A peptide vaccine that prevents experimental autoimmune myasthenia gravis by specifically blocking T cell help

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ABSTRACT **Myasthenia gravis (MG) and its animal model, experimental autoimmune (EA) MG, are caused by T cell-dependent autoantibodies that react with the nicotinic acetylcholine receptor (AChR) on muscle and interfere with neuromuscular transmission. Thus, selective inactivation of $CD4^+$ AChR-specific T helper cells should lower AChR Ab levels and ameliorate disease. In the Lewis rat model of EAMG, α chain residues 100–116 of the AChR represent the dominant T cell epitope, which is important in helping Ab responses to this autoantigen. In the present report, we have applied a new design technique that requires no knowledge of Ag receptor sequences on errant T cells in order to develop a synthetic peptide vaccine against T cells reactive with the aforementioned T cell epitope. Immunization with the peptide *1)* induced polyclonal and monoclonal Ab, which inhibited AChR 100–116 stimulation of AChR-sensitized lymphocytes and recognized Vβ15 containing T cell receptors on AChR 100–116-specific T cell lines and clones; *2)* lowered AChR Ab levels; *3)* reduced the loss of muscle AChR; and *4)* lessened the incidence and severity of EAMG. These findings suggest a new strategy for the functional abrogation of epitope-specific T cells that could have potential application to human autoimmune diseases.—Araga, S., Xu, L., Nakashima, K., Villain, M., Blalock, J. E. A peptide vaccine that prevents experimental autoimmune myasthenia gravis by specifically blocking T cell help. *FASEB J.* 14, 185–196 (2000)**

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In contrast to commonly used global immunosuppressive techniques with their deleterious side effects, 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 such specific 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/clonotypic Ab, and the use of the resulting monoclonal (m) anti-Id Ab in patients often requires 'humanization.' We have reported a technique that overcomes these problems by actively inducing anti-Id Ab with a peptide immunogen rather than Id Ab (1, 2). Furthermore, this procedure requires no *a priori* knowledge of Id Ab sequence, only delineation of the disease-causing epitope. The design of such peptide immunogens rests with an evolving view of the role of the pattern of amino acid hydropathy in protein and peptide structure.

Although the rules governing the folding and interaction of peptides and proteins are complex and have not yet been fully deciphered, accumulating evidence suggests that a simple binary code of polar and nonpolar amino acids arranged in the appropriate order is an important driving force for gross shape and rudimentary function (for review, see ref 3). Only the sequence location, not the identity, of the polar and nonpolar amino acids must be explicitly specified for the formation of a stable structure or biologically active peptide. Such coding has been successfully used to produce biologically active analogs of corticotropin (ACTH) and growth hormone-releasing hormone (4, 5), to design proteins that fold into compact 3-helical structures (6), and to develop computer programs that simulate or predict some aspects of protein folding (7). Considering that 20 different amino acids are encompassed by the binary code, one would expect a marked degree of sequence degeneracy for a given shape, since any one of a number of specific polar or nonpolar amino acids could occupy a given position

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in the sequence. Indeed, experimental evidence has confirmed that gross shape is degenerate with regard to sequence in that any number of different primary amino acid sequences with the same binary code can fold into compact α-helical structures (6).

If the gross shape of a peptide or protein is determined by its binary code, then exactly inverting a particular binary code may result in inverted or complementary shape because the same driving force is involved, yet in reversed orientation (for review, see ref 3). Inversion of the binary code of one sequence relative to another can be achieved by computer programs designed for this task (8–10) or by simple reliance on an interesting characteristic of the genetic code (11, 12). In the latter instance, since A and U are complementary and in the second codon position specify hydrophilic and hydrophobic R groups, respectively, amino acid sequences deciphered from noncoding strands of DNA will have exactly inverted patterns of hydropathy relative to those of coding strands (for review, see ref 3).

Such peptides specified by complementary nucleotide sequences (13) or designed by simply inverting the hydropathic pattern (9) are termed complementary or antisense peptides and have characteristics suggestive of complementary structure. For instance, in almost 40 different systems, complementary peptides were observed by us and others to bind one another with high specificity and moderate affinity (for review, see ref 3, 14). Additional evidence of complementary structure includes using this concept to locate the interactive sites of ligands and receptors by identification of complementary sequences or inverted patterns of hydropathy (15–20), to generate interacting pairs of monoclonal idiotypic (Id) and anti-Id antibodies (Ab) with complementary combining sites by immunization with pairs of complementary peptides (1, 2, 21–28), to produce Ab to receptor binding sites by immunization with complementary peptides for the receptor's ligand (1, 2, 12, 13, 29–37), and to design novel ligands (38, 39). Most recently, novel hormone receptors have been cloned and their binding sites localized using this principle (17, 18).

One of the aforementioned methods of exploring the structural relationship between complementary peptides has used Ab (1, 2, 21–28, 35, 40–42). This approach was based on the premise that if complementary peptides have complementary shapes, then they in turn should induce the formation of interacting pairs of Id and anti-Id Abs whose combining sites are complementary. Using different complementary peptide pairs, polyclonal as well as monoclonal, Id and anti-Id Abs have now been generated using this procedure (1, 2, 21–28, 35, 40–42). In a comparative study, the sole distinguishing characteristics between anti-Id Abs produced by this technique vs. classical immunization with Id Ab was the absolute number of hybridoma clones secreting Ab, and consequently the total number secreting anti-Id Ab (22). This may reflect the better immunogenicity of the complementary peptide compared to the syngeneic Ab. Monoclonal anti-Id Abs produced by this methodology have also been shown to suppress hybridoma production of Id Ab *in vitro* (22). These immunological characteristics of complementary peptides provided the rationale for the design of an efficacious vaccine for a model of an autoimmune disease (26).

Specifically, myasthenia gravis (MG) and its animal model, experimental autoimmune (EA) MG, are caused by interference with neuromuscular transmission by auto-Ab against the nicotinic acetylcholine receptor (AChR) on muscle (43, 44). Elucidation of the amino acid residues (α61–76) of the main immunogenic region (MIR) of the AChR α subunit against which EAMG-inducing Ab are directed afforded a target for our approach (45, 46). We found that active immunization with a peptide specified by a nucleotide sequence complementary to that of the MIR, and thus having an inverted binary code, induced the expected polyclonal and monoclonal anti-Id Ab, lowered AChR Ab levels, and prevented the development of EAMG (26). Thus, the use of such a peptide vaccine circumvented the dilemma as to which AChR Id Ab to use as an antigen (Ag), and because of active immunization with the peptide, likely caused an anti-Id Ab response that matches the complexity of the Id Ab response to the MIR itself.

The production of high-affinity immunogobulin G (IgG) isotype anti-AChR Ab requires specific T cell help via recognition of AChR peptides in association with MHC class II molecules (47). Selective inactivation of disease-specific $CD4^+$ lymphocytes therefore represents another means of intervention in auto-Ab production in MG and EAMG. In fact, treatment with anti-CD4 or anti-MHC class II Ab can prevent or ameliorate EAMG (48, 49). Although more specific than immunosuppressive agents such as steroids or cyclosporine, these Abs still lack a desired degree of specificity. As a test of whether our approach would be successful with a T cell epitope and result in Ab reactive with AChR-specific T cell receptors (TCR), we have designed a peptide (termed RhCA 611–001) with an inverted pattern of hydropathy relative to that of the immunodominant T cell epitope (AChR α chain residues 100–116) in the Lewis rat (50) (**Fig. 1**). Immunization with this peptide *1)* induced polyclonal and mAb, which inhibited the proliferation of AChR-sensitized lymphocytes stimulated with AChR 100–116 peptide and recognized a Vβ15-containing TCR on AChR 100–116-specific T cell lines and clones; *2)* lowered AChR Ab levels; *3)* reduced the

A

AChR 100-116 NH_2-F A I V H M T K L L L D Y T G K I

+RNA 5'- TTT-GCC-ATT-GTT-CAC-ATG-ACC-AAA-CTG-CTT-TTG-GAT-TAT-ACG-GGA-AAA-ATA-3'
-RNA 3'-AAA-CGG-TAA-CAA-GTG-TAC-TGG-TTT-GAC-GAA-AAC-CTA-ATA-TGC-CCT-TTT-TAT-5'

RhCA 611-001 K G N N V H G F Q K Q I I R S F Y-NH_2

B

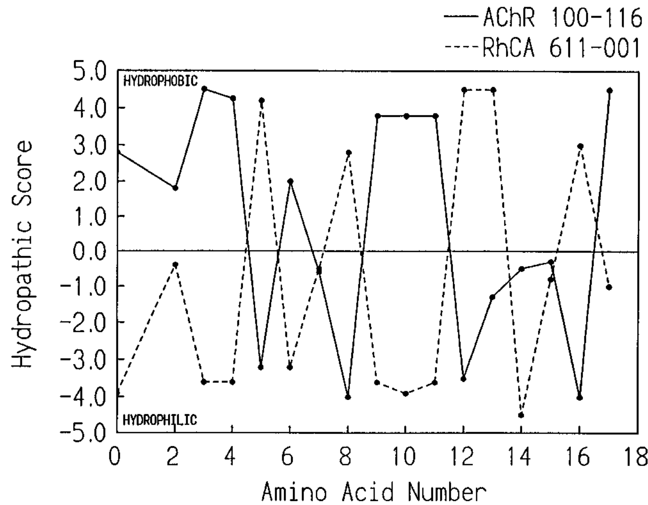

Figure 1. *A)* Nucleotide and amino acid sequences of *Torpedo* AChR 100–116 and RhCA 611–001. *B)* Comparison of the hydropathy plots of AChR 100–116 and RhCA 611–001.

loss of AChR; and *4)* blocked the development and lessened the severity of EAMG.

MATERIALS AND METHODS

Acetylcholine receptor

AChR was purified from *Torpedo californica* electroplax organs (Pacific Bio-Marine, Venice, Calif.) by using detergent solubilization and affinity chromatography on cobra toxin-Sepharose 4B as described by Froehner and Rafto (51). The affinity column was prepared by coupling 50 mg of *Naja naja siemensis* toxin to Sepharose 4B (52). Purified AChR was analyzed for the presence of four intact subunits by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and was quantified by binding ^{125}I-α-bungarotoxin (α-BGT) (Amersham, Arlington Heights, Ill.). The specific binding activity of this preparation was 5.8 pmol ^{125}I-α-BGT bound per gram of protein. Excessive Triton X-100 was removed from the purified AChR by passing through an Extracti-Gel D column (Pierce, Rockford, Ill.) and purified AChR was stored at −80°C until used.

Measurement of muscle AChR content

After removal of skin, head, paws, tail, viscera, and fat, rat carcasses were weighed and frozen on dry ice and stored at −70°C. For determination of AChR content, the carcasses were minced and homogenized in 250 ml of 10 mM Na phosphate pH 7.5, 100 mM NaN_3, 10 mM iodoacetamide, and 1 mM phenymethylsulfonyl fluoride (buffer A) in a blender. A 20 g sample of the homogenate was centrifuged at 30,000 × *g* for 30 min and the resulting pellet was resuspended in 15 ml buffer A with 2% Triton X-100. Detergent extraction was performed for 4 h at 4°C on a shaker. After centrifugation, the volumes of supernates were measured and 250 μl/tube in triplicate of each sample supernatant was labeled with 0.35 μl of ^{125}I-α- BGT (Amersham, IM 109, 100 μCi/200 μl, 200 Ci/mmol) overnight at 4°C. Parallel aliquots were labeled in the presence of 2×10^{-3} M each of acetylcholine chloride and neostigmine bromide to block specific binding. Each tube was incubated with 5 g/ml each of two mAb (mAb35 and JEB4) against AChR. After overnight incubation, 200 g/ml goat-anti-rat IgG (Sigma, R 5005) was added and incubated for 4 h at 4°C. After centrifugation for 30 min, the precipitate was washed once with 1 ml phosphate-buffered saline (PBS) containing 0.5% Triton X-100, and 0.02% NaN_3. Precipitates were counted in a gamma counter. The percentage of AChR loss was calculated by comparison of picomoles of ^{125}I-α-BGT precipitated/20 g muscle in *Torpedo* AChR-immunized animals with that in nonimmunized controls.

Peptide synthesis

AChRα 100–116, AChRα 61–76, and RhCA 611–001 were synthesized on a Biosearch Peptide Synthesizer, Model 9500, using f-moc chemistry and were purified by reverse-phase high performance liquid chromatography. An octameric form of RhCA 611–001 was synthesized by the same methods as above except for using the multiple antigenic peptide (MAP) resin $Fmoc_8Lys_4$-Lys_2- Cys(Acm)-β Ala WANG resin (AminoTech, Ontario, Canada). MAP peptide denoted PBM 9–1 was similarly synthesized, purified, and used as a control. PBM 9–1 has an inverted binary code relative to the first nine residues of human myelin basic protein (MBP) and has the sequence NH_2-Arg-Ser- Leu-Leu-Ser-Gly-Gly-Leu-Pro-NH_2 (40).

Immunization

Female Lewis rats, age 6 wk, were obtained from the Charles River Laboratories (Wilmington, Mass.). Before challenging

with purified native *Torpedo* AChR, rats were preimmunized twice (with a 2 wk interval in between) with 50 g/injection of the MAP forms of RhCA 611–001 or PBM 9–1 or with PBS. The primary injection was in complete Freund's adjuvant (CFA) and the booster was in incomplete Freund adjuvant (IFA). Equivalent results were obtained using TiterMax (CytRx Corporation, Norcross, Ga.). One week after the last MAP peptide injection, rats were challenged with various doses of purified native *Torpedo* AChR as described previously (26).

Clinical scoring

Rats were observed daily and weighed three times a week. Clinical signs were scored by a certified clinical neurologist on a scale ranging from 0 for normal: 1 for weak grip and cry to 3 for severe, generalized weakness (53). Serum was collected from the tail vein under ether anesthesia and samples were stored at −20°C until used.

Antibody assay

Ab against the *Torpedo* AChR was determined by an indirect enzyme-linked immunoassay (ELISA) after coating ELISA plates with 1 μg/ml AChR (26). Ab titers were calculated by linear regression estimation from a dose response curve using 0.5 $\log_{10}$ dilutions of sera.

Preparation of rat polyclonal Abs to RhCA 611–001 or PBM 9–1

Three 10-wk-old female Lewis rats/group were immunized (100 μg/rat) with the MAP forms of the peptides, RhCA 611–001 or PBM 9–1, emulsified with an equal volume of CFA, followed by three immunizations with octameric forms of MAP RhCA 611–001 or MAP PBM 9–1 (emulsified with an equal volume of IFA) at 3 wk intervals. Ab titer was checked by an indirect ELISA as above for AChR except that plates were coated with 10 μg/ml of peptides. Blood was collected by cardiac puncture under ether anesthesia. Sera were pooled, divided into several tubes, and stored at −20°C until used.

mAb

An mAb (denoted CTCR8, IgG 2b/κ) was obtained from rats immunized with MAP RhCA 611–001 according to published procedures (54). Rat spleen cells were fused with mouse myeloma cell line X63 Ag8.653 (kindly provided by Dr. J. F. Kearney, University of Alabama at Birmingham) by using a 50% polyethylene glycol solution (Sigma, St. Louis, Mo.). Hybridoma cells were selected for monoclonality by three serial limiting dilutions and grown in protein-free media (PFHM-II, Life Technologies). CTCR8 had reactivity against monomeric as well as octameric RhCA 611–001. A control mAb, F28C4 (IgG-2a/λ), against human MBP 1–9 was kindly provided by Drs. John N. Whitaker and Shan-Ren Zhou (University of Alabama at Birmingham) and has been described (40). MAbs were purified by ammonium sulfate precipitation from protein-free medium followed by dialysis against PBS.

Sensitized T cell preparation

Lewis rats (female, 8 wk old) were immunized into the rear foot pads with 50 μg of purified native *Torpedo* AChR emulsified with an equal volume of CFA. Two weeks after immunization, rats were killed under ether anesthesia; lymph nodes were then removed aseptically. The lymphocytes were collected by passing through a stainless mesh. The tissue debris and dead cells were removed by Ficoll-Hypaque centrifugation and resuspended in RPMI 1640, 10% fetal calf serum, 5×10^{-5} M 2-mercaptoethanol, 100 unit/ml penicillin, 100 mg/ml streptomycin, 0.25 mg/ml fungizon, 10 mM HEPES pH 7.0, 10 mM nonessential amino acids, 20 mM L-glutamine, and 10 mM sodium pyruvate. Macrophages were depleted by using a plastic plate adherence method.

Irradiated spleen cell preparation

Normal Lewis rats (8 wk old) were killed and spleens were removed aseptically. The splenocytes were collected by Ficoll-Hypaque centrifugation and suspended in the above media. The irradiated splenocytes (3000 rad) were incubated with 10 μg/ml of AChR 100–116 peptide for 1 day. After washing twice with complete media, the irradiated splenocytes were used as educated or peptide-loaded, antigen-presenting cells (APC). In all experiments, APC, which were not peptide loaded and mixed with sensitized lymphocytes, gave [^{3}H]TdR levels that were 1–2% of APC loaded with AChR 100–116.

Inhibition of AChR-sensitized lymphocytes by anti-RhCA 611–001 Ab

The sensitized T cells (2×10^5/well) and educated APC (5×10^{5}/well) were coincubated with the indicated concentrations of either anti-PBM 9–1 serum or anti-RhCA 611–001 serum for 4 days at 37°C in 5% CO_2 incubator. Cultures for proliferation assay were harvested after 4 days, with the final 16 h composed of pulse labeling with 0.5 μCi/culture of [^{3}H]TdR (Amersham).

Inhibition of AChR-sensitized lymphocytes by mAb CTCR8

To test the inhibition of proliferation by mAb CTCR8, the following two experiments were done. *1)* Educated APC (5×10^5/well) and sensitized lymphocytes (2×10^5/well) were coincubated with the indicated concentrations of mAb CTCR8 or F28C4 for 3 days, followed by the aforementioned proliferation assays. *2)* Before coincubation of educated APC and sensitized lymphocytes, educated APC or sensitized lymph node cells were preincubated with the indicated concentration of mAb CTCR8 or F28C6 for 1 h at room temperature, washed with complete media twice, followed by the proliferation assay.

***Torpedo* AChR 100–116-specific T cell line**

This protocol followed previously published techniques (25, 55). Lewis rats were immunized subcutaneously (s.c.) with 50 μg of purified native *Torpedo* AChR emulsified with an equal volume of CFA. Nine days later, their draining lymph nodes were removed and single-cell suspensions were made. The lymph node cells were stimulated in the presence of 200 μg/ml *Torpedo* AChR 100–116 peptide and irradiated APC for 3 days, followed by several days coincubation with irradiated APC alone. After three such passages, the T cell line was expanded with AChR 100–116 peptide and irradiated APC in the presence of 10% IL-2 without concanavalin A (Collaborative Research). A control T cell line that is ovalbumin specific was made in the same way only using immunization with ovalbumin (50 μg) s.c. in CFA.

Immunoblotting with CTCR8

AChR 100–116-specific T cells or ovalbumin-specific T cells were lysed in 10 mM Tris-HCl buffer, pH 7.4, containing 1% (w/v) Nonidet P-40 (Sigma), 150 mM NaCl, 1 mM diethylenediamine tetra-acetic acid (EDTA) supplemented with the protease inhibitors 2 μg/ml leupeptin (Boehringer Mannheim, Mannheim, Germany), 2 μg/ml aprotinin (Sigma), 4 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, hydrochloride (AEBSF; Boehringer Mannheim), and 20 M iodoacetamide (Sigma) (56). The lysate was then treated with streptavidin agarose (Life Technologies, Gaithersburg, Md.) to preclear the lysates. The precleared lysate was reduced with 100 mM dithiothreitol (DTT, Life Technologies). We used this preparation as total soluble membrane proteins. Part of the lysate of AChR 100–116-specific T cells was dialyzed against 10 mM Tris-HCl buffer, pH 7.4, containing 1% (w/v) Nonidet P-40, 150 mM NaCl, 1 mM EDTA to remove DTT. The lysate was immunoprecipitated with mAb R73 against rat TCRβ constant domain (57) (IgG1; Serotec, England) using agarose anti-mouse Ig (Sigma) to remove TCRβ molecules. We used this preparation as R73-precleared lysate. The total soluble membrane samples and R73-precleared lysate were analyzed on a 10% acrylamide gel and electrotransferred to polyvinyllidene difluoride membrane (Bio-Rad, Richmond, Calif.). After blocking with 10% skim milk in PBS, membranes were incubated with either biotin-labeled mAb R73 or biotin-labeled mAb CTCR8 Ab at the concentration of 10 μg/ml in PBS containing 0.1% Tween. After washing with PBS/Tween, membranes were incubated with alkaline phosphatase-labeled streptavidin. Finally, bands were detected with CDP-Star Western blot chemiluminescence reagent (DuPont, Wilmington, Del.) to expose to X-rays films. The blot of soluble membrane proteins from ovalbumin-specific T cells was checked by reactivity against either mAb R73 or mAb CTCR8. This was followed by an ordinary Western blotting procedures as mentioned above. For the Western procedures, a blotter-mini 10 apparatus (Cosmo Bio, Tokyo, Japan) was used to apply the mAbs. This apparatus has 10 slots to fit a 10-well comb. The blotted membrane is sandwiched between two acrylic plates so that different mAb can be applied to the same membrane.

Preparation and flow cytometric analysis of T cell clones

T cell clones were prepared using lymphocytes from the T cell line isolated on a Ficoll-Pack density gradient on the third day after stimulation with AChR 100–116-loaded, irradiated APC. T cells were cloned by limiting dilution and expanded by stimulation with antigen (200 μg/ml AChR 100–116) -loaded, irradiated APC (4×10^6 cells/ml) in the presence of rat T-STIM (10%) (Collaborative Research). Established clones were stored in liquid nitrogen until used (55). T cell clones were analyzed using FACScan (Becton Dickinson, Calif.) with propidium iodide (PI) and FITC-labeled anti-CD3 (W3/13), anti-CD4 (W3/25), or anti-CD8 (OX-8) (Serotec). For CTCR8 staining, T cell lines were stained with PI and biotin-CTCR8, followed by streptavidin-FITC. For biotinylation, a CTCR8 solution was oxidized with sodium metaperiodate (Sigma) at a concentration of 10 mM in labeling solution (0.1 M sodium acetate, pH 5.5) for 20 min at 4°C in the dark. The oxidation reaction was stopped by adding glycerol, followed by dialysis against labeling solution at 4°C overnight. Finally, biotin-LC-hydrazide (Pierce) was added at a final concentration of 5 mM for 2 h at room temperature. Biotin-labeled sample was dialyzed against PBS containing 0.1% sodium azide and stored at −4°C until used. All T cell lines were phenotyped to be $CD3^+$, $CD4^+$, and $CD8^-$ (data not shown). Several clones of the AChR 100–116-specific T cell lines were recognized by CTCR8 (**Table 1**).

TCR Vβ chain analysis

TCR Vβ chain usage was analyzed by the reverse transcription polymerase chain reaction (RT-PCR) method. RNA was prepared from cloned T cell lines with TRIZOL LS reagent (Life Technologies). cDNA was synthesized from total RNA using SuperScript II RNase H^- reverse transcriptase (Life Technologies) and a random hexamer. The cDNA was then amplified using an antisense Cβ and specific primers for Vβs (Vβ 1, Vβ 2, Vβ 3.3, Vβ 4, Vβ 5.1, Vβ 6, Vβ 7, Vβ 8.1, Vβ 8.2, Vβ 8.3, Vβ 9, Vβ 10, Vβ 11, Vβ 12, Vβ 13, Vβ 14, Vβ 15, Vβ 16, Vβ 17, Vβ18, Vβ19, and Vβ 20) (58) in a typical PCR reaction for a total of 40 cycles consisting of 94°C/1 min, 54°C/1 min, and 72°C/1 min, ending with a 10 min extension at 72°C. PCR products were size-selected using a 2% agarose gel. PCR products were directly ligated into pGEM T vector (Promega, Madison, Wis.). Inserted V-D-J products were sequenced with an AmpliCycle sequencing kit (Perkin Elmer, N.J.). V-D-J genes were identified by comparisons to previously published sequences in the EMBL-GDB (European Bioinformatics Institute) and LASL-GDB (GenBank, National Center for Biotechnology Information) using Genetic MaC/CD software (Software Development Co., Ltd., Tokyo, Japan). Rearranged V-D-J sequences of each T cell clone were determined by sequencing at least ten isolates.

Statistical analysis

All statistical analyses were done with a 2 × 2 G test, two-tailed *t* test, and Fisher's exact test.

RESULTS

Peptide vaccine design

The sequence of the peptide RhCA 611–001 was derived by 5′ to 3′ assignment of amino acids to the nucleotide sequence complementary to that of *Torpedo* AChR α chain mRNA encoding amino acids 100–116 (59), the immunodominant T cell epitope in the Lewis rats (50) (Fig. 1*A*). Figure 1*B* shows that the binary code or pattern of hydropathy of RhCA 611–001 is exactly inverted relative to that of the T cell epitope. To preclude coupling to a carrier protein as well as to test a peptide formulation that could potentially be used in humans, a MAP form of RhCA 611–001 was synthesized. MAP, which is essentially nonantigenic, consists of a core matrix made up of three levels of lysine with eight amino termini for subsequent synthesis of antigens. MAP resin coupled peptides have a molecular weight of more than 10,000 and can be used directly as immunogens.

Ab-mediated blockade of lymphocyte proliferation

Figure 2 shows that rat antisera to RhCA 611–001 caused a dose-dependent inhibition of the prolifer-

TABLE 1. *Vβ-Dβ-Jβ nucleotide and predicted amino acid sequences of AChRα 100–116-specific T clones*

Clone	Proliferative response to AChRα 100–116[a]	% CTCR8-stained T cells[b]	Vβ (usage)[c]	N/Dβ/N	Jβ (usage)
F3a	+	85	TGTGGTGCT (15)	AGAGACAGC	AATGAAAGATTGTTTTTCGGCCATGGAACC-AAGCTGTCTGTCCTG (1.4)
			C G A	R D S	N E R L F F G H G T K L S V L
E4a	+	91	TGTGGTGCT (15)	CGACAGGGGGCCGAC	ACAGAAGTTTTCTTTGGTAAAGGAACGAGA-CTGACAGTTGTA (1.1)
			C G A	R Q G A D	T E V F F G K G T R L T V V
G1a	+	72	TGTGGTGCT (15)	CGACAGGGGGCCGAC	ACAGAAGTTTTCTTTGGTAAAGGAACGAGA-CTGACAGTTGTA (1.1)
			C G A	R Q G A D	T E V F F G K G T R L T V V
C2	+	76	TGTGGTGCT (15)	AGAGACAGC	AATGAAAGATTGTTTTTCGGCCATGGAACC-AAGCTGTCTGTCCTG (1.4)
			C G A	R D S	N E R L F F G H G T K L S V L
D2	+	68	TGTGGTGCT (15)	CGACAGGGGGCCGAC	ACAGAAGTTTTCTTTGGTAAAGGAACGAGA-CTGACAGTTGTA (1.1)
			C G A	R Q G A D	T E V F F G K G T R L T V V
F3b	+	6	TGTGGTGCT (15)	AGGAACGGACAGGGC-CCC	CATGAGCAGTATTTCGGTCCCGGCACCAAG-CTCACGGTTTTA (2.7)
			C G A	R N G Q G P	H E Q Y F G P G T K L T V L
E4b	+	3	TGTGGTGCT (15)	AGCGAGGGGGGGGGT-ACT	ACAGACAAGATATATTTTGGCTCAGGAACC-AGACTGACCGTTCTC (2.3)
			C G A	S E G G G T	T D K I Y F G S G T R L T V L
G1b	+	4	TGTGGTGCT (15)	AGGGAAGGCAGGCCC	TATGACCAGTATTTCGGTCCCGGCACCAAG-CTCACGGTTTTA (2.7)
			C G A	R E G R P	Y D Q Y F G P G T K L T V L
C4	+	6	TGTGGTGCT (15)	AGCGAGGGGGGGGGT-ACT	ACAGACAAGATATATTTTGGCTCAGGAACC-AGACTGACCGTTCTC (2.3)
			C G A	S E G G G T	T D K I Y F G S G T R L T V L

[a] + indicates a stimulation index ≥ 3. [b] T cell clones are prepared using Ficoll-Pack density gradients on the third day after stimulation with AChR 100–116 peptide and irradiated APC. T cells were analyzed using FACScan flow cytometric analysis (Becton Dickinson) with propidium iodide (PI) and FITC-labeled anti-CD3 (W3/13), anti-CD4 (W3/25), or anti-CD8 (OX-8) (Serotec). All clones were $CD4^+$, $CD3^+$, $CD8^-$. For CTCR8 staining, T cells were stained with PI and biotin-CTCR8, followed by streptavidin-FITC. [c] TCR Vβ chain usage was analyzed by the RT-PCR method. RNA was prepared from cloned T cells with TRIZOL LS reagent (Gibco/BRL). The cDNA was synthesized from the total RNA preparation using SuperScript II RNASE H^- reverse transcriptase (Gibco/BRL) with random hexamers. cDNA was amplified using an antisense Cβ and specific primers for Vβs (Vβ1, Vβ2, Vβ3.3, Vβ4, Vβ5.1, Vβ6, Vβ7, Vβ8.1, Vβ8.2, Vβ8.3, Vβ9, Vβ10, Vβ11, Vβ12, Vβ13, Vβ14, Vβ 15, Vβ16, Vβ17, Vβ18, Vβ19, and Vβ20) in a typical PCR reaction for a total of 40 cycles consisting of 94°C/1 min, 54°C/1 min, and 72°C/1 min, ending with a 10 min extension at 72°C. PCR products were size-elected using a 2% agarose gel. PCR products were directly ligated into pGEM-T vector (Promega). Inserted-V-D-J products were sequenced with AmpliCycle sequencing kits (Perkin Elmer). V-D-J sequences were analyzed by comparison to previously published sequences in the EMBL-GDB (European Bioinformatics Institute) and LASL-GDB (GenBank, National Center for Biotechnology Information) using Genetic Mac/CD software (Software Development Co., Ltd., Tokyo, Japan). The V-D-J rearrangement for each T cell clone was determined by sequencing at least 10 recombinants.

ation of AChR-sensitized lymphocytes in response to a peptide representing AChR α chain residues 100–116 (termed AChR 100–116). The inhibition was apparently complement-independent since heat-inactivated antisera were equally effective (Fig. 2). Specificity of the inhibition was demonstrated by the inability of normal rat sera (data not shown) or rat antisera to a control MAP peptide (PBM 9–1) with an inverted hydropathic profile relative to MBP residues 1–9 to block AChR 100–116-induced proliferation.

To conclusively demonstrate that the peptide was able to induce Ab capable of blocking T lymphocyte proliferation in response to AChR 100–116, hybridomas were made from rats immunized with RhCA 611–001. One such hybridoma, CTCR8, secreted Ab reactive with monomeric as well as the MAP form of RhCA 611–001. CTCR8, but not an mAb to MBP 1–9 (F28C4), caused a dose-dependent inhibition of AChR 100–116-induced proliferation of AchR-sensitized lymphocytes (data not shown). To determine the cellular site of action of CTCR8, sensitized cells or AChR 100–116-loaded or educated APC were pretreated with the mAb. **Figure 3** shows that AChR-sensitized lymphocytes only respond to APC if the cells have been loaded with AChR 100–116. As previously demonstrated (47), the response to AChR 100–116 is specific and dominant since AChR 61–76-loaded APC cause minor proliferation. The response to AChR 100–116 is blocked by pretreatment of the sensitized lymphocyte, but not the APC, with CTCR8. This indicates that the responding T cell and not the APC is primarily affected by the mAb. Not unexpectedly, continuous treatment of the mix-

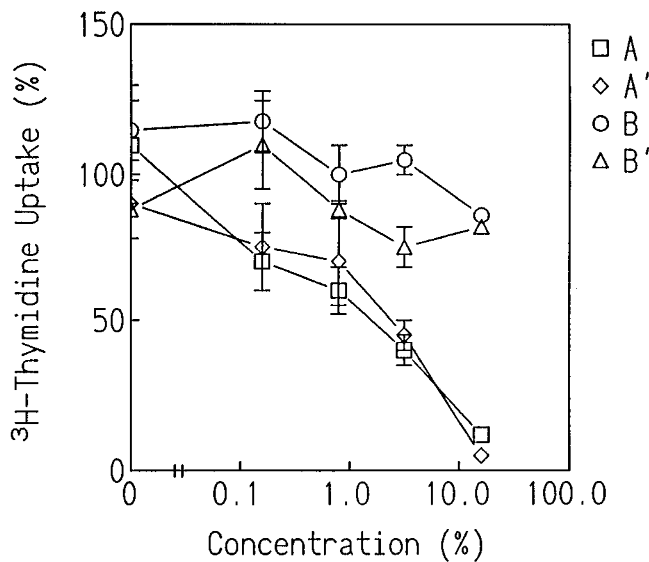

Figure 2. Inhibition of AChR 100–116 stimulation of AChR-sensitized lymphocyte proliferation by Ab to RhCA 611–001. *Torpedo* AChR-sensitized lymphocytes, depleted of macrophages, were coincubated with AChR 100–116 educated macrophages and the indicated dilutions of rat anti-sera to RhCA 611–001 (A, heat-inactivated; A′, non-heat-inactivated) or to PBM 9–1 (B, heat-inactivated; B′, non-heat-inactivated). Proliferation assays were done as described in Materials and Methods. Each point represents the mean ± SE. 100% of ^{3}H-thymidine uptake represents 58,447 cpm.

ture of sensitized lymphocytes and APC with CTCR8 was more effective than a relatively brief (1 h) pretreatment; a control mAb, F28C4, was without effect (Fig. 3).

CTCR8 recognizes a Vβ15-bearing TCR

To test whether CTCR8 was able to react with an AChR-specific TCR, we developed a CD4$^+$ AChR 100–116-specific T cell line. By Western blotting, CTCR8 (**Fig. 4**, lane 2) recognized a protein that comigrated under reducing conditions with that recognized by mAb R73 (Fig. 4, lane 1) against the rat TCR β chain constant (57). The molecular masses (39 kDa) of the proteins recognized by R73 as well as CTCR8 are consistent with that reported for the rat TCR β chain (60). The epitope recognized by CTCR8 was also located on the TCR β chain, since T cell lysates precleared with R73 mAb were not recognized by CTCR8 (Fig. 4, lane 3). The clonotypic nature of the cell surface protein recognized by CTCR8 was demonstrated by the ability of R73 mAb (**Fig. 5**, lane 1) but not CTCR8 (Fig. 5, lane 2) to recognize TCRβ from a rat CD4$^+$ ovalbumin-specific T cell line.

To ascertain whether there was restricted V region usage in the TCR that was recognized by CTCR8, AChR 100–116 reactive T cell clones were estab-

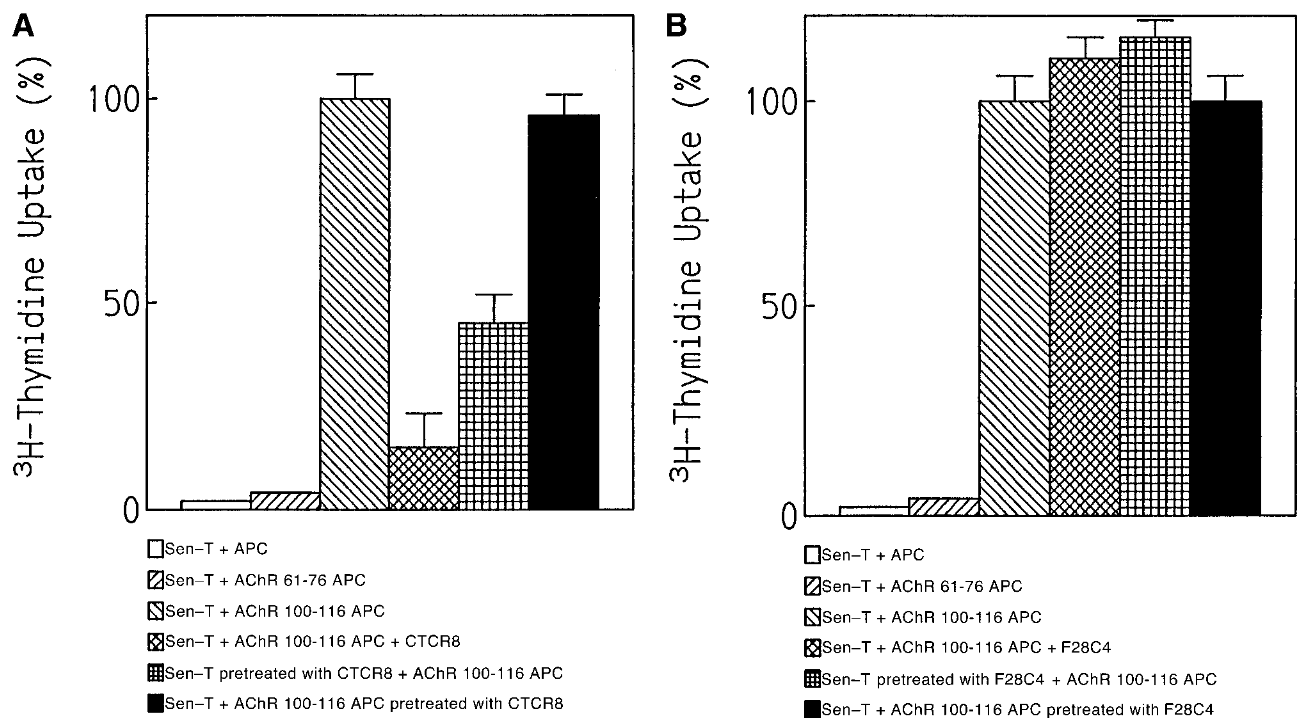

Figure 3. Inhibition of AChR 100–116-specific lymphocyte-proliferation by pretreatment of APC or AChR-sensitized lymphocytes with CTCR8. *Torpedo* AChR-sensitized lymphocytes, depleted of macrophages (sen-T), were coincubated with AChR 100–116 or AChR 61–76 educated (ed) irradiated APC. In each assay, sen-T or ed APC were preincubated or coincubated with 15 μg/well of either mAb CTCR8 (*A*) or mAb F28C (*B*), followed by the proliferation assay described in Materials and Methods. APC represents irradiated APC nonloaded with AChR 100–116 or 61–76 and gave a background incorporation of 1847 cpm. Each point represents mean ± SE. 100% of ^{3}H-thymidine uptake represents 94,670 cpm.

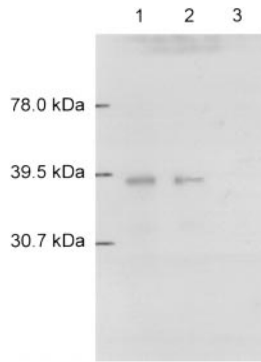

Figure 4. Immunoblots of membrane proteins from an AChR 100–116 T cell line. Membranes were blotted as described in Materials and Methods and stained with mAbs R73 (lane 1) or CTCR8 (lane 2). Lane 3 is an R73 precleared lysate that is developed with CTCR8.

lished from the aforementioned T cell line. All clones were found to use Vβ15 in their T cell receptors, although they showed differences in D region sequences and Jβ usage (Table 1). Three sets of clones (F3a and C2; E4a, Gla, and D2; E4b and C4) were found to be of identical lineage since they had identical VDJ junctional sequences. FACS analysis of each clone with biotinylated CTCR8, followed by FITC streptavidin, showed that five of the nine clones representing two lineages (i.e., D region sequences RDS and RQGAD) were positively stained by the mAb whereas four of the nine clones representing three original clones (D region sequences RNGQGP, SEGGGT, and REGPR) were CTCR8 negative.

Collectively, these findings suggest that immunization with RhCA 611–001 can induce polyclonal and monoclonal Ab that block the proliferation of AChR 100–116-specific T cells by recognition of their TCR. These results also confirm those of Smith et al. (61) that Vβ15 predominates in the T cell response to AChR7 100–116. In that study, three of four AChRα 100–116-specific T cells used Vβ15; like our clones recognized by CTCR8, all used Jβ 1.2 or 1.4. Indeed, one of the three clones had the same sequence as our RDS (N/Dβ/N) group.

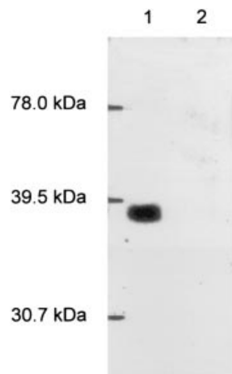

Figure 5. Immunoblots of membrane proteins from an ovalbumin-specific T cell line. Membranes were blotted as described in Materials and Methods and stained with mAbs R73 (lane 1) or CTCR8 (lane 2).

Prevention of EAMG

If Ab to RhCA 611–001 is directed to the TCR of AChR 100–116-specific T cells, and assuming this T cell epitope is important to the AChR Ab response, vaccination with RhCA 611–001 should lower AChR Ab levels and prevent EAMG. This was tested by immunization with the peptide prior to challenge with purified *Torpedo* AChR. RhCA 611–001 vaccination markedly lowered (80 to 90% inhibition) AChR Ab levels (**Fig. 6**). This effect seemed specific since the control peptide, PBM 9–1, did not alter AChR Ab titers. Not unexpectedly, increasing doses of AChR caused more robust Ab levels and antagonized the effect of RhCA 611–001 vaccination.

In association with the lower AChR Ab levels, there was a significant inhibition of EAMG incidence (**Fig. 7*A***), clinical severity (Fig. 7*B*), and mortality (Fig. 7*C*) in the RhCA 611–001-vaccinated animals compared with the controls, respectively. In contrast, vaccination with the control peptide PBM 9–1 showed the same incidence, severity, and mortality as the PBS control (Fig. 7*A–C*). Thus, the beneficial effect seems to be specific for RhCA 611–001. The effects are disease as well as peptide specific, since an AChR complementary peptide was previously shown to have no effect on the model of multiple sclerosis (experimental allergic encephalomyelitis) in Lewis rats (62).

To assure that the clinical improvement reflected

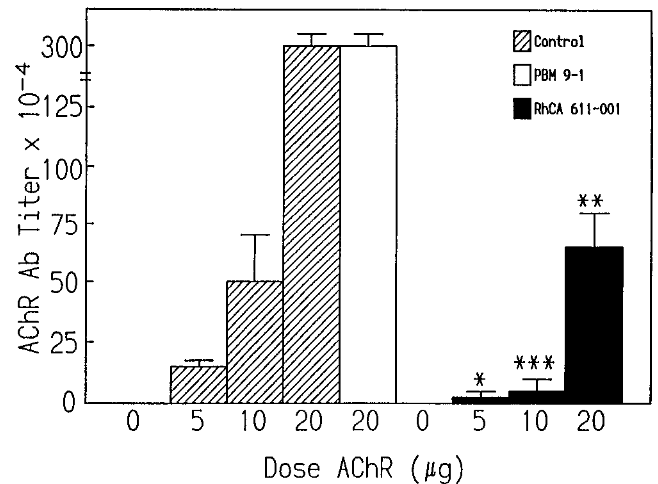

Figure 6. Effect of increasing doses of *Torpedo* AChR on the development of anti-AChR Ab in Lewis rats preimmunized with RhCA 611–001, PBS (control), or PBM 9–1 (control peptide). As described in Materials and Methods, AChR Ab levels were determined at 12 wk postimmunization with AChR. The mean Ab titers ± SE are presented. *$P<0.01$; **$P<0.005$; ***$P<0.0001$.

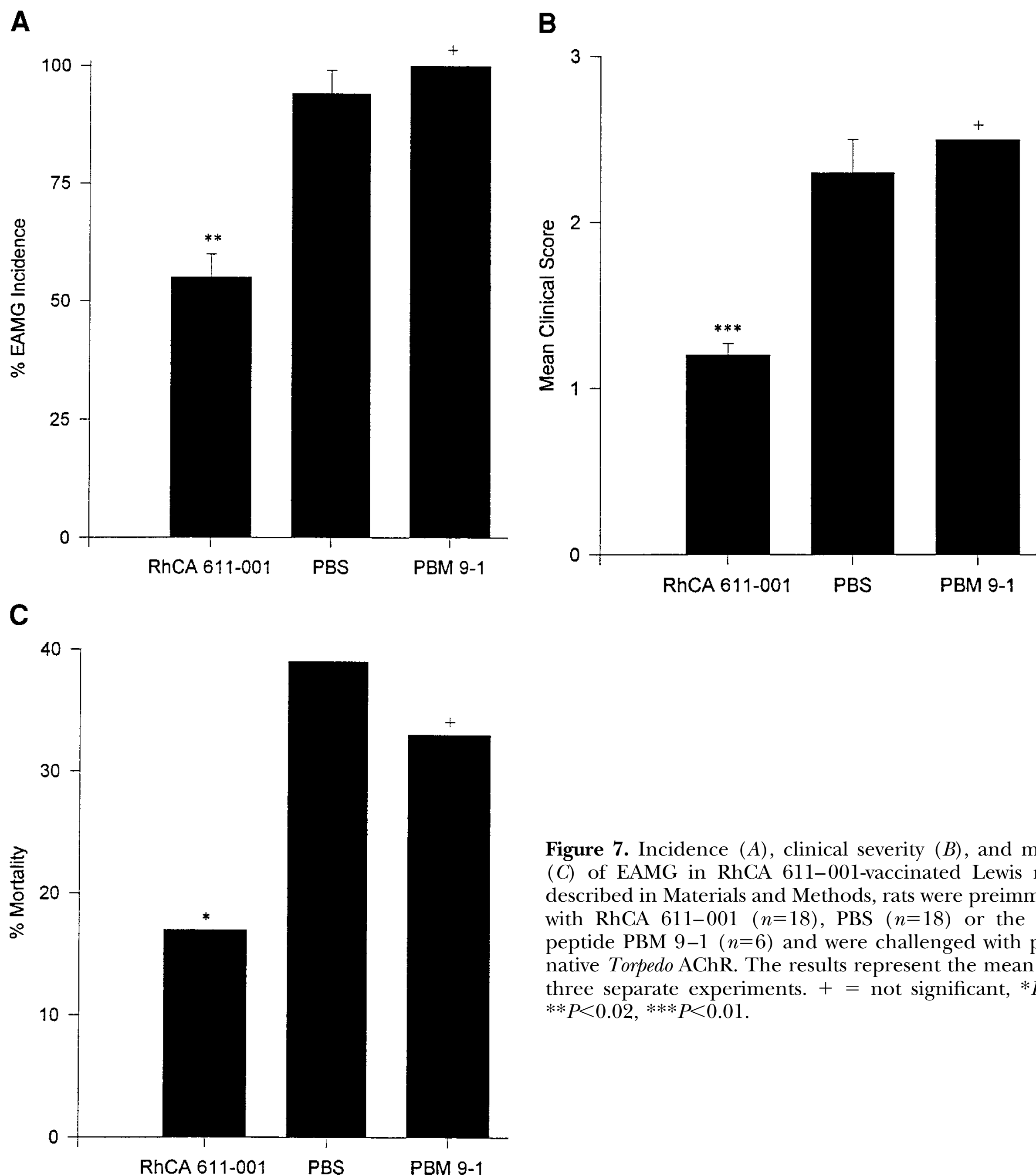

Figure 7. Incidence (*A*), clinical severity (*B*), and mortality (*C*) of EAMG in RhCA 611–001-vaccinated Lewis rats. As described in Materials and Methods, rats were preimmunized with RhCA 611–001 (n=18), PBS (n=18) or the control peptide PBM 9–1 (n=6) and were challenged with purified native *Torpedo* AChR. The results represent the mean ±SE of three separate experiments. + = not significant, *P<0.05, **P<0.02, ***P<0.01.

a diminished loss of AChR in RhCA 611–001-immunized animals, we quantified the amount of AChR in rat carcasses 5 wk after challenge with AChR. **Figure 8** shows the expected and previously observed (63) 50–60% loss of muscle AChR in control animals. In contrast, RhCA 611–001-vaccinated animals displayed only a 35% loss of muscle AChR. The ~40% prevention of AChR loss due to RhCA 611–001 vaccination correlates very well with the 50% improvement in clinical score (Fig. 7*B*). Thus, the clinical results would appear to mirror a diminished loss of muscle AChR.

DISCUSSION

This report describes a new procedure to evoke anti-clonotypic/TCR Ab responses that might be useful in treating autoimmune diseases. The procedure requires no knowledge of the Ag receptor sequences on the errant T and B lymphocytes, only the primary structure of the disease-causing epitope. As described earlier, epitope sequence information, together with new knowledge of the role of the precise pattern of amino acid hydropathy in protein and peptide shape or structure, allows one to con-

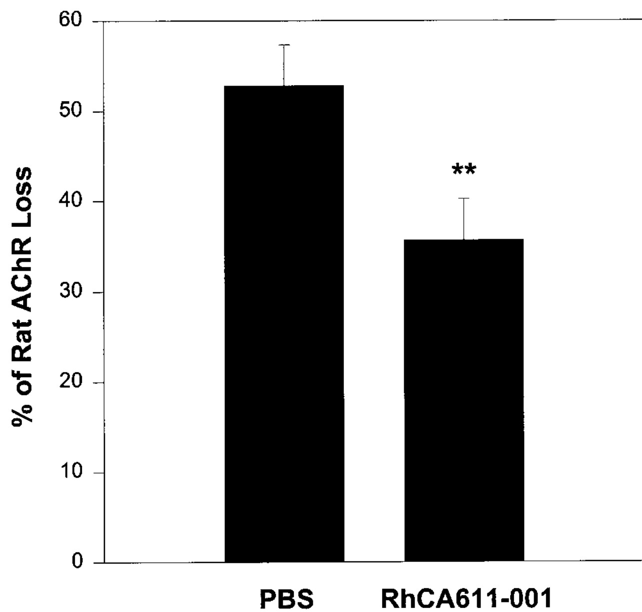

Figure 8. Reduction in AChR loss by RhCA 611–001 vaccination. Rat AChR content was measured at 5 wk after immunization with *Torpedo* AChR. The AChR content in complete carcasses was measured by RIA (as described in Materials and Methods) and expressed as the percentage of the AChR content of PBS/CFA-immunized control rats that were not exposed to *Torpedo* AChR. Each bar represents the mean of 10 rats ± SD. $^{**}P<0.01$.

struct peptides that presumably assume shapes or structures complementary to the disease-associated epitope. As a consequence, vaccination with the novel peptides lead to the production of anti-idiotypic and anti-clonotypic Ab whose combining sites are complementary to and therefore reactive with Ag receptors on disease epitope-specific T and B cells. As yet unresolved is the question of whether such peptide vaccines also elicit regulatory T cells. The utility of this approach to reduce the autoimmune Ab response to a B cell epitope was previously demonstrated (26). Since autoimmune diseases can be of B and/or T cell origin, with the two populations extensively interacting, we were interested in whether the procedure would be successful in specifically altering the function of autoreactive T cells. Of course, future applications to human autoimmune diseases require the capability to manipulate either arm of the immune system. The elucidation of 8 chain residues 100–116 of the AChR as the dominant Lewis rat T cell epitope provided a target for such a test in the model of MG, EAMG. The results are entirely consistent with the idea that peptide vaccines can be designed that evoke specific and inhibitory Ab responses against autoreactive epitope-specific T cells, since a peptide designed to have an inverted code of hydropathy relative to AChR 100–116: *1)* induced polyclonal and mAb (CTCR8), which inhibited the proliferation of AChR 100–116-specific lymphocytes and recognized a Vβ15- containing TCR on AChRα 100–116-specific T cell lines and clones; *2)* lowered AChR Ab levels; *3)* prevented muscle AChR loss; and *4)* reduced the incidence and lowered the severity of EAMG.

Aside from the obvious practical aspects of these findings, the procedure offers a means to probe some rather interesting questions in autoimmunity. For instance, although AChRα 100–116-specific T cell clones were demonstrated to help AChR Ab production, the relationship between specific disease-causing Ab and its helper T cell epitope has remained obscure (47). In other words, which T cell epitopes facilitate the production of disease-causing Ab? In the present instance, the results clearly show that the T cell epitope AChR 100–116 is involved in the disease process, probably through effects on Ab production, because reduction of the T cell response to this epitope lessens the incidence and severity of EAMG and lowers AChR Ab levels. However, undoubtedly there are other disease-associated T cell epitopes because the incidence of EAMG was not totally ablated nor was it lowered to the 25% level observed when the Ab response to the MIR was abrogated (26, 27). Elucidation of these additional T cell epitopes could well lead to more effective 'polyvalent' peptide vaccines. Ourselves and others have observed that there is not a good correlation between overall AChR Ab levels and disease state, because many Ab to the AChR are not directed to disease-associated B cell epitopes (26, 64–67). This observation was borne out in the present study by the observation that AChR Ab levels were affected more profoundly by RhCA 611–001 immunization than was EAMG. Such a result is in accord with the findings of Yeh and Krolick (47) that AChR 100–116-specific T cells probably help Ab production to multiple and not exclusively disease-associated B cell epitopes on the AChR.

Although the present and earlier studies have concentrated on MG, a model of a T cell-dependent, B cell-mediated autoimmune disease, recent studies have demonstrated that the procedure described here is also effective against two animal models of the human T cell-mediated autoimmune diseases: multiple sclerosis (24, 25, 62) and Guillain-Barré syndrome (68). mAb CTCR8 that recognized AChR 100–116-specific T cells in the present report had no effect on rat T cells specific for the peripheral myelin protein P2 residues 60–70 in the model of Guillain-Barré syndrome (68). Collectively, these findings suggest a new strategy for the functional abrogation of epitope-specific T cells that could have potential application to human autoimmune diseases. FJ

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