

Phase 2 Trial of a DNA Vaccine Encoding Myelin Basic Protein for Multiple Sclerosis

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Objective: To evaluate the efficacy and safety of BHT-3009 in relapsing-remitting multiple sclerosis (MS) and to confirm that BHT-3009 causes immune tolerance.

Methods: BHT-3009 is a tolerizing DNA vaccine for MS, encoding full-length human myelin basic protein. Relapsing-remitting MS patients were randomized 1:1:1 into three groups: placebo, 0.5mg BHT-3009, or 1.5mg BHT-3009, given intramuscularly at weeks 0, 2, 4, and every 4 weeks thereafter until week 44. The primary end point was the 4-week rate of occurrence of new gadolinium-enhancing lesions on brain magnetic resonance images from weeks 28 to 48. Protein microarrays were used to measure levels of anti-myelin autoantibodies.

Results: Compared with placebo, in the 267 patient analysis population the median 4-week rate of new enhancing lesions during weeks 28 to 48 was 50% lower with 0.5mg BHT-3009 ($p = 0.07$) and during weeks 8 to 48 was 61% lower with 0.5mg BHT-3009 ($p = 0.05$). The mean volume of enhancing lesions at week 48 was 51% lower on 0.5mg BHT-3009 compared with placebo ($p = 0.02$). No significant improvement in magnetic resonance imaging lesion parameters was observed with 1.5mg BHT-3009. Dramatic reductions in 23 myelin-specific autoantibodies in the 0.5mg BHT-3009 arm were observed, but not with placebo or 1.5mg BHT-3009.

Conclusions: In relapsing-remitting MS patients, treatment with the lower dose (0.5mg) of BHT-3009 for 44 weeks nearly attained the primary end point for reduction of the rate of new enhancing magnetic resonance imaging lesions ($p = 0.07$) and achieved several secondary end points including a reduction of the rate of enhancing magnetic resonance imaging lesions from weeks 8 to 48 ($p = 0.05$). Immunological data in a preselected subgroup of patients also indicated that treatment with 0.5mg induced antigen-specific immune tolerance. The greater dose was ineffective.

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Antigen-specific tolerance leading to therapy of organ-specific inflammatory diseases such as multiple sclerosis (MS) could fundamentally alter the course of these autoimmune diseases. The prevailing hypothesis to describe the pathogenesis of MS is that the destruction of myelin within the central nervous system is largely due to antigen-specific autoimmunity.^{1–3} If a therapy could tolerize in an antigen-specific manner, the autoimmune process might be halted and the remainder of the immune system would remain intact to continue to protect against cancer and infection.

One such antigen-specific approach that appears promising is DNA vaccination.^{4,5} We previously published the results of a phase I/II trial using a DNA

plasmid vaccine encoding myelin basic protein (MBP), termed *BHT-3009*.⁶ The trial was performed in a cohort of 30 relapsing-remitting MS (RRMS) or secondary-progressive MS patients, and a total of 4 doses of BHT-3009 were administered. We demonstrated that the approach was safe and produced antigen-specific immune tolerance. The primary end point of safety and tolerability was achieved, and favorable trends in the reduction of gadolinium (Gd)-enhancing lesion activity on brain magnetic resonance imaging (MRI) was demonstrated. Furthermore, we observed a pronounced decrease in activity of myelin-specific CD4+ Th1 cells from peripheral blood and reduction in myelin-specific autoantibody titers in ce-

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Members of the BHT-3009 Study Group are listed in the Appendix on page 619.

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rebrospinal fluid (CSF) with BHT-3009 treatment. Our hypothesis is that after the DNA plasmid is injected, it produces MBP within antigen-presenting cells of the immune system, which then causes the MBP-reactive pathogenic T cells to become tolerized, thus attenuating the autoimmune disease process. A surrogate measure of clinical benefit is a reduction of lesion activity on brain MRI. In a clinical trial reported in 2000, therapy with an altered peptide ligand of a portion of MBP induced an increase in T cells reactive to MBP with concomitant worsening of MRI activity and disease.^{7,8} Given this evidence that MBP indeed is a relevant target of the autoimmune response in MS, we hypothesized, therefore, that tolerization to MBP with the native MBP protein encoded by BHT-3009, as opposed to the altered form in the altered peptide ligand, would be beneficial.

Having demonstrated safety and antigen-specific immune tolerance, we wanted to test BHT-3009 further in a larger number of patients and for a longer duration of treatment. We conducted a randomized, placebo-controlled phase 2 trial in 289 RRMS patients treated for a total of 44 weeks with BHT-3009. In this trial, we tested the hypothesis that BHT-3009 would decrease central nervous system inflammation as assessed by brain MRI. We further examined whether BHT-3009 would alter pre-existing immune responses to myelin autoantigens by examining the spinal fluid for autoantibodies in a subset of 80 patients who agreed to have lumbar punctures performed before therapy and again at week 44.

Patients and Methods

Patients

The protocol was reviewed and approved by each country's regulatory agency, 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.

Patients were enrolled at 43 clinical sites in 11 countries throughout Europe, Asia, and North America. Eligible patients were 18 to 55 years of age, had a definite diagnosis of RRMS by the McDonald criteria, and screening disability score on the Kurtzke expanded disability status scale (EDSS) between 0 and 3.5 inclusive.^{9,10} Furthermore, eligible patients had one or more relapses within the previous year and were clinically stable without relapse for at least 50 days before screening. Patients were excluded if they had more than five Gd-enhancing lesions on screening MRI, were treated with high-dose corticosteroids within 50 days before screening, were treated with β -interferon or glatiramer acetate within 180 days before screening or for a greater than 180-day lifetime total, or were ever treated with natalizumab.

Study Design

After providing informed consent, eligible patients were randomized in one of three arms in a 1:1:1 ratio to receive in-

tramuscular injections of placebo, 0.5mg BHT-3009, or 1.5mg BHT-3009. Placebo consisted of saline injections, which could not be differentiated either visually or by injection-site reactions from the BHT-3009 preparations. After a patient passed the screen, randomization was performed by a third-party organization (ClinPhone, East Windsor, NJ), and both the sponsor and all personnel at the clinical sites were blinded to this randomization process.

BHT-3009 was administered during an induction phase at weeks 0, 2, and 4 relative to randomization, and then during a maintenance phase every 4 weeks through week 44, for a total of 13 doses. Patients then returned 4 weeks after the last dose for a final visit (week 48).

Safety evaluations were performed at baseline and at weeks 2, 4, and every 4 weeks thereafter until week 48. At each safety evaluation visit, assessment of the patient included history and physical examination, vital signs, adverse event monitoring, hematology, blood chemistries, and urinalysis (every 8th week after week 8). In addition, EDSS and the Multiple Sclerosis Functional Composite assessment were performed at screening and weeks 20, 40, and 48. The National Cancer Institute Common Terminology Criteria for Adverse Events criteria 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.

Magnetic Resonance Imaging

MRI of the brain with injection of Gd was performed according to a standardized protocol using a 1.5-Tesla magnet at each site. MRI was performed during screening and at weeks 8, 16, 28, 32, 36, 40, 44, and 48. A central MRI reading unit qualified each participating site's MRI department 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.

End Points

The primary end point was the rate of occurrence of new Gd-enhancing lesions on brain MRIs performed every 4 weeks from weeks 28 to 48, inclusive. Key secondary end points include total number and volume of new Gd-enhancing lesions (weeks 8–48), T2 lesion volume change from baseline to week 48, mean 4-week rate of new T2 lesions performed every 4 weeks from week 28 through 48, time to first relapse, and the proportion of patients with worsening Multiple Sclerosis Functional Composite on week 48 compared with baseline.

Statistical Methods

The primary efficacy analysis was conducted after the last patient completed the Week 48 visit. The analysis population was predefined in the protocol as comprising all patients randomized who completed at least 10 doses of study drug, had fewer than or equal to 5 Gd+ lesions at screening, and had at least 3 postenrollment MRIs in weeks 28 to 48. This analysis population consisted of 267 of the 289 patients initially randomized. The test of the superiority of either of the two doses of BHT-3009 to placebo was performed on the

mean 4-week rate of new Gd-enhancing lesions, using a two-sided Wilcoxon rank sum test using PROC STATXACT (SAS) stratified by pooled center and the number of Gd-enhancing lesions on the baseline MRI scan (categorized as 0, 1–2, >2 lesions). Furthermore, unstratified Hodges–Lehmann estimates of the treatment differences and their confidence intervals were performed.

The total number of new Gd-enhancing lesions between visits 10 (week 28) and 15 (week 48) inclusive was analyzed using a generalized linear model (with a negative binomial distribution) and the log link function, with treatment group and pooled center as factors, the natural log of the number of Gd-enhancing lesions on the baseline MRI scan as covariate, and the natural log of the number of scans available for analysis as an offset variable. Where the number of lesions at baseline was zero, this was approximated by natural log (0.1). This analysis was performed in the SAS System with the PROC GENMOD procedure.

The superiority of either dose of BHT-3009 to placebo was examined via null hypotheses of the form: $H_0 = \text{BHT-3009 does not differ from placebo}$; and $H_1 = \text{BHT-3009 differs from placebo}$. The two null hypotheses with their corresponding alternatives each specified a different dose of BHT-3009, namely, 0.5 and 1.5mg. Overdispersion was taken into account and was estimated via the deviance. Wald χ^2 tests were used to assess the difference in the least-squares means between each of the two active treatment groups and placebo. The estimates of these differences were presented, together with their 95% confidence intervals. Hochberg's multiple-test procedure was used to account for multiplicity in the calculation of confidence intervals. Goodness-of-fit tests were performed using the ASSESS option of the PROC GENMOD procedure.

Analysis of toxicity was conducted on the safety population, comprising all patients who received at least one dose of study drug and had any follow-up data. A data analysis plan was prepared and submitted to the US Food and Drug Administration and other competent authorities before data lock and analysis.

Protein Microarray Assays

Myelin protein arrays, each containing 132 myelin and control peptides printed in duplicate, were produced by JPT Peptide Technologies GmbH (Berlin, Germany). As described previously, arrays were blocked with phosphate-buffered saline containing 3% fetal calf serum and 0.05% Tween 20, probed with 1:15 dilutions of CSF, and bound autoantibody detected using Cy-3-labeled goat anti-human IgG/M antiserum (Jackson ImmunoResearch, West Grove, PA).¹¹ Arrays were scanned with the GenePix 4000B scanner (Molecular Devices Corporation, Sunnyvale, CA), and GenePix Pro 5.0 software (Molecular Devices Corporation) was used to quantitate net median pixel intensities for each peptide. Baseline CSF anti-MBP antibody reactivity is presented as a heat map of reactivity in which individual patients are ordered based on the sum of \log_2 (median fluorescence intensity/300) of the anti-MBP peptide reactivity, and the peptides ordered based on the sum of \log_2 (median fluorescence intensity/300) of all of the patients' reactivities. Antibody reactivities exhibiting significant differences between pretreat-

ment and posttreatment samples were identified using significance analysis of microarrays (SAM; false discovery rate, $q < 0.1$).¹²

Results

Demographics

A total of 373 patients were screened for this clinical trial from the period of February 2006 to June 2006. Two hundred eighty-nine patients were randomized into 1 of 3 arms: 96 to placebo, 104 to 0.5mg BHT-3009, and 89 to 1.5mg BHT-3009. Of these patients, the 267 patient analysis population was predefined in the protocol and comprised all patients randomized who completed at least 10 doses of study drug, had fewer than or equal to 5 Gd+ lesions at screening, and had at least 3 post-enrollment MRIs in weeks 28 to 48. In this population, 87 were randomized to placebo, 96 to 0.5mg BHT-3009, and 84 to 1.5mg BHT-3009. There were no significant differences in the baseline demographic, clinical, or MRI characteristics in these three arms (Table 1).

There were 22 patients who differed between the 289 randomized patients and the 267 patients of the analysis population. Of these 22 patients, 14 were those in which either no drug or insufficient doses of drug were given (6 in placebo, 3 in 0.5mg BHT-3009, and 5 in 1.5mg BHT-3009). One additional patient (in the 0.5mg BHT-3009 arm) had an insufficient number of MRI scans performed. The remaining seven patients did not meet the protocol-defined inclusion criteria either because they had more than five Gd+ lesions at screen (three patients in placebo arm and three patients in 0.5mg BHT-3009 arm) or had an EDSS greater than 3.5 (one patient with EDSS of 4.0 in the 0.5mg BHT-3009 arm). Because these patients were approximately equally distributed among the arms, it was decided that the analysis was not compromised by their exclusion.

Safety and Tolerability

No substantial difference in the distribution of adverse events was observed among all three treatment arms (Table 2). Of these, a total of seven serious adverse events were reported in the placebo arm, five in the 0.5mg BHT-3009 arm, and four in the 1.5mg BHT-3009 arm. The majority of adverse events were judged to be mild or moderate. Severe or worse adverse events were observed in 11 patients who received placebo, 7 who received 0.5mg BHT-3009, and 8 who received 1.5mg BHT-3009. Routine clinical laboratory testing (including blood chemistries, hematology, and urinalysis) did not identify any significant abnormal trends over time or with dose.

Table 1. Baseline Patient Characteristics

Characteristics	Placebo	BHT-3009	
		0.5mg	1.5mg
n	87	96	84
Female sex	62 (71.3%)	72 (75.0%)	51 (60.7%)
Age			
<40 yr, n	53 (60.9%)	60 (62.5%)	48 (57.1%)
Mean, yr	37.2	35.6	36.8
Median, yr	37.0	34.5	37.0
Range, yr	18–54	18–54	18–54
Race			
White	86 (98.9%)	96 (100.0%)	84 (100.0%)
Mean time from first symptoms, mo	74.9	84.9	71.0
Mean time from diagnosis, mo	35.1	43.8	37.0
Relapses			
≥1 in last year, n	84 (96.6%)	91 (94.8%)	83 (98.8%)
Mean number of relapses in last 2 years	1.9	2.0	2.1
Mean time since last relapse, mo	5.9	6.1	5.0
EDSS score			
Mean	2.48	2.43	2.44
Median	2.50	2.50	2.50
MRI parameters			
Gd+ lesions			
Mean number	0.7	0.8	1.0
Mean volume, mm ³	54.4	52.0	96.2
Mean T2 lesion volume, mm ³	6,852.7	6,416.4	7,383.6
Mean T1 hypointense lesion volume, mm ³	2,127.5	1,905.2	1,982.1

EDSS = expanded disability status scale; MRI = magnetic resonance imaging.

Table 2. Adverse Events during Treatment (% of Patients)

Characteristics	Placebo	BHT-3009	
		0.5mg	1.5mg
n	95	104	87
At least one AE	78.9%	83.7%	89.7%
Nervous system	49.5%	51.9%	56.3%
Infections	47.4%	51.9%	51.7%
General and injection-site reaction	33.7%	33.7%	32.2%
Musculoskeletal	17.9%	18.3%	21.8%
Gastrointestinal	16.8%	15.4%	11.5%
Psychiatric	14.7%	8.7%	9.2%
Injuries	5.3%	7.7%	12.6%
Renal and urinary	5.3%	8.7%	11.5%
Skin	8.4%	7.7%	6.9%
Eye	8.4%	5.8%	8.0%
Ear	5.3%	6.7%	8.0%
Respiratory	6.3%	8.7%	3.4%
Reproductive	3.2%	3.8%	5.7%
Investigations (laboratory test, blood pressure, etc)	3.2%	5.8%	2.3%

Only the adverse events (AEs) occurring in greater than 5% of patients in any one of the three groups is listed.

Magnetic Resonance Imaging End Points

We achieved favorable changes on several MRI end points with the 0.5mg dose of BHT-3009 (Table 3). The 1.5mg dose of BHT-3009 did not produce significant changes by any MRI parameter.

On the primary end point of the 4-week rate of new enhancing lesions per patient in the last 6 months of the study (weeks 28–48 after enrollment), the median rate in the 0.5mg BHT-3009–treated arm was 50% lower than in the placebo arm (0.167 vs 0.333, respectively; $p = 0.07$). Several secondary MRI end points also demonstrated favorable changes with the 0.5mg dose of BHT-3009 (see Table 3). The median of the 4-week rate of new enhancing lesions per patient in the entire study (weeks 8–48) was 61% lower in the 0.5mg BHT-3009 treatment arm compared with the placebo arm (0.148 vs 0.375, respectively; $p = 0.05$). The median number of new enhancing lesions per patient in the last 6 months was 50% lower in the 0.5mg BHT-3009 treatment arm than in the placebo arm (1.0 vs 2.0, respectively; $p = 0.07$), and in the entire study was 67% lower with 0.5mg BHT-3009 compared with placebo (1.0 vs 3.0, respectively; $p = 0.05$).

The mean volume of enhancing lesions at week 48 was 51% lower on 0.5mg BHT-3009 compared with placebo (56.4 vs 116.3mm³, respectively; $p = 0.02$). In other MRI parameters, favorable but nonstatistically significant improvement was observed. The mean T2 lesion volume of the end point MRI, defined as the last MRI available at week 40 or later, was 7,110.1 mm³ on placebo compared with 6,361.0mm³ on 0.5mg BHT-3009 ($p = 0.11$). The median percentage change in T1 hypointense lesion volume from baseline to the end-point MRI was 0% on placebo compared with –3.76% on 0.5mg BHT-3009 ($p = 0.08$). No significant differences were observed in T2 lesion number.

Clinical Outcomes

No significant differences were observed in any group in the relative risk for relapse, annualized rate of relapse, or time to first relapse. The mean annualized rate of confirmed relapses in all randomized patients was 0.44 in the placebo arm, 0.46 in the 0.5mg BHT-3009 arm, and 0.60 in the 1.5mg BHT-3009 arm. Similarly, no significant differences were noted in the disability

Table 3. Magnetic Resonance Imaging Findings

Findings	Placebo	0.5mg BHT-3009	1.5mg BHT-3009	<i>p</i>	
				Placebo vs 0.5mg BHT-3009	Placebo vs 1.5mg BHT-3009
4-Week rate of new enhancing lesions/patient (weeks 28–48)					
n	87	96	84		
Mean ± SD	0.838 ± 1.266	0.757 ± 1.619	1.235 ± 2.215		
Median	0.333	0.167	0.372	0.07	1.0
4-Week rate of new enhancing lesions/patient (weeks 8–48)					
n	87	96	84		
Mean ± SD	0.818 ± 1.071	0.714 ± 1.488	1.190 ± 2.029		
Median	0.375	0.148	0.350	0.05	0.78
Number of new enhancing lesions/patient (weeks 28–48)					
n	87	96	84		
Mean ± SD	4.3 ± 6.38	4.0 ± 8.78	6.4 ± 11.23	0.21	0.13
Median	2.0	1.0	2.0	0.07	0.94
Number of new enhancing lesions/patient (weeks 8–48)					
n	87	96	84		
Mean ± SD	5.8 ± 7.54	5.1 ± 11.05	8.5 ± 14.44	0.17	0.14
Median	3.0	1.0	2.5	0.05	0.81
Enhancing lesion volume (week 48), mm ³					
n	81	91	80		
Mean ± SD	116.2 ± 293.0	56.4 ± 208.7	134.3 ± 322.5	0.02	0.43

SD = standard deviation.

assessment parameters (EDSS and Multiple Sclerosis Functional Composite) among the three treatment arms.

Baseline Autoantibody Profile of Patients

To determine whether the anti-myelin autoantibody profile differed among patients at the beginning of the trial and whether this profile predicted response to study drug, we obtained CSF from 80 patients via a preestablished protocol that was approved by local ethics committees and national authorities. Specimens were obtained by lumbar puncture at baseline and at the conclusion of the trial, and analyzed for autoantibody levels by protein microarray as described in Patients and Methods. The baseline levels of anti-MBP autoantibodies in the CSF of each patient was quantified, and the patients were prospectively sorted based on their degree of reactivity before trial unblinding (Fig 1). As shown in Figure 1, nearly all patients had some degree of anti-MBP autoantibody reactivity. There was, however, a broad range in the degree of reactivity to individual MBP epitopes, with some patients reactive to multiple MBP peptides (patients on far right of Fig 1) and some reactive to only a few peptides (patients on the far left of Fig 1).

In determining whether the baseline antibody profile predicted response to study drug, we prospectively examined those patients who had the greatest degree of anti-MBP reactivity. In the upper half of the anti-MBP reactive patients ($n = 13$ on placebo, $n = 11$ on 0.5mg BHT-3009, and $n = 14$ on 1.5 mg BHT-3009), there was a significantly lower number of new Gd-enhancing lesions per patient in week 28 to 48

MRIs with 0.5mg BHT-3009 compared with placebo (mean \pm standard deviation, 2.5 ± 4.03 on 0.5mg BHT-3009 vs 3.3 ± 4.59 on placebo; $p = 0.02$). In contrast, in this subgroup, there was no significant difference between 1.5mg BHT-3009 and placebo.

BHT-3009 Decreases Autoantibody Reactivity

In the 80 patients who contributed CSF for protein array analysis at baseline, we also obtained follow-up CSF at week 44 for repeat protein array analysis. This allowed us to determine whether treatment with BHT-3009 had an effect on the levels of anti-myelin autoantibodies. Treatment with the 0.5mg BHT-3009 dose was associated with a significant decrease in the autoantibody titers to 23 myelin autoantigens (Fig 2), whereas treatment with placebo did not result in a statistically significant net change in any of the anti-myelin autoantibodies measured. Not only did antibody titers to MBP peptides decrease with 0.5mg BHT-3009, but titers of autoantibodies binding to α B-crystallin, proteolipid protein (PLP), myelin oligodendrocyte glycoprotein, myelin-associated oligodendrocytic basic protein, and oligodendrocyte-specific protein (OSP) also decreased in a statistically significant manner as determined by the SAM statistical algorithm. In contrast, treatment with 1.5mg BHT-3009 was associated with an increase in titers to four PLP peptide epitopes (see Fig 2).

Discussion

In this phase 2 trial of a DNA vaccine for autoimmune diseases, we have demonstrated that the lower 0.5mg dose of BHT-3009 was safe and provided favorable

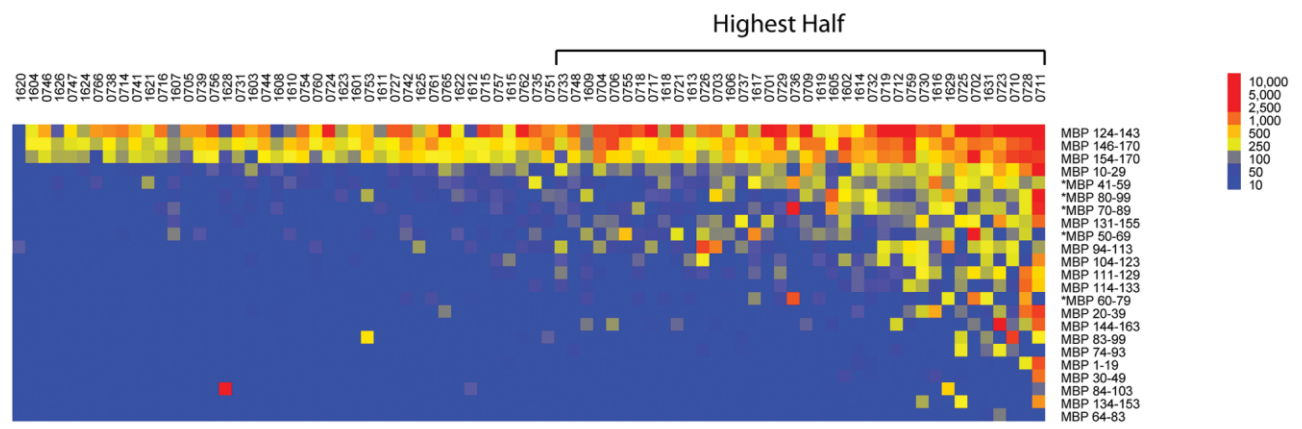


Fig 1. Anti-myelin basic protein (MBP) antibody profiles in baseline cerebrospinal fluid (CSF) samples. Myelin peptide arrays were probed with 1:15 dilutions of CSF samples, and antibody binding was detected with Cy3-conjugated goat anti-human IgG- and IgM-specific secondary antibodies. Arrays were scanned, and antibody reactivity quantitated. The heat map presents the myelin array results for anti-MBP peptide antibodies, with patients (columns) and peptides (rows) ordered by their respective net reactivities. The magnitude of reactivity is represented by the color scale, with blue representing lack of reactivity, yellow intermediate, and red strong reactivity. The prospectively identified highest half of the anti-MBP-reactive patients is indicated. MBP peptide sequences are derived from and numbered based on the 18.5 kD isoform (genbank accession number AAA59562), with the exception of peptides denoted by "*"s which are numbered based on the 21.5 kD isoform (genbank accession number AAA59564).

changes on several measures of brain lesion activity by MRI. In fact, there were 61% fewer lesions on the week 8 to 48 MRIs with 0.5mg BHT-3009 compared with placebo ($p = 0.05$). We did not detect any effect on the 1-year relapse rate, which is not surprising because the trial was short and was not powered for this clinical outcome. It is interesting to note that the rates seen in all three groups of approximately 0.4 relapse/year is substantially lower than that seen in other pivotal trials for drugs thus far approved for use in RRMS.¹³

When examining those patients who had the greatest degree of pretreatment anti-MBP antibodies in their spinal fluid as predefined prospectively before data lock, highly significant improvement in MRI activity was achieved with the 0.5mg dose ($p = 0.02$). Given that BHT-3009 encodes for and tolerizes to MBP, it is not surprising that the best responders to BHT-3009 are those patients with the greatest degree of reactivity against MBP. We further demonstrated that the 0.5mg dose produced favorable changes in the autoantibody profiles within the CSF when pretreatment and post-treatment levels were compared. Autoantibody levels in the CSF to 23 myelin autoantigens including MBP decreased substantially in nearly all of those patients treated with 0.5mg BHT-3009 but not in those treated with placebo.

The changes in multiple autoantibodies with BHT-3009 are similar to our observations in the experimental autoimmune encephalomyelitis model and in the phase I/II trial of BHT-3009.^{6,11} In that trial, we observed decreases in the peripheral anti-myelin T-cell activity that extended beyond MBP-specific T cells to PLP-specific T cells, and we also noted decreases to multiple anti-myelin autoantibodies in the CSF, including myelin oligodendrocyte glycoprotein, PLP, and α B crystallin, beyond the anti-MBP-specific autoantibodies. Thus, this phenomenon of expansion of the tolerogenic response, sometimes termed *bystander suppression*, is consistently observed in both the phase I/II and this much larger phase 2 trial. Because we observe bystander suppression, we believe that in both trials we achieved a dominant form of tolerance most likely mediated by the generation of antigen-specific regulatory T cells against MBP. Regulatory lymphocytes such as Th3 CD4+ cells, observed experimentally in antigen-specific tolerance, may mediate this effect.¹⁴ Regulatory T cells against MBP could traffic to a lesion within the brain and downregulate the activity of other myelin-reactive T cells, and alternatively or in addition, cause resident myelin antigen-presenting microglia to promote tolerance. We further believe that antigen-specific regulatory T cells could also downregulate the autoantibody response as measured here on protein arrays by interfering with the T-cell help required for B-cell

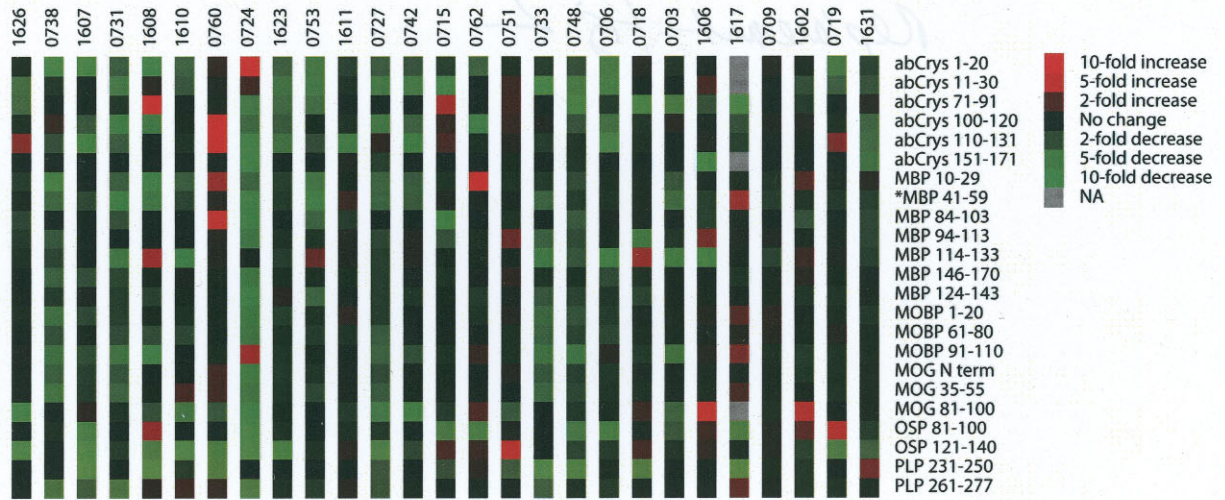
maturation. Alternatively, we have not ruled out a direct effect on B cells by the DNA vaccine. Recent reports suggest that B cells contain endosomal receptors that can sense and respond to DNA sequences directly.¹⁵

In contrast, in the 1.5mg BHT-3009-treated group, there were no substantial signs of improvement of any MRI or clinical end point compared with placebo. Furthermore, in the CSF autoantibody profiles, there were no significant decreases, but instead there were increases in titers of four anti-PLP autoantibodies. This result is similar to the clinical results of other antigen-specific therapies in which greater doses produced no improvement or unfavorable results.⁷ Although many were removed to promote tolerance, the BHT-3009 plasmid, like all other plasmids, contains some residual consensus immunostimulatory CpG motifs. CpG sequences bind Toll-like receptor 9, and thereby induce interferon- γ production.¹⁶ Recombinant interferon- γ when delivered to human MS patients exacerbated disease.¹⁷ It is possible that the 1.5mg dose delivered sufficient numbers of CpG motifs to overcome the tolerogenic effect of the MBP-encoding BHT-3009 plasmid. Given these results, we are currently exploring the possibility of testing an even lower dose of BHT-3009 to determine whether efficacy can be further enhanced.

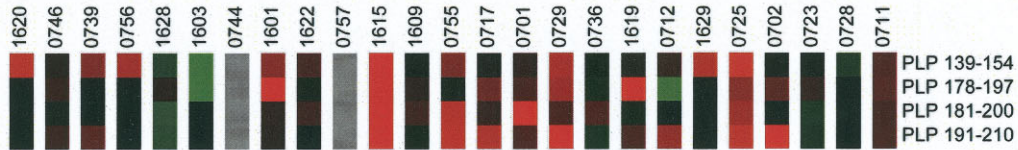
BHT-3009 appears to act by tolerizing the immune system in such a way that the ongoing autoimmune response against MBP and other myelin-specific antigens is downregulated. By administering one of the key antigenic targets in the pathogenesis of the disease in a tolerogenic manner by way of a DNA vaccine, the immune system becomes tolerized to that antigen. This concept of induction of tolerance to self has been observed in many systems, although induction of tolerance in human autoimmune disease has proved to be an elusive goal.¹⁸ The observation that chronic low doses of an antigen may, in fact, be better at tolerizing than high doses has been well described as “low-zone tolerance.”¹⁹ For example, Zinkernagel²⁰ reports that a single dose of LCMV peptide causes immune priming, whereas multiple doses induce immunological tolerance.

In conclusion, in RRMS patients, treatment with the lower dose, 0.5mg, of BHT-3009 for 44 weeks nearly attained the primary end point for reduction of the rate of new enhancing MRI lesions ($p = 0.07$) and achieved several secondary end points including a reduction in the rate of enhancing MRI lesions from weeks 8 to 48 ($p = 0.05$). Immunological data in a preselected subgroup of patients also indicated that treatment with 0.5mg induced antigen-specific immune tolerance. Measurement of myelin antibodies in the spinal fluid before and after treatment from a predefined subset of patients suggested that treatment at 0.5mg, but not at 1.5mg, induced antigen-specific

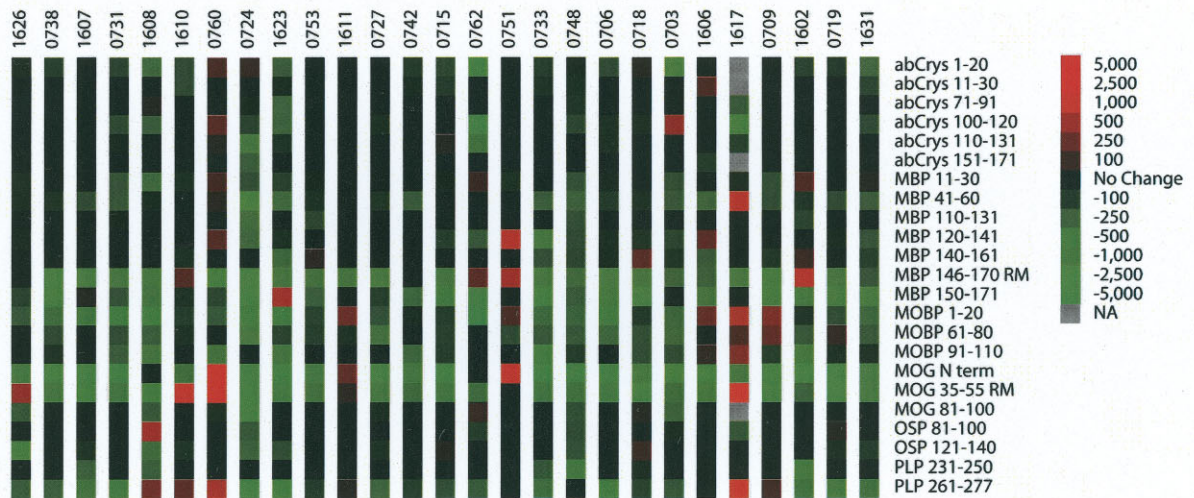
A. 0.5 mg BHT-3009



B. 1.5 mg BHT-3009



C. 0.5 mg BHT-3009



D. 1.5 mg BHT-3009

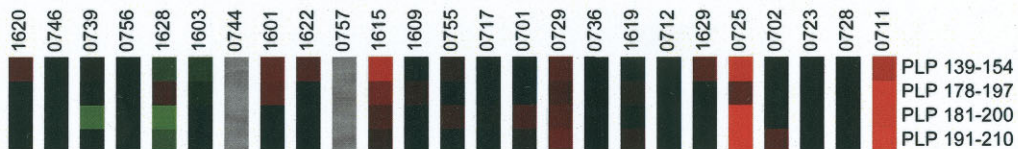


Figure 2

immune tolerance. The greater dose was ineffective. These data support the observation that increased MBP reactivity is associated with worsening of MRI-related activity in MS.⁷ From that observation, one might predict that tolerizing to MBP should reduce MRI activity, as was demonstrated in this clinical trial. Furthermore, there were no safety or tolerability issues of concern with this dose. The data on the high anti-MBP-reactive patients suggest that we may be able to identify those patients who will experience the greatest benefit to BHT-3009. These results justify additional clinical studies of BHT-3009 in larger trials to examine whether this approach can provide clinically relevant benefit, for example, in relapse rate reduction. If so demonstrated, BHT-3009 could play a substantial role in the treatment of MS by providing a method of treating a fundamental cause of the disease, the myelin-specific autoimmune response. Of potential additional importance, this approach represents a platform for tolerizing inflammatory lymphocytes in other autoimmune diseases and in transplant rejection.

Appendix

In addition to the authors, the following are members of the BHT-3009 Study Group: Ivan Milanov and Dimitar Georgiev (Specialized Hospital for Active Treatment in Neurology and Psychiatry “Sv Naum,” Sofia, Bulgaria); Penko Shotekov (Multiprofile Hospital for Active Treatment “Alexandrovska,” Sofia, Bulgaria); Paraskeva Stamenova (Multiprofile Hospital for Active Treatment “Tzaritza Yoanna,” Sofia, Bulgaria); Slobodan Vojinovic (Clinical Centre of Nis, Department of Neurology, Bulevar Zorana, Serbia); Petr Kanovsky (Neurologicka klinika FN, Olomouc, Czech Republic); David Dolezil (Neurologicka klinika FN, Ostrava, Czech Republic); Edvard Ehler (Neurologie KN Pardubice, Czech Republic); Otakar Keller (Neurologie FTNsP Prague, Czech Republic); Pavel Stourac (Neurologicka klinika FN Brno, Czech Republic); Juha-Pekka Erälänna (Suomen Erkikois-

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◀ Fig 2. Treatment with 0.5mg BHT-3009 is associated with a reduction in anti-myelin antibody titers. Myelin array analysis was performed to quantify anti-myelin peptide antibodies in baseline and posttreatment samples in patients treated with 0.5mg BHT-3009 (A, C), 1.5mg BHT-3009 (B, C), or placebo. Antibodies with pretreatment to posttreatment changes within each treatment group are represented either as fold change in intensity (A, B) or as magnitude change (C, D), with increases false colored red, no change false colored black, and decreases false colored green. Gray represents samples with no data available. Significance analysis of microarrays (SAM) was applied to identify statistical differences in antibody reactivity between the pretreatment and posttreatment samples, and identified 23 myelin peptides (listed to right of heat map) that exhibited overall significant changes (all were decreases) in the posttreatment as compared with pretreatment CSF samples in patients treated with 0.5mg BHT-3009 (false discovery rate, $q < 0.1$). The same analysis identified four anti-proteolipid protein (PLP) antibodies that exhibited statistical changes (all were increases) after treatment with 1.5mg BHT-3009. There were no statistically significant differences between prereactivity and post-reactivity in placebo-treated patients. MBP peptide sequences are derived from and numbered based on the 18.5 kD isoform (genbank accession number AAA59562), with the exception of peptides denoted by “*” which are numbered based on the 21.5 kD isoform (genbank accession number AAA59564). MOG = myelin oligodendrocyte glycoprotein; MOBP = myelin-associated oligodendrocytic basic protein; OSP = oligodendrocyte-specific protein.

Romford, Essex, United Kingdom); Basil Sharrack (Royal Hallamshire Hospital, Neurology Department, Sheffield, Syorks, United Kingdom); Lloyd Kasper (Dartmouth-Hitchcock Medical Center and Dartmouth College, Lebanon, NH).

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