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Induction of Antigen-Specific Tolerance in Multiple Sclerosis After Immunization With DNA Encoding Myelin Basic Protein in a Randomized, Placebo-Controlled Phase 1/2 Trial

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Objective: To assess safety and immune modulation by BHT-3009, a tolerizing DNA vaccine encoding full-length human myelin basic protein, in patients with multiple sclerosis (MS).

Design: The study was a randomized, double-blind, placebo-controlled trial. Subjects receiving placebo were crossed over into an active arm after treatment unblinding.

Setting: The trial was conducted at 4 academic institutions within North America.

Patients: Thirty patients with relapsing-remitting or secondary progressive MS who were not taking any other disease-modifying drugs were enrolled in the trial. Further, the patients were required to have either 1 to 5 gadolinium-enhancing lesions on screening brain magnetic resonance imaging (MRI), a relapse in the previous 2 years, or disease worsening in the previous 2 years.

Interventions: BHT-3009 was administered as intramuscular injections at weeks 1, 3, 5, and 9 after randomization into the trial, with or without 80 mg of daily oral atorvastatin calcium in combination. Three dose levels of BHT-3009 were tested (0.5 mg, 1.5 mg, and 3 mg).

Main Outcome Measures: The primary outcome measures were safety and tolerability of BHT-3009. Secondary outcome measures included the number and volume of gadolinium-enhanced lesions on MRI, relapses, and analysis of antigen-specific immune responses.

Results: BHT-3009 was safe and well tolerated, provided favorable trends on brain MRI, and produced beneficial antigen-specific immune changes. These immune changes consisted of a marked decrease in proliferation of interferon-γ–producing, myelin-reactive CD4+ T cells from peripheral blood and a reduction in titers of myelin-specific autoantibodies from cerebral spinal fluid as assessed by protein microarrays. We did not observe a substantial benefit of the atorvastatin combination compared with BHT-3009 alone.

Conclusion: In patients with MS, BHT-3009 is safe and induces antigen-specific immune tolerance with concordant reduction of inflammatory lesions on brain MRI.

Trial Registration: clinicaltrials.gov Identifier: NCT00103974.

Arch Neurol. 2007;64(10):(doi:10.1001/archneur.64.10.nct70002)

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MULTIPLE SCLEROSIS (MS) is a chronic demyelinating disease characterized by a coordinated immune attack on the myelin sheath in the central nervous system (CNS), leading to eventual damage of the underlying axon. There are elements of an antigen-specific adaptive immune response in MS involving T cells and antibodies, as well as many aspects of an innate immune response that includes the presence in brain lesions of mediators such as complement and microglia, which process myelin-derived autoantigens.[1] The cause of MS is unknown, but substantial evidence exists that autoreactive immune cells and antibodies that recognize specific myelin antigens, such as myelin basic protein (MBP), as well as a subset of inflammatory cytokines, such as interferon-γ or more recently IL-17 (interleukin 17), play a fundamental role in its pathogenesis.[2,3] There are no approved MS therapies aimed specifically toward down-regulating antigen-

specific autoreactive immune cells. Development of a therapy that could down-regulate or tolerize the pathogenic antigen-specific autoimmune cells in MS provides the propitious opportunity of maximizing efficacy while minimizing potential adverse effects.

One such approach involves the use of plasmid DNA vaccines encoding 1 or more myelin antigens. Initially, intramuscularly injected plasmid DNA was demonstrated to be a relatively efficient method of gene transfer without the potential adverse consequences associated with viral-mediated gene transfer.[4] It was then quickly discovered that plasmid DNA could also be used as a vaccine because immune responses were generated against the encoded protein product of the DNA plasmid.[5] Recent work by ourselves and others has demonstrated that DNA vaccines can be used instead to down-regulate or alter an ongoing immune response in various animal models of autoimmunity, providing the framework for the human studies we describe herein.[6-9]

We present herein the results of the first DNA vaccine, to our knowledge, used to treat autoimmune disease in humans. We have engineered a DNA vaccine called BHT-3009 encoding full-length human MBP and have treated 30 patients with relapsing-remitting (RRMS) or secondary progressive MS (SPMS) in a randomized, multicenter, double-blind, placebo-controlled, dose-escalation phase 1/2 trial. The results reported herein demonstrate that BHT-3009 is safe and well tolerated and produced favorable brain magnetic resonance imaging (MRI) and immune response changes.

METHODS

SUBJECTS

After providing informed consent, patients were enrolled at 4 clinical sites in North America. The protocol was reviewed and approved by both the Canadian and US regulatory agencies as well as ethics committees at each site. An independent data and safety monitoring board oversaw the study. This study was performed according to the Declaration of Helsinki guidelines.

Eligible patients were 18 years or older and had a definite diagnosis of MS by the McDonald criteria, RRMS or SPMS, and a screening disability score on the Kurtzke Expanded Disability Status Scale (EDSS) between 2.5 and 6.5, inclusive. Further, eligible patients had either (1) 1 to 5 gadolinium-positive lesions on screening MRI, (2) a clinical relapse within 2 years prior to screening, or (3) disease worsening in the previous 2 years. Patients were excluded if treated with high-dose corticosteroids in the month prior to screening, glatiramer acetate within 12 months prior to screening, or interferon-β within 1 month prior to screening.

STUDY DESIGN

A total of 30 patients with RRMS or SPMS were enrolled in 1 of 3 arms: placebo, BHT-3009 alone, or BHT-3009 plus atorvastatin calcium (Lipitor; Pfizer Inc, New York, New York). Patients were divided into 3 dose cohorts of BHT-3009: 0.5 mg, 1.5 mg, or 3 mg per dose. After a subject passed the screen, randomization was performed by a third-party organization (Pacific Data Designs, San Francisco, California). Both the sponsor and all personnel at the clinical sites were blinded to this randomization process.

BHT-3009 was administered intramuscularly at weeks 1, 3, 5, and 9 after randomization. Oral atorvastatin calcium was administered as an immunomodulatory agent at a dose of 80 mg once daily, beginning 2 days before the first BHT-3009 dose and continuing until the treatment blind was broken (at week 13). The BHT-3009 and atorvastatin combination arm was tested because of the theoretical benefit of adding a helper T cell subtype 2 promoting adjuvant. Preclinical studies have shown that atorvastatin by itself can treat animal models of MS by promoting helper T cell subtype 2 deviation of the autoimmune response.[10] Placebo consisted of either saline injections or placebo tablets, as appropriate. Magnetic resonance imaging and other safety evaluations were performed twice at baseline and at weeks 5, 9, 13, 26, 38, and 50. Patients initially randomized to placebo were rerandomized into one of the active treatment arms at week 13 (crossover patients). These patients were dosed and evaluated in an analogous way to those originally randomized to one of the active treatment arms, except in an open-label manner.

At each safety evaluation visit, assessment of the patient included history and physical examination (including injection-site examination after receiving dose), vital signs, adverse-event monitoring, hematologic testing, blood chemistries, and urinalysis. In addition, EDSS assessment, an electrocardiogram, and blood tests for measurement of antinuclear antibody titers and anti–double-stranded DNA levels were performed at screening and at week 13. The National Cancer Institute Common Terminology Criteria for Adverse Events were used for scoring the severity of adverse events. Patients who were experiencing a relapse were evaluated and received standard of care using the best judgment of the treating physician.

END POINTS

The primary outcome measure was safety as determined by the number of adverse events, neurologic assessments of relapses and disability, MRI measurements of gadolinium-enhancing and T2 lesions, and standard laboratory evaluation of hematological, renal, and liver function. A relapse was defined as the appearance or reappearance of 1 or more neurologic abnormalities persisting for at least 48 hours and immediately preceded by a period of relatively stable or improving disease for at least 30 days. Other outcome measures included the assessment of the impact on antigen-specific immune response as measured by T-cell activity assays on peripheral blood and autoantibody measurements on cerebral spinal fluid (CSF), when available.

MAGNETIC RESONANCE IMAGING

Magnetic resonance imaging of the brain with injection of gadolinium was performed according to a standardized protocol. A central MRI reading unit (NeuroRx Research, Montreal, Quebec, Canada) qualified each participating site's MRI departments before the study opened at the site. The MRI reading unit evaluated MRIs for quality and measured the study end points according to standardized protocols without knowledge of the patients' treatment assignments.

T-CELL ASSAYS

Fresh peripheral blood mononuclear cells (PBMCs) were isolated by standard Ficoll gradient techniques at baseline, week 9, and week 50 in a subset of patients in the trial. On the day of collection and immediately on purification of the PBMCs, they were incubated with a variety of myelin antigens and 5,6-carboxyfluorescein diacetate succinimidyl ester, a vital dye used to measure proliferation. Three positive controls were used: anti-

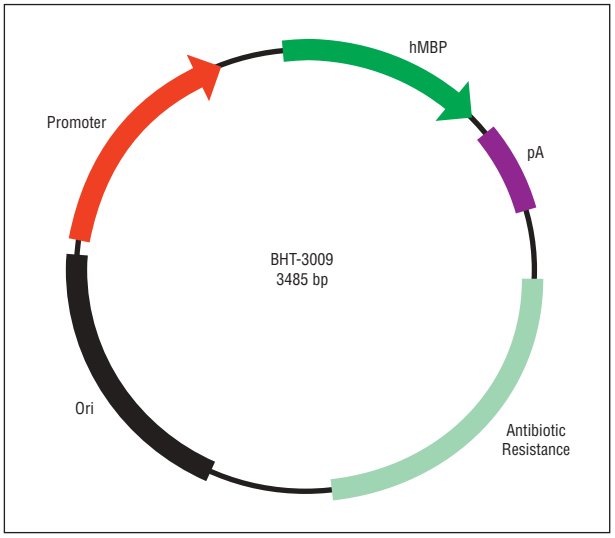

Figure 1. Structure of BHT-3009. The molecular structure of the 3485–base pair (bp) BHT-3009 plasmid is shown. The plasmid encodes for the 18.5-kD isoform of full-length human myelin basic protein (hMBP), which is driven by a eukaryotic promoter (promoter). Other standard elements on the plasmid include a poly A (pA) region for the termination of transcription and a bacterially active origin of replication (ori) along with an antibiotic resistance gene driven by a prokaryotic promoter for propagation and manufacture of the plasmid in *Escherichia coli*.

CD3, tetanus toxoid, and glatiramer acetate. The PBMCs were cultured for 10 days, then stimulated with phorbol 12-myristate 13-acetate and ionomycin in the presence of brefeldin A, after which the cells were stained with anti-CD3, anti-CD4, and an intracellular cytokine stain for IFN-γ or IL-4. Cells were acquired on a fluorescent-activated cell sorter and the data were analyzed by gating on lymphocytes and CD4+ cells.

PROTEIN MICROARRAY ASSAYS

Custom myelin peptide arrays, each containing 127 myelin and control peptides printed in duplicate, were produced by JPT Peptide Technologies GmbH (Berlin, Germany). As previously described, arrays were blocked with phosphate-buffered saline containing 3% fetal calf serum and 0.05% Tween 20, probed with 1:10 dilutions of CSF, and autoantibody binding detected using Cy-3–labeled goat-antihuman IgG/M antiserum samples (Jackson Immunoresearch, West Grove, Pennsylvania).[11] Arrays were scanned with the GenePix 4000B scanner (Molecular Devices Corporation, Sunnyvale, California), and GenePix Pro 5.0 software (Molecular Devices Corporation) was used to quantitate net median pixel intensities for each peptide. Two arrays were probed with each CSF sample, and the heatmap presents reactivities for all peptides for which there was more than a 2-fold and more than a 25–fluorescence unit difference between the pretreatment and posttreatment median values.

RESULTS

DNA VACCINE CONSTRUCTION

We isolated the full-length complementary DNA encoding the 18.5-kDa isoform of human MBP from a human brain complementary DNA library. This was cloned into a modified expression plasmid driven by a eukaryotic promoter. The expression plasmid was created as a derivative of the pVAX1 plasmid, where certain immunostimulatory CpG motifs were removed and immunoinhibitory GpG motifs were included. We have previously shown that oligonucleotides containing these GpG motifs either alone or combined with DNA vaccine plasmids were effective in treating animal models of several prototypic autoimmune diseases.[12,13] The plasmid thus constructed, BHT-3009 was then manufactured under good manufacturing practice conditions and formulated in a phosphate-buffered saline solution containing calcium (**Figure 1**).

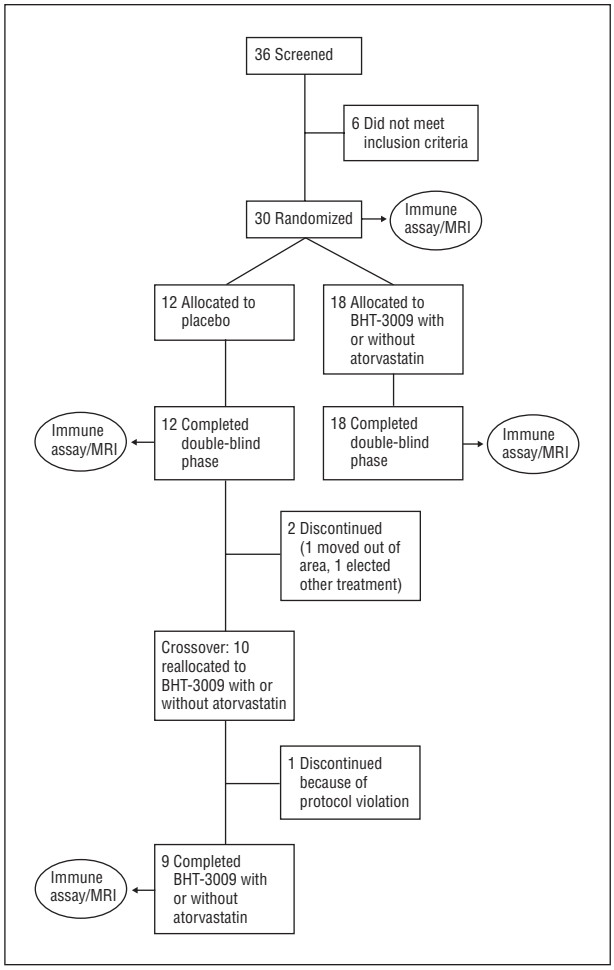

Figure 2. Flow of subjects through the protocol. Rectangles indicate the flow of subjects through the protocol. The ovals indicate the major points when samples were obtained for immune assays and magnetic resonance imaging (MRI) evaluations. The MRI evaluations were performed at additional points as detailed in the "Methods" section of the text. Atorvastatin was given as atorvastatin calcium.

DEMOGRAPHICS AND TRIAL DESIGN

The flow of subjects through the study is illustrated in **Figure 2**. The first subject was screened in June 2004, and the last was screened in December 2005. The first patient completed dosing (ie, week 13) in October 2004, and the last patient completed dosing in July 2006. The demographic composition of the 3 treatment arms was balanced among the groups. A total of 11 patients with RRMS, 13 patients with SPMS without relapses, and 6 patients with SPMS with relapses were enrolled in the

Table 1. Disposition of Patients

	0.5-mg Dose			1.5-mg Dose			3-mg Dose			
	Placebo	BHT-3009	BHT-3009 + atv	Placebo	BHT-3009	BHT-3009 + atv	Placebo	BHT-3009	BHT-3009 + atv	Total
No. of Patients at Initial Randomization										
RRMS	0	2	2	0	0	2	2	0	3	**11**
SPMS w/ relapse	0	1	0	1	2	1	0	1	0	**6**
SPMS w/o relapse	4	0	1	3	1	0	2	2	0	**13**
Total	**4**	**3**	**3**	**4**	**3**	**3**	**4**	**3**	**3**	**30**
No. of Crossover Patients Initially Randomized to Placebo										
RRMS	NA	0	0	NA	0	0	NA	1	1	**2**
SPMS w/ relapse	NA	0	0	NA	0	0	NA	0	0	**0**
SPMS w/o relapse	NA	2	2	NA	1	1	NA	1	1	**8**
Total	NA	**2**	**2**	NA	**1**	**1**	NA	**2**	**2**	**10**

Abbreviations: atv, atorvastatin calcium; NA, not applicable; RRMS, relapsing-remitting multiple sclerosis; SPMS, secondary progressive multiple sclerosis; w/, with; w/o, without.

Table 2. Treatment-Related AE

	No. (%)			
	BHT-3009	BHT-3009 + atv	Total BHT-3009[a]	Placebo
No. of patients	14	14	28	12
Patients with AE	8 (57.1)	6 (42.9)	14 (50.0)	8 (66.7)
Total AE	16	13	29	29
Patients who experienced indicated AE				
Injection site	2 (14.3)	3 (21.4)	5 (17.9)	4 (33.3)
Gastrointestinal				
Diarrhea	1 (7.1)	0	1 (3.6)	1 (8.3)
Gas/abdominal pain, discomfort	0	1 (7.1)	1 (3.6)	1 (8.3)
Upset stomach	1 (7.1)	0	1 (3.6)	0
Constipation	0	0	0	1 (8.3)
Sour taste	0	0	0	1 (8.3)
Sore throat	1 (7.1)	0	1 (3.6)	0
Constitutional				
Fatigue	1 (7.1)	1 (7.1)	2 (7.1)	2 (16.7)
Dizziness/imbalance	0	1 (7.1)	1 (3.6)	1 (8.3)
Sweats	1 (7.1)	0	1 (3.6)	0
Weakness	0	1 (7.1)	1 (3.6)	1 (8.3)
Insomnia	0	0	0	1 (8.3)
Other				
Gynecologic	1 (7.1)	0	1 (3.6)	1 (8.3)
Urinary urgency	0	0	0	1 (8.3)
Pain	0	2 (14.3)	2 (7.1)	0
Headache	0	0	0	4 (33.3)
Contusion	1 (7.1)	0	1 (3.6)	0
Urinary tract infection	0	0	0	1 (8.3)
Palpitations	1 (7.1)	0	1 (3.6)	0

Abbreviations: AE, adverse events; atv, atorvastatin calcium; NA, not applicable.
[a] Both BHT-3009 only and BHT-3009 + atv treatment arms are combined within this column.

study. Patients were randomized without regard to their clinical subtypes, and thus, the final distribution of patients by subtype of MS is shown in **Table 1**. The median age of 50.1 years and the mean EDSS score of 4.6 indicated the patient population had moderately advanced disease.

ADVERSE EVENTS AND CLINICAL RELAPSES

Adverse events that were considered to be treatment related or possibly treatment related are tabulated in **Table 2**. All adverse events were considered to be mild to moderate in severity and brief. No adverse events grade higher than 3 was considered to be treatment related. Furthermore, the percentage of patients with adverse events in the BHT-3009 treatment arm was no greater than that with placebo. Routine clinical laboratory testing did not demonstrate any significant abnormalities, with the exception of low-density lipoprotein cholesterol levels, which were reduced to lower than 50 mg/dL (to convert to micromoles per liter, multiply by 0.0259) in 9 of 13 patients randomized to the BHT-3009 plus atorvastatin arm.

Table 3. MRI Gd-Positive Lesion Number and Volume

Variable	Sample Size	Baseline	During Treatment	Change (%)
Gd-positive lesion[a]				
Placebo	12	0.63 (15/24)	1.12 (38/34)	+0.49 (+79)
BHT-3009	9	0.88 (15/17)	0.32 (8/25)	–0.56 (–64)
BHT-3009 + atv	9	0.81 (13/16)	0.81 (22/27)	No change
BHT-3009[b]	14	0.95 (20/21)	0.78 (29/37)	–0.17 (–18)
BHT-3009 + atv[b]	14	0.62 (13/21)	0.67 (28/42)	+0.05 (+8)
Gd-positive lesion, cm[3][c]				
Placebo	12	0.0393	0.0874	+0.0481 (+122)
BHT-3009	9	0.1545	0.0270	–0.1275 (–83)
BHT-3009 + atv	9	0.0916	0.0626	–0.0289 (–32)
BHT-3009[b]	14	0.1410	0.0873	–0.0537 (–38)
BHT-3009 + atv[b]	14	0.0698	0.0472	–0.0225 (–32)

Abbreviations: atv, atorvastatin calcium; Gd, gadolinium; MRI, magnetic resonance imaging.
[a] Values in parentheses indicate total number of Gd-positive lesions over the total number of MRIs, which in most cases included 2 per patient at baseline and 3 per patient during treatment. Values outside the parentheses indicate the total number of lesions divided by the total number of MRIs.
[b] Includes data for crossover patients who were initially randomized to placebo. The last single scan before crossover was used as the baseline scan on these patients.
[c] Values indicate the total volume of lesions divided by the total number of MRIs.

A total of 8 clinical relapses were reported during the entire trial. Only 1 of these relapses occurred while taking the study drug (5 days after the first dose of BHT-3009). After treatment of the relapse with corticosteroids, this patient remained in the study and continued dosing with BHT-3009 in accordance with the protocol, with no additional relapses. All other relapses occurred after the last dose of BHT-3009 (range of 5 to 29 weeks after last dose of BHT-3009).

MRI DATA

Contrast-enhanced brain MRI was obtained at baseline and during treatment as described in the "Methods" section. No statistically significant increases in the number of gadolinium-enhancing lesions, volume of gadolinium-enhancing lesions, and volume of T2 lesions were observed with either BHT-3009 alone or in combination with atorvastatin compared with placebo. Rather, there was a trend toward a reduction in lesion activity after treatment relative to the placebo group (**Table 3**). The gadolinium lesion number and volume increased in the placebo arm relative to baseline, whereas the gadolinium lesion number decreased with BHT-3009 alone and the gadolinium lesion volume decreased with both BHT-3009 alone and with atorvastatin (Table 3). These lesion changes were favorable with BHT-3009 but did not reach statistical significance.

ANTIGEN-SPECIFIC PERIPHERAL IMMUNE RESPONSE

At 1 clinical site, the peripheral immune response of myelin antigen–specific CD4+ T cells was measured throughout the course of the study (**Figure 3** and **Figure 4**). This was measured with a flow cytometer–based proliferation assay as described in the "Methods" section.

Using this assay, data at baseline and after treatment with BHT-3009 were obtained from a total of 6 patients. Of these, 5 patients were found to have significant proliferation of IFN-γ–producing CD4+ T cells reactive to myelin antigens at baseline, and all had a decline in proliferation with BHT-3009 (Figure 4). No such decrease was observed in their response to tetanus toxoid or to global T-cell responses, such as with anti-CD3 or glatiramer acetate, indicating that antigen-specific tolerance had occurred in these 5 patients. This antigen-specific decrease in T-cell activity persisted up to 50 weeks after randomization in several patients.

Further, in none of the patients, including the single patient who had no activity at baseline, was there a significant induction of myelin antigen–specific T-cell proliferation at any point after BHT-3009 treatment. Taken together, these data thus demonstrate that BHT-3009 did not induce a cellular immune response to MBP or any of the other myelin antigens tested and suggest that it is an effective agent for the induction of antigen-specific tolerance to myelin proteins in patients with MS.

ANTIGEN-SPECIFIC IMMUNE RESPONSE WITHIN THE CNS

To measure the antigen-specific immune response in the CNS as opposed to peripheral blood, patients were given the option of volunteering to undergo lumbar punctures for CSF collection. Five patients volunteered during the screening period and 3 of these patients had a follow-up lumbar puncture after completing dosing with BHT-3009. The CSF was analyzed for autoantibody titers using a protein microarray as described in the "Methods" section.

The results shown in **Figure 5** demonstrate that treatment with BHT-3009 significantly decreased autoantibody titers in CSF to multiple myelin antigens in all 3 patients who had follow-up lumbar punctures. Importantly, this reduction in autoantibody titers was not restricted to MBP but was also observed with other antigens, such as proteolipid protein (PLP), suggesting that BHT-3009 can cross-tolerize to other antigenic components of the myelin sheath. Although these specific re-

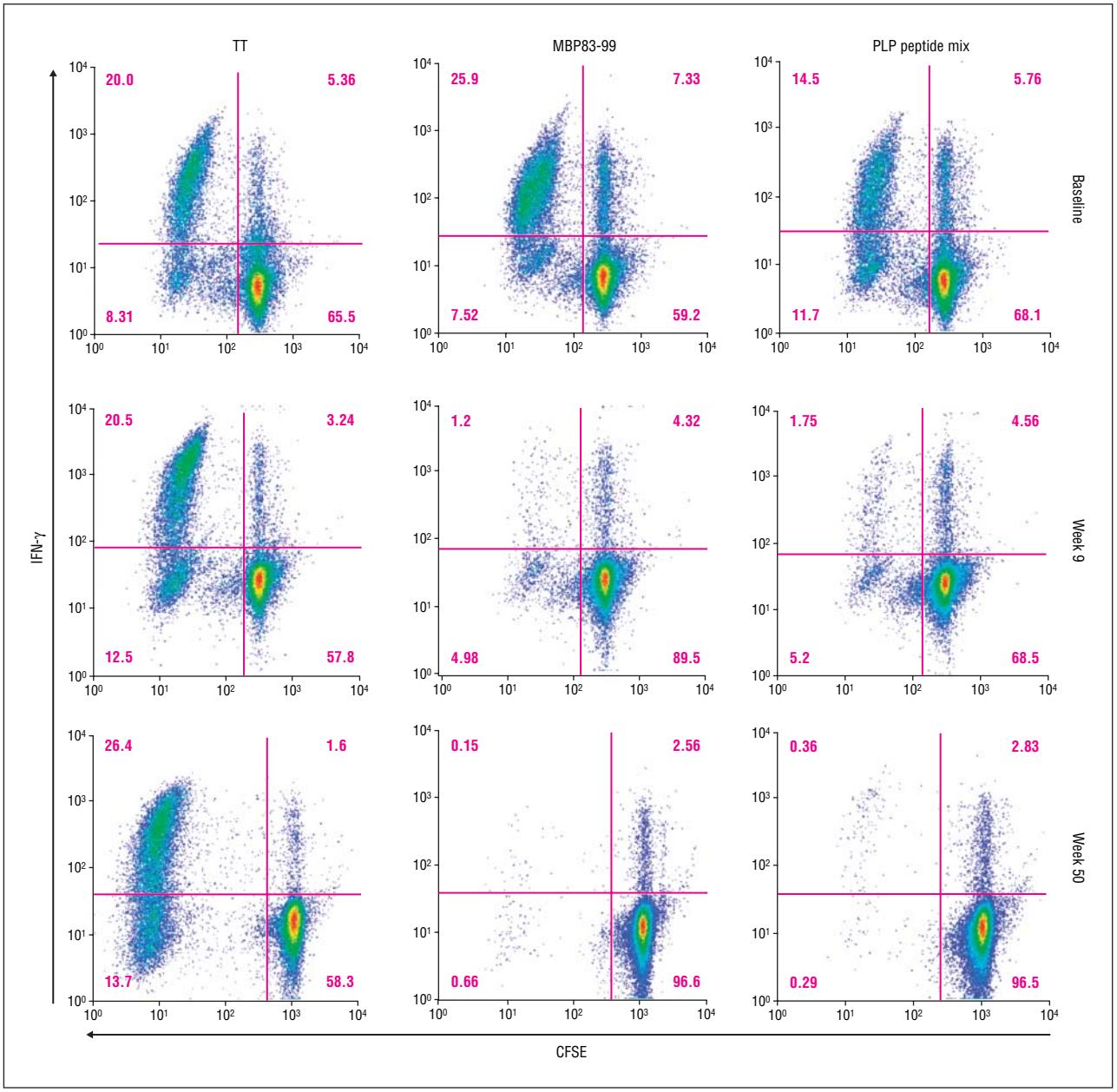

Figure 3. Example of decreased T-cell response with BHT-3009. An example of one patient whose myelin basic protein (MBP)– and proteolipid protein (PLP)–specific T-cell proliferative response decreased in response to BHT-3009 is shown. Proliferation was measured using a dye dilution method with the vital dye 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE). Peripheral blood mononuclear cells were incubated with a variety of antigens and controls, but for simplicity, only the responses to tetanus toxoid (TT), MBP83-99 peptide, and a PLP peptide mix are shown. The upper 3 panels correspond to the baseline response; the middle 3, to the week 9 response; and the bottom 3, to the week 50 response. Proliferating interferon (IFN)-γ–positive CD4+ T cells are shown in the upper left quadrant of each fluorescent-activated cell sorter plot. Numbers in red indicate the percentage of cells in each quadrant. A dramatic decrease in IFN-γ–positive cells specific for MBP and PLP is demonstrated by week 9 and persists to week 50. Importantly, the response to TT is unchanged with dosing, confirming the antigen-specific nature of BHT-3009.

sults were not repeated with conventional enzyme-linked immunosorbent assay, we have previously shown that this assay correlates well with and is more sensitive than enzyme-linked immunosorbent assay.[14]

COMMENT

The results reported herein demonstrate that BHT-3009 is safe and well tolerated, produced favorable trends in MRI brain lesions, and was associated with antigen-specific down-regulation of autoimmune activity in both blood and CSF. The combined delivery of self-antigens along with backbone modifications of the CpG content in the DNA plasmid may be sufficient to cause the down-regulation of the autoimmune process, leading to antigen-specific tolerance and less disease activity. The BHT-3009 plasmid encodes MBP, one of the major autoantigens putatively involved in MS pathogenesis. Further, the plasmid backbone has been modified in such a way as to reduce the number of immunostimulatory CpG motifs and increase the number of immunoinhibitory GpG motifs. Preclinical experiments indicated that this combination

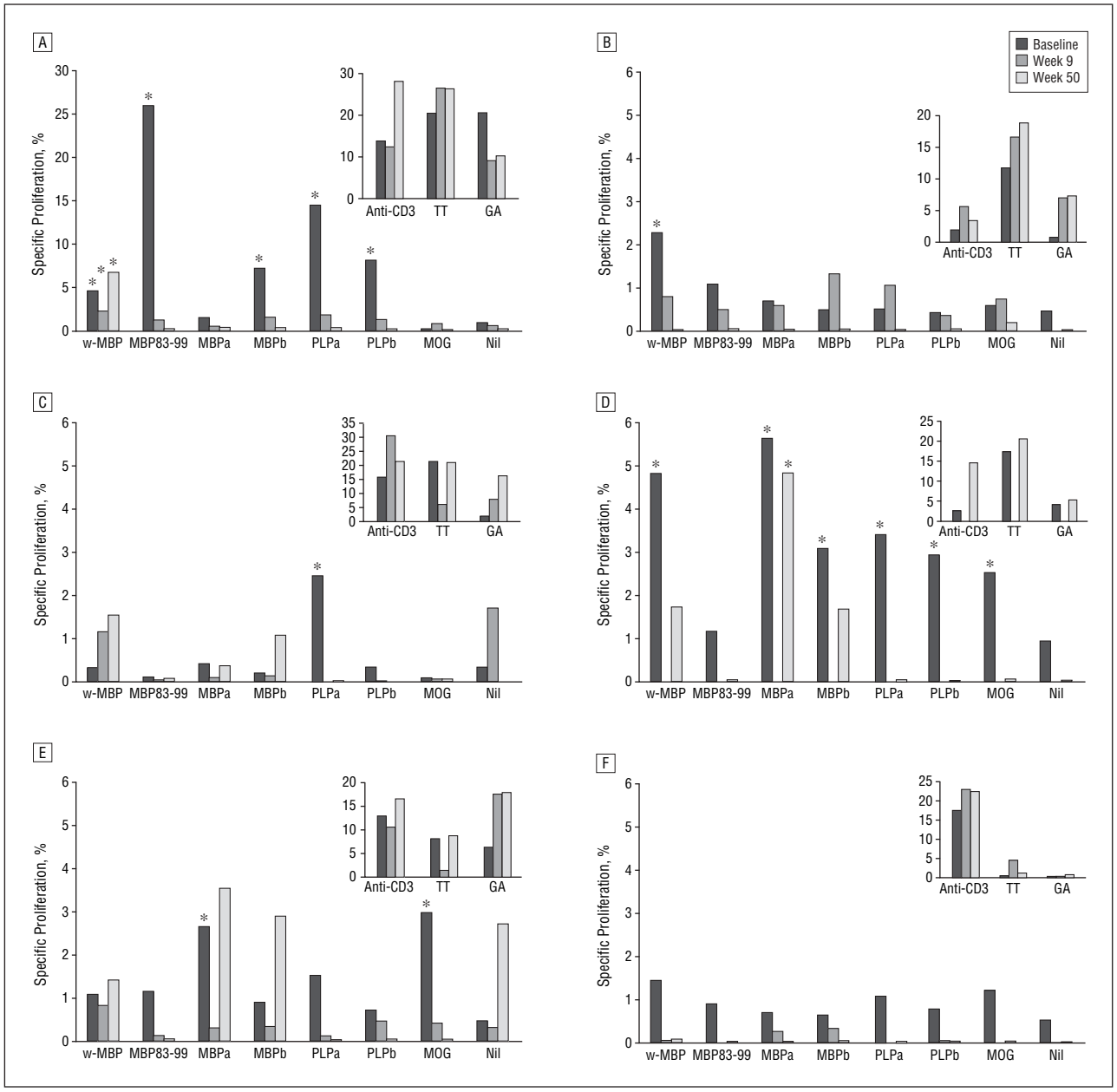

Figure 4. Reduction in antigen-specific T-cell activity with BHT-3009. Shown are the percentages of specific interferon (IFN)-γ–positive CD4+ T-cell responses (y-axis) to various antigens (x-axis) at several points for 6 patients: patient 003 (A), patient 002 (B), patient 028 (C), patient 011 (D), patient 027 (E), and patient 020 (F). For each patient, the right panel represents T-cell responses to the positive controls (anti-CD3, tetanus toxoid [TT], and glatiramer acetate [GA]). The left panel shows the responses to various myelin antigen peptides or whole myelin basic protein (w-MBP). The peptides used in the assay are the following: MBP83-99; a mixture of MBP14-33 and MBP114-126 (MBPa); a mixture of MBP111-129 and MBP145-164 (MBPb); a mixture of proteolipid protein (PLP) 191-210, PLP184-199, and PLP178-197 (PLPa); a mixture of PLP139-154, PLP89-106, and PLP42-59 (PLPb); a mixture of myelin-oligodendrocyte glycoproteins MOG35-55, MOG145-160, and MOG1-20 (MOG); and no peptide (nil). Samples were drawn at 3 different points: baseline, week 9, or week 50. Week 9 data for the patient in part D was not analyzable because of poor assay conditions. Patients in panels A, B, and C were in the BHT-3009 plus atorvastatin calcium arm, and patients in panels D, E, and F were in the BHT-3009 arm. The only patient among these who experienced a relapse was patient 028 (C), who had a relapse 7 weeks and 29 weeks after the last dose of BHT-3009. Positive responses are indicated with asterisks (except in the case of the positive controls where this is not indicated because the majority of data points are positive). For a particular response to be considered positive, 2 criteria had to be met: an absolute proliferation greater than 2% and a stimulation index greater than 2.0 (stimulation index is defined as the specific proliferation with a given antigen divided by that in the nil). For part E, all of the myelin antigen–specific proliferation at week 50 is judged to be negative because although many samples have a greater than 2% specific proliferation, the stimulation indexes are less than 2.0 because of the relatively high proliferation in the nil sample. Sample F is negative for all myelin antigens. During the study, the responses to the myelin antigens generally decreased in each case, whereas the positive control responses remained relatively stable, reinforcing the antigen-specific nature of the action of BHT-3009.

of full-length self-antigen delivery and an immunoinhibitory DNA backbone could lead to favorable immunological changes in patients with MS.

Statins, a class of lipid-lowering drugs that includes atorvastatin, have proven efficacious in some animal models of autoimmunity and in early-stage human trials.[10,15] We tested whether high-dose, daily oral atorvastatin could potentiate the efficacy of BHT-3009. The clinical, MRI, and immunological data indicate that combining oral atorvastatin with our BHT-3009 vaccine provided little if any additional benefit to what was observed with BHT-3009 alone.

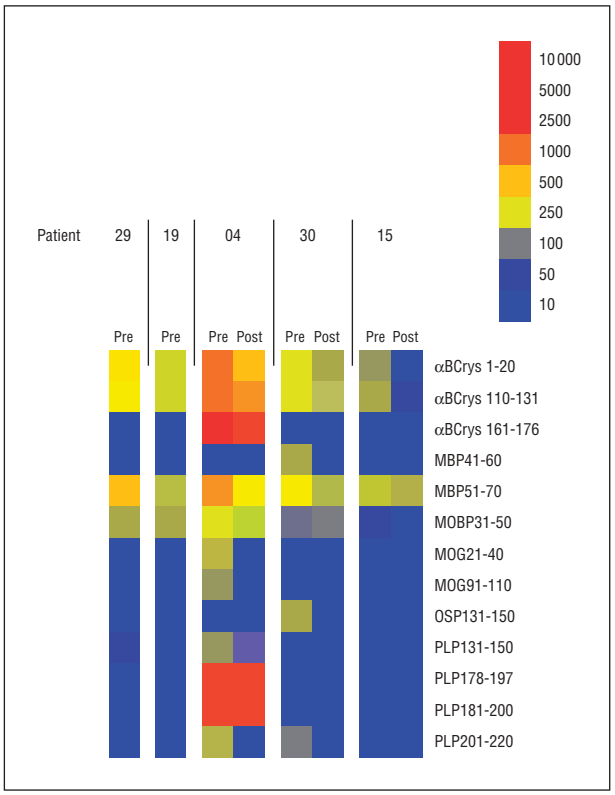

Figure 5. Decrease in cerebral spinal fluid (CSF) autoantibodies. The CSF was obtained from 5 patients at baseline (pre) and in 3 patients at 13 weeks after the first dose with BHT-3009 (post). Autoantibody reactivity to various myelin peptides (far right column) was measured by protein array and represented by the heatmap, with red being the highest reactivity and blue, the lowest. Only antigens with more than a 2-fold and more than a 25–fluorescence unit difference in reactivity between a pretreatment and posttreatment sample in any patient are shown. In all 3 patients where both predosing and postdosing CSF samples were analyzed, there is a decrease in the reactivity of myelin-reactive autoantibodies. All 3 of these patients were randomized to the BHT-3009–only arm (ie, without atorvastatin calcium). αBCrys indicates αB-crystallin; MBP, myelin basic protein; MOG, myelin-oligodendrocyte glycoprotein; MOBP, myelin-associated oligodendrocytic basic protein; OSP, osteopontin; PLP, proteolipid protein.

In this trial, we demonstrated based on every clinical and paraclinical parameter that BHT-3009 is safe and well tolerated. There were no increases in clinical relapses, disability, drug-associated laboratory abnormalities, adverse events, or the number and volume of contrast-enhancing lesions on brain MRI with BHT-3009 treatment compared with placebo. In fact, there was a trend toward a decrease in the number and volume of contrast-enhancing lesions in the brain in patients treated with BHT-3009 compared with placebo. One consideration in interpreting the MRI data is that a relatively higher number of patients with SPMS without relapses were randomized to placebo, and thus, conclusions solely based on the MRI data should be avoided.

Because of the unique antigen-specific nature of the mechanism of action of BHT-3009, we also carried out immunological assays in several patients, analyzing T-cell and autoantibody responses in blood and CSF, respectively. In all 5 patients who had MBP- or PLP-specific activity at baseline, the antigen-specific autoreactive T-cell response diminished with BHT-3009 by week 9, as measured by the proliferation of IFN-γ–producing CD4+ T cells. In no case was MBP- or PLP-specific T-cell activity induced with BHT-3009, providing further evidence that our DNA vaccine approach leads to tolerance rather than active immunization. Using protein microarrays, we demonstrated that compared with the screening samples, there was a relative decrease in the myelin-reactive autoantibody titers in the CSF of 3 individuals. Further, the data demonstrate that this decrease in myelin-reactive autoantibody titers extends beyond MBP to other myelin antigens, such as PLP, myelin-oligodendrocyte glycoprotein, and αB-crystallin.

This expansion of the tolerogenic response beyond the antigen encoded by BHT-3009 is a demonstration of a phenomenon known as "bystander suppression" or "infectious tolerance."[16] Bystander suppression is well described in animal models of autoimmunity and has been observed in patients undergoing allergen desensitization. There are 2 mechanisms by which bystander suppression and thus tissue-specific tolerance may have occurred with BHT-3009 treatment: induction of regulatory T cells or via dendritic cell–mediated cross-tolerance. Regulatory T cells generated against MBP could traffic to a lesion within the CNS and have beneficial effects either directly on other myelin-reactive T cells or on resident myelin antigen–presenting microglia, rendering them tolerance promoting. An exhaustive search for regulatory T cells was not conducted in the trial, and thus, we can neither exclude nor conclude that regulatory T cells are induced by BHT-3009. Animal model studies are being conducted in addition to assays in humans as part of an ongoing, larger phase 2b trial to examine this possibility.

The other mechanism by which bystander suppression can be incurred is via dendritic cells. It has recently been demonstrated that a variety of CNS myelin antigens, including MBP and PLP, can be found within cervical draining lymph nodes of both primates with experimental autoimmune encephalomyelitis and humans with MS.[17] It has been proposed that dendritic cells within cervical lymph nodes can present multiple myelin antigens and thus are central to the observed phenomenon of epitope spreading. Further, it is argued that these multiple myelin antigen–presenting dendritic cells should play an essential role in any approach designed to reverse epitope spreading (ie, bystander suppression) within the peripheral immune system because they can potentially cross-tolerize to multiple antigens simultaneously. BHT-3009 is administered within the deltoid muscle of the arm, and because we know from preclinical studies that expression of BHT-3009 can be detected in draining lymph nodes, BHT-3009 is likely to be distributed to and expressed within dendritic cells of cervical lymph nodes. Thus, BHT-3009 may tolerize to MBP-reactive T cells circulating through these lymph nodes, but by virtue of these multiple myelin antigen–presenting dendritic cells now rendered tolerance promoting by BHT-3009, other myelin-reactive T cells (eg, PLP-reactive T cells) may also become tolerized.

We have demonstrated in this first, to our knowledge, in-human trial of a DNA vaccine for autoimmune disease that the approach is safe and well tolerated. We describe evidence for induction of favorable trends on brain MRI, indicating a reduction in the inflammatory

response in the CNS. BHT-3009 reduced antigen-specific immune responses both in the peripheral immune system and the CNS, including reductions in proliferation of IFN-γ–producing, myelin-reactive CD4+ T cells and levels of CSF antibodies directed against myelin. Based on these encouraging results, a 1-year, double-blind, randomized, placebo-controlled phase 2b trial of BHT-3009 in approximately 290 patients with RRMS has commenced. If successful in MS, antigen-specific DNA vaccines can be developed for prevention or treatment of related diseases, such as type 1 diabetes mellitus, systemic lupus erythematosus, rheumatoid arthritis, and myasthenia gravis.

Accepted for Publication: May 17, 2007.
Published Online: August 13, 2007 (doi:10.1001/archneur.64.10.nct70002).
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Financial Disclosure: Dr Vollmer has received honoraria for consultation and lectures related to this research and a research grant to conduct this study from Bayhill Therapeutics, Inc. Ms Gianettoni and Dr Valone have received salary, stock, and/or stock options from Bayhill Therapeutics, Inc. Drs Robinson and Steinman have received consulting fees, stock, and/or stock options from Bayhill Therapeutics, Inc. Dr Utz is a cofounder of and consultant at Bayhill Therapeutics, Inc, and receives stock and consulting fees. Dr Steinman is a cofounder of and consultant and director at Bayhill Therapeutics, Inc. Dr Garren is a cofounder of and has received salary, stock, and/or stock options from Bayhill Therapeutics, Inc.
Funding/Support: The work described in this article was funded by Bayhill Therapeutics, Inc.
Additional Contribution: Brian Kidd, BS, analyzed the CSF protein arrays.

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