Experimental Autoimmune Encephalomyelitis in Lewis rats: IFN- β Acts As a Tolerogenic Adjuvant for Induction of Neuroantigen-Dependent Tolerance

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Experimental Autoimmune Encephalomyelitis in Lewis rats: IFN-β Acts As a Tolerogenic Adjuvant for Induction of Neuroantigen-Dependent Tolerance

Mark D. Mannie, Derek J. Abbott, and J. Lori Blanchfield

Cytokine-Ag fusion proteins represent a novel approach for induction of Ag-specific tolerance and may constitute an efficient therapy for autoimmune disease. This study addressed whether a fusion protein containing rat IFN-β and the encephalitogenic 73–87 determinant of myelin basic protein (i.e., the neuroantigen, or NAg) could prevent or treat experimental autoimmune encephalomyelitis (EAE) in Lewis rats. The optimal structure of the fusion protein was comprised of the rat IFN-β cytokine as the N-terminal domain with an enterokinase (EK) linker to the NAg domain. Both cytokine and NAg domains had full biological activity. Subcutaneous administration of 1 nmol of IFNβ-NAg fusion protein in saline on days −21, −14, and −7 before encephalitogenic challenge on day 0 resulted in a substantial attenuation of EAE. In contrast, administration of IFN-β or NAg alone did not affect susceptibility to EAE. The covalent attachment of IFN-β and NAg was not necessary, because separate injections of IFN-β and NAg at adjacent sites were as effective as injection of IFNβ-NAg for prevention of disease. When treatment was initiated after disease onset, the rank order of inhibitory activity was as follows: the IFNβ-NAg fusion protein ≥ a mixture of IFN-β plus NAg > IFN-β > NAg. The novel finding that IFN-β acts as a tolerogenic adjuvant as well as a tolerogenic fusion partner may have significance for development of tolerance vaccines. The Journal of Immunology, 2009, 182: 5331–5341.

Multiple sclerosis (MS) is a demyelinating inflammatory disease of the CNS myelin that afflicts well over 2 million people in the Western world (1–3). Although the etiology of MS is currently unknown, substantial evidence indicates that autoimmune responses may be important for initiation and progression of the disease. IFN-β is currently used as a mainstream therapy for MS (4–6). IFN-β is an effective disease-modifying treatment that markedly reduces clinical disease measured by magnetic resonance imaging, although the clinical benefit is limited to an ~30% reduction in the attack rate. Aside from the modest efficacy of IFN-β, the drug has additional disadvantages, including high costs and substantial interpatient variability in tolerability and efficacy. Also, IFN-β most likely inhibits MS through modulation of general regulatory pathways rather than by specifically disabling pathogenic clonotypes. Because IFN-β inhibits autoimmune responses without causing immunological tolerance, IFN-β must be chronically self-administered as an injection for a lifetime. In contrast, Ag-specific therapies may provide substantial advantage, particularly in the possibility that a time-limited series of administrations may cause an enduring cessation of autoaggressive immunological attacks. A central goal of contemporary immunological research is the induction of Ag-specific tolerance as a means to reestablish self tolerance to autoantigens in human autoimmune diseases.

Experimental autoimmune encephalomyelitis (EAE) is a widely studied animal model of MS and has helped to shape our current understanding of the pathophysiology of MS (7). Like MS, EAE is inhibited by administration of IFN-β. A substantial number of studies have shown that type I IFNs effectively inhibit EAE or experimental autoimmune neuritis when IFN-α/β is administered postimmunization during the induction or effector phases of disease (8–18). The inhibitory mechanism of IFN-β is associated with altered immunoregulation, but no evidence exists to indicate that IFN-β causes an Ag-specific immunological tolerance. Endogenous IFN-β also appears to limit encephalitogenic responses because EAE is exaggerated in mice genetically deficient in IFN-β (19, 20) or the type I IFN receptor (21). Indeed, expression of the type I IFN receptor on myeloid cells appears to be pivotal in the mechanism by which IFN-β controls encephalitogenic responses. Ag-specific therapies are advantageous compared with general immunosuppressive strategies because the former have the potential to cause specific immunological tolerance (22–24). Several Ag-specific therapies are being developed, including those based on altered peptide ligands, DNA vaccines, and mucosal Ag delivery. Cytokine-Ag fusion proteins were originally developed for vaccination against cancer and infectious agents but have also been explored as tolerogenic vaccines based on the use of inhibitory, antiinflammatory, or tolerogenic cytokines as the cytokine fusion partner (25, 26). Two cytokine-Ag fusion proteins in which the IL-2 or IL-16 cytokines were fused to the encephalitogenic determinant of myelin basic protein (MBP; i.e., IL2-neuroantigen (NAg) or NAg-IL16) have successfully been used to prevent a subsequent encephalitogenic sensitization and to treat ongoing...
EAE. Herein, we describe a third tolerogenic cytokine-Ag fusion protein based on the use of IFN-β as the N-terminal cytokine domain and the major 73–87 determinant of MBP as the Ag domain. This IFN-β-NAg strategy was superior to an IFN-β-based therapy because synergy of the IFN-β and NAg domains facilitated an inhibitory memory capable of preventing a subsequent encephalitogenic challenge. Unlike the IL2-NAg and NAg-IL16 fusion proteins, covalent attachment of IFN-β and NAg was not necessary because separate injections of IFN-β and NAg at adjacent sites were as effective as injection of the fusion protein for prevention of disease. These data indicate that when used to prevent a subsequent attack, IFN-β acts as a tolerogenic adjuvant to promote resistance to CNS autoimmunity. When used to treat an ongoing attack, the IFN-β and NAg halted disease progression and facilitated recovery.

Materials and Methods

Recombinant protein structure

The IFNβ-NAg fusion protein was expressed by use of a recombinant baculovirus as described previously (27). The IFNβ-NAg fusion protein consisted of the following order from the N terminus to C terminus (see Table I). Rat IFN-β cytokine was the N-terminal domain (NM_019127), which was fused by a G-D-D-D-D-K-G enterokinase (EK) linker to the dominant 73–87 encephalitogenic epitope (P-Q-K-S-Q-R-S-Q-D-E-N-P-V-V-H) of guinea pig myelin basic protein (GPMBP; accession no. P25188, www.ncbi.nlm.nih.gov/protein) followed by five additional H residues to comprise a C-terminal 6-histidine (6his) tag. The native IFN-β signal sequence directed secretion of the fusion protein as a biologically active protein into the supernatant of baculovirus-infected Sf9 cells. The IFNβ-NAg fusion gene and five additional IFN-β-based fusion genes were cloned in pVAX1 and pCEP4 expression plasmids (Invitrogen) and were assembled by overlap extension PCR, and the resulting fusion genes were inserted into the expression plasmid by directional, restriction endonuclease (PstI and HindIII) sites (Table I). The genes for these fusion proteins were inserted into the expression plasmid by directional, restriction endonuclease-free, whole plasmid PCR. The inserts were subjected to forward and reverse DNA sequencing to verify the predicted DNA sequence.

Purification of fusion proteins

Expression supernatants were concentrated on YM10 ultrafiltration membranes and were subjected to consecutive affinity chromatography steps (27). The first affinity chromatography step was based on the use of a single-chain Fv anti-6his Ab fused to two tandem chitin-binding domains (scFv-CBD2) (28). This recombinant protein was immobilized on a chitin resin column by stable binding of the tandem chitin-binding domains to the chitin bead matrix. Immobilization of the scFv anti-6his single-chain Ab onto chitin columns enabled purification of recombinant proteins bearing C-terminal 6-histidine tags from concentrated baculovirus supernatants. These columns were maintained in TBST buffer (50 mM Tris-HCl, 500 mM NaCl, 0.1 mM EDTA, 1% Triton X-100, 0.01% Na azide (pH 8.0)). Before each use, columns were equilibrated in MBS buffer (20 mM MES, 500 mM NaCl, 0.1 mM EDTA (pH 6.5)), and concentrated expression supernatants were passed through the column to trap the 6his-tagged protein. The fusion proteins were eluted in CAPS (N-cyclohexyl)-3-amino propanesulfonic acid) buffer (50 mM CAPS, 500 mM NaCl, 0.1 mM EDTA (pH 10.0)), concentrated, and directly applied to Ni-NTA agarose columns (Qiagen) followed by extensive washing of the resin (50 mM NaH2PO4, 500 mM NaCl, 10 mM imidazole (pH 8.0)). IFNβ-NAg or IFN-β was eluted by acid elution (pH 4.5) and was concentrated and diafiltrated in Millipore Ultra-15 centrifugal filter devices. Protein quantity was assessed by the BCA protein assay (Pierce) and by absorbance at 280 nm. Purity was assessed by SDS-PAGE.

Animals and reagents

A colony of Lewis rats was maintained at East Carolina University School of Medicine. Animal care and use was approved by the Institutional Animal Care and Use Committee and was performed in accordance with approved institutional guidelines. The synthetic peptide gp69–88 (YGGSLPQKSQRSQDENPVVHF) was obtained from Controllable Biologicals. The purity of gp69–88 was >98%. The peptide was freely soluble and was routinely reconstituted in saline. The OX6 anti-I-A (RT1B) IgG1, the R73 anti-TCRβ IgG1, the ox18 anti-IL-4 IgG1, and the ox18 anti-IL-2 IgG1 were obtained from the European Collection of Cell Cultures. The B5 IgM anti-MHC-I (RT1Aa1b) mAb was purchased from BioXcell. FITC-conjugated goat anti-mouse IgG1 was purchased from SouthernBiotec.

Cell lines and culture conditions

The RSL.11 MBP-specific clone was a stable, IL-2–dependent line derived from Lewis rats sensitized with rat MBP in CFA (29). The RSL.11 clone was a transformed variant of the RSL.1 clone. The R1T cell clone used in the described experiments was referred to as R1-trans. The R1-trans clone derived from Lewis rats that constitutively expressed MHC class II glycoproteins (MHC-II), B7.1, and B7.2 (29, 30). The BN-GP T cell line was a transformed variant of a IL-2–dependent, MBP–specific clone derived from Brown Norway rats (30, 31). These lines originated as primary T cell lines that exhibited a quiescent resting phase during long-term maintenance in IL-2 but spontaneously transformed to a constitutive blasticogenic, proliferative phenotype. These variant T cell lines were IL-2–dependent and did not revert to a resting phase during maintenance culture in IL-2. The Cona.SD9 clone was isolated from Lewis rats and was specific for conalbumin (Sigma-Aldrich) (32, 33). CTL T cells represented an IL-2–dependent line of murine T cells (34). Assays were performed in complete RPMI 1640 medium (10% heat-inactivated FBS, 2 mM glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, 2 mM L-glutamine (Whittaker Bioproducts) (Sigma-Aldrich)). T cell lines were propagated in complete RPMI 1640 supplemented with recombinant rat IL-2 (0.4% (v/v) S9 supernatant) (35).

Measurement of Ag-specific proliferation and IL-2 production

Cultures were pulsed with 1 μCi of [3H]thymidine (6.7 Ci/mmol; New England Nuclear) during the last day of a 3- or 4-day culture. Cultures were harvested onto filters by use of a Tomtec Mach III harvester. [3H]thymidine incorporation into DNA was measured by use of a Wallac 1450 MicroBeta Plus liquid scintillation counter. Error bars represent SDs of triplicate or quadruplicate sets of wells.

Flow cytometric analysis

T cells were incubated with a 1/20 titration of a concentrated supernatant containing the designated Ab for 45 min at 4°C. The cells were washed twice and were incubated for 45 min with a FITC-conjugated secondary Ab. Dead cells were excluded from analysis by forward vs side scatter profiles. Data were acquired with a FACScan flow cytometer (BD Biosciences) and were analyzed with the CellQuest software program.

Induction of EAE, tolerance induction, and clinical assessment of EAE

EAE was induced in Lewis rats by injection of an emulsion containing 50 μg of dihydrofolate reductase (DHFR)-NAg (25, 26) in CFA (200 μg of Mycobacterium tuberculosis) in a total volume of 0.1 ml. DHFR-NAg comprised of the mouse DHFR as the N-terminal domain and the encephalitogenic gp69–87 peptide of GPMBP as the C-terminal domain. The DHFR-NAg fusion protein was equivalent to gp69–88 for induction of EAE (data not shown). The emulsion was injected s.c. in two 0.05-ml volumes on either side of the base of the tail. To determine whether IFNβ-NAg prevented active induction of EAE, rats were given injections of 1 nmol of IFNβ-NAg in saline on days −21, −14, and −7, and then 7 days after the last injection, rats were challenged with DHFR-NAg in CFA (day 0) to induce EAE. Alternatively, rats were challenged with DHFR-NAg in CFA and then were treated with IFNβ-NAg or control reagents on day 9 (1 nmol), day 10 (1 nmol), and day 12 (0.5 nmol) or as otherwise designated.

The following scale was used to score EAE: paralysis in the distal tail, 0.25; limp tail, 0.5; ataxia, 1.0; hind leg paresis, 2.0; full hind leg paralysis, 3.0. Ataxia was scored as an uneven or wobbly gait. Hind leg paresis was scored as the retention of some voluntary ambulatory movement in the hind limbs but without the ability to ambulate upright.

Statistical analysis

Mean cumulative score, mean maximal score, and mean number of days with severe EAE were analyzed by parametric ANOVA. The mean cumulative score was calculated by summing the daily scores for each rat and then averaging the cumulative scores to obtain the mean cumulative score. The mean maximal score was calculated by averaging the most severe score of EAE for all rats in each group. Means were reported together with the SD. Median cumulative score and median maximal score were listed as the median values for all rats in each group and
FIGURE 1. The IFNβ-NAg Histagged fusion protein was biologically active and was purified on nickel resins. A. To assess whether IFNβ-NAg induced class I MHC expression, thymocytes (5 × 10⁷/ml in complete RPMI 1640) were cultured with or without IFNβ-NAg for 2 days and then were stained with B5 (anti-MHC-I), OX18 (anti-MHC-I), R73 (anti-TCRβ), or OX81 (anti-IL4; isotype control) and were analyzed by flow cytometry. B. To assess IFN-β-mediated antiproliferative activity, supernatants from two independently derived IFNβ-NAg expression systems as well as supernatants from several control expression systems were added at designated titrations (x-axis) to IL-2-stimulated cultures of BN-GP T cells. C. To assess the specificity of antiproliferative activity, baculovirus supernatants containing IFNβ-NAg were added at designated titrations to cultures of IL-2-stimulated rat (RIT, BN-GP, RsL.11T, RsL.11, or Conal.8D9) or mouse (CTLL) T cells. Proliferative assays (B and C) were pulsed with [3H]thyminidine on the second day of a 3-day culture. D. Shown is a representative SDS-PAGE analysis of purified IFNβ-NAg. These data are representative of at least three independent experiments.

were analyzed by nonparametric ANOVA based on ranked data. ANOVA was interpreted with the Bonferroni post hoc test. One-way ANOVA was used to assess data from a single experiment, whereas two-way ANOVA (variable vs experiment) was used to assess data compiled from two separate experiments. "Incidence of severe EAE" was analyzed pairwise with the Fisher's exact test. Severe EAE was defined as the incidence of hind leg paresis (EP; 2.0) or full hind limb paralysis (P; 3.0) (see Tables II–V), unless designated otherwise (see Table VI).

Results

Biological activity of the cytokine domain

The main objective of the study was to test IFN-β as a fusion partner for generation of tolerogenic cytokine-NAg vaccines. The initial question was whether the cytokine and NAg domains of an IFNβ-NAg fusion protein were biologically active. IFNβ-NAg fusion proteins and relevant controls were expressed in baculovirus and by transient transfection of HEK293 cells. The biological activity of the IFN-β domain of the fusion protein was assessed by assays measuring IFN-β-mediated induction of MHC-I and antiproliferative activity (Figs. 1 and 2, A–C). The activity of the NAg domain was measured in NAg-specific T cell proliferation assays (Fig. 2, D and E). The IFNβ-NAg fusion protein was comprised of rat IFN-β as the N-terminal domain and a C-terminal domain containing an EK linker, the NAg domain (the encephalitogenic 73–87 epitope of MBP), and a C-terminal 6-histidine sequence (Table I). This fusion protein had a predicted molecular mass of 22,922 Da with four potential N-linked glycosylation sites.

Two identical baculovirus expression systems of IFNβ-NAg were independently derived (IFNβ-NAg.1 and IFNβ-NAg.4) to test biological activities of the cytokine and NAg domains. To measure the ability of IFNβ-NAg to induce MHC-I, thymocytes were cultured with or without a 1% titration of a baculovirus expression supernatant containing the IFNβ-NAg.1 fusion protein. After 2 days of culture, the thymocytes were analyzed by flow cytometry for expression of MHC-I. Supernatants containing the IFNβ-NAg.1 (Fig. 1A, top) or IFNβ-NAg.4 (not shown) induced the expression of MHC-I on immature thymocytes. Mock baculovirus supernatants lacked activity (Fig. 1A, bottom). Although the IFN-β domain of IFNβ-NAg augmented MHC-I expression, this domain did not affect TCRβ expression (Fig. 1A) or other markers such as CD4, Thy1.1, LFA-1, MHC-II, CD2, CD5, CD28, CD45, or CD48 (not shown). These data indicated that the IFN-β domain of the IFNβ-NAg fusion protein was biologically active.

Baculovirus expression supernatants containing the IFNβ-NAg fusion protein also efficiently caused death of the BN-GP T cell line (Fig. 1B). Expression supernatants containing IFNβ-NAg inhibited the IL-2-dependent proliferation of BN-GP T cells by induction of cell stasis followed by extensive cell death (not shown). Both IFNβ-NAg fusion proteins were active at titrations of 10⁻⁴.
whereas baculovirus supernatants containing other cytokine-NAg fusion proteins, including IL1RA-NAg, IL2-NAg, IL4-NAg, IL10-NAg, IL13-NAg, and NAg-IL16, as well as mock baculovirus supernatants, had no activity. These control cytokine-NAg fusion proteins were active in bioassays specific for the respective cytokine domains (26, 27). IFNβ-H9252-NAg caused cell death independently of the NAg domain, because BN-GP T cells did not recognize the 73–87 peptide sequence of MBP (not shown). IFNβ-H9252-NAg also directly caused the death of other IL-2-dependent T cell lines, including the NAg-specific RsL.11 clone as well as a transformed variant of the RsL.11 clone (RsL.11T) and a conalbumin-specific clone (Conal.8D9) (Fig. 1C). IFNβ-H9252-NAg-mediated killing of RsL.11 T cells was entirely dependent on the cytokine domain because killing was not blocked by anti-MHC-II mAb (not shown). IFNβ-H9252-NAg did not kill a transformed variant of another NAg-specific clone (R1T). These data indicated that IFN-β was able to kill certain rat T cell lines but not others. IFNβ-H9252-NAg did not kill murine CTLL T cells even though rat IFN-β is able to efficiently kill mouse T cells (not shown). These data show that the IFNβ-NAg fusion protein had high levels of IFN-β-specific biological activity.

Table I. Rat IFNβ-NAg fusion proteins

<table>
<thead>
<tr>
<th>Used in Tables</th>
<th>Name Used in Tables II–VI</th>
<th>N- to C-terminal Order of Domains</th>
<th>Biological Activity of IFN-β in Expression Supernatants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>ss-IFNβ-NAg-6his</td>
<td>Potent activity</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>ss-IFNβ-6his</td>
<td>Potent activity</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>ss-IFNβ-NAg-6his</td>
<td>Weak activity</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>ss-IFNβ-6his</td>
<td>Weak activity</td>
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</tr>
<tr>
<td>No</td>
<td>ss-6his-NAg-IFNβ</td>
<td>Weak activity</td>
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ss, native signal sequence of rat IFN-β (MANKWTLHIAFLLLCCSTTLALS); IFN-β, rat IFN-β cytokine (accession no. NP_062000, www.ncbi.nlm.nih.gov/protein); EK, enterokinase linker (GDDDDDKG); NAg, neuroantigen (the encephalitogenic 73–87 peptide of MBP; PQKSQRSQDENPVH); 6his, 6-histidine sequence. The NAg in the native ss-IFNβ-NAg-6his and ss-6his-NAg-IFNβ had a native 4 aa extension at the N-terminus (YGSLPQKSQRSQDENPVH), which does not affect encephalitogenic activity. The term “NAg” also refers to the synthetic gp69–88 peptide (YGSLPQKSQRSQDENPVH). The NAg in all cases contains the full-length encephalitogenic determinant of MBP (PQKSQRSQDENPV) in Lewis rats (43). whereas baculovirus supernatants containing other cytokine-NAg fusion proteins, including IL1RA-NAg, IL2-NAg, IL4-NAg, IL10-NAg, IL13-NAg, and NAg-IL16, as well as mock baculovirus supernatants, had no activity. These control cytokine-NAg fusion proteins were active in bioassays specific for the respective cytokine domains (26, 27). IFNβ-NAg caused cell death independently of the NAg domain, because BN-GP T cells did not recognize the 73–87 peptide sequence of MBP (not shown). IFNβ-NAg also directly caused the death of other IL-2-dependent T cell lines, including the NAg-specific RsL.11 clone as well as a transformed variant of the RsL.11 clone (RsL.11T) and a conalbumin-specific clone (Conal.8D9) (Fig. 1C). IFNβ-NAg-mediated killing of RsL.11 T cells was entirely dependent on the cytokine domain because killing was not blocked by anti-MHC-II mAb (not shown). IFNβ-NAg did not kill a transformed variant of another NAg-specific clone (R1T). These data indicated that IFN-β was able to kill certain rat T cell lines but not others. IFNβ-NAg did not kill murine CTLL T cells even though rat IFN-β is able to efficiently kill mouse T cells (not shown). These data show that the IFNβ-NAg fusion protein had high levels of IFN-β-specific biological activity.
The C-terminal 6-histidine sequence of the IFNβ-NAg fusion was accessible and enabled affinity purification of the fusion protein from expression supernatants (Fig. 1D).

Optimization of rat IFN-β fusion proteins

The IFNβ-NAg fusion protein had the domain structure IFNβ-EK-NAg-6his (Table I) and was successfully expressed in a baculovirus expression system. We attempted to express a control IFN-β protein in baculovirus that was comprised of an IFNβ-6his sequence. However, this protein was expressed insufficiently (not shown). To assess an optimal structure for IFNβ-NAg and a control IFN-β protein, six different fusion proteins were expressed by transient transfection of HEK293 cells (Table I and Fig. 2A). Optimal expression was achieved when the IFN-β protein did not have non-native C-terminal or N-terminal additions. Optimal expression was also observed in proteins that included an EK linker separating the N-terminal cytokine domain and the C-terminal domain. That is, expression of IFN-β, IFNβ-EK-6his, or IFNβ-EK-NAg-6his was optimal and resulted in essentially equal IFN activity. In contrast, fusion proteins lacking the EK linker such as IFNβ-6his or IFNβ-NAg-6his were not efficiently expressed. The lack of an EK linker may be why the IFNβ-6his was not efficiently produced by the baculovirus expression system. An additional fusion protein (6his-NAg-IFNβ) was also expressed in which the N-terminal IFN-β signal sequence was directly fused to a 6his-NAg domain, and the C-terminal domain was comprised of the mature IFN-β cytokine. This ordering of domains was successfully used to construct the NAg-IL16 fusion protein, but the 6his-NAg-IFNβ fusion protein was not efficiently expressed. In conclusion, an EK linker appeared to facilitate the efficient expression of biologically active, stable IFN-β fusion proteins.

The finding that IFN-β fused to either an EK-NAg-6his C terminus or EK-6his C terminus had equal cytokine activity (Fig. 2A) was reinforced by testing independently derived expression plasmids encoding IFNβ-EK-NAg-6his or IFNβ-EK-6his proteins (Fig. 2B). The two purified proteins (IFNβ-EK-NAg-6his vs IFNβ-EK-6his) also exhibited essentially equipotent cytokine activity (Fig. 2C). Both IFN-β fusion proteins exhibited half-maximal inhibition at concentrations in the 1–10 pM range. These potencies were equal or superior to those reported for commercial IFN-β preparations. IFNβ-EK-NAg-6his proteins purified from baculovirus expression supernatants, however, were less active. The amino acid sequences of the IFNβ-EK-NAg-6his protein expressed in the HEK and baculovirus systems were identical. The main structural correlates of this activity difference appeared to involve distinct patterns of glycosylation (not shown).

Biological activity of the NAg domain

The activity of the NAg domain was confirmed by assaying Ag-specific proliferation of the MBP-specific RsL.11 T cell clone in the presence of splenic APC (Fig. 2D). The stimulatory activity of purified IFNβ-EK-NAg-6his (IFNβ-NAg) was completely inhibited by the OX6 anti-class II MHC mAb. These data indicated that the NAg peptide of the IFNβ-NAg was efficiently processed and presented by MHC-II to an Ag-specific T cell clone. The proliferative response was dependent on the NAg domain. Five independently purified preparations of IFNβ-EK-NAg-6his exhibited similar stimulatory activity, whereas six independently purified preparations of IFNβ-EK-6his were devoid of stimulatory activity (Fig. 2E). The IFNβ-EK-NAg-6his protein, at low concentrations, appeared more potent than did intact GPMBP (Fig. 2D) or the synthetic gp69–88 peptide (not shown). These data provide evidence that the cytokine domain may facilitate presentation of the NAg by professional APC, as was described for the IL4-NAg, IL2-NAg, and NAg-IL16 fusion proteins (25–27). Although the antigenic potency of IFNβ-NAg appeared greater than GPMBP as measured by the concentration eliciting a half-maximal response, the peak proliferative response stimulated by IFNβ-NAg was

Table II. The IFNβ-NAg fusion protein ameliorates a subsequent encephalitogenic challenge

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incidence of EAE</th>
<th>Mean Cumulative Score</th>
<th>Median Cumulative Score</th>
<th>Mean Maximal Score</th>
<th>Median Maximal Score</th>
<th>Incidence of Severe EAE</th>
<th>Mean No. Days with Severe EAE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>13 of 13</td>
<td>9.8 ± 2.4</td>
<td>9.3</td>
<td>2.9 ± 0.3</td>
<td>3.0</td>
<td>13 of 13</td>
<td>3.2 ± 0.8</td>
</tr>
<tr>
<td>NAg</td>
<td>15 of 15</td>
<td>8.4 ± 2.7</td>
<td>8.5</td>
<td>2.7 ± 0.6</td>
<td>3.0</td>
<td>14 of 15</td>
<td>2.6 ± 1.1</td>
</tr>
<tr>
<td>IFNβ-NAg</td>
<td>9 of 9</td>
<td>3.4 ± 2.7</td>
<td>2.5</td>
<td>1.4 ± 0.9</td>
<td>1.0</td>
<td>4 of 9</td>
<td>1.0 ± 1.3</td>
</tr>
</tbody>
</table>

* Rats were pretreated with saline (first row), with 1 nmol of NAg (synthetic peptide gp69–88) in saline (second row), or with 1 nmol of IFNβ-NAg in saline (third row) on days −21, −14, and −7 and were challenged with 50 μg of DHR-NAg in CFA on day 0. EAE was scored at 24-h intervals.

† The incidence of severe EAE was scored as the incidence of EP or P (partial or full hind limb paralysis). The incidence of severe EAE in rats treated with IFNβ-NAg was significantly less than those for rats pretreated with saline (p = 0.0048) or NAg (p = 0.0147). The mean number of days afflicted with severe EAE in rats pretreated with IFNβ-NAg was significantly less than those for rats treated with saline (p < 0.0001) or NAg (p = 0.003).

FIGURE 3. The IFNβ-NAg fusion protein prevented the subsequent induction of EAE, whereas neither IFN-β nor NAg alone had tolerogenic activity. Shown are disease time courses for experiments shown in Table II (A) and pooled data from experiments 1 and 2 of Table III (B). Rats were treated with baculovirus-derived (A) or HEK293-derived (B) fusion proteins.
substantially less than that stimulated by GPMBP. The latter observation may reflect the cytotoxic or antiproliferative activity of the IFN-β domain in this concentration range.

**IFNβ-NAg fusion proteins prevented EAE**

Treatment of rats with a baculovirus-derived preparation of IFNβ-NAg substantially inhibited EAE induced by a subsequent encephalitogenic challenge of DHFR-NAg in CFA. Rats were administered 1 nmol of either IFNβ-NAg or NAg in saline s.c. on days −21, −14, and −7 followed by an encephalitogenic challenge on day 0 (Table II). IFNβ-NAg significantly inhibited the cumulative and maximal EAE scores. This fusion protein also significantly decreased the incidence of severe paralytic disease (i.e., a partial or full hind limb paralysis) and reduced the mean number of days afflicted by severe EAE. However, IFNβ-NAg pretreatment did not significantly modulate the day of disease onset (not shown). Rats treated with NAg alone did not show any significant attenuation of disease compared with saline-treated control rats. The time course of disease is shown in Fig. 3A. These data show that IFNβ-NAg can be used as a pretreatment to inhibit subsequent induction of EAE. To our knowledge, this observation is unique and is the first demonstration that an IFN-β-based reagent can inhibit EAE when delivered exclusively as a prechallenge treatment regimen.

![Table III](http://www.jimmunol.org/)

**Vaccination with the IFNβ-NAg fusion protein prevented the subsequent induction of EAE**

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Treatment a</th>
<th>Incidence of EAE</th>
<th>Mean Cumulative Score b</th>
<th>Median Cumulative Score b</th>
<th>Mean Maximal Score b</th>
<th>Median Maximal Score b</th>
<th>Incidence of Severe EAE b</th>
<th>Mean No. Days with Severe EAE b</th>
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<td>18.1 ± 1.8</td>
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<td>2.8 ± 0.3</td>
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<td>IFNβ</td>
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<td>22.0 ± 2.6</td>
<td>21.3</td>
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<td>IFNβ-NAg</td>
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<td>6.3 ± 6.7</td>
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<td>1.3 ± 1.2</td>
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<td>0.6 ± 1.3</td>
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<td>1.6 ± 0.8</td>
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<td>1.0 ± 0.7</td>
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<td>3.2 ± 0.7</td>
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<td>22.5</td>
<td>2.9 ± 0.4</td>
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<td>3.4 ± 0.8</td>
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<td>7.0 ± 5.3</td>
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<td>1.5 ± 0.9</td>
<td>1.5</td>
<td>4 of 8</td>
<td>0.8 ± 1.0</td>
</tr>
</tbody>
</table>

aFor experiments 1 and 2, rats were pretreated with 1 nmol of NAg in saline (first row), with separate injections of 1 nmol of NAg and 1 nmol of IFNβ at a distance of <0.5 cm apart near the base of the tail (IFNβ + NAg; second row), or with 1 nmol of IFNβ-NAg (third row) on days −21, −14, and −7. All injections were s.c. in saline. Rats were challenged with 50 μg of DHFR-NAg in CFA on day 0. EAE was scored at 12-h intervals.

bThe time course of clinical signs from combined data of experiments 1 and 2 are shown in Fig. 3B and were compiled for statistical analysis. The mean and median cumulative or maximal scores of rats treated with IFNβ-NAg were significantly less than the respective scores for rats treated with IFNβ or NAg (p ≤ 0.001). The incidence of severe EAE (partial or full hind limb paralysis) in rats pretreated with IFNβ-NAg (4 of 8) was significantly less than the combined incidence of severe EAE in rats treated with IFNβ or NAg (16 of 16; p = 0.0066). The mean number of days afflicted with severe EAE in rats pretreated with IFNβ-NAg was significantly less than those for rats treated with IFNβ or NAg (p < 0.001).

<table>
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<tr>
<th>Expt.</th>
<th>Treatment a</th>
<th>Incidence of EAE</th>
<th>Mean Cumulative Score b</th>
<th>Median Cumulative Score b</th>
<th>Mean Maximal Score b</th>
<th>Median Maximal Score b</th>
<th>Incidence of Severe EAE b</th>
<th>Mean No. Days with Severe EAE b</th>
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<td>3.2 ± 0.4</td>
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<td>9.0 ± 5.9</td>
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<td>2.0 ± 1.1</td>
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<td>1.5 ± 1.0</td>
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<td>1.1 ± 1.2</td>
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<td>2.7 ± 0.5</td>
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<td>8.8 ± 4.9</td>
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<td>2.1 ± 1.1</td>
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<td>1.2 ± 1.2</td>
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<td>IFNβ-NAg</td>
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<td>7.2 ± 6.4</td>
<td>3.0</td>
<td>1.5 ± 1.4</td>
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<td>3 of 7</td>
<td>0.9 ± 1.2</td>
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<td>3.0 ± 0.5</td>
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<td>IFNβ + NAg</td>
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<td>8.9 ± 5.2</td>
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<td>IFNβ-NAg</td>
<td>14 of 14</td>
<td>7.6 ± 6.2</td>
<td>5.5</td>
<td>1.5 ± 1.1</td>
<td>1.5</td>
<td>7 of 14</td>
<td>1.0 ± 1.2</td>
</tr>
</tbody>
</table>

aFor experiments 1 and 2, rats were pretreated with 1 nmol of NAg (first row), with separate injections of 1 nmol of NAg and 1 nmol of IFNβ at a distance of <0.5 cm apart near the base of the tail (IFNβ + NAg; second row), or with 1 nmol of IFNβ-NAg (third row) on days −21, −14, and −7. All injections were s.c. in saline. Rats were challenged with 50 μg of DHFR-NAg in CFA on day 0. EAE was scored at 12-h intervals.

bThe time course for experiments 1 and 2 are shown in Fig. 4, A and B, respectively, and were compiled for statistical analysis. For rats treated with the combination of IFNβ + NAg or the IFNβ-NAg fusion protein, the mean and median cumulative scores (p < 0.001, p < 0.001), the mean (p = 0.013, p < 0.001) and median (p = 0.005, p = 0.001) maximal scores, the mean percentage weight loss (p = 0.003, p = 0.003); the incidence of severe EAE (p = 0.0149, p = 0.0013), and the mean number of days with severe EAE (p < 0.001, p < 0.001) were significantly less than the respective scores for rats treated with NAg.

Transfection of HEK293 cells was used to express IFNβ-NAg (IFNβ-EK-NAg-6his) and IFNβ (IFNβ-EK-6his) to assess whether the NAg domain of the IFNβ-NAg fusion protein was needed for inhibition of EAE (Table III). Again, rats were treated with 1 nmol of the fusion proteins or NAg on days −21, −14, and −7 followed by an encephalitogenic challenge on day 0. IFNβ-NAg significantly inhibited the cumulative and maximal disease scores, decreased the incidence of severe EAE, and reduced the mean number of days rats were afflicted by severe paralysis. In contrast, IFNβ was without activity and did not measurably affect the course of EAE (Fig. 3B). Thus, when treatment was completed 1 wk before encephalitogenic challenge, IFNβ did not persist to have any enduring effect on EAE. We conclude that the beneficial activity of IFNβ-NAg was not due to either IFNβ or NAg alone; instead, a synergistic activity of IFNβ and NAg was needed for modulation of EAE.

Previous studies of IL2-NAg and NAg-IL16 fusion proteins showed that covalent linkage of the cytokine and NAg domains was necessary for inhibition of EAE (25, 26). That is, administration of a fused cytokine-NAg protein was tolerogenic, whereas administration of cytokine and NAg as separate molecules to the same inoculation site was without activity. To assess whether covalent linkage of IFNβ and NAg was necessary for the inhibitory
activity of IFNβ-NAg, rats were injected either with IFNβ-NAg or with the combination of IFNβ and NAg as separate molecules (Table IV and Fig. 4). Treatments were administered on days −21, −14, and −7 followed by an encephalitogenic challenge on day 0. Unlike what was observed for the IL2-NAg and NAg-IL16 fusion proteins, IFNβ-NAg treatment or the combined treatment (IFNβ- plus NAg) were both equally effective for inhibition of EAE. Either treatment reduced cumulative and maximal scores and decreased the duration of severe paralytic EAE. This finding distinguished the tolerogenic mechanism of IL2-NAg and NAg-IL16 from that of IFNβ-NAg due to the differential requirement for covalent linkage of cytokine and NAg. The tolerogenic activity of IFNβ-NAg appeared to require presentation of the relevant Ag in lymphatic tissues conditioned by IFNβ-but did not require a strict intramolecular linkage of cytokine and NAg.

**IFNβ-NAg fusion proteins were used to treat ongoing EAE**

To assess whether the IFNβ-NAg fusion protein would inhibit ongoing EAE when treatment with IFNβ-NAg was initiated after disease onset (Table V), rats were matched for clinical intensity of EAE on day 9 after encephalitogenic challenge. Matched groups of rats were treated with IFNβ-NAg or controls on days 9, 10, and 12 (experiment 1) or on days 9, 10, 12, and 14 (experiment 2). IFNβ-NAg treatment substantially inhibited the cumulative and maximal disease scores, decreased the incidence of severe EAE, and reduced the number of days that rats were afflicted with severe paralytic EAE. Rats treated with IFNβ-alone showed an initial suppression of EAE (Fig. 5). However, cessation of IFNβ-treatment on day 12 (Fig. 5A) or day 14 (Fig. 5B) was associated with disease rebound to the extent that no significant depression of overall disease was noted for IFNβ-treated rats (Table V). Disease scores for rats treated with NAg alone were not significantly different from the EAE scores of rats treated with saline. The effect of these treatment modalities on EAE severity were mirrored by parallel changes in the percentage maximal weight loss. For example, the time course of weight loss correlated closely with the EAE time course and maximal disease scores (experiment 2 of Table V and Fig. 5, B and C). Based on these data, we conclude that IFNβ-NAg was also an effective inhibitor of EAE when delivered after onset of EAE.

Three experiments were performed to assess whether the covalent linkage between IFNβ- and NAg was necessary for effective inhibition of EAE. In addition, IFNβ-NAg treatment was evaluated to determine if IFNβ-NAg was more effective than NAg or IFNβ alone. In all cases, IFNβ-NAg treatment was equal to or more effective than the individual components in the treatment regimen.

**Table V. Treatment with the IFNβ-NAg fusion protein halts progression of clinical EAE**

<table>
<thead>
<tr>
<th>Expt</th>
<th>Treatmenta</th>
<th>Mean Cumulative Scoreb</th>
<th>Median Cumulative Scoreb</th>
<th>Mean Maximal Scoreb</th>
<th>Median Maximal Scoreb</th>
<th>% Mean Maximal Weight Lossb</th>
<th>Incidence of Severe EAEb</th>
<th>Mean No. Days with Severe EAEb</th>
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<tr>
<td>1</td>
<td>Saline</td>
<td>17.6 ± 3.0</td>
<td>19.5</td>
<td>3.0 ± 0.0</td>
<td>3.0</td>
<td>14.5 ± 2.9</td>
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<td>2.6 ± 0.6</td>
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<tr>
<td></td>
<td>NAg</td>
<td>10.0 ± 4.9</td>
<td>11.9</td>
<td>2.1 ± 1.4</td>
<td>2.0</td>
<td>11.5 ± 4.1</td>
<td>4 of 6</td>
<td>1.4 ± 1.1</td>
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<td>IFNβ-</td>
<td>11.1 ± 5.1</td>
<td>10.3</td>
<td>2.2 ± 0.8</td>
<td>2.0</td>
<td>10.6 ± 5.3</td>
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<td>1.4 ± 1.1</td>
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<td>0.5</td>
<td>3.8 ± 2.0</td>
<td>1 of 6</td>
<td>0.1 ± 0.2</td>
</tr>
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<td>Saline</td>
<td>17.6 ± 3.9</td>
<td>17.3</td>
<td>3.0 ± 0.0</td>
<td>3.0</td>
<td>20.3 ± 1.8</td>
<td>7 of 7</td>
<td>2.5 ± 0.7</td>
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<td>NAg</td>
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<td>17.5</td>
<td>3.0 ± 0.0</td>
<td>3.0</td>
<td>18.4 ± 1.3</td>
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<td>2.2 ± 0.8</td>
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<td>IFNβ-</td>
<td>11.2 ± 5.9</td>
<td>8.3</td>
<td>2.0 ± 1.0</td>
<td>2.0</td>
<td>20.7 ± 1.9</td>
<td>3 of 5</td>
<td>1.3 ± 1.4</td>
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<td>IFNβ-NAg</td>
<td>2.7 ± 1.5</td>
<td>2.3</td>
<td>0.7 ± 0.8</td>
<td>0.3</td>
<td>6.4 ± 5.0</td>
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<td>0.1 ± 0.2</td>
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<td>18.0</td>
<td>3.0 ± 0.0</td>
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<td>17.9 ± 3.7</td>
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<td>2.6 ± 0.6</td>
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<td>3.0</td>
<td>14.6 ± 4.7</td>
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<td>1.8 ± 1.0</td>
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<td>IFNβ-</td>
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<td>8.3</td>
<td>2.1 ± 0.8</td>
<td>2.0</td>
<td>15.2 ± 6.5</td>
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<td>1.4 ± 1.2</td>
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<td>IFNβ-NAg</td>
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<td>0.5</td>
<td>5.0 ± 3.7</td>
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<td>0.1 ± 0.2</td>
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</table>

aFor experiments 1 and 2, rats were challenged with 50 μg of DHFR-NAg in CFA on day 0. On day 9, rats were matched for clinical signs of EAE and were randomly assigned to groups that were injected s.c. with 1 nmol of NAg, 1 nmol of IFNβ, or 1 nmol of IFNβ-NAg. For experiment 1, matched groups that received NAg, IFNβ, or IFNβ-NAg (n = 6; mean cumulative score, 0.33) were each comprised of four rats exhibiting distal limp tail (dLT; 0.25) and two rats exhibiting limp tail (LT; 0.5). A fourth group (n = 5; mean cumulative score, 0.2; 0.25, 0.25, 0.25, 0.25, 0) was treated with saline. Each group was treated on day 9 (1 nmol), day 10 (1 nmol), and again on day 12 (0.5 nmol). For experiment 2, matched groups that received NAg, IFNβ, or IFNβ-NAg (n = 5; mean cumulative score, 0.35) were each comprised of three rats exhibiting distal limp tail (dLT; 0.25) and three rats exhibiting limp tail (LT; 0.5). A fourth group (n = 7; mean cumulative score, 0.36; 0, 0.25, 0.25, 0.25, 0.5, 1, 1) was treated with saline. Each group was treated with 1 nmol on days 9, 10, 12, and 14.

bThe disease time course for experiments 1 and 2 are shown in Fig. 5, A and B, respectively, and were compiled for statistical analysis. The time course of weight loss for experiment 2 is shown in Fig. 5C. Compared to rats treated with IFNβ-NAg, the mean and median cumulative scores (p < 0.001), the mean and median maximal scores (p ≤ 0.001), the mean maximal weight loss (p < 0.001), the incidence of severe EAE (p < 0.001, p = 0.0089; p = 0.03), and the mean number of days with severe EAE (p = 0.01, p < 0.001, p = 0.007) were significantly less than the respective values for rats treated with saline, NAg, or IFNβ, respectively. Compared to rats treated with IFNβ, the mean (p = 0.003) and median (p = 0.022) cumulative scores, the mean (p = 0.035) and median (p = 0.018) maximal EAE scores, and the mean number of days with severe EAE (p = 0.009) were significantly less than the respective scores for rats treated with saline.
highly effective in halting progression of EAE and resulted in reduced cumulative and maximal scores, a lower incidence of severe EAE, and a reduced duration of severe EAE. The pooled administration of IFN-β and NAg was also effective in blunting progression of EAE as assessed by the same disease measures. However, the IFNβ-NAg fusion protein was more consistent than was the combination of cytokine and NAg (experiment 2, Fig. 6C). Analysis of the three experiments together (Table VI) supported the following rank order of activity: the IFNβ-NAg fusion protein ≥ a mixture of IFN-β plus NAg > IFN-β > NAg. Possibly, the two treatment modalities (IFNβ-NAg vs IFN-β plus NAg) appear equal during less aggressive disease due to the plateau effect of full recovery, whereas the fusion protein may have superior activity in blocking a more aggressive attack. Analysis of weight loss supported this proposition. In all three experiments of Table VI, the IFNβ-NAg fusion protein was more effective in preventing EAE-associated weight loss than was the combined IFN-β and NAg treatment (experiment 1, mean, 9.7% vs 16.7%, p = 0.0387; experiment 2, mean, 8.7% vs 15.1%, p = 0.0042; experiment 3, mean, 8.5% vs 13.2%, p = 0.0407; unpaired t test). Even when IFNβ-NAg and the combined IFN-β plus NAg treatment both fully blocked progression of EAE (experiment 1, Fig. 6A), the IFNβ-NAg had superior activity in preventing weight loss (Fig. 6B).

Concerted cytotoxic action of IFN-β and NAg

Previous studies indicated that the IL2-NAg and NAg-IL16 fusion proteins required covalent linkage of cytokine and NAg domains for tolerogenic efficacy (25, 26). A requirement for covalent linkage most likely reflected a mechanism whereby the cytokine domain targets Ag to particular APC subsets by a mechanism of receptor-mediated endocytosis to achieve enhanced Ag presentation. This study indicated that the cytokine-Ag link is less important for the inhibitory action of IFNβ-NAg, particularly when the IFN-β and NAg are given before encephalitogenic sensitization. The mechanism of cooperative action of IFN-β and NAg may reflect cytotoxic actions of the two reagents acting in concert.

Table VI. Treatment with IFNβ-NAg or a mixture of IFN-β and NAg attenuates active EAE

<table>
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<tr>
<th>Exp.</th>
<th>Treatment</th>
<th>Mean Cumulative Score</th>
<th>Median Cumulative Score</th>
<th>Mean Maximal Score</th>
<th>Median Maximal Score</th>
<th>Incidence of Severe EAE</th>
<th>Mean No. Days with Severe EAE (A-P)</th>
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<td>1.5 ± 1.1</td>
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<td>IFN-β + NAg</td>
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<td>0.5 ± 0.9</td>
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<td>1.8</td>
<td>0.4 ± 0.1</td>
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<td>0.3 ± 0.7</td>
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<td>1.4 ± 0.9</td>
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<td>1.0 ± 0.7</td>
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<td>1.1 ± 1.1</td>
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<td>0.4 ± 0.6</td>
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<td>0.4 ± 0.1</td>
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<td>0 of 7</td>
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<td>NAg</td>
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<td>IFNβ-NAg</td>
<td>1.4 ± 0.8</td>
<td>1.4</td>
<td>0.4 ± 0.4</td>
<td>0.3</td>
<td>1 of 18</td>
<td>0.1 ± 0.4</td>
</tr>
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</table>

aDHFR-NAg-sensitized rats were matched for clinical signs of EAE and were treated with 3 nmol of the following reagents: NAg, IFN-β, a mixture of IFN-β plus NAg, or the IFNβ-NAg fusion protein (s.c. in saline) on days 10, 11, and 13 (experiment 1), on days 9, 10, 12, and 14 (experiment 2), or on days 9, 10, and 12 (experiment 3).

bThe time course of EAE for experiments 1–3 are shown in Fig. 6A. C, and D, respectively. The time course of weight loss for experiment 1 is shown in Fig. 6B. The three experiments (last four rows of Table VI) were compiled and analyzed. Compared to rats treated with IFNβ-NAg, the mean and median cumulative scores (p ≤ 0.005), the mean and median maximal score (p ≤ 0.002), the incidence of severe EAE (p < 0.0001, p = 0.0012), and the mean number of days with severe EAE (p = 0.001, p = 0.0036) were significantly less than the respective values for rats treated with NAg or IFN-β, respectively. Compared to rats treated with the combination of IFN-β plus NAg, the mean (p = 0.001) and median (p = 0.032) cumulative scores, the mean (p = 0.011) and median (p = 0.019) maximal scores, the incidence of severe EAE (p = 0.021), and the mean number of days with severe EAE (p = 0.039) were significantly less than the respective values for rats treated with NAg. For these three experiments, severe EAE was defined by scores from 1.0 to 3.0.
stimulates proliferation of R1T (but not the irradiated responders). In the presence of Ag, R1T APC were killed by responders during MHC-II-restricted Ag presentation, thereby resulting in diminished IL-2-dependent growth. As shown in Fig. 7A, IFN-β inhibited growth of R1T cells across a concentration range of 10 pM to 10 nM, and NAg also inhibited R1T growth across a concentration range of 10 nM to 10 μM. When IFN-β and NAg were combined in the same culture, the inhibitory effects were cooperative. Normalization of these data showed that the NAg had essentially the same inhibitory activity in the presence of each IFN-β concentration (Fig. 7B) and vice versa; that is, IFN-β had essentially the same proportional inhibitory activity in the presence of each NAg concentration (not shown). We conclude that IFN-β and NAg stimulated independent cytotoxic mechanisms that acted in concert to abrogate this T cell response.

Discussion
IFN-β is currently one of the most universally used therapeutics for treatment of MS. The cytokine decreases the frequency of clinical exacerbations, reduces the cumulative disease burden as assessed by magnetic resonance imaging, and may slow progression of disability. However, beneficial effects of chronic IFN-β administration are partial, are subject to considerable patient-to-patient variability, and are difficult to assess in terms of efficacy in any given patient. This study focused on the therapeutic action of IFN-β in a rat model of EAE with the aim of testing new approaches for IFN-β-based therapies of CNS autoimmune disease. The study uncovered two important findings. First, cytokine-Ag fusion proteins that incorporated rat IFN-β as the N-terminal domain and a major NAg (the 69–88 encephalitogenic determinant of MBP) as a C-terminal domain effectively prevented a subsequently induced attack of EAE. Notably, the covariant attachment was not needed for preventative efficacy because a combination of IFN-β and NAg was as effective as the IFN-β-NAg fusion protein for disease prevention. Second, when treatment was initiated after disease onset, the IFN-β-NAg was highly effective for disease suppression. The combination of IFN-β and NAg was also effective but to a lesser extent than that of the intact fusion protein. These two findings may provide the basis for a new Ag-specific treatment of MS. Disadvantages of Ag-specific therapy include uncertainty regarding the identity of etiological autoantigens coupled with the concern that the use of autoantigen in any therapy may aggravate autoimmunity or cause anaphylaxis. However, Ag-specific therapies have major countervailing advantages. For example, a main advantage is that Ag-specific therapy will be key to achieving long-lasting immunological tolerance and thereby may obviate the need for global or nonspecific immunosuppression.

The first observation was that IFN-β-NAg prevented a subsequent bout of EAE (Tables II–IV and Figs. 3 and 4). The tolerogenic activity of IFN-β-NAg was notable because the fusion protein was effective at low doses (total dose of 3 × 1 nmol in saline given at weekly intervals), and the same dosage of either IFN-β or NAg did not protect against disease. IFN-β-NAg elicited an inhibitory activity that persisted during the 1-wk interval between the last s.c. injection of IFN-β-NAg and the encephalitogenic challenge. Previous studies have shown that IFN-β is inhibitory in EAE. The distinction, however, is that these studies were based on the administration of a type I IFN concurrent with or subsequent to an encephalitogenic challenge (8–18). Studies of mice with a targeted deficiency in the IFN-β gene or in the type I IFN receptor gene showed that the beneficial effect of IFN-β is exerted during the effector phase of EAE and most likely involves expression of the type I IFN receptor on myeloid cells (19–21). The data shown in Tables II–IV represent a novel observation indicating that an
IFNβ-NAg fusion protein can be used effectively before challenge as part of a tolerogenic regimen. In the present study, IFNβ-NAg was most likely cleared from the system so that no significant IFNβ-activity remained at the time of encephalitogenic challenge or much less during active disease. Thus, the inhibitory activity of IFNβ-NAg was clearly NAg-dependent and was most likely manifested as an immunological tolerance specific for the major encephalitogenic determinant of MBP.

Importantly, the requirement for covalent linkage of the cytokine-NAg domains observed for the IL16-NAg (25) and IL2-NAg (26) fusion proteins was not apparent for IFNβ-NAg. Rather, the tolerogenic activity achieved by equal mixtures of IFNβ- and NAg was essentially equivalent to that of IFNβ-NAg (Table IV and Fig. 4). These data indicate that tolerance mediated by IFNβ-NAg did not require a strict targeting of the NAg domain to type I IFN receptor-bearing APC. Rather, IFNβ appeared to have broader inhibitory actions such that any Ag presented in the vicinity of IFNβ may be subjected to those inhibitory influences. In contrast, the IL2-NAg and NAg-IL16 fusion proteins efficiently targeted the covalently tethered NAg to the MHC-II-α-processing compartment of APC. In the presence of IL-2-"MHC-II+" T cell APC, IL2-NAg potentiated the presentation of NAg by >1000-fold compared with NAg alone (26). This potentiation required the covalent attachment of the IL-2 cytokine to NAg. Likewise, the IL2-NAg and NAg-IL16 fusion proteins required covalent linkage of the cytokine and Ag domains to exhibit tolerogenic activity for prevention or treatment of EAE (25, 26). The interpretation was that the cytokine domain interacted with cell surface receptors on APC and biologically conditioned those APC while simultaneously targeting the attached NAg domain for presentation by those APC. The distinct requirements for cytokine-NAg linkage may be of clinical significance. Inclusion of selected myelin Ags may enable a transformation of the standard IFNβ therapy for MS from a nonspecific mechanism to one that also efficiently induces Ag-specific tolerance.

The second observation was that IFNβ-NAg and, to a lesser extent, the combination of IFNβ and NAg inhibited the effector phase of EAE when treatment was initiated after disease onset (Tables V and VI and Figs. 5 and 6). IFNβ was also highly suppressive, but paralytic disease emerged when treatment was initiated after disease onset (Tables V and VI and Figs. 5 and 6). The latter observation may reflect induction of type I IFN receptors on particular APC subsets or emergence of APC subsets bearing high concentrations of IFNα/β receptors within inflammatory environments. In this case, the IFNβ-NAg may elicit regulatory activities by those APC while simultaneously targeting high concentrations of NAg to those APC, thereby facilitating efficient NAg presentation by those conditioned APC.

The mechanism by which NAg and IFNβ may interact to inhibit EAE is currently unknown. We used an in vitro T cell cytotoxicity assay to model potential interactions of NAg and IFNβ. In this assay, NAg was presented by MHC-II+ T cell APC to (irradiated) MBP-specific responders that in turn killed RI T cells by a mechanism dependent on Ag recognition (38). The observation was that both NAg and IFNβ strongly inhibited IL2-dependent growth of RI T cells and that these inhibitory mechanisms were cooperative (Fig. 7). That is, in the presence of either low or high concentrations of IFNβ, NAg had the same proportional killing activity (Fig. 7B) and vice versa; in the presence of either low or high concentrations of NAg, IFNβ had the same proportional inhibitory activity. The data in Fig. 7A are the same as in Fig. 7B normalized to 100% for each IFNβ concentration. Taken together, the combined action of IFNβ and NAg resulted in profound inhibition. The cytotoxicity of IFNβ was selective for certain lines but did not affect other T cell lines. Indeed, we successfully derived continuous T cell lines in the presence of IL-2 and high concentrations of IFNβ (data not shown). The selective cytotoxicity of IFNβ may represent a complementary or integrated component of immunoregulatory mechanisms that culminate in altered polarization of DC or T cells to suppress disease (17, 39–42).

Construction of the IFNβ-based fusion proteins deserves mention. The IFNβ-NAg and IFNβ proteins had a domain structure of IFNβ-EK-NAg-6his and IFNβ-EK-6his, respectively (Table I), in which the EK linker is an EK cleavage site. The necessity of the EK linker as opposed to the direct linkage of cytokine and NAg domains without a linker was previously investigated for IL2-NAg and IL4-NAg. In both cases, the cytokine-EK-NAg-6his fusion protein was equivalent to the cytokine-NAg-6his fusion protein measured by the biological activity of either the cytokine or NAg domain and in regard to the ability of the cytokine domain to target the NAg domain to specialized subsets of APC (26, 27). However, the IFNβ-NAg fusion protein was different. The IFNβ-EK-NAg-6his and IFNβ-EK-6his fusion proteins were either more efficiently expressed or more active compared with control proteins lacking the EK linker (Fig. 2A). Why the EK linker was needed for IFNβ-based fusion proteins is not currently known, although this information may be useful in the future design of IFNβ-based fusion proteins.

In summary, IFNβ was highly effective as a tolerogenic fusion partner or as a tolerogenic adjuvant and was used in concert with an encephalitogenic myelin Ag to constitute an effective tolerogenic vaccine for prevention and treatment of EAE. This information may lay the foundations of a new platform for developing Ag-specific, IFNβ-based therapies for MS.

Disclosures
The authors have no financial conflicts of interest.