Augmented Anti–Acetylcholine Receptor Response Following Long-Term Penicillamine Administration

Christopher T. Bever, Jr, MD,* Kenneth L. Dretchen, PhD,† George J. Blake, PhD,† Hai W. Chang, PhD,‡ Audrey S. Penn, MD,‡ and Richard Asofsky, MD*

Because of the association of D-penicillamine (DP) therapy with myasthenia gravis, we have studied long-term DP treatment in five inbred strains of mice with doses comparable to those used in patients with rheumatoid arthritis. No clinical weakness or anti–acetylcholine receptor (AChR) antibody developed with up to 6 months of treatment, but augmented responses did occur to challenge with purified AChR in adjuvant. Anti-AChR antibody titers in C57BL/6 and C3H/He mice were significantly higher after challenge with AChR in DP-treated than in control mice. Augmented anti-AChR titers were not seen in strain A mice, but after 6 months of DP treatment increased susceptibility developed to the induction of experimental autoimmune myasthenia gravis. Nine weeks after challenge with purified AChR, 10 of 11 mice developed clinical weakness, leading to death in 6. Results of edrophonium testing were positive in 5 of 6 mice, and electrophysiological abnormalities were demonstrated in 3 of the surviving mice.

Long-term DP treatment is associated with augmented anti-AChR antibody responses in C3H/He and C57BL/6 mice, and increased susceptibility to experimental autoimmune myasthenia gravis in strain A mice.

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Therapy with D-penicillamine (DP) has been associated with a reversible myasthenia gravis (MG)-like illness in patients receiving the drug for scleroderma [25], rheumatoid arthritis [5], and Wilson's disease [10]. The drug-induced disease is indistinguishable from spontaneously occurring MG in its clinical and electrophysiological features [5], the presence of anti–acetylcholine receptor (AChR) antibodies [18], and the presence of thymic hyperplasia [18]. The drug-induced disease can be distinguished from spontaneously occurring MG only by the resolution of symptoms 3 to 6 months after DP therapy has been stopped.

The mechanism of induction of anti-AChR antibodies in DP-induced MG, as in spontaneously occurring MG, is unknown. The association of DP therapy with other autoimmune diseases, including pemphigus [24], systemic lupus erythematosus [12], Goodpasture's syndrome [23], and polymyositis [22], has led to the hypothesis that DP causes an alteration in immunity predisposing to autoimmunity. The ability of DP to reduce disulfides in purified AChR has led to the hypothesis that DP can react with AChR in situ and cause an antigenic alteration, leading to a loss of tolerance [3]. Because an experimental model of DP-induced MG would facilitate investigation of these alternatives, we have undertaken a study of mice receiving long-term treatment with DP.

Methods

Female mice, strains A, BALB/c, C3H/He, C57BL/6, and DBA/1, were obtained from Jackson Laboratories, Bar Harbor, Maine. DP hydrochloride was obtained from Sigma Chemical, St. Louis, Missouri, and alpha-bungarotoxin labeled with iodine 125 was obtained from New England Nuclear, Boston, Massachusetts.

Ten mice of each strain received 1 mg of DP(40 mg per kilogram of body weight per day) in 0.1 ml of normal saline by intraperitoneal injection on 5 days of each week during the treatment period. Ten control mice of each strain were identically housed and treated except that they received injections of saline alone. Treatment durations of both 2 and 6 months were examined.

AChR challenge was conducted with AChR obtained from *Torpedo californica* electric organ by affinity chromatography as described previously [8, 9]. AChR(10 μg per mouse) was emulsified with an equal volume of Freund's complete adjuvant and injected intraperitoneally one week after the last DP injection.

From the *Laboratory of Microbial Immunity, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, the †Department of Pharmacology, Georgetown University, Washington, DC, and the ‡H. Houston Merritt Center for Muscular Dystrophy, Columbia-Presbyterian Medical Center, New York, NY.

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Address reprint requests to Dr Penn, Department of Neurology, College of Physicians and Surgeons, 630 W 168th St, New York, NY 10032.

All mice were evaluated periodically both during the DP treatment and after the AChR challenge. Examination routinely included study of repetitive grasping, forced running, and inverted hanging. Edrophonium chloride testing (using 40 μg/kg by intraperitoneal injection) was not found to be useful in evaluating mild weakness but was used to investigate weakness in severely affected mice. Test results were considered positive if a mouse unable to walk or hang from a cage lid was able to do so 1 to 5 minutes after the injection.

Serum samples were obtained periodically during the treatment period and after AChR challenge, and anti-AChR titers were determined by a method that has been reported in detail [21]. In brief, purified AChR from *T. californica* was incubated with [^{125}I]alpha-bungarotoxin and then added to aliquots of test serum diluted serially. After overnight incubation at 4°C, goat antimouse immunoglobulin was added. After a second incubation the resulting pellet was washed and counted in a gamma counter. Titers were determined from four or more points on the titration curve by linear regression analysis. Log mean titers were determined for each group, and the means were then compared using the Student *t* test. Assays on all control and experimental sera of each strain were run simultaneously with the same batch of reagents so that titers were comparable.

Mice were killed by cervical dislocation, and the hemidiaphragm, with attached phrenic nerves, was removed. The tissue was placed in a Lucite chamber, the nerve covered with mineral oil, and the muscle bathed with oxygenated mouse Ringer's solution maintained at 31°C. As previously described [4], the muscle fibers were cut 1 to 2 mm from their point of insertion into the tendons. This procedure reduced the resting membrane potential to between 30 and 40 mV, and no mechanical response was obtained upon stimulation of the phrenic nerve. Individual muscle fibers were impaled with glass micropipettes filled with 3 M potassium chloride (5 to 10 megaohms). Miniature end plate potentials (MEPPs) and end plate potentials (EPPs) were evoked by stimulation of the nerve at supramaximal voltage for 0.02 ms at frequencies ranging from 2 to 50 Hz and recorded on FM tape for later analysis on a PDP-11 computer. Because MEPP amplitudes are affected by resting membrane potential (RMP), which varied from fiber to fiber, analysis involved the calculation of the ratio of MEPP amplitudes to RMP. EPPs were analyzed for amplitude and decrement in response to repetitive stimulation of the nerve. After control responses were obtained, edrophonium 10 μM was introduced into the bathing media and the experimental procedure repeated.

Results

In the first experiment six strains of mice were treated for 6 months with DP. At the end of this period there was no evidence of weakness in any of the mice and no measurable anti-AChR antibody. None of the mice were killed for electrophysiological testing at that time.

Seven days after DP treatment was terminated, all mice were challenged with 10 μg of AChR. Figure 1 gives the log mean anti-AChR titers measured 10 days after challenge. In all strains other than A, mean titers among the DP-treated mice were approximately twice those among the control mice. This difference was statistically significant for both the C3H/He and the C57BL/6 mice.

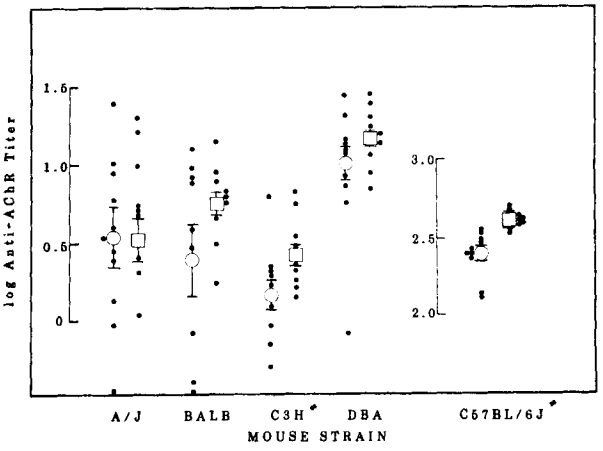

Fig 1. Anti–acetylcholine receptor (AChR) titers in penicillamine-treated and control mice after challenge with AChR. Open circles indicate mean values for controls, and open squares indicate mean value for treated mice. Bars indicate standard errors. The difference between treated animals and controls was significant at the 0.05 level for C3H and the 0.001 level for C57BL/6 strains.

Table 1. Alterations in Experimental Autoimmune Myasthenia Gravis in Strain A Mice after Long-Term Penicillamine Treatment

Finding	No. of Mice: Treated (n = 11)	No. of Mice: Control (n = 10)
Clinical weakness	10/11	0
Positive edrophonium chloride test	5/6	0
Died	6/11	0
Decreased MEPPs	3/4	0/3

MEPPs = miniature end plate potentials.

Nine weeks after AChR challenge, weakness was noted in several of the DP-treated strain A mice. Ultimately, 10 of the 11 mice in this group developed at least transient weakness (Table 1). In 6 mice the weakness was severe and led to death. Edrophonium chloride testing was completed in 6 mice (3 died before they could be tested) and gave positive results in 5. Electrophysiological studies were done on 3 mice with mild weakness and 1 without weakness, as well as on control and normal mice. The DP-treated mice had significantly diminished MEPP amplitudes compared with the controls, and the 3 weak mice had significantly greater decrements at 50 Hz stimulation than the controls (Table 2). This decrement was reversed by edrophonium (Fig 2). Examination of the control mice individually showed that one had a significant decre-

Table 2. Summary of Single-Unit Electrophysiological Findings in Strain A Mice

Group	MEPP/RMP (×100) Mean	MEPP/RMP (×100) SD	EPP_5/EPP_1 (×100) Mean	EPP_5/EPP_1 (×100) SD	Fibers Studied
Normal	1.23	0.37	73.2	7.01	8
Control					
1	1.62	0.26	84.2	12.54	7
2	1.23	0.26	74.6	8.56	10
3	1.21	0.53	66.8	6.04	6
Pooled	1.35	0.19	75.2	7.12	. . .
Treated					
1	0.95[a]	0.36	63.4[b]	6.30	5
2	1.06[b]	0.40	62.7[a]	12.5	5
3	0.80[a]	0.35	69.1[c]	5.9	9
4	1.22	0.37	85.1	12.0	12
Pooled	1.01[c]	0.15	70.1	9.0	. . .

Significantly different from pooled control values: [a]$p = 0.005$; [b]$p = 0.02$; [c]$p = 0.05$.

MEPP = miniature end plate potential; RMP = resting membrane potential; EPP = end plate potential.

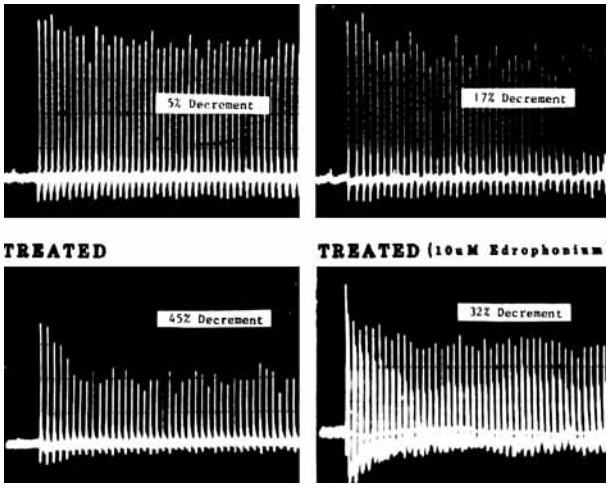

Fig 2. Comparison of decrements in single fibers from normal, control, and treated mice, showing edrophonium reversal of the decrement at 50 Hz stimulation.

ment at 50 Hz stimulation compared with the other two, but this decrement was not significant when compared with findings in the normal mice and was not correlated with a significant decrease in MEPP amplitude. Antibody titers against AChR from both *T. californica* and denervated rat muscle (data not shown) were determined in these strain A mice both 10 days after challenge and at the time that weakness developed, but no correlation with treatment group or clinical status was found.

Table 3. Anti–Acetylcholine Receptor Titers after 2 Months of Penicillamine Treatment and AChR Challenge

Strain	Treatment	Mean Titer (nM/ml)	Log (mean titer)	Log (SD)	No. of Animals
C57BL/6	DP	6.14[a]	0.79[a]	0.31[a]	10
	Saline	1.72[a]	0.24[a]	0.56[a]	10
A	DP	3.57	0.55	0.39	10
	Saline	1.95	0.29	0.54	9

[a]Significantly different from control group ($p < 0.05$).

DP = D-penicillamine.

In the second experiment mice were treated for only 2 months and then challenged with purified AChR. Titers measured ten days after challenge showed significant augmentation in C57BL/6 mice but not in strain A animals (Table 3). One month after challenge 2 of 10 DP-treated A mice developed weakness, 1 clearly responsive to edrophonium. Both mice recovered within 2 days, and postmortem electrophysiological studies showed no significant abnormalities (data not shown). Antibody titers to purified AChR from *T. californica* and denervated rat muscle AChR were determined (data not shown), but no correlation with clinical status was found.

Discussion

Although the importance of anti-AChR antibodies in MG is well established, the factors leading to autoantibody production are not known. Because DP-induced MG is one of the few instances in which a cause has been implicated in autoantibody production, a number of investigators have studied the experimental effects of DP in vitro and in vivo.

Several investigations have addressed the question of whether DP interacts directly with the AChR, a possibility that was raised by the disulfide reducing abilities of DP [15] and the presence of a disulfide near the ACh binding site of the AChR [16]. DP has been shown to reduce disulfides in purified AChR from *T. californica* and to alter the kinetics of ACh binding [3], but no alterations in MEPPs or evoked potentials were observed with short-term high levels of DP in vitro [1] and in vivo [7]. These findings support the clinical impression that DP-induced MG is an immunologically mediated attack on the AChR rather than a direct chemical interference with AChR function, and leave open the possibility that DP chemically alters AChR and causes antigenic alteration, leading to a loss of tolerance.

Two groups have studied long-term treatment of guinea pigs with doses of DP (150 to 600 mg/kg per day) 10 to 40 times the dose used in humans. These animals developed decreased MEPP amplitudes and

edrophonium-reversible decremental responses at 50 Hz stimulation evident on electromyography [7, 17, 19]; one study found significant elevations in anti-AChR titers, but none of the animals developed clinical symptoms of myasthenia. At the higher doses of DP studied, the animals developed a systemic illness and were found to have polymyositis [17] and hepatitis [6, 19] pathologically. Both polymyositis [22] and hepatitis [26] have been reported rarely in patients taking DP, but the severity of muscle and hepatic abhormalities in the guinea pig model have led some to question its relevance to human DP-induced MG [19].

We have studied mice treated with doses of DP comparable to those used in humans and demonstrated augmented responses to challenge with AChR. Anti-AChR titers were higher in DP-treated BALB/c, C3H/He, C57BL/6, and DBA/1 mice than in controls. The differences were statistically significant in C57BL/6 and C3H/He mice; in the C57BL/6 mice this difference was found after as little as 2 months of treatment.

Strain A mice showed an increased sensitivity to experimental MG after 6 months of DP treatment. The clinical weakness that developed in 10 of these mice was edrophonium responsive and was associated with characteristic electrophysiological abnormalities not present in the controls. The failure to show significant decrements at rates of stimulation lower than 50 Hz without provocative measures is consistent with previous observations of murine EAMG [14, 20]. Antibodies to AChR were present in all mice challenged with AChR (including the controls), but no correlation could be found between titer and clinical status. Previous studies of murine EAMG have failed to demonstrate a good correlation between anti-AChR titer and clinical status [2, 14, 20]. The fact that the increased sensitivity to EAMG was not seen in one of the strains previously reported to be a high responder for EAMG [2, 13] is analogous to the situation seen in human DP-induced MG. Whereas spontaneously occurring MG seems to be associated with HLA-B8, Dw3, DP-induced MG seems to be associated with HLA-Bw35, DR1 [11].

These results cannot distinguish altered immunoregulation from antigenic alteration, but further investigation of the low-dose treatment of mice (immunologically well-characterized animals) may provide support for one hypothesis or the other. If responses to other antigens are similarly augmented and measurable differences in other immunological variables are found, support will be lent to some form of altered immunoregulation with a predisposition to autoimmunity. An alteration limited to the response to AChR, however, would support some AChR-specific mechanism, such as chemical alteration with loss of tolerance. These issues are currently under investigation.

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