A Fusion Protein Consisting of IL-16 and the Encephalitogenic Peptide of Myelin Basic Protein Constitutes an Antigen-Specific Tolerogenic Vaccine That Inhibits Experimental Autoimmune Encephalomyelitis[1]

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To test a novel concept for the generation of tolerogenic vaccines, fusion proteins were constructed encompassing a tolerogenic or biasing cytokine and the major encephalitogenic peptide of guinea pig myelin basic protein (GPMBP; i.e., neuroantigen or NAg). The cytokine domain was predicted to condition APC while simultaneously targeting the covalently linked encephalitogenic peptide to the MHC class II Ag processing pathway of those conditioned APC. Rats were given three s.c. injections of cytokine-NAg in saline 1–2 wk apart and then at least 1 wk later were challenged with NAg in CFA. The rank order of tolerogenic activity in the Lewis rat model of EAE was NAgIL16 > IL2NAg > IL1RA-NAg, IL13NAg ≥ IL10NAg, GPMBP, GP69–88, and saline. NAgIL16 was also an effective inhibitor of experimental autoimmune encephalomyelitis when administered after an encephalitogenic challenge during the onset of clinical signs. Covalent linkage of the NAg and IL-16 was required for inhibition of experimental autoimmune encephalomyelitis. These data identify IL-16 as an optimal cytokine partner for the generation of tolerogenic vaccines and indicate that such vaccines may serve as Ag-specific tolerogens for the treatment of autoimmune disease. *The Journal of Immunology,* **2007, 179: 1458–1465.**

New strategies are needed to elicit Ag-specific immunological tolerance as a means for the treatment of autoimmune disease (1–4). As opposed to generalized immunosuppression, Ag-specific regimens of tolerance induction may have improved efficacy because anti-inflammatory activity would be focused on the small percentage of relevant pathogenic T cells, obviating the need for global immune suppression. Ag-specific regimens may require temporary rather than chronic administration, may be effective at lower doses, may require local rather than systemic application, and thereby may exhibit superior efficacy and cost-effectiveness with fewer adverse side effects.

This study focused on a novel approach for the induction of Ag-specific tolerance in the Lewis rat model of experimental autoimmune encephalomyelitis (EAE)[3]. Fusion proteins were generated that contained an anti-inflammatory or tolerogenic cytokine together with the dominant encephalitogenic determinant of guinea pig myelin basic protein (GPMBP). The basic premise was that cytokine-neuroantigen (NAg) fusion proteins would target NAg to particular types of APC by cytokine receptors on those APC. The cytokine moiety of the fusion protein was predicted to modulate APC function to engender inhibitory or tolerogenic APC activities and simultaneously load the NAg into MHC class II Ag-processing pathways for presentation by those APC. NAg-specific Th cells that recognize the NAg presented by these tolerogenic APC were predicted to be rendered nonresponsive or to differentiate into regulatory T cells. This approach was thereby designed to focus the nonspecific inhibitory activities of particular cytokines onto rare NAg-specific Th cells to prevent CNS pathology. Fusion proteins incorporating IL-1RA, IL-2, IL-10, IL-13, and IL-16 were tested and the results indicate that fusion proteins incorporating IL-16 were the most effective tolerogens in EAE. Tolerance induction was Ag-specific rather than purely a cytokine-mediated effect, because covalent linkage of cytokine and NAg was required for tolerance induction.

Materials and Methods

Animals and reagents

Lewis rats (Harlan Sprague Dawley) were maintained at East Carolina University School of Medicine (Greenville, NC). Animal care and use was approved by the university's Institutional Animal Care and Use Committee and was performed in accordance with approved institutional guidelines. Myelin basic protein (MBP) was purified from guinea pig spinal cords (Rockland). Fusion proteins used in this study are shown in Table I. Derivation of baculovirus expression systems, protein expression, and protein purification for IL1RA-NAg, IL2NAg (IL2.7 and IL2Ekdel, where Ekdel denotes deletion of the enterokinase cleavage site), IL10NAg, IL13NAg (5, 6), NAgIL16 (NAgIL16S and NAgIL16L), and IL16S (M. D. Mannie, manuscript in preparation) were described elsewhere. The synthetic peptide GP69–88 (YGSLPQKSQRSQDENPVVHF) was obtained from Quality Controlled Biochemicals. The anti-I-A OX6 IgG1 mAb (specific for a monomorphic determinant of the rat MHC class II glycoprotein I-A) was concentrated from hybridoma supernatants by ultrafiltration through Amicon spiral wound membranes (100-kDa exclusion) and was used at a final titration of 1/40.

Cell lines and culture conditions

The RsL.11 MBP-specific clone was derived from Lewis rats sensitized with rat MBP in CFA (7). Assays were performed in complete RPMI 1640 medium (10% heat-inactivated FBS, 2 mM glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin (BioWhittaker), and 50 μM 2-ME (Sigma-Aldrich)).

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[3] Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; DHFR, dihydrofolate reductase; Ekdel, deletion of the enterokinase cleavage site; GPMBP, guinea pig myelin basic protein; MBP, myelin basic protein; MHCII, MHC class II glycoprotein; NAg, neuroantigen.

T cell lines were propagated in complete RPMI 1640 supplemented with recombinant rat IL-2 (0.4% (v/v) Sf9 supernatant) (8). Con A (Sigma-Aldrich) was used at a final concentration of 2.5 μg/ml.

Proliferative assays

RsL.11 T cells (2.5×10^4/well) and irradiated splenocytes (5×10^5/well) were cultured in 200 μl of complete RPMI 1640 in 96-well flat-bottom plates at 37°C and 5% CO_2 with designated Ags. Cultures were pulsed with 1 μCi of [^{3}H]thymidine (6.7 Ci/mmol; New England Nuclear) after 48 h of a 3-day assay. T cells were harvested onto filters by use of a Tomtec Mach III harvester. [^{3}H]Thymidine incorporation into DNA was measured by use of a Wallac 1450 MicroBeta Plus liquid scintillation counter. Error bars represent SD values of triplicate or quadruplet sets of wells.

Tolerance induction

To determine whether cytokine/NAg proteins prevented the active induction of EAE in Lewis rats, rats were given three s.c. injections of a given cytokine/NAg protein (1-nmol dose or at designated dosages) in saline at 1- to 2 wk intervals as designated. At least 7 days after the last injection of the respective fusion protein, rats were challenged with NAg in CFA (day 0) to induce EAE. NAgIL16 (and controls) were also tested to determine whether the fusion protein could ameliorate EAE when administered after challenge. Rats were sensitized with NAg in CFA on day 0 and given a total of two injections (i.v. or s.c. as designated) of 5 nmol of NAgIL16 in saline on day 8 and then once again on either day 11 or 12.

Induction and clinical assessment of EAE

EAE was induced in Lewis rats by the injection of an emulsion containing 50 μg of GPMBP in CFA (200 μg of *Mycobacterium tuberculosis*) or with 50 μg of a dihydrofolate reductase (DHFR)-NAg fusion protein in CFA (6). DHFR-NAg was comprised of the mouse DHFR as the N-terminal domain and the encephalitogenic GP69–87 peptide of GPMBP as the C-terminal domain. The following scale was used to assign intensity of EAE: 0.25, paralysis in the distal tail; 0.5, limp tail; 1.0, ataxia; 2.0, hind leg paresis; and 3.0, full hind leg paralysis. 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. Rats were scored at approximate 24-h intervals (Tables II, III, IV, and VI; Fig. 2) or at 12-h intervals (Table V and Fig. 4).

Statistical analysis

Mean cumulative score, mean maximal intensity, mean day of onset, and the mean number of days with severe EAE were analyzed with parametric ANOVA. Calculations of the mean cumulative score and mean maximal intensity were based on the assumption that the ordinal scale used for assessment of EAE (0, 0.25, 0.5, 1.0, 2.0, and 3.0) was an approximate quantitative representation of disease severity. Based on this assumption, the mean cumulative score was calculated by summing the daily scores for each rat and then averaging the cumulative scores for each rat to obtain the mean cumulative score for the group. The mean maximal intensity 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 intensity were listed as the median values for all rats in each group and were analyzed by nonparametric (based on ranked data) ANOVA or a nonparametric independent samples *t* test. Data compiled from two experiments (Tables II and IV) were analyzed by two-way parametric or nonparametric ANOVA (experiment no.1 vs experiment no.2 and comparison of treatment groups). Otherwise, multiple comparisons were analyzed with one-way parametric ANOVA or nonparametric ANOVA. ANOVA was interpreted with the Bonferroni post hoc test. "Incidence of EAE" or "incidence of severe EAE" was analyzed pair-wise with the Fisher's Exact test. Severe EAE was defined as the incidence of hind leg paresis (2.0) or full hind limb paralysis (3.0) unless designated otherwise (Table VI). Mean severity of EAE (*y*-axis of Figs. 3 and 4) was the mean of clinical scores for all rats within a treatment group on a given day.

Results

Antigenic activity of purified NAgIL16

Purified NAgIL16L was essentially equipotent compared with GPMBP (Fig. 1, *A* and *B*). These data indicated that the NAg in NAgIL16 was as accessible to Ag-processing and presentation as the NAg in GPMBP. Both NAgIL16L and GPMBP were substantially more potent than the DHFR-NAg fusion protein (Fig. 1*A*). Proliferative responses by the MBP-specific RsL.11 T cell clone to these fusion proteins were completely inhibited in the presence of the anti-MHC class II (MHCII) mAb OX6. Thus, the stimulatory activity of the NAgIL16 fusion protein was due to a mechanism of MHC-restricted Ag recognition rather than an Ag-independent, cytokine-mediated effect on T cell growth. Purified IL16S, like the respective baculovirus supernatant (data not shown), had no stimulatory activity (Fig. 1*B*). The purity of the IL-16 fusion proteins was estimated to be >98% (data not shown). Different purified NAgIL16L preparations had similar antigenic potency and therefore lacked any substantial degree of batch to batch variability (Fig. 1*B*).

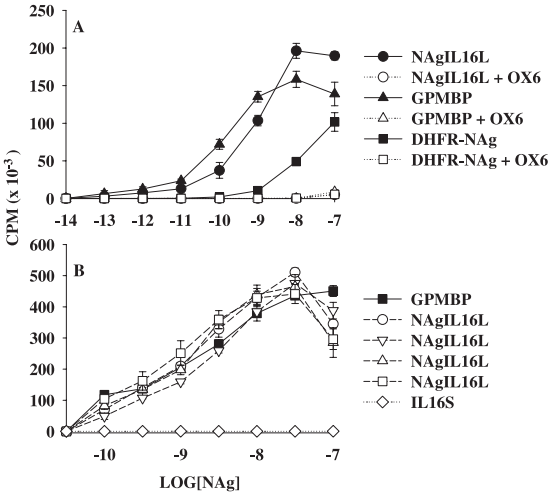

FIGURE 1. MHC-restricted antigenic activity of purified IL-16 fusion proteins. The MBP-specific clone of RsL.11 T cells (2.5×10^4/well) and irradiated splenic APC (5×10^5/well) were cultured with designated concentrations of purified NAgIL16L, GPMBP, or DHFR-NAg (*x*-axis) in the presence or absence of the anti-MHCII anti-I-A (RT1B) IgG1 mAb OX6 (*A*) or cultured with designated concentrations of GPMBP, IL16S, or different purified preparations of NAgIL16L (*x*-axis) (*B*). These data are representative of three experiments.

Comparative tolerogenic activity of cytokine-NAg fusion proteins

This research was designed to test the hypothesis that cytokine-NAg fusion proteins (Table I) may act as effective tolerogens in EAE. Like the IL2NAg fusion protein, NAgIL16 was predicted to have preferential interactions with T cells (9) and might target NAg for presentation by activated MHCII$^+$ T cells in vivo. These fusion proteins (Table II; experiments no. 1 and no. 2) were injected s.c. in saline at a dose of 1 nmol every 1–2 wk for a total of three injections. Seven days after the last injection, rats were actively challenged with NAg. Two independently derived forms of NAgIL16 (NAgIL16S and NAgIL16L) and two independently derived forms of IL2NAg (IL2.7 and IL2Ekdel) exhibited suppressive activity. NAgIL16 was a highly effective tolerogen that significantly ameliorated all measures of EAE severity, delayed disease onset, and reduced the incidence of severe EAE compared with rats pretreated with equal doses of NAg (GPMBP or GP69–88 peptide). Although IL2NAg was not as efficient as NAgIL16, the IL2NAg proteins were effective tolerogens compared with GPMBP or the synthetic peptide GP69–88 as revealed by significantly reduced cumulative and maximal intensity scores and a delayed disease onset.

To address the question of whether NAgIL16 and IL2NAg fusion proteins exhibited unique tolerogenic activity, we tested other fusion proteins including IL1RA-NAg, IL10NAg (IL10.6), and IL13NAg (IL13.6) (Table II; experiment no.3). Again, the fusion proteins were administered in three injections (days −21, −14,

Table I. *Rat cytokine-NAg fusion proteins*

Descriptor	Name	N- to C-terminal Order of Domains
IL1RA-NAg[a]	IL1RA-NAg4	Native ss-IL1RA-EK-NAg-6His
IL2NAg[a]	IL2.7	Native ss-IL2-EK-NAg-6His
IL2NAg[b]	IL2Ekdel	Native ss-IL2-NAg-6His
IL10NAg[a]	IL10.6	Native ss-IL10-EK-NAg-6His
IL13NAg[a]	IL13.6	Native ss-IL13-EK-NAg-6His
NAgIL16[c]	NAgIL16S	HBM ss-7His-NAg-C-terminal IL16
IL16 alone[c]	IL16S	HBM ss-7His-C-terminal IL16
NAgIL16[c]	NAgIL16L	HBM ss-7His-NAg-C-terminal IL16

[a] The IL1RA-NAg4 protein consisted of the native IL1RA signal sequence (ss), the mature IL-1RA cytokine, a GDDDDKG enterokinase (EK) domain, the major encephalitogenic peptide of GPMBP (PQKSQRSQDENPVVH), and a six-histidine (6His) C-terminal tag. IL2.7, IL10.6, and IL13.6 had a parallel structural design comprised of an N-terminal cytokine domain and a C-terminal EK-NAg-6His domain. These latter fusions also had the native signal sequence of the respective cytokine.

[b] Rat IL2Ekdel had a deletion of the enterokinase domain but was otherwise identical to the IL2.7 fusion protein.

[c] NAgIL16S and IL16S were cloned before the rat IL-16 sequence was available, and the cloning was accomplished by use of primers based on the mouse IL-16 sequence (GenBank accession no. AF006001). These proteins have a C-terminal serine of the mouse sequence, whereas the remainder of the protein encompasses the native rat sequence (GenBank accession XP_218851). The native C-terminal amino acid in rat IL-16 is a leucine. NAgIL16S protein consisted of the honey bee mellitin (HBM) signal sequence (MKFLVNVALVFMVVYISYIYA), a seven-histidine (7His) tag, the encephalitogenic 69–87 peptide of GPMBP (YGSLPQKSQRSQDEN PVVH), and the C-terminal 118-aa sequence of rat IL-16 with a C-terminal serine. NAgIL16L was identical to NAgIL16S except for the C-terminal substitution of leucine. IL16S had a modified HBM signal sequence (MAFLVNVALVFMVVYI SYIYA), a seven-histidine tag, and the C-terminal 118-aa sequence of rat IL-16 with a C-terminal serine.

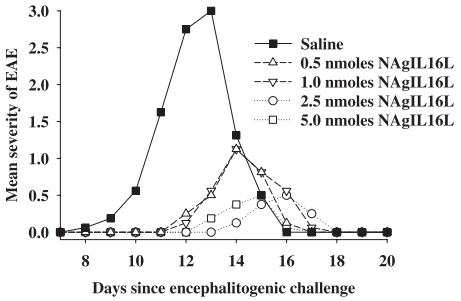

FIGURE 2. Higher doses of NAgIL16L induced more efficient tolerance. On days −21, −14, and −7 ($n = 4$ for each group) rats were injected with the designated doses of NAgIL16L and then were challenged with 50 μg of DHFR-NAg in CFA on day 0. The mean cumulative score ($p < 0.001$; all groups), the mean maximal intensity ($p = 0.009$, $p = 0.049$, $p = 0.001$, and $p = 0.001$, respectively), the mean day of onset ($p < 0.001$; all groups), and the mean number of days with severe EAE ($p < 0.001$; all groups) of rats treated with 0.5, 1.0, 2.5, or 5.0 nmol NAgIL16L were significantly different from the respective values of the control group (saline). The incidence of severe EAE for rats treated with 2.5 or 5.0 nmol of NAgIL16L (0 of 4) was significantly less than the respective incidence of rats treated with saline (four of four) ($p = 0.0286$) (Fisher's exact test).

and −7; 1 nmol/injection), and 7 days were allowed to elapse between the last pretreatment and the encephalitogenic challenge. The IL10.6 did not exhibit tolerogenic activity whereas the IL13.6 and IL1RA-NAg4 fusions proteins decreased the cumulative score compared with rats pretreated with saline. However, the tolerogenic

Table II. *Vaccination with IL-16NAg and IL-2NAg fusion proteins protects against the subsequent active induction of EAE*

Experiment No.	Treatment[a]	Incidence of EAE	Mean Cumulative Score	Median Cumulative Score	Mean Maximal Intensity	Median Maximal Intensity	Mean Day of Onset	Incidence of Severe EAE	Mean No. Days with Severe EAE
1	Saline	10 of 10	9.4 ± 3.1	9.5	2.5 ± 0.7	3.0	10.6 ± 1.3	9 of 10	3.0 ± 1.5
	GPMBP	7 of 7	8.3 ± 4.1	8.3	2.6 ± 0.8	3.0	10.4 ± 0.8	6 of 7	2.4 ± 1.4
	IL2.7	7 of 8	4.4 ± 3.3	3.9	1.6 ± 1.1	1.5	12.1 ± 1.2	4 of 8	1.3 ± 1.4
	NAgIL16S	7 of 8	3.2 ± 2.5	2.8	1.3 ± 1.0	1.0	12.6 ± 1.0	3 of 8	0.6 ± 0.9
2	Saline	6 of 6	9.2 ± 0.7	9.1	3.0 ± 0.0	3.0	10.0 ± 1.5	6 of 6	2.8 ± 0.4
	GP69–88	7 of 7	8.5 ± 1.4	8.5	2.9 ± 0.4	3.0	10.4 ± 0.5	7 of 7	2.6 ± 0.5
	IL2EKdel	9 of 9	5.1 ± 2.6	4.8	2.1 ± 0.7	2.0	12.2 ± 0.7	8 of 9	1.8 ± 1.0
	NAgIL16L	9 of 9	2.4 ± 1.2	2.0	1.1 ± 0.7	1.0	12.2 ± 0.4	3 of 9	0.4 ± 0.7
1 and 2	Saline	16 of 16	9.3 ± 2.4	9.4	2.7 ± 0.6	3.0	10.4 ± 1.4	15 of 16	2.9 ± 1.2
	NAg	14 of 14	8.4 ± 3.0	8.3	2.7 ± 0.6	3.0	10.4 ± 0.6	13 of 14	2.5 ± 1.0
	IL2NAg[b]	16 of 17	4.8 ± 2.9	4.8	1.8 ± 0.9	2.0	12.2 ± 0.9	12 of 17	1.5 ± 1.2
	NAgIL16[c]	16 of 17	2.8 ± 1.9	2.8	1.2 ± 0.8	1.0	12.4 ± 0.7	6 of 17	0.5 ± 0.8
3	Saline	28 of 28	10.4 ± 3.0	9.9	2.9 ± 0.4	3.0	10.5 ± 1.4	28 of 28	3.4 ± 1.2
	GP69–88	21 of 21	8.5 ± 3.4	9.0	2.5 ± 0.8	3.0	10.5 ± 1.7	18 of 21	2.5 ± 1.4
	IL10.6	8 of 8	7.3 ± 3.8	7.5	2.3 ± 1.0	2.5	11.3 ± 1.3	7 of 8	2.4 ± 1.4
	IL13.6	9 of 9	6.8 ± 2.5	7.8	2.6 ± 0.9	3.0	11.2 ± 0.7	7 of 9	2.0 ± 1.2
	IL1RA-NAg	7 of 7	6.2 ± 3.1	6.5	2.0 ± 1.0	2.0	12.3 ± 1.7	5 of 7	1.6 ± 1.3
	IL2Ekdel[d]	8 of 8	4.8 ± 2.4	5.1	1.8 ± 0.8	2.0	12.8 ± 1.3	6 of 8	1.4 ± 1.1

[a] Rats were pretreated with saline or 1 nmol of the designated protein on days −31, −17, and −7 (experiment no. 1) or on days −21, −14, and −7 (experiment no. 2 and no. 3). Rats were then challenged with 50 μg of GPMBP (experiment no. 1 and no. 3) or DHFR-NAg (experiment no. 2) in CFA on day 0.

[b] Combined experiments no. 1 and no. 2 (IL2NAg). The mean cumulative score ($p < 0.002$ and $p < 0.001$), the median cumulative score ($p = 0.004$ and $p < 0.001$), the mean maximal intensity ($p = 0.013$ and $p = 0.013$), the median maximal severity ($p = 0.003$ and $p = 0.002$), the mean day of onset ($p < 0.001$ and $p < 0.001$) of IL2NAg-treated rats were significantly different from the respective measures for NAg or saline-treated rats (two-way ANOVA).

[c] Combined experiments no. 1 and no. 2 (IL16NAg). The mean cumulative score, the median cumulative score, the mean maximal intensity, the median maximal severity, the mean day of onset, and the mean number of days with severe EAE of NAgIL16-treated rats were significantly different from those for NAg or saline-treated rats ($p < 0.001$) (two-way parametric ANOVA). The incidence of severe EAE of NAgIL16-treated rats was significantly less than that for NAg-treated rats ($p = 0.0008$, Fisher's exact test).

[d] Experiment no. 3. The mean cumulative score (NS, $p < 0.001$), the median cumulative score ($p = 0.005$ and $p < 0.001$), the mean maximal intensity (NS, $p = 0.008$), the median maximal severity ($p = 0.05$ and $p = 0.002$), and the mean day of onset ($p = 0.004$ and $p = 0.004$), and the mean number of days with severe EAE (NS, $p = 0.001$) of IL2NAg-treated rats were significantly different from the respective measures for NAg or saline-treated rats. IL1RA-NAg ($p = 0.027$ and $p = 0.032$) and IL13NAg ($p = 0.045$ and $p = 0.025$) respectively reduced the mean and median cumulative scores of EAE compared to rats pretreated with saline (ANOVA).

Table III. *Covalent tethering of IL-16 and NAg was necessary for tolerogenic activity*

Treatment[a]	Incidence of EAE	Mean Cumulative Score	Median cumulative Score	Mean Maximal Intensity	Median Maximal Intensity	Mean Day of Onset	Incidence of Severe EAE	Mean No. Days with Severe EAE
Saline alone	6 of 6	10.5 ± 4.2	10.1	2.8 ± 0.4	3.0	10.3 ± 0.5	6 of 6	2.3 ± 0.8
NAg	7 of 7	9.0 ± 2.0	9.0	2.7 ± 0.5	3.0	10.3 ± 0.5	7 of 7	3.0 ± 1.2
IL16S	8 of 8	12.5 ± 2.6	11.5	3.0 ± 0.0	3.0	9.9 ± 1.0	8 of 8	3.5 ± 1.1
IL16S and NAg	7 of 7	9.0 ± 2.1	9.0	2.9 ± 0.4	3.0	10.3 ± 0.5	7 of 7	3.0 ± 0.8
NagIL16[b]	9 of 9	2.2 ± 1.7	1.5	0.8 ± 0.7	0.5	13.2 ± 0.8	2 of 9	0.2 ± 0.4

[a] Rats were pretreated with saline, 1 nmol of NAgIL16L, 1 nmol of GP69–88 (NAg), 1 nmol of IL16S (no NAg), or the combination of GP69–88 and IL16S. Rats (fourth row from the top) were treated with separate injections of 1 nmol of IL16S and 1 nmol of GP69–88 at a distance of <0.5 cm apart near the base of the tail. Rats were pretreated on days −21, −14, and −7 and challenged with 50 μg of DHFR-NAg in CFA on day 0.

[b] The mean cumulative score, the median cumulative score (on ranks), the mean maximal intensity, the median maximal intensity (on ranks), the mean day of onset, and the mean number of days afflicted with severe EAE of rats pretreated with NAgIL16L were significantly different from the respective scores of the rats treated with saline, GP69–88, IL16S alone, or the combination of IL16S and GP69–88 as separate molecules ($p < 0.001$; ANOVA). The incidence of severe EAE in NAgIL16-treated rats was significantly lower than the incidence of rats treated with the combination of IL16 and NAg ($p = 0.0032$; Fisher's exact test).

activities of IL1RA-NAg and IL13NAg were substantially less compared with that of IL2NAg. Overall, the rank order of tolerogenic activities for the six fusion proteins in the Lewis rat model of EAE was NAgIL16 > IL2NAg > IL1RA-NAg, IL13NAg ≥ IL10NAg, GP69–88, GPMBP, and saline. Two IL4NAg fusion proteins were also tested and were found to lack suppressive activity (6).

Dose dependence of NAgIL16-mediated inhibition of EAE

Cytokine-Ag fusion proteins may elicit a balance of effector and regulatory cells, and this balance may vary with dosage. Tolerogenic fusion proteins would predictably cause tolerance by dose-dependent mechanisms. Higher doses should induce more profound tolerance. However, paradoxical dosage effects are possible, and higher doses may more efficiently prime effector cells and promote immunity. To test the effect of dosage for NAgIL16-mediated tolerance, rats were pretreated with NAgIL16 at four different doses (Fig. 2). Rats were injected with saline or 0.5, 1.0, 2.5, or 5.0 nmol of NAgIL16 on days −21, −14, and −7 and then were challenged with 50 μg of DHFR-NAg in CFA on day 0. All four dosages of NAgIL16 resulted in significant tolerance, and the two highest two doses elicited the most profound tolerogenic response. These data indicate that the tolerogenic activity of NAgIL16 was dose dependent and that optimal tolerance may be induced with doses of 2.5–5.0 nmol.

Linkage of IL-16 and NAg was required for tolerance

The tolerogenic activity of the NAgIL16 fusion protein was contingent upon the covalent linkage of IL-16 and NAg (Table III). Rats pretreated with NAgIL16 exhibited reductions in all measures of EAE severity, including significantly reduced incidence of severe EAE. In contrast, rats pretreated with a combination of IL16 and GP69–88 as separate molecules or with either agent alone were fully susceptible to EAE. Thus, physical linkage of IL-16 and NAg was required for tolerance induction. Similar studies showed that physical linkage of IL-2 and NAg was also required for the tolerogenic activity of the IL2NAg fusion proteins (6).

NAgIL16 was an effective inhibitor when administered during the onset of EAE

The NAgIL16 fusion protein was highly effective when delivered systemically at the onset of the effector phase (Table IV and Fig. 3). Rats challenged on day 0 with DHFR-NAg in CFA were treated with 5 nmol of NAgIL16L i.v. on day 8 and then were given an equivalent i.p. injection 3–4 days later. A dosage of 5 nmol was

Table IV. *NAgIL16L treatment causes the antigen-specific inhibition of the encephalitogenic effector phase*

Experiment No.	Treatment[a]	Incidence of EAE	Mean Cumulative Score	Median Cumulative Score	Mean Maximal Intensity	Median Maximal Intensity	Mean Day of Onset	Incidence of Severe EAE	Mean No. of Days Afflicted with Severe EAE
1	GP69–88	4 of 4	8.9 ± 2.6	8.5	3.0 ± 0.0	3.0	10.5 ± 0.6	4 of 4	2.3 ± 0.5
	NagIL16L	5 of 5	1.5 ± 0.9	1.0	0.5 ± 0.3	0.5	11.6 ± 2.1	0 of 5	0.0
2	GP69–88	8 of 8	7.4 ± 2.1	6.8	2.9 ± 0.4	3.0	9.5 ± 1.2	8 of 8	2.4 ± 1.2
	IL-16	8 of 8	7.3 ± 1.1	7.3	3.0 ± 0.0	3.0	10.0 ± 0.9	8 of 8	2.1 ± 0.4
	Both	8 of 8	6.8 ± 2.3	7.1	2.6 ± 0.5	3.0	10.1 ± 0.8	8 of 8	2.0 ± 0.8
	NAgIL16L	6 of 8	2.5 ± 2.5	1.6	1.0 ± 1.0	0.8	10.5 ± 0.5	2 of 8	0.4 ± 0.7
1 and 2	GP69–88	12 of 12	7.9 ± 2.2	6.8	2.9 ± 0.3	3.0	9.8 ± 1.1	12 of 12	2.3 ± 1.0
	IL-16	8 of 8	7.3 ± 1.1	7.3	3.0 ± 0.0	3.0	10.0 ± 0.9	8 of 8	2.1 ± 0.4
	Both	8 of 8	6.8 ± 2.3	7.1	2.6 ± 0.5	3.0	10.1 ± 0.8	8 of 8	2.0 ± 0.8
	NAgIL16[b]	11 of 13	2.1 ± 2.0	1.6	0.8 ± 1.0	0.8	11.0 ± 1.5	2 of 13	0.2 ± 0.6

[a] For both experiments, rats were challenged with 50 μg DHFR-NAg in CFA on day 0. On day 8, rats were treated with intravenous injections of 5 nmol of GP69–88, 5 nmol of IL-16, a combined solution of 5 nmol of IL-16 and 5 nmol of GP69–88, or 5 nmol of NAgIL16L as designated. These same injections (5 nmol) for each group were repeated by i.p. injection on day 12 (experiment no. 1) or 11 (experiment no. 2).

[b] Combined experiments no. 1 and no. 2. The mean cumulative score ($p < 0.001$), the median cumulative score (on ranks) ($p \leq 0.002$), the mean maximal intensity ($p < 0.001$), the median maximal intensity (on ranks) ($p < 0.001$), and the mean number of days afflicted with severe EAE ($p < 0.001$) of rats treated with NAgIL16L were significantly different from those treated with GP69–88, IL16S alone, or the combination of IL16S and GP69–88 as separate molecules (ANOVA). The incidence of severe EAE in NAgIL16-treated rats was significantly lower than the incidence of rats treated with the combination of IL16 and NAg ($p = 0.0002$; Fisher's exact test).

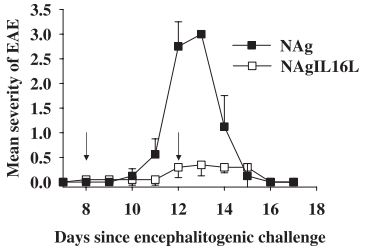

FIGURE 3. Systemic administration of NAgIL16L inhibited the effector phase of EAE. Data presented in tabular form (experiment no.1 of Table IV) are shown as a time course of EAE. Rats were challenged with 50 μg of DHFR-NAg in CFA on day 0. Rats were then treated with 5 nmol of NAg (i.e., synthetic peptide GP69–88) or NAgIL16L i.v. on day 8 and by i.p. injection on day 12 (arrows). The mean and median cumulative scores ($p < 0.001$ and $p = 0.002$), the mean and median maximal intensity scores ($p < 0.001$ and $p = 0.002$), the incidence of severe EAE ($p = 0.0079$), and the mean number of days afflicted with severe EAE ($p < 0.001$) for NAgIL16-treated rats were significantly less than the respective values for NAg-treated rats (independent samples *t* test and Fisher's exact test).

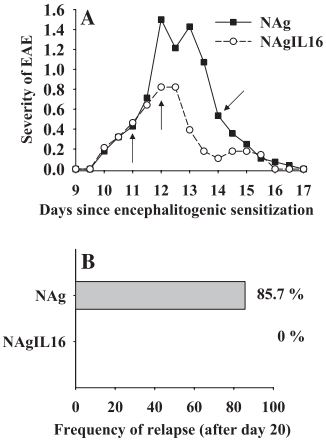

FIGURE 4. Administration of NAgIL16 during clinical disease halts EAE progression and prevents subsequent relapse. Data presented in tabular form (Table V, experiment 2) are shown as a time course of EAE. On day 11, rats were matched for clinical signs of EAE and randomly assigned to one of two groups that were injected with either NAg (GP69–88) or NAgIL16L. *A*, Treatments were on day 11 (5 nmol in saline i.v.), day 12 (5 nmol in saline i.p.), and day 14 (2 nmol in saline i.v.) (see arrows). The cumulative EAE severity per day for NAgIL16-treated rats was significantly less than that for NAg-treated rats on days 13, 13.5, and 14 ($p = 0.013$, 0.005, and 0.009, respectively). The mean cumulative scores (tallied after the first treatment on day 11) also were significantly different ($p < 0.001$) (Mann-Whitney Test). *B*, The frequency of relapses (onset after day 21) for NAg-treated rats (six of seven, 85.7%) was significantly higher than that for NAgIL16-treated rats (zero of seven) ($p = 0.0047$, Fisher's exact test).

used because we reasoned that a systemic administration of fusion protein would be required to affect the clonally expanded encephalitogenic T cell repertoire that by day 8 had a systemic distribution. Furthermore, as shown in Fig. 2, use of a 5.0-nmol dose may provide optimal tolerance. The two injections blocked progression to severe EAE. The effectiveness of this postchallenge treatment regimen was contingent upon covalent linkage of the NAg and IL16 domains of the fusion protein. Compared with control rats that received equivalent dosages of GP69–88 alone, IL-16 alone, or the combination as separate molecules, rats receiving NAgIL16L exhibited a significant reduction in all measures of EAE severity, including a significantly reduced incidence of severe EAE. These data indicate that NAgIL16 regulates events that are necessary for the effector phase of EAE.

To assess the potential clinical significance, experiments were performed to assess whether NAgIL16 could blunt an encephalitogenic attack when the advent of clinical EAE preceded the first NAgIL16 treatment (Table V and Fig. 4). Rats challenged with DHFR-NAg were allowed to progress to partial or full paralysis of the tail (0.25–0.5 scores), and were divided into two groups that exhibited essentially equivalent categories of paralysis. Two treatments of NAgIL16 (experiment no. 1) on days 10 and 11 (5 nmol i.v. and i.p., respectively) resulted in the virtually complete abrogation of EAE by day 13, but then residual disease re-emerged in two of eight rats, progressing to a score of 2.0. Compared with NAg-treated control rats, NAgIL16 inhibited all measures of cumulative and maximal intensity disease, including the severity of EAE within a 2.5 day window following the second treatment (Table V, sixth and seventh columns from the left). In experiment 2, treatments of NAgIL16 on days 11 and 12 (5 nmol) essentially abrogated the disease as before, and a third treatment on day 14 (2 nmol) prevented any re-emergence of EAE. Treatment with NAgIL16 also inhibited the spontaneous relapse of EAE, which occurred in six of seven NAg-treated rats during days 22–30 (Fig. 4*B*). In conclusion, NAgIL16 had an acute inhibitory effect that suppressed an ongoing encephalitogenic attack and facilitated recovery.

Table V. *NAgIL16 treatment inhibits progression of EAE when administered after disease onset*

Experiment No.	Treatment[a]	Incidence of EAE	Mean Cumulative Score	Median Cumulative Score	Mean Cumulative Score (2.5-day Window)	Median Cumulative Score (2.5-day Window)	Mean Intensity Score	Median Intensity Score	Incidence of Severe EAE
1	NAg	7 of 7	13.3 ± 7.0	12.5	4.9 ± 2.5	4.5	2.1 ± 1.0	2.0	5 of 7
	NAgIL16	8 of 8	6.7 ± 5.4	4.5	1.2 ± 0.7	1.0	1.0 ± 0.7	1.0	2 of 8
2	NAg	7 of 7	9.1 ± 1.6	8.3	3.6 ± 1.8	3.8	2.0 ± 0.8	2.0	5 of 7
	NAgIL16	7 of 7	3.5 ± 1.5	3.3	1.0 ± 0.5	0.8	0.9 ± 0.6	0.5	1 of 7
1 and 2	NAg	14 of 14	11.2 ± 5.4	10.0	4.3 ± 2.2	4.4	2.0 ± 0.9	2.0	10 of 14
	NAgIL16[b]	15 of 15	5.2 ± 4.2	3.8	1.1 ± 0.6	0.8	1.0 ± 0.6	1.0	3 of 15

[a] Rats were challenged with 50 μg of DHFR-NAg in CFA on day 0. Rats were randomly assigned to one of two treatment groups based on the expression of distal limp tail (0.25) or limp tail (0.5) immediately before the first treatment. In experiment no. 1 (NAg group: 0.25, $n = 2$ and 0.5, $n = 5$; NAgIL16 group: 0.25, $n = 2$ and 0.5, $n = 6$) treatments were administered on day 10 (5 nmol i.v.) and day 11 (5 nmol i.p.). In experiment no. 2 (NAg group: 0.25, $n = 2$ and 0.5, $n = 5$; NAgIL16 group: 0.25, $n = 1$ and 0.5, $n = 6$), NAg and NAgIL16 were administered on day 11 (5 nmol i.v.), day 12 (5 nmol i.p.), and day 14 (2 nmol i.v.). In experiments no.1 and no. 2, rats were scored on approximate 12-h intervals.

[b] The mean and median cumulative score, the mean and median cumulative scores for a 2.5-day window following the second treatment (days 12–14.5 and days 13–15.5 for experiments 1 and 2 respectively), and the mean and median maximal intensity scores for NAgIL16-treated rats were significantly less than those for NAg-treated rats ($p < 0.001$; independent samples *t* test). Differences in median values were assessed by comparison of ranked data. The incidence of severe EAE in NAgIL16-treated rats was significantly less than the respective incidence in NAg-treated rats ($p = 0.0092$; Fisher's exact test).

Table VI. *A prechallenge and postchallenge NAgIL16 treatment regimen inhibits disease incidence*

Treatment[a]	Incidence of EAE	Mean Cumulative Score	Median Cumulative Score	Mean Maximal Intensity	Median Maximal Intensity	Mean Day of Onset	Incidence of Severe EAE	Mean No. of Days Afflicted with	
								Mild EAE	Severe EAE
Saline	9 of 9	8.5 ± 2.1	8.5	2.7 ± 0.5	3.0	8.8 ± 0.7	9 of 9	2.6 ± 1.0	3.8 ± 0.8
GP69–88	9 of 9	6.9 ± 3.3	6.0	2.8 ± 0.7	3.0	10.3 ± 1.4	9 of 9	2.4 ± 1.0	2.9 ± 1.4
NAgIL16L[b]	2 of 9	0.3 ± 0.8	0.0	0.3 ± 0.7	0.0	14.0 ± 2.8	1 of 9	0.3 ± 0.7	0.1 ± 0.3

[a] Rats were pretreated with 2 nmol of NAgIL16L (bottom row), 2 nmol of GP69–88 (middle row), or saline (top row) on days −21, −14, and −7. Rats were challenged with 50 μg of DHFR-NAg in CFA on day 0 and then treated on days 8 and 11 with i.p. injections of 5 nmol of the respective antigen or saline.

[b] The incidence of EAE ($p = 0.0023$) and the incidence of severe EAE ($p = 0.0004$) of rats treated with NAgIL16L was significantly less than the respective incidences in rats treated with GP69–88 (Fisher's exact test). The mean cumulative score ($p < 0.001$), the median cumulative score ($p < 0.001$ on ranks), the mean maximal intensity ($p < 0.001$), the median maximal intensity ($p < 0.001$ on ranks), the mean day of onset ($p = 0.006$, $p < 0.001$), and the mean number of days with mild EAE ($p = 0.004$ and $p < 0.001$), or severe EAE ($p < 0.001$) of rats treated with NAgIL16 were significantly reduced compared to the respective scores for rats treated with GP69–88 or saline (ANOVA). Mild EAE was defined as clinical scores of 0.25 or 0.5, and severe EAE was defined as clinical scores of 1.0, 2.0, or 3.0. Note that for all other tables, severe EAE was defined as clinical scores of 2.0 or 3.0.

NAgIL16 also had longer term tolerogenic activity that prevented a subsequent relapse.

NAgIL16 inhibits incidence of EAE

Prechallenge (Tables II and III) and postchallenge (Table IV and V) NAgIL16 treatment regimens independently reduced the severity of EAE, but these regimens did not reduce the incidence of EAE. In contrast, a combined prechallenge and postchallenge treatment regimen showed substantially stronger inhibitory activity (Table VI) than either prechallenge or postchallenge regimens alone. Rats that were treated with NAgIL16 before (days −21, −14, and −7 with 2 nmol per injection) and after (days 8 and 11 with 5 nmol) challenge were largely protected from EAE. These data indicate that successive NAgIL16 treatments had a cumulative tolerogenic effect with sufficient tolerogenic efficacy to inhibit the incidence of EAE. An IL16NAg fusion protein may thereby represent a unique candidate for the induction of Ag-specific tolerance as a treatment for autoimmune disease. NAgIL16 was not encephalitogenic and did not cause adverse reactions at the injection site. Also, multiple injections of NAgIL16 did not result in anaphylactic symptoms or any evidence of a more intense rebound disease as was observed in Lewis rats given multiple injections of IFN-β (10).

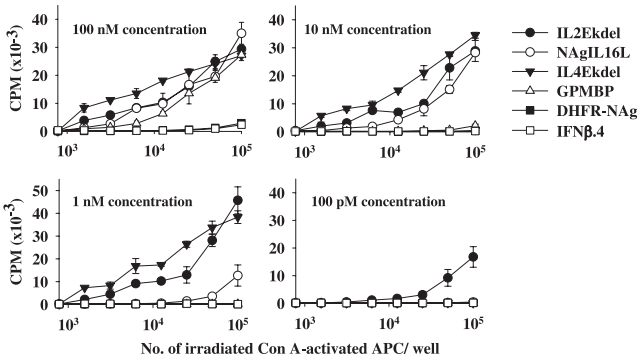

FIGURE 5. The IL-16 domain of NAgIL16 targeted NAg to Con A-activated splenic APC. Lewis rat splenocytes were depleted of adherent APC in a 2-day culture in complete RPMI 1640. Nonadherent splenocytes were dispensed into 24-well plates and activated with 2.5 μg/ml Con A in the presence of designated concentrations of IL2NAg (IL2Ekdel), NAgIL16L, IL4NAg (IL4Ekdel), GPMBP, DHFR-NAg, or IFNβ-NAg (IFNβ.4). After 3 days of activation, splenic APC were extensively washed, irradiated (3000 rads of gamma irradiation), and added to 96-well plates at designated densities (*x*-axis) in the presence of RsL.11 T cells (2.5×10^4/well) without any addition of soluble Ag. Cultures were pulsed with [^{3}H]thymidine during the last day of a 3-day culture. These data were representative of three experiments.

The IL16 domain facilitates presentation of NAg by Con-A activated splenic APC

Previous research showed that both the IL-2 and IL-4 cytokines targeted NAg to APC for enhanced presentation (6). The IL-16 moiety of the NAgIL16L fusion protein also potentiated the presentation of NAg (Fig. 5). Splenic APC were depleted of adherent APC by a 2-day culture in complete RPMI 1640 medium. Nonadherent splenocytes were then cultured with 100 nM, 10 nM, 1 nM, or 100 pM of designated fusion proteins in the presence of 2.5 μg/ml Con A. After 3 days of culture, splenic lymphoblasts were washed, irradiated, and used at designated cell densities as APC (*x*-axis) to stimulate the GPMBP-specific RsL.11 T cell line. Thus, Ag was incorporated into MHC class II glycoproteins during a 3-day culture in the presence of Con A, and then Ag presentation was measured in a subsequent 3-day culture in the absence of soluble Ag. Proliferation of RsL.11 T cells in the final 3-day culture was measured by the addition of 1 μCi of [^{3}H]thymidine during the last day of culture. Proliferation was due to NAg-specific stimulation of RsL.11 T cells because the addition of the OX6 anti-MHCII mAb to a parallel set of cultures abrogated the proliferative response (data not shown).

The results show that a concentration of 1 nM NAgIL16L elicited stronger Ag presentation than 10 nM GPMBP or 100 nM DHFR-NAg. These data indicated that the IL-16 moiety potentiated Ag presentation by at least 10-fold compared with NAg alone. As expected, the IL2Ekdel and IL4Ekdel fusion proteins also strongly augmented Ag presentation. Indeed, either IL2Ekdel or IL4Ekdel at 1 nM was as active as 100 nM GPMBP. In contrast, a purified IFNβ-NAg fusion protein (IFNβ.4) did not result in detectable Ag presentation by Con A-activated splenocytes. Previous data showed that the neuroantigenic activity of IFNβ.4 equaled that of GPMBP or NAgIL16 in cultures of professional splenic APC and RsL.11 T cells (not shown). Overall, the rank order of activity in this assay for the targeting of NAg to Con A-activated splenic T cells was as follows: IL2NAg > IL4NAg > NAgIL16 > GPMBP > DHFR-NAg and IFNβ.4. These findings support the hypothesis that IL-2 and IL-16 may target covalently tethered NAg to activated T cells to potentiate the presentation of NAg by activated T cells.

Discussion

The purpose of this study was to test the concept of a tolerogenic vaccine. The prediction was that a fusion protein containing a

tolerogenic cytokine and the major encephalitogenic determinant of GPMBP (i.e., the NAg) would represent a qualitatively superior tolerogen compared with Ag alone. The covalently linked cytokine was predicted to provide two critical activities. First, the cytokine was predicted to bind the respective receptors on particular subsets of APC and condition those APC to express tolerogenic, anti-inflammatory, or inhibitory activity. Secondly, the binding of the cytokine domain to the cytokine receptor on those APC was predicted to target the covalently tethered Ag to the MHCII Ag processing pathway of those APC. Such a mechanism would ensure presentation of the covalently tethered NAg predominantly on cytokine-conditioned APC. Six cytokines were tested as potential candidates, including IL1-RA, IL-2, IL-4, IL-10, IL-13, and IL-16. IL-1RA and IL-16 were predicted to have pronounced anti-inflammatory activity (11–15). IL-4 and IL-13 were predicted to drive immune deviation toward a nonpathogenic Th2 lineage (16, 17). IL-2 and IL-10 are required for self-tolerance and therefore were predicted to facilitate Ag presentation in the context of a regulatory T cell response (18–21). Furthermore, IL-2 and IL-16 were chosen based on the prediction that these cytokines may target Ag to $MHCII^+$ T cells in accordance with the hypothesis that T cell-mediated Ag presentation, under certain circumstances, may lead to tolerance (22–26).

A previous study focused on IL2NAg and IL4NAg fusion proteins (6). These fusion proteins targeted the covalently tethered NAg to distinct subsets of APC. In assays measuring the MHCII-restricted proliferative responses of a MBP-specific T cell clone, IL2NAg was at least 1,000 times more potent than NAg in the presence of $MHCII^+$ T cells, and IL4NAg was ~100-fold more active than NAg in the presence of splenic APC. The enhanced antigenic potencies of both IL2NAg and IL4NAg were contingent upon the covalent linkage of the respective cytokine with NAg. When administered s.c. in saline either before or after the encephalitogenic challenge, IL2NAg attenuated the severity of EAE. In contrast, IL4NAg and NAg lacked tolerogenic activity. The tolerogenic activity of IL2NAg in vivo was contingent upon the covalent linkage of IL-2 and NAg, because injection of IL-2 and NAg as separate molecules had no effect. Therefore, the tolerogenic activity of IL2NAg could not be explained by the independent or combined actions of IL-2 and NAg as separate molecules. Rather, the tolerogenic activity required a linked activity entirely consistent with a mechanism in which IL-2 targeted the NAg to a MHCII Ag-processing pathway. These data were consistent with the possibility that the IL-2 domain may target NAg to cell surface IL-2 receptors on a $MHCII^+$ APC subset, perhaps expressing IL-2 dependent cytotoxic activity.

A central finding reported herein was that NAgIL16 exhibited efficient tolerogenic activity. IL-16 is a highly conserved, species cross-reactive cytokine (27) that binds to CD4 or other cell surface receptors (28) to facilitate chemotaxis of T cells, monocytes, eosinophils, and dendritic cells (29). IL-16 received considerable attention due to reports that IL-16 inhibits HIV replication (30–33). IL-16 is synthesized as a large precursor and is cleaved into an N-terminal portion that is translocated into the nucleus and a C-terminal protein that constitutes the active secreted IL-16 (34, 35). IL-16 exhibits anti-inflammatory activities. IL-16 has been reported to bind with high affinity to the D4 domain of CD4 to inhibit CD4 interactions with MHCII (36). IL-16 has also been shown to inhibit mixed lymphocyte reactions by a mechanism that is reversed by anti-IL-16 mAb or soluble CD4 (37). IL-16 also inhibits T cell proliferation stimulated by anti-CD3 mAb (38) and is an anti-inflammatory mediator in rheumatoid synovitis (15). That is, in NOD-SCID mice engrafted with a human inflamed rheumatoid synovium, the adoptive transfer of a human $CD8^+$ T cell blocked production of the proinflammatory mediators TNF-α, IFN-γ, and IL-1. The inhibitory activity of $CD8^+$ T cells was blocked by an anti-IL-16 mAb and was mimicked by the administration of IL-16.

NAgIL16 was more effective than IL2NAg for the inhibition of EAE (Table II), and the NAgIL16 or IL2NAg fusion proteins were more effective than the other tested cytokine-NAg fusion proteins (Table II). Based on this study and a previous study (6), the rank order of tolerogenic activity in the Lewis rat model of EAE was NAgIL16 $>$ IL2NAg $>$ IL1RA-NAg, IL13NAg $\geq$ IL4NAg, IL10NAg, GPMBP, GP69–88, and saline. The baculovirus expression systems for the cytokine-NAg fusion proteins IL1RA-NAg, IL2NAg, IL4NAg, IL10NAg, and IL13NAg were described elsewhere (5, 6). These fusion proteins expressed the predicted cytokine-specific activities, and the NAg domain appeared fully accessible to Ag processing and presentation on MHCII glycoproteins (Fig. 1) (5, 6).

In both prechallenge and postchallenge treatment regimens, covalent linkage of NAg and IL-16 was required for inhibition of EAE (Tables III-IV). The NAg or IL-16 domains, delivered independently or delivered together as separate molecules, did not affect EAE. The requirement for the covalent NAg-IL16 linkage implied a mechanism of Ag targeting and was consistent with the observation that the IL16 domain of NAgIL16 facilitated the presentation of the tethered NAg by Con A-activated splenic T cells (Fig. 5). The requirement for covalent linkage also indicated that the induction of tolerance by NAgIL16 was Ag-specific rather than a cytokine-mediated, Ag-nonspecific effect because the administration of cytokine alone had no inhibitory effect. The cytokine may target APC subsets that are naturally tolerogenic or the cytokine domain may elicit expression of anti-inflammatory or tolerogenic activity by those APC. Like many cytokines that exhibit pleiotropic activity, IL-16 also appears to possess proinflammatory activities that may facilitate EAE (39–41). Hence, another possibility one might consider is that fusion proteins such as NAgIL16 and IL2NAg may target NAg for MHCII-restricted presentation among activated encephalitogenic $MHCII^+$ T cells to cause the NAg-specific fratricidal elimination of T cells responsible for EAE.

The IL-16 fusion proteins used in this study were highly soluble proteins that were secreted into the supernatants of infected insect cells. These proteins were readily purified on Ni-NTA resins and remained soluble during all phases of the purification. The fusion proteins were highly soluble in saline and had no tendency toward precipitation. Indeed, when injected systemically by either i.v. or i.p. routes during the onset of clinical EAE, NAgIL16 strongly inhibited disease progression (Tables IV to VI and Figs. 3 and 4). Subcutaneous administration of NAgIL16 did not cause any signs of inflammation at the injection site. When injected i.v. in NAgIL16-sensitized rats (Tables IV to VI), NAgIL16 did not elicit an anaphylactic or other notable adverse reaction. Our experience was that the NAgIL16 fusion proteins were highly soluble, readily purified, stable, and safe when delivered by local or systemic routes.

In conclusion, this study validates the concept of a tolerogenic vaccine and identifies IL-16 as an optimal cytokine partner for the generation of tolerogenic vaccines. NAgIL16 was highly effective as a tolerogenic vaccine that prevented EAE (Tables II and III and Fig. 2) and an effective inhibitor of EAE when administered after an encephalitogenic challenge during onset of clinical signs (Tables IV–V & Figs. 3–4), and it completely inhibited EAE in a majority of rats when administered both before and after challenge (Table VI). Thus, tolerogenic vaccines may acutely inhibit inflammatory attacks and may be beneficial when administered during remission or relapse for the prevention of subsequent autoimmune attacks. Overall, tolerogenic vaccines may become useful as highly

effective, Ag-specific tolerogens for the treatment of autoimmune disease.

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Disclosures

The authors have no financial conflict of interest.

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