panel indicates the percentage of positive pixels per area of the photomicrograph.

mimic ligand-expressing arenavirus, unlike HI-BeAn, is not capable of initiating autoimmune disease (4). Interestingly, HI-BeAn-infected mice reinfected with TMEV encoding an irrelevant OVA epitope also developed a more severe clinical disease, indicating that bystander activation may also restimulate autoreactive T cells that are initially activated by HI-BeAn virus infection. This is significant, as anecdotal evidence suggests that relapse episodes in MS can be triggered by various upper respiratory virus infections (2, 9). The present study also advances previous findings in which mice primed with a plasmid coding for PLP could render mice susceptible to a mild disease following a subsequent rechallenge with CFA (35). This highlights the difficulties faced when investigating the role of viruses in MS pathogenesis, as relapses may be induced by otherwise innocuous or irrelevant viral infections.

Significantly, an initial infection of the CNS with the OVA-BeAn virus followed by a secondary infection with the HI-BeAn virus slightly accelerated the clinical disease. However, reinfection with the HI-BeAn virus did not increase the severity of autoimmune demyelinating disease as significantly as when the initial infection was with the HI-BeAn virus. Although the proliferative responses to $PLP_{139-151}$ were similar between the OVA-BeAn/HI-BeAn- and HI-BeAn/HI-BeAn-infected groups, the clinical disease was more severe for the HI-BeAn/HI-BeAn-infected group (Fig. 4a). Importantly, we observed that IFN-γ secretion, assessed by a $PLP_{139-151}$ rechallenge in vitro, was significantly higher for the HI-BeAn/HI-BeAn-infected group (Fig. 4f), indicating that the enhanced disease severity correlates with the ability of the *H. influenzae* mimic peptide to efficiently cross-activate pathogenic $PLP_{139-151}$ Th1 cells, which are essential to the initiation and progression of CNS autoimmune disease. Thus, this suggests that molecular mimicry boosts the autoreactive Th1 cell population, resulting in a more severe autoimmune demyelinating disease. Therefore, a non-mimic-encoding pathogen may only actively participate in disease pathogenesis once autoimmune cells are activated, whether by molecular mimicry or other mechanisms, e.g., epitope spreading (17). Further evidence to support this conclusion can be drawn from the observation that preinfection with a persistent virus which expressed a nonself, nonmimic epitope (OVA-BeAn) or a transient infection with ΔCla-BeAn did not predispose mice to exacerbated autoimmune disease upon $HI_{574-586}$ immunization (5). However, mice preinfected with HI-BeAn and subsequently immunized with the *H. influenzae* peptide showed an exacerbated disease compared to that after *H. influenzae* immunization or HI-BeAn infection alone. Furthermore, infection with HI-BeAn and immunization with an irrelevant peptide (OVA) did not exacerbate the disease. Therefore, it appears that one requirement for autoimmune disease is an infection of the target organ, i.e., the CNS, with the mimic-expressing virus.

Interestingly, there were significant differences in the numbers of inflammatory cells present in the spinal cord and brain in mice receiving secondary infections. With mice infected with HI-BeAn/HI-BeAn, we observed a significant number of $CD4^+$ T cells and macrophages present in the spinal cord, consistent with the severe clinical disease. Furthermore, the presence of inflammatory infiltrates in the cerebellum and brain stem was only observed in mice from the HI-BeAn/HI-BeAn-infected group with severe clinical disease. Therefore, the increased clinical severity may have been due to damage in these regions of the CNS. The cerebellum is involved in the coordination of voluntary motor movement, balance, equilibrium, and muscle tone. Therefore, inflammation in these structures is likely to induce a further loss of coordination of motor movement (asynergia), movement tremors (intention tremor), staggering, wide-based walking (ataxic gait), and weak muscles (hypotonia). Overall, mice that received secondary infections had a more severe disease which correlated with more infiltrating cells in the brain and spinal cord.

Our results suggest that molecular mimicry could be an important factor in the pathogenesis of MS by expanding a population of T cells that recognize self-epitopes. These autoreactive cells may then be further expanded to different degrees by differing stimuli, including reinfections with viruses or bacteria, which may or may not encode epitope mimics of relevant autoantigens via direct and/or bystander mechanisms. The potential requirements for target organ infection and persistence of the pathogen in the initiation of autoimmune disease by infection-induced molecular mimicry require further study. Furthermore, while the identification of potential mimic epitopes from pathogens by the use of computer searches is useful, it is important to determine whether the epitopes are naturally processed and presented in the infected host and if they can stimulate pathological autoimmune responses in the context of a variety of innate immune stimuli. Although the recombinant Theiler's virus model of molecular mimicry allows the determination of various conditions required for the induction of CNS autoimmunity, we are currently testing whether the infection of SJL mice with viable wild-type *H. influenzae* versus a *sppA*-deficient mutant (lacking the protease IV protein, which contains the $PLP_{139-151}$ mimic epitope) induces $PLP_{139-151}$-specific T-cell responses and/or clinical disease as the definitive test of infection-induced molecular mimicry. Collectively, these studies present compelling evidence that virus-induced molecular mimicry can both induce and enhance Th1-mediated CNS autoimmune disease in a model of MS, which may have important implications for the initiation and relapse phases of MS pathogenesis.

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