The Role of Adjuvants in the Efficacy of a Peptide Vaccine for Myasthenia Gravis

JAMES L. MCANALLY, LIKANG XU, MATTEO VILLAIN, AND J. EDWIN BLALOCK[1]

Department of Physiology and Biophysics and the Center for Neuroimmunology, University of Alabama at Birmingham, Birmingham, Alabama 35294

Myasthenia gravis (MG) and its animal model, experimental autoimmune (EA) MG, are caused by interference with neuromuscular transmission by autoantibodies against the nicotinic acetylcholine receptor (AChR) on muscle. Previously, we have shown that two peptides, denoted RhCA 67-16 and RhCA 611-001, designed to be complementary in structure to the main immunogenic region and the dominant Lewis rat T cell epitope (α-chain residues 100–116) of the AChR, respectively, are effective vaccines that prevent EAMG in rats by inducing anti-idiotypic/clonotypic antibodies (Ab) and lowering levels of AChR Ab. These studies employed keyhole limpet hemocyanin (KLH) as a carrier and complete Freunds adjuvant (CFA). In advance of a clinical trial the present study tested the efficacy of RhCA 611-001 when combined with different adjuvants that are approved for use in humans. Adjuvants chosen for comparison were incomplete Freunds adjuvant (IFA) and aluminum hydroxide (Alum). As a second goal we evaluated diphtheria toxin (DT) as an alternative carrier protein to KLH. Alum was found to be an effective adjuvant, particularly when used with the peptide conjugated to DT. This combination of carrier and adjuvant provided protection against EAMG comparable with that observed with CFA and KLH. Using enzyme-linked immunosorbent assays for Ab against RhCA 611-001, it was found that disease protection is qualitatively, but not quantitatively, related to the anti-peptide Ab response. Our results demonstrate a vaccine formulation that should be useful in the first soon-to-be-conducted clinical trials of peptide vaccines to specifically correct aberrant T and B cell responses in an autoimmune disease. [Exp Biol Med Vol. 226(4):307–311, 2001]

Key words: adjuvant; complementary peptide vaccine; myasthenia gravis; autoimmunity

Myasthenia gravis (MG) and its animal model, experimental autoimmune (EA) MG, are caused by the production of T cell-dependent autoantibodies against the nicotinic acetylcholine receptor (AChR) on muscle cells (1, 2). This aberrant immune response leads to a decrease in the number of available AChRs at the postsynaptic muscle membrane, which manifests itself as the fatigability and weakness that characterize this debilitating disease. Although current therapy can strikingly improve the prognosis of patients with MG, this therapy involves the use of global immunosuppressive agents, the side effects of which are very deleterious. In contrast to global immunosuppression, an ideal therapy for any autoimmune disease would be one that specifically corrects the errant immune response.

In theory, anti-idiotypic (Id) antibodies (Ab) reactive with disease-causing Id Ab or clonotypic T cells represent ideal therapeutic agents. In practice, however, it is difficult to know the appropriate Id Ab or clonotypic T cell to use for the induction of anti-Id Ab because disease-causing Abs and T cells are usually of oligoclonal origin at best. We have previously reported a technique that circumvents this problem by actively inducing anti-Id Ab with a peptide immunogen rather than Id Ab or cloned T cells (3, 4). This approach is based on accumulating evidence that suggests that the gross architecture of a peptide or protein is in part determined by its pattern of amino acid hydropathy. Consequently, inverting a particular hydropathy pattern can result in a second peptide or protein with a complementary surface contour to the first since the hydrophobic effect is involved in reversed orientation (5, 6). Such peptides with inverted hydropathic patterns are termed complementary peptides and have characteristics expected of complementary structure. For instance, we, and many others, have shown that in almost 40 different systems complementary peptides bind one another with specificity and moderate affinity (6, 7). There is also substantial additional evidence of such complementary structure. This includes the ability to locate the interactive sites of ligands and receptors by identification of complementary sequences (8–12) and the ability to generate interacting pairs of monoclonal idiotypic and Id Ab with complementary combining sites by immunization with

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[1] To whom requests for reprints should be addressed at University of Alabama, Department of Physiology and Biophysics, 1918 University Blvd., MCLM 896, Birmingham, AL 35294–0005. E-mail: blalock@physiology.uab.edu

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pairs of complementary peptides (3, 4, 13–20). In addition, Abs to ligand-binding sites on receptors have been obtained by immunization with complementary peptides for the receptor's ligand and novel hormone receptors have been cloned with their binding sites located using this principle (12, 21–27). Most recently, the algorithm of inversion of hydropathy patterns was used to target a *de novo* designed peptide to a predetermined site on a protein (6). At the site of interaction, the protein/peptide complex had complementary surface contour.

The immunologic characteristics of complementary peptides have provided the rationale for the design of efficacious vaccines for models of autoimmune disease (18, 28). Of particular interest are two complementary peptides that target both the B cells and the T cells responsible for the aberrant autoimmune response in MG. The B cell vaccine, denoted RhCA 67-16, contains a peptide sequence complementary to residues 61 to 76 of the AChR α-subunit, an epitope shown to be the main immunogenic region (MIR). The T cell vaccine, denoted RhCA 611-001, contains a complementary peptide to α-chain residues 100 to 116, an epitope shown to be the dominant Lewis rat T cell epitope. Both peptides have been shown to be effective vaccines that prevent EAMG in the rat by eliciting anti-Id/clonotypic Abs, which lower AChR Ab levels (18, 28). These studies in Lewis rats employed keyhole limpet hemocyanin (KLH) as a carrier and complete Freunds adjuvant (CFA).

In addition to the rat model of EAMG, naturally occurring autoimmune MG has been described in dogs. Canine MG is very similar to human MG in clinical signs, methods of diagnosis, and modes of therapy (29, 30). Preliminary studies have shown profound functional improvement and return of AChR Ab levels to normal in existing cases of canine MG after immunization with RhCA 67-16 (B cell vaccine) and RhCA 611-001 (T cell vaccine) (Blalock JE, unpublished data). In these canine studies the T and B cell vaccines were conjugated to KLH, and both vaccines utilized TiterMax as an adjuvant.

In anticipation of clinical trials and considering that neither CFA nor TiterMax can be used in humans, this study evaluates the efficacy of RhCA 611-001 when administered to test animals with human-approved adjuvants. Adjuvants chosen for comparison were alum, CFA, and incomplete Freunds adjuvant (IFA). We also evaluated diphtheria toxoid (DT) as an alternative carrier protein to KLH. The efficacy of each combination in preventing EAMG was compared with the previously established success of using RhCA 611-001 with KLH as a carrier protein in CFA.

Materials and Methods

Acetylcholine Receptor. AChR was purified from *Torpedo californica* electroplax organs (Pacific BioMarine, Venice, CA) by using detergent solubilization and affinity chromatography on cobra toxin-Sepharose 4B as described by Froehner and Rafto (31). The affinity column was prepared by coupling 50 mg of *Naja naja siemensis* toxin to Sepharose 4B (32). Purified AChR was analyzed for the presence of four intact subunits by SDS-PAGE and was quantified by binding ^{125}I-α-bungarotoxin (α-BGT; Amersham, IL). The specific binding activity of this preparation was 5.8 pmol ^{125}I-α-BGT bound per 1 μg of protein. Excessive Triton X-100 was removed from the purified AChR by passing through an Extracti-Gel D column (Pierce, IL) and purified AChR was stored at −80°C until it was used.

Peptide Synthesis. RhCA 611-110 was synthesized on a Biosearch Peptide Synthesizer (model 9500) using f-moc chemistry and was purified by reverse-phase high performance liquid chromatography. Purified peptide was then conjugated to either KLH or DT as a carrier peptide.

Immunization. Female Lewis rats, age 6 weeks, were obtained from the Charles River Laboratories (Wilmington, MA). After a 1-week period of rest, the rats began a series of three immunizations with RhCA 611-001 conjugated to either KLH or DT, emulsified in one of the three adjuvants. The immunizations were given at 2-week intervals, each consisting of two 25-μg subcutaneous injections along the back and at the base of the tail. In the groups using CFA, the second and third immunizations replaced CFA with IFA. The control group was immunized with only phosphate-buffered saline (PBS) emulsified in CFA for the first immunization, and in IFA for the second and third immunizations. One week after the second peptide immunization, the rats were given the first challenge with purified native Torpedo AChR in TiterMax (CytRx Corporation, Norcross, GA). This first challenge was repeated twice for a total of three challenges, each separated by 2 weeks. Each AChR challenge was administered as 10 μg injected to both hind footpads (5 μg per hind footpad).

Clinical Scoring. In previous studies, a clear correlation has been established between clinical presentation of the disease and objective measures such as concentration of disease-causing Ab and concentration of AChR on muscle (28). With this in mind, clinical scoring was used to evaluate the efficacy of the different adjuvants and carrier proteins in this experiment. Rats were observed daily. Clinical signs were scored on a scale ranging from 0 for normal, 1 for weak grip and cry, to 3 for severe generalized weakness. A score of 4 indicates the death of an animal (33).

Ab Response. Ab against the RhCA 611-001 peptide was determined by indirect ELISA. A positive response was defined as greater than two standard deviations above the mean value measured in 10 rats before immunization.

Statistical Analysis. The level of significance of the differences of the average clinical severities between each group and the PBS control group was measured by the two-tailed Student's *t* test.

Results

Clinical measurements of disease severity indicate that IFA is less effective in eliciting protection when compared with CFA (Table I). Alum, however, was found to be an

Table I. Prevention of AChR-Induced EAMG by Complementary Peptide Vaccine RhCA 611-001 with Different Combinations of Carrier Protein and Adjuvant

Group	Number of rats with EAMG/total rats (%)	Clinical severity					Average severity	% mortality
		0	+	++	+++	++++		
RhCA611-001-KLH in CFA	4/10 (40)	6	3	1			0.50 ± 0.22***	0
RhCA611-001-KLH in IFA	6/7 (86)	1	4		1	1	1.57 ± 0.53	14
RhCA611-001-KLH in Alum	6/10 (60)	4	4	1	1		0.90 ± 0.31*	0
RhCA611-001-DT in CFA	4/10 (40)	6	3		1		0.60 ± 0.31**	0
RhCA611-001-DT in IFA	8/9 (89)	1	6	1		1	1.33 ± 0.37	11
RhCA611-001-DT in Alum	5/10 (50)	5	5				0.50 ± 0.17***	0
PBS	10/10 (100)		5	1	2	2	2.10 ± 0.41	20

Note. Clinical signs were scored on a scale ranging from 0 for normal, 1 for weak grip and cry, to 3 for severe generalized weakness. A score of 4 indicates the death of an animal. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.005$ compared with PBS control as determined by Student's t test.

effective adjuvant, particularly when used with the DT-conjugated peptide. This combination of carrier and adjuvant provided protection against EAMG comparable with that observed with CFA and KLH. Indeed, these combinations resulted in decreased incidence, severity, and mortality associated with the disease.

Enzyme-linked immunosorbent assay was used to measure the Ab response against RhCA 611-001. In each animal group, at least 80% of the test animals showed a measurable Ab response during the course of the experiment (Fig. 1). The groups immunized with DT-conjugated peptides showed a faster response, with 80% of each group showing a measurable Ab response by 3 weeks, and 100% by 5 weeks. In comparison, although 80% of the animals in each group receiving KLH-conjugated peptide did ultimately demonstrate an Ab response, this response was slower to develop. It is tempting to speculate that the faster peak response to DT/alum compared with KLH/alum might be responsible for the slightly greater efficacy of the former (Table I).

Despite the high prevalence of measured antibody response in all groups, disease severity varied both between and within animal groups (Table I). From this it was concluded that disease protection is qualitatively, but not quantitatively, related to the anti-peptide Ab response. This may be related to the ability of some, but not all, adjuvant/carrier combinations to elicit a good anti-Id Ab response (34).

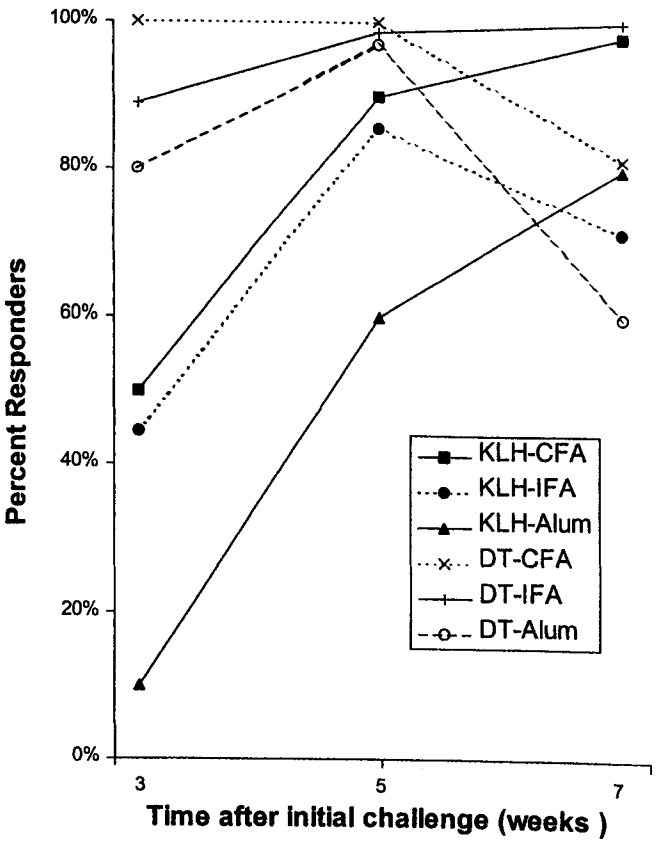

Figure 1. Percentage of animals from each group showing a measurable antibody response to immunization. Positive response was measured by indirect ELISA and was defined as greater than two standard deviations above the mean value measured in 10 rats before immunization.

Discussion

As one of the most well-defined forms of autoimmune disease, MG has been extensively studied as a model for autoimmune disorders. Despite great advancements in the understanding and treatment of autoimmune diseases, to date none of these diseases has ever been cured. Many different strategies have been researched regarding the use of vaccines in the treatment of autoimmune diseases, with some proving more effective than others (35–38). However, none involve the present technique of actively eliciting an endogenous anti-Id Ab response.

Because at present we can not predict who will develop MG or any other autoimmune disease, an effective vaccine must be therapeutic and not merely preventative. A therapeutic effect of the current strategies has been measured as a reversal of clinical disease progression and a reduction in disease-causing Ab levels in both the rat model of EAMG (28; Xu L, Villain M, Galin FS, Araga S, Blalock JE, unpublished data), as well as in naturally occurring canine MG (Blalock JE, unpublished data). Each of these studies clearly demonstrates benefit after the onset of disease.

If the vaccines discussed above prove successful in

human trials, they could potentially represent the first true cure for an autoimmune disease. Moreover, the methodology of using inverted hydropathy in the development of epitope-specific peptide vaccines could serve as a template for the correction of other autoimmune disorders. Indeed, this seems to be borne out by the recent successful use of this approach to treat an animal model of Giullain-Barre' syndrome (39).

The results of these experiments support the conclusion that these novel peptide vaccines are effective when used with human-compatible adjuvants. This conclusion represents one of the final steps in the preparation for the first soon-to-be-conducted clinical trials of peptide vaccines designed to specifically correct aberrant T and B cell responses in an autoimmune disease. In a more general sense they point to the importance of a particular adjuvant and carrier in determining peptide vaccine efficacy as opposed to simply eliciting an Ab response. In this regard it is interesting to note that DT/alum gave a faster peak response than KLH/alum and DT/alum was slightly more effective against EAMG.

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