Induction of a non-encephalitogenic type 2 T helper-cell autoimmune response in multiple sclerosis after administration of an altered peptide ligand in a placebo-controlled, randomized phase II trial

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In this 'double-blind', randomized, placebo-controlled phase II trial, we compared an altered peptide ligand of myelin basic protein with placebo, evaluating their safety and influence on magnetic resonance imaging in relapsing–remitting multiple sclerosis. A safety board suspended the trial because of hypersensitivity reactions in 9% of the patients. There were no increases in either clinical relapses or in new enhancing lesions in any patient, even those with hypersensitivity reactions. Secondary analysis of those patients completing the study showed that the volume and number of enhancing lesions were reduced at a dose of 5 mg. There was also a regulatory type 2 T helper-cell response to altered peptide ligand that cross-reacted with the native peptide.

Multiple sclerosis (MS) is thought to be a chronic demyelinating disease characterized by a coordinated inflammatory attack on the myelin sheath in the central nervous system, with ensuing damage to the underlying axon[1–3]. The cause of MS is unknown[4–6]. However, at the site of demyelination there is evidence that peptides encompassing the immunodominant epitope of myelin basic protein, amino acids 83–99 (called $MBP_{(83–99)}$ here), are bound to the DR2 molecule expressed on inflammatory cells[7]. Moreover, at the site of MS brain plaques in patients who have human leukocyte antigen DR2, there are T-cell receptor (TCR) rearrangements characteristic of T-cell clones reactive to $MBP_{(83–99)}$ bound to human leukocyte antigen DR2 (refs. 8–10). The T-cell response is characterized by a type 1 T helper-cell (T_H1) phenotype[11]. The ability of these cells to recognize peptide antigen has been extensively studied using altered peptide ligands (APLs), in which the native peptide is modified by amino-acid substitutions at essential contact residues for the TCR (refs, 12–14). An APL derived from $MBP_{(83–99)}$ was used to treat experimental autoimmune encephalomyelitis (EAE) in rodents. A single amino-acid substitution of Ala for Lys at position 91 in the core sequence of $MBP_{(83–99)}$ changes the cellular response to MBP considerably[15,16]. Thus, APL conferred protection from the development of EAE, if given at the time of autoimmunization with the native peptide, and even reversed established paralytic disease, possibly through the decreased production of tumor necrosis factor α and interferon (IFN)-γ, two pro-inflammatory cytokines involved in the pathogenesis of EAE (ref. 15). Further modifications of the $MBP_{(83–99)}$ peptide with a substitution of Ala for Lys at position 91 led to the APL called NBI 5788, consisting of D-Ala83-Lys84-Leu89-Ala91-MBP(83–99).

Phase I studies demonstrated that NBI 5788 was generally well tolerated, with transient injection site reactions reported as the

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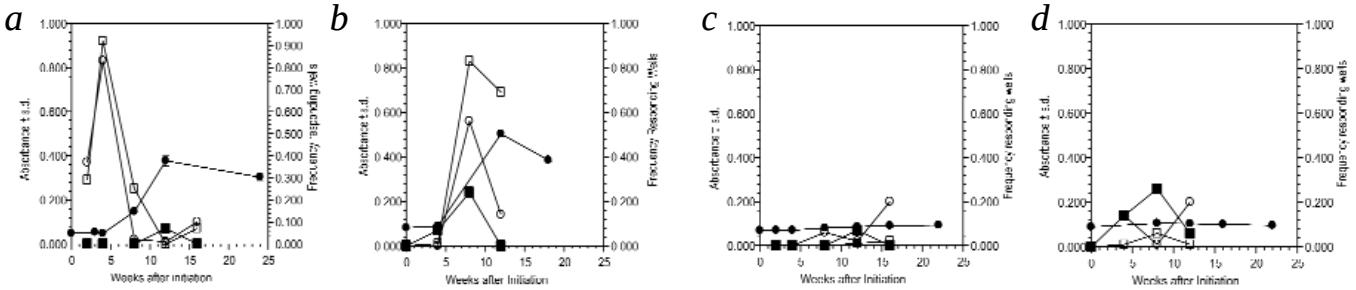

Fig. 1 Effects of weekly administration of NBI 5788 or placebo on peripheral blood cell cytokine response and production of antibodies against NBI 5788. Patients received 50 mg NBI 5788 (*a* and *b*) or placebo (*c* and *d*) weekly. Serum was collected and diluted 1:25 with assay buffer, and the level of antibody against NBI 5788 was determined by ELISA. Data (l) represent mean ± standard deviation. For cytokine production, peripheral blood cells were isolated and cultured in the presence of 10 μM $MBP_{(83–99)}$ or medium[19], and the presence of IFN-γ (■), IL-5 (○) or IL-13 (□) was assessed in supernatants by ELISA. Data represent the frequency of cytokine-producing wells in the NBI 5788 cultures minus the medium control.

most common adverse event (75% for NBI 5788; 40% for placebo). Magnetic resonance imaging (MRI) analysis showed that three of five patients treated with placebo and six of twenty patients treated with APL had an increased total number of gadolinium-enhancing lesions, compared with baseline values[17]. Immunological analysis demonstrated that cytokine secretion by APL-reactive T-cell lines from patients with MS treated with NBI 5788 was more like a type 2 T helper-cell (T_H2) response than that of T-cell lines from untreated patients with MS (ref. 18). Thus, APL seemed to induce an immune response to itself with cross-reactivity to native peptide, and this immune response had a T_H2-like phenotype. Here we report the results of a subsequent larger, multi-center, placebo-controlled, 'double-blind' trial (in which neither the patients nor the investigators knew the treatment being given).

Demographics and trial design

The trial was initiated in fourteen centers in seven countries. The study (planned to include 144 patients) was designed such that patients (36 per group) received either placebo or 5, 20 or 50 mg APL weekly, by subcutaneous injection in physiologic buffer, for 4 months. The demographic composition of the four treatment groups was balanced among the groups (http://www.neurocrine.com/). We monitored MRI activity monthly and compared this to two baseline scans. We then determined the number of new gadolinium-enhancing lesions, as well as the volume of the enhancing lesions.

Incidence of clinical relapses in patients receiving APL

Analysis of the entire group of patients (n = 142) who entered the trial showed that there was no subtsantial difference in the frequency (19–26%) or the number (n = 7–9) of relapses in any of the groups receiving APL, compared with those receiving placebo (Table I). Most patients had no relapses at all. There was no clustering of relapses in any of the treatment groups at any particular time after the injections began (http://www.neurocrine.com/). Thus, over the 4-month course of this phase II study, there was no indication that APL changed the frequency of either clinical relapse or score on the expanded disability status scale (EDSS).

Incidence and characterization of the hypersensitivity reactions

The data and safety monitoring board (which was established to oversee the NBI 5788 clinical studies) recommended, approximately midway through the phase II study, that the administration of APL be stopped. This was because of the appearance of a systemic hypersensitivity-type reaction in 13 of 142 patients: 10 during the 'double-blind' phase and 3 during the 'open-label' extension of the study (during which the identity of the drug was known to the patients and investigators. The symptoms, which were self-limiting and usually resolved in 2 hours, included itching (n = 11), paresthesias in the extremities (n = 4), macules on the trunk (n = 1), exanthematous rash (n = 5), dyspnea (n = 2), nausea (n = 2), abdominal pain (n = 1), eosinophilia within a week (n = 2) and hives (n = 4). In addition, one hypotensive episode and one syncopal episode were reported.

There did seem to be a correlation between the systemic hypersensitivity and number of injections, with most of the reactions occurring after more than 10 injections of the peptide (Table 2). One adverse event occurred after only two injections, which was in contrast to previous experiences with the peptide in phase I trials[17]. No one discontinued the drug at a dose of 5 mg because of an adverse reaction in the 'double-blind' phase. However, during the 'open-label' extension, two patients receiving the 5-mg dose experienced hypersensitivity reactions, which lead to discontinuation of drug treatment. The remainder of the immediate hypersensitivity reactions occurred with the higher doses. At the time the trial was discontinued, 53 patients had fully completed the 4-month 'double-blind' phase of the trial, whereas another 79 patients were continuing to receive medication, or were in the 1-month baseline period. Only 10 patients terminated the study because of adverse events.

Measurement of serum antibodies against NBI 5788 showed increased immunoglobulin (Ig) G antibodies (mainly IgG_1) in the patients experiencing the hypersensitivity-like reactions (5

Table 1 Patients with MS relapses during the 'double-blind' study

Variable	Group	5 mg	20 mg	50 mg	Placebo	Total
	Size	36	36	35	35	142
Number of Relapses	0	29 (81%)	29 (81%)	27 (77%)	26 (74%)	
	1	6 (17%)	7(19%)	7(20%)	7 (20%)	
	2	1 (3%)	0	0	2 (6%)	
	3	0	0	1 (3%)	0	
Total relapses[a]		7 (19%)	7 (19%)	8 (23%)	9 (26%)	

[a]Total number of patients with MS relapses.

mg (n = 2), Optical density (O.D.) 0.5775; 20 mg (n = 5), O.D. 0.888 ± 0.365; 50 mg (n = 4), O.D. 0.819 ± 0.283) compared with baseline values (n = 11; O.D. 0.097 ± 0.024) . The antibodies against peptide generated by treatment with APL also reacted to the native $MBP_{(83–99)}$ peptide by enzyme-linked immunosorbent assay (ELISA), but not to the same magnitude. However, there was no substantial reactivity to whole myelin basic protein (MBP) in these samples compared with the reactivity of pretreatment specimens.

To determine whether patients with hypersensitivity reactions showed worsening by MRI, we analyzed the number of new enhancing lesions on the scan at 4 months. In general, there was no worsening by MRI with hypersensitivity. Three patients' MRI scans improved after hypersensitivity reactions, two patients never had lesions before or after the hypersensitivity reaction and three patients' MRI scans worsened after a hypersensitivity reaction.

Table 2 Summary of immediate-type hypersensitivity reactions

Patient identification number	Weekly dose (mg)	Number of injections before hypersensitivity reaction[a]	Antibodies against drug[b] (Week collected)
119	50	15	0.969 ± 0.012 (18)
503	50	15	n.d.
219	50	13	0.782 ± 0.003 (16)
129	50	10	1.086 ± 0.004 (18)
115	50	2	0.438 ± 0.013 (4)
603	20	25	n.d.
121	20	16	0.923 ± 0.035 (17)
204	20	14	1.447 ± 0.012 (17)
217	20	13	0.525 ± 0.001 (16)
518	20	13	0.948 ± 0.011 (16)
131	20	7	0.598 ± 0.008 (31)
125	5	27	0.681 ± 0.008 (27)
117	5	7[c]	0.474 ± 0.013 (24)

[a]Week hypersensitivity reaction first noted. [b]Total IgG antibodies against NBI 5788, as determined using a 1:25 dilution of the serum in an ELISA. Data represent the mean absorbance ± s.e.m. [c]Patient completed 16 weeks of placebo treatment, and enrolled in 'open-label' extension, receiving 5 mg weekly. n.d., not done.

Analysis of the immune response

We evaluated the ability of NBI 5788 to elicit cellular (T-cell-derived cytokines) or humoral (B cell-derived antibody) responses in a cohort of patients (all participants from one center; n = 7) who received either 50 mg (patients 210 and 215) or placebo (patients 212 and 213). At varying times after administration of APL or placebo, we obtained blood samples and isolated serum and peripheral blood mononuclear cells. We then cultured the cells in 96-well plates, in the presence of interleukin (IL)-7 (to expand memory cells), with either medium alone, native $MBP_{(83–99)}$ or NBI 5788 (ref. 19). After 6 days, we removed the supernatants and assessed the frequency of antigen-specific cytokine responses (IFN-γ, or T_H1-like; IL-5 and IL-13, orT_H2-like) of each patient, by ELISA (Figs. 1 and 2).

Patients with MS do not show much responsiveness to APL before treatment[18]. However, after administration of 50 mg NBI 5788 weekly, there was a robust T_H2-like immune response in both patients (Fig. 1*a* and *b*), whereas there were no substantial cytokine responses in the placebo group (Fig. 1*c* and *d*). Both of the patients receiving 50 mg generated substantial frequencies of cells producing IL-5 and IL-13, which peaked around 4–8 weeks from baseline and decreased thereafter. This decrease in cytokine production occurred despite the continuation of dosing in these individuals through week 16. There was not much production of IFN-γ in response to APL at any time during treatment with NBI 5788.

Because T_H2 cell responses are associated with the promotion of humoral responses, the strong induction of T_H2 cytokines by APL indicated that antibodies against APL might be present, as seen in patients with hypersensitivity reactions (Table 1). Indeed, serum levels of antibody against APL (measured by ELISA) in the patients receiving 50 mg weekly were demonstrably higher than those of control patients given placebo. These antibodies were mainly IgG_1 with no increase in IgE levels (data not shown). The kinetics of antibody production were delayed compared with those of the cellular response, with peak antibody production occurring around week 12. This increase in IgG1 antibodies did coincide with the onset of the hypersensitivity reactions in some of the patients. However, the level of antibody produced in this cohort of patients did not approach that in the 13 who experienced hypersensitivity symptoms (Table 2).

An essential feature of APL therapy was to induce an antigen-

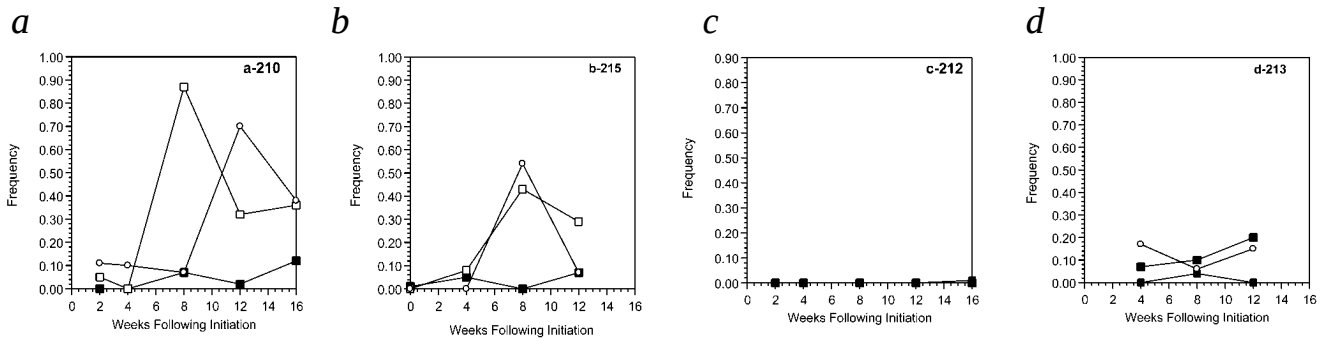

Fig. 2 Effects of weekly administration of NBI 5788 or placebo on peripheral blood cell cytokine response to MBP(83–99). Peripheral blood cells were isolated from patients receiving either 50 mg NBI 5788 (*a* and *b*) or placebo (*c* and *d*) weekly, and were cultured in the presence of 10 μM $MBP_{(83–99)}$ or medium[19], and the presence of IFN-γ (■), IL-5 (○) or IL-13 (□) was assessed in supernatants by ELISA. Data represent the frequency of cytokine-producing wells in the MBP(83–99) cultures minus the medium control.

Table 3 Change in number of new enhancing lesions at 4 months in patients receiving 16 injections

Group	Decrease	No change	Increase	Total
5 mg	8 (57%)*	5 (36%)	1 (7%)	14
20 mg	2 (18%)	3 (36%)	5 (45%)	11
50 mg	1 (11%)	7 (77%)	1 (11%)	9
Placebo	4 (25%)	8 (50%)	4 (25%)	16

Data represent number of new enhancing lesions (in parenthesis, percent).

specific T_H2 protective response that was cross-reactive with the native antigen. Therefore, we determined the ability of NBI 5788 therapy to induce cytokine responses to the native $MBP_{(83–99)}$ autoantigen. As with APL responsiveness, patients treated with 50 mg APL mounted a strong T_H2 cytokine response to the native antigen, with a negligible IFN-γ response (Fig. 2*a* and *b*). $MBP_{(83–99)}$ responses were delayed relative to those of NBI 5788, in which $MBP_{(83–99)}$ responses peaked from 8 to 12 weeks (Fig. 2*a* and *b*), whereas APL responses peaked from 5 to 7 weeks (Fig. 1*a* and *b*). We obtained similar results with the one patient in the group receiving 20 mg (data not shown). However, in two individuals receiving 5 mg, there was no increase in the cellular or humoral response to NBI 5788 or the native MBP peptide (data not shown).

Analysis of MRI data

The primary efficacy criterion in this trial was the number of new enhancing lesions appearing on a MRI scan. New enhancing lesions were defined as lesions not present on a previous MRI scan. The immunological data indicated that a T_H2 shift in the response to MBP began between 8 and 12 weeks (Figs. 1 and 2). Thus, we analyzed MRI scans at sequential time points, but concentrated on the results at the scan following the 4-month 'double-blind' phase.

Fifty-three patients received all sixteen injections required for the 'double-blind' phase of the study. At the time the study was terminated, 79 patients were continuing in the study, and only 10 patients had terminated the study because of adverse events. Among the patients who received all 16 injections and had the fourth-month MRI evaluation, the group receiving 5 mg NBI 5788 had a higher frequency of patients with a decrease in new enhancing lesions at 4 months than did the groups receiving the higher doses of NBI 5788 or the group receiving placebo (Table 3).

There were no substantial changes in the number of new enhancing lesions from baseline to 4 months at doses of 20 or 50 mg, although with 50 mg there was only one new enhancing lesion at baseline or at the end of the study (data not shown). At a dose of 5 mg, there was a change in the total number of enhancing lesions and in the volume of enhancing lesions in a comparison of baseline volumes and those at 4 months (Table 4). The mean change in the volume of enhancing lesions from baseline two to the volume after 4 months of treatment was a decrease of 207 ± 471 ml in the group receiving 5 mg, compared with an increase of 291 ± 698 ml in the placebo group ($P < 0.0289$ for two treatments using baseline one, or $P < 0.0304$ using baseline two; Mann Whitney). There were no substantial changes in the volume of enhancing lesions or the total number of enhancing lesions at doses of 20 and 50 mg. Using the Bonferonni correction for multiple comparisons in our exploratory secondary analysis of the 5-mg dose, the corrected *P* value for the change in volume of enhancing lesions at 5 mg compared with placebo from baseline one to 4 months was $P < 0.086$, and for baseline two to 4 months, it was $P < 0.09$.

We next determined whether patients who had contrast-enhancing lesions at baseline responded differently to APL than to placebo. Including those scans in the group receiving 5 mg with enhancing lesions at baseline one or two (called 'active'), eleven of twelve had 'active' scans at baseline one or two and a reduction in the volume of contrast enhancing lesions at 4 months, and one had an increase. This is in contrast to the placebo group with 'active' scans at baseline one or two, in which four of eight had an increase in the volume of enhancing lesions with placebo and four of eight had a reduction in this volume of enhancing lesions with placebo ($P < 0.10$; Fisher's exact test). At a dose of 20 mg, three patients with 'active' scans at baseline one or two had an increase in the volume of enhancing lesions at 4 months, whereas two patients with 'active' baseline scans had a reduction in the volume of enhancing lesions. At a dose of 50 mg, four patients had 'active' scans at baselines one or two, and one had an increase in the volume of enhancing lesions from baseline to 4 months, whereas three showed a decrease in the volume of enhancing lesions. In conclusion, patients who had 'active' scans at baseline did not show worsening at any dose of APL, and the 5-mg dose showed a trend toward reduction in the volume of enhancement.

Discussion

Most organ-specific diseases that are considered autoimmune, such as MS, rheumatoid arthritis and juvenile diabetes, are thought to be 'driven' by T_H1 immune responses, for which the cytokines IFN-γ and tumor necrosis factor α are essential. In contrast, most allergic conditions are 'driven' by T_H2 responses, for which the cytokines IL-4, IL-5 and IL-13 dominate the immune response. A T_H1 inflammatory response toward various myelin antigens[20–23] is characteristic of MS. Systemic injection of IFN-γ led to worsening of MS in a clinical trial that was halted because of an increased number of exacerbations[24]. Evidence for T_H1 responses to MBP can be detected among both cerebrospinal fluid and peripheral blood lymphocytes[25,26]. Immunohistochemical evidence of T_H1 cytokines has been demonstrated in MS lesions[27–30].

Given that MS may be a T_H1-mediated autoimmune disease,

Table 4 Analysis of the group receiving 5 mg, for patients receiving all 16 injections

Variable[a]	Group	Baseline	4-Month	Change (%)
New enhancing lesions				
	5 mg	1.6	0.9	–0.7 (–44%)
	Placebo	1.5	0.9	–0.6 (–40%)
Total enhancing lesions				
	5 mg	2.5	0.9	–1.6 (–64%)
	Placebo	3.0	2.3	–0.7 (–23%)
Volume of enhancing lesions				
	5 mg	416	209	–207 (–50%)
	Placebo	256	547	+291 (+114%)*

[a]Mean value of new enhancing lesions, total enhancing lesions and total volume of enhancing lesions per patient. *, $P < 0.0289$ (Mann-Whitney for two treatments), differences were not significant at doses of 20 and 50 mg, compared with placebo; correcting for multiple comparisons, $P < 0.087$.

and that APLs were effective in treating EAE by inducing a shift away from T_H1 cytokines toward a T_H2 response[13,15,16,31–34], we designed an APL for $MBP_{(83–99)}$. $MBP_{(83–99)}$ is a target of the T- and B-cell response in MS, and $MBP_{(83–99)}$ has been identified in MS lesions associated with DR2 on astrocytes and microglia[7]. The results of our phase II clinical trial indicate that APL was able to induce a T_H2 type response to MBP, and as a result, some patients (9%) developed immediate-type hypersensitivity reactions probably mediated by this T_H2 immune response.

APL induced a T_H2 response in T cells reactive to MBP. APLs have been designed based on $MBP_{(83–99)}$, and antagonize the TCR to $MBP_{(83–99)}$ in patients with MS (ref. 34), and 'drive' the immune response toward T_H2 (ref. 18). Here the kinetics of induction of the T_H2 response to APL and to native MBP are relevant: Whereas the T_H2 response to APL arose within a week and waned by a month, the T_H2 response to MBP had a latency of 6–10 weeks (Figs. 1 and 2). This may help explain why the trend toward a reduction in new enhancing lesions was not seen until the MRI scan at 4 months. This is similar to what has been seen after treatment with the synthetic copolymer glatiramer acetate, in which a decrease in gadolinium-enhancing lesions became prominent after 6 months of treatment[35] Glatiramer acetate acts as an APL in blocking the immune response to $MBP_{(82-99)}$ (ref. 36). In fact, it induces a nonspecific T_H2 response in patients, through the induction of cross-reactive cells[37]. This may contribute to the efficacy seen with the drug.

The immunological studies presented here indicate that NBI 5788 induced a T_H2 response with IL-5 and IL-13 cytokines 'driven' initially by APL itself, and then spreading with a latency of another 4–8 weeks to the self antigen MBP. Immediate-type hypersensitivity reactions have also been reported for glatiramer acetate (http://www.copaxone.com/professional/home.html). Approximately 10% of the patients in clinical trials who received glatiramer acetate experienced a reaction immediately after injection at least once; these reactions included a constellation of symptoms such as flushing, chest pains, palpitations, anxiety, shortness of breath, constriction of the throat and urticaria. There are possible similarities between APL, NBI 5788 and glatiramer acetate in both mechanism of action and side effects related to a T_H2 response.

Immunization against peptides from the TCR induces a T_H2 response toward the TCR in some patients with MS (ref. 38). A 'double-blind', placebo-controlled clinical trial with TCR peptides in 23 DR2-positive patients with MS showed that this treatment was non-toxic. Furthermore, the peptide stabilized clinical progression in 6 of 23 patients with MS who responded to vaccination, whereas the non-responders underwent continuous clinical decline. Vaccination with TCR peptides seems to induce TCR peptide-specific T cells that preferentially produce IL-4 and IL-10 but not IFN-γ. TCR peptide-specific T cells induced by vaccination suppressed *in vitro* proliferation of MBP-reactive T cells and production of IFN-γ (ref. 38, 39). Vasculitic lesions were seen in that trial, after induction of a T_H2 response. Direct induction of T_H2 cells responding to myelin proteins was not demonstrated there, however[38].

APL provides a new class of therapeutic reagents for treatment of T_H1-mediated autoimmune diseases. Regulation of the strength of the T_H2 response will be an essential consideration in future studies, as optimization of the suppression of autoimmunity should be balanced against untoward hypersensitivity reactions 'driven' by T_H2 cytokines. Our study indicates that it is possible to induce T_H2 cells directed toward self antigens, but this T_H2 response may in some cases lead to immediate hypersensitivity reactions. The T_H2 response to myelin antigens may have beneficial effects, however, in reducing the development of new lesions within the central nervous system.

Methods

Overall study design. This was a multi-center, multinational, randomized, 'double-blind', parallel-group, placebo-controlled study in patients with relapsing-remitting MS, with 142 patients randomly assigned to the 'double-blind' phase. The study consisted of three phases.

First, the baseline phase consisted of 1 month immediately proceeding the 'double-blind' phase. Two baseline MRI scans (1 month apart) at the beginning and at the end of the 1-month period were obtained during this phase, according to a standardized MRI protocol.

For the subsequent 'double-blind' phase, patients were randomly assigned in equal numbers at visit 1 ('week 0') to receive weekly subcutaneous injections, starting on the same day, of 5, 20 or 50 mg NBI 5788 or matching placebo for 4 months (weeks 0–16), once weekly (7 ± 2 d). NBI 5788 and placebo were in single-use vials for injection after reconstitution with diluent (sterile water for injection); these were supplied as sterile, white lyophilized (freeze-dried) powder containing 0, 5, 20 or 50 mg of NBI 5788 with D-mannitol present as an inactive ingredient to adjust the isotonicity. Serial MRI scans were obtained according to a standardized MRI protocol during this phase. A trained physician not otherwise involved in the care of the patients documented EDSS scores and relapses, and the safety of the NBI 5788 treatment was monitored.

Finally, a long-term extension phase was provided to allow for the treatment of patients who completed all visits and treatment of the 'double-blind' phase and who could benefit from treatment with NBI 5788. During this phase, patients were treated with 'open-label' supplies of 5 mg NBI 5788 weekly (that is, both patients and investigators knew the treatment) and the safety and efficacy of the drug were monitored using clinical and 'paraclinical' (MRI) assessments. All MRI scans obtained during the course of the study were evaluated centrally at McConnell Brain Imaginng Centre, Montréal. An external data safety monitoring board reviewed clinical and MRI data during the study and provided an independent assessment of overall safety and tolerability.

MRI acquisition protocol. All scans were acquired either on a GE Signa or a Siemens Vision 1.5T MR imager, depending on the acquisition site. A total of six MRI scans were acquired in each scanning session, each with a slice thickness of 3 mm: sagittal 'scout'; Proton density (PD) /T_2-weighted axial spin echo (time to resonance Repetition time (TR) , 3250 ms; time to echo (TE), 30/90 ms); T_1-weighted axial spin-echo (TR, 350 ms; TE, 20 ms); fast fluid attenuation inversion recovery (TR, 7500 ms; TE, 120 ms; time to initiation, 2500 ms); Pre-contrast flow-saturated T_1-weighted axial spin echo (TR, 800 ms; TE, 25 ms); and post-contrast flow saturated T_1-weighted axial spin echo (same as pre-contrast, started exactly 10 min after administration of 0.1 mmol/kg gadolinium Diethylenetriamine pentaacetic acid (DTPA).

Definitions of MRI parameters. Enhancing lesions were enhancements seen in the post-contrast scan not visible in the pre-contrast scan acquired in the same session. New enhancing lesions were enhancing lesions seen in a particular scan not already present in the previous scan of the same patient. The primary efficacy parameter was the number of new enhancing lesions. Secondary efficacy parameters were the total number of enhancing lesions, the volume of enhancing lesions and the volume of all lesions visible on non-contrast scans.

MRI analysis protocol. For each patient scan, the efficacy parameters were quantified at the McConnell Brain Imaging Center of the Montreal Neurological Institute. The enhancing lesions were identified and counted using Display, a computerized visualization tool developed at the Brain Imaging Center (http://www.bic.mni.mcgill.ca/software). This program offers simultaneous display of three orthogonal views (transverse, sagittal and coronal), and thus allows the operator to verify the continuity of labeled regions in three dimensions. Before analysis, all three-dimensional MRI scans were explicitly registered to each other and to a stereotaxic coordinate sys-

tem[40], thus minimizing misalignment due to between-scan patient motion. As a result, the operator had the ability to switch between the pre- and post-gadolinium scan, as well as between scans of the current session and those of the previous one, while remaining at the same anatomical location.

The enhancing lesions seen in each individual pre-/post-gadolinium scan pair were first labeled ('painted') and counted by trained operators. Subsequently, the obtained results were explicitly verified by a radiologist. During verification, the radiologist established the number of new enhancing lesions by comparing the current scans to those obtained in the patient's previous session. Furthermore, the enhancing lesion counts (total and new) were verified fully automatically using computer analysis of the lesions 'painted' by the operators. Any discrepancies between computer-obtained counts and those obtained by the radiologist were explicitly checked and corrected if necessary. The volumes of lesions visible in the non-contrast scans (acquisition sequences two to four) were quantified fully automatically using the Intensity normalized stereotactic environment for classification of tissue (INSECT) processing pipeline developed at the Brain Imaging Center[41].

Key inclusion criteria. To be included in the study, patients were required to have a clinically definite and/or laboratory-supported definite diagnosis of MS with a relapsing–remitting course, one or more documented relapses in the past 1 year but no evidence of relapse for at least 30 d before entry and during the baseline phase, and an EDSS score of 0–6.0. Patients had to be 18–55 years of age, and were required to give informed consent according to national legal requirements.

Female patients had to be either post-menopausal, surgically incapable of bearing children or practicing an acceptable method of birth control (such as hormonal contraceptives, intrauterine device, spermicide or barrier). Females of childbearing potential were required to have a negative pregnancy test immediately before entry. Male patients were required to use barrier contraception.

Patients could have no treatment with corticosteroids or ACTH in the previous 30 d, no treatment with other immunosuppressive medications such as cyclophosphamide, azathioprine, methotrexate, mitocxantrone, linomide, cyclosporine or deoxyspergualine, or with IFN β, copolymer 1 or myloral during the previous 6 months, and no treatment with MBP APL, lymphoid irradiation or cladribine.

Concomitant medications/therapies that are part of standard care for patients with MS were allowed. Use of immunosuppressives, β-interferons and other therapies as specified in the exclusion criteria were prohibited during the baseline, 'double-blind' and extension phases.

'Blinding' and randomization. The investigator site and MRI reading center personnel, as well as personnel involved in the monitoring or conducting of the study were 'blinded' to the study drug codes. To avoid potential bias due to possible 'unblinding' adverse events, neurological assessments were made by a trained neurologist 'blinded' to treatment identity and not involved in the treatment of the patient.

Definition of relapse. A relapse was defined as the appearance or reappearance of one or more neurologic abnormalities persisting for at least 48 h and immediately preceded by a relatively stable neurologic state of at least 30 d or improving neurologic state of at least 14 d. A relapse was confirmed by the investigator only when the patient's symptoms were accompanied by objective changes on the neurologic examination consistent with an increase of at least a half a step on the EDSS, two points on one of the seven functional systems or one point on two or more of the functional systems. Events associated with fever were excluded. A change in bowel/bladder or cognitive function was not solely responsible for changes in either the EDSS or the functional system scores. MS relapses were confirmed by the investigator, with an EDSS score obtained at the time the relapse was diagnosed.

Detection of IgG and IgE antibodies against NBI 5788 in serum samples from patients with MS. ELISA was used to detect IgG and IgE antibodies against NBI 5788. A 96-well Nunc-Immuno Plate–MaxiSorp Surface (Fisher Scientific, Pittsburgh, Pennsylvania) was coated overnight at 4 °C with 100 μl/well NBI 5788 at a concentration of 1 μg/ml in 0.1 M sodium bicarbonate buffer, pH 9.5. The plate was then washed three times with 0.05% Tween-20 in PBS (PBST; Sigma), and the remaining reactive sites on the plate were blocked for 1 h at room temperature with 200 μl/well of 1% BSA in PBS. After three washes with PBST, 100 μl/well of serum sample diluted 1:25 in 1% BSA in PBS was added in duplicates and the plate incubated at room temperature for 1 h. After being washed times with PBST, the plate was then incubated for 1 h at room temperature with 100 μl/well horseradish peroxidase-conjugated goat antibody against human IgG or goat antibody against human IgE (Sigma) diluted 1:5,000 in 1% BSA in PBS. After five washes with PBST, 100 μl/well of 3,3′,5,5′-tetramethyl-benzidine substrate solution (Sigma) was added, followed by incubation for 2 min in the dark at room temperature to develop the color. The reaction was stopped by the addition of 100 μl/well 0.5 M H_2SO_4, and the absorbance was measured at 450 and 655 nm.

IL-7 memory cell assay and cytokine detection. Human MS patient peripheral blood mononuclear cells (10^5 cells/well) from each time point were isolated and plated in a 96-well tissue-culture plate in the presence of 10 ng/ml IL-7 and with medium alone (12 wells) or 10 μg/ml $MBP_{(83–99)}$ peptide (42 wells) or NBI 5788 APL (42 wells), according to a published method[19]. After 6 d, an aliquot of medium from each well was collected for cytokine analysis by ELISA, and proliferation of the remaining cells was assessed by ^{3}H-thymidine incorporation. Conditioned medium from each well was assessed in duplicate for each cytokine (IFN-γ, IL-5 and IL-13). The mean of cytokine or proliferation values from the 12 wells with medium alone were used as the denominator in the calculation of the stimulation index (SI; individual well value/mean 'medium-alone' value) for each well of the plate, including each of the 12 wells containing medium alone. Positive wells contained an SI of at least 2. Where 'medium-alone' values were consistently 0 (such as with measurements of IFN-γ), the lower limit of detection in the ELISA was used as the criterion for positive wells, provided each value of the duplicate was positive. Frequencies were calculated by dividing the number of positive wells in each group (medium alone, $MBP_{(83–99)}$ and NBI 5788) by either 12 for the 'medium-alone' group or 42 for the groups treated with $MBP_{(83–99)}$ and NBI 5788. Data represent the frequency of APL or MBP peptide minus the medium control.

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