Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 4810–4814, August 1982
Neurobiology

Anti-idiotypic route to anti-acetylcholine receptor antibodies and experimental myasthenia gravis

(agonist/combining site/anti-idiotypic response/ligand)

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Communicated by Harry Grundfest, April 26, 1982

ABSTRACT ***trans*-3,3′-Bis[α-(trimethylammonio)methyl]azobenzene bromide (BisQ) is a potent agonist of the acetylcholine receptor (AcChoR) of *Electrophorus electricus*. BisQ is highly constrained, suggesting that its structure is complementary to the combining site of the AcChoR when the latter is in its activated state. Antibodies produced in rabbits to a conjugate of bovine serum albumin and a derivative of BisQ mimicked the binding characteristics of the AcChoR with respect to the order of binding of a variety of agonists and to the preferred recognition of decamethonium ion (an agonist) over hexamethonium ion (an antagonist). Immunization of three rabbits with purified anti-BisQ yielded antisera having binding characteristics of anti-AcChoR in that, by complement fixation and enzyme immunoassay, cross-reactions with receptor preparations from rat, *Torpedo*, and eel could be demonstrated in sera of all three rabbits immunized. Two of the three rabbits showed signs of muscle weakness similar to that seen after immunization with the AcChoR. One of the rabbits was injected intramuscularly with neostigmine and showed temporary improvement. Another showed post-tetanic exhaustion of hindlimb muscles after stimulation of the sciatic nerve at 50 Hz. Antibodies reactive with the AcChoR, therefore, were elicited by immunization with an antibody to a potent ligand of the AcChoR without the necessity of isolating the receptor itself. A similar mechanism may play a part in the etiology of at least some autoimmune diseases in which antibodies to various other receptors are involved.**

In 1971, we described the synthesis and biological activity of 3,3′-bis[α-(trimethylammonio)methyl]azobenzene bromide (BisQ), a highly active agonist of the acetylcholine receptor (AcChoR) of *Electrophorus electricus* (1, 2). This compound is photochromic and exists in either a *trans* or a *cis* configuration, the relative concentrations of which can be controlled by exposure to light of selected wavelengths. The *trans* isomer, at a concentration of 60 nM, produced a 50% depolarization of electroplax preparations of *Electrophorus electricus*; maximal depolarization was −60 mV at about 40 nM. The *cis* isomer showed no activity; i.e., it was neither an agonist nor an antagonist. The depolarizing activity of *trans*-BisQ was found to be about 500-fold greater than that of carbamoylcholine, 50-fold greater than that of acetylcholine, and about 20-fold greater than that of decamethonium (3, 4), making it one of the most potent agonists of the *Electrophorus electricus* system.

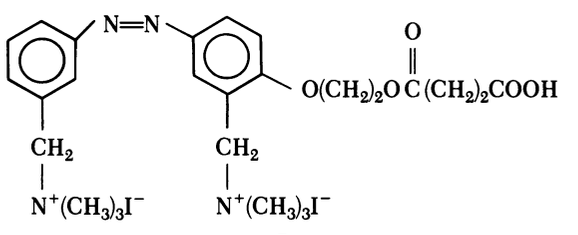
trans-3,3′-BisQ is a structurally constrained molecule (5) having a planar configuration. Unlike a flexible molecule, which can assume many conformations in solution, a structurally constrained molecule has few, if any, degrees of freedom. Hence, it can be either highly active or of little activity as a physiological effector molecule, depending on whether its conformation is appropriately complementary to the binding site of the target receptor. The extremely high activity of *trans*-BisQ as an agonist suggested that its structure was complementary to the combining site of the AcChoR when the latter was in its *activated* state—i.e., the state associated with opening of the ion channels (6).

Macromolecules that bind the same ligand but have different biological functions (7–9) show homology in their binding specificities. It was therefore reasoned that the combining sites of antibodies raised to *trans*-BisQ have binding specificities similar to that of the AcChoR when the latter is in the activated state. Presumably, then, these antibodies should be able to bind agonists of the AcChoR and to distinguish between agonists and antagonists. In the first part of this paper, we show that this is indeed the case.

The above findings suggested that anti-idiotypic antibodies (10) directed to determinants of the combining sites of the anti-*trans*-3,3′-BisQ antibodies should share characteristics of anti-AcChoR antibodies, i.e., the anti-idiotypic antibodies should bind the AcChoR. Rabbits immunized with AcChoR show signs of experimentally induced autoimmune myasthenia gravis (11, 12). Moreover, anti-AcChoR antibodies circulate in patients who have myasthenia gravis. We might expect then that rabbits immunized to produce anti-idiotypic antibodies to anti-*trans*-BisQ would also show signs of autoimmune myasthenia gravis and have circulating AcChoR-reactive antibodies. Observations are presented supporting this inference.

METHODS

A BisQ derivative was prepared that could be covalently linked to proteins to produce an immunogenic molecule. It was 4-(succinoyloxyethyloxy)-3,3′-bis[α-(trimethylammonio)methyl]azobenzene (compound **I**). Because of space limitations, its synthesis will be described elsewhere. Its structure is shown below.



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Abbreviations: BisQ, 3,3′-bis[α-(trimethylammonio)methyl]azobenzene bromide; AcChoR, acetylcholine receptor; P_i/NaCl, phosphate-buffered saline.

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3,3′-Bis[(α-tri[^{3}H]methylammonio)methyl]azobenzene Iodide ([^{3}H]BisQ Iodide). 3,3′-[^{3}H]BisQ iodide was prepared as described (13) for 3,3′-[^{14}C]BisQ using C^3H_3I having an activity of 3.0 Ci/mmol (21.1 mCi/mg; 1 Ci = 3.7×10^{10} becquerels; Amersham).

Preparation of the Immunizing Conjugate. I was linked to bovine serum albumin (and to rabbit serum albumin) by the mixed anhydride technique (14). Approximately 8 molecules of I were linked to the albumin carrier, as determined spectrophotometrically (ϵ_{324nm} = 20,000).

Preparation of An Affinity Column for Purification of Anti-*trans*-3,3′-BisQ. The affinity column was prepared by the mixed anhydride procedure starting with 17.8 mg of I and the appropriate quantities of tributylamine and isobutylchloroformate in dimethylformamide. The solution was added in three portions to 4 ml of aminohexyl-Sepharose (Bio-Rad) suspended in 10 ml of dimethylformamide. The reaction mixture was stirred for 24 hr in a cold room. The yellow modified Sepharose derivative was recovered by centrifugation and washed exhaustively with phosphate-buffered saline (P_i/NaCl).

Purification of Anti-3,3′-*trans*-BisQ Antibody. The BisQ affinity column was washed with 0.2 M glycine·HCl (pH 2.2) until no absorption at 324 nm could be detected and then thoroughly with P_i/NaCl. Serum (42 ml) shown to have anti-3,3′-*trans*-BisQ activity by tube precipitation with a rabbit serum albumin conjugate of I was passed twice through the column in the cold room at a rate of about 20 ml/hr. The column was washed exhaustively with P_i/NaCl, and specific antibody was eluted with 0.2 M glycine·HCl (pH 2.8), and the eluate was immediately brought to neutral pH with saturated K_2HPO_4. The purified antibody precipitated with a rabbit serum albumin conjugate of I.

Immunization Procedures. New Zealand White rabbits were immunized intradermally at multiple sites along the back with antigen at 1 mg/ml in complete Freund's adjuvant. For the preparation of anti-3,3′-*trans*-BisQ, booster injections were administered at 3- to 4-wk intervals. For the preparation of antibodies to anti-BisQ, the sehedule was varied depending on the signs shown by the rabbits.

Radioimmunoassay for Anti-3,3′-*trans*-BisQ. The relative activities of various AcChoR-reactive compounds were determined by a competitive binding assay with *trans*-[^{3}H]BisQ (ca. 4,000 cpm; 3.5 Ci/mmol) as the primary ligand; antibody–ligand complexes were separated from free ligand by $(NH_4)SO_4$ precipitation (15, 16). Supernatant fluids were assayed. Eserine at 0.2 mM was present to prevent hydrolysis of susceptible esters (e.g., acetylcholine) by acetylcholinesterase possibly present in the antisera. In some experiments, 50 μM diphenylcarbamoyl fluoride (17) was also present.

Measurement of AcChoR-Reactive Activity in Sera. Complement fixation was carried out in 0.1 M Tris·HCl according to the procedure of Wasserman and Levine (18). The enzyme immunoassay procedure was a modification of the procedure of Norcross *et al.* (19). Coating of the plastic wells was by incubation of the receptor in 0.1 M $NaHCO_3$ (pH 9.3) for 3 hr at 37°C. The receptor preparations used included purified eel (gifts from H. W. Chang and J. Lindstrom), purified *Torpedo* (12), and purified preparations from denervated rat and rabbit hindleg muscle (20). The wells were washed with 0.01 M phosphate buffer/0.15 M NaCl, pH 7.3/0.05% Tween 20 (P_i/NaCl/Tween) as described (21), twice after coating and four times after each incubation with antibody. The putative anti-receptor antibody was incubated for 1.5 hr at 37°C; the enzyme-labeled goat anti-rabbit antibody was incubated for 1 hr at 37°C. For the alkaline phosphatase-labeled antibody, the substrate was *p*-nitrophenylphosphate. For the peroxidase-labeled antibody, *o*-phenylenediamine was used as the substrate (21). The peroxidase label was more satisfactory because blank values were lower and the results were quantitatively more reproducible.

For the inhibition studies (by excess receptor and BisQ), inhibitor in P_i/NaCl/Tween was incubated with antibody for 1 hr at 25° prior to the assay. Control antibody was incubated similarly in P_i/NaCl/Tween. The wells contained soluble receptor (at the concentration used for coating but in P_i/NaCl/Tween) throughout the incubation with antibody. In the case of inhibition studies with BisQ, the wells were coated with receptor in 0.1 M $NaHCO_3$ containing BisQ at 1 mg/ml and then the incubation with antibody was carried out in the presence of BisQ (1 mg/ml) in P_i/NaCl/Tween.

RESULTS

Binding of Anti-3,3′-*trans*-BisQ to Various Ligands. Anti-3,3′-BisQ antibodies were elicited in rabbits by immunization with a conjugate consisting of bovine serum albumin linked to a suitable derivative of BisQ. Binding to the antisera was determined by a competitive radioimmunoassay using 3,3′-*trans*-[^{3}H]BisQ as the primary ligand. The results are shown in Table 1. The order of binding activities of the various ligands (agonists) was generally similar to that seen in experiments with AcChoR, although exact comparisons were not possible because many of the latter were made with electroplax or membrane preparations rather than free soluble AcChoR (3, 4). There were some additional striking findings: (*i*) The IC_{50} value for *trans*-BisQ (0.15 μM) was close to the 50% point on the dose–activity curve in *Electrophorus electricus* electroplax preparations (0.06 μM) (1). (*ii*) Decamethonium ion, an agonist, bound about 500 times better than hexamethonium ion, an antagonist. *A priori*, one would not expect an antibody to distinguish so markedly between two such similar molecules.

Characteristics of the Response to Immunization with Anti-BisQ. Anti-3,3′-*trans*-BisQ was purified on an affinity column and used to immunize three rabbits. Primary and booster immunizations were done by multiple intradermal injection in complete Freund's adjuvant. Rabbits were bled at various times (Table 2), and the animals were observed for signs of muscle weakness as they moved in their cages or on a smooth floor. Limb strength was tested by repeatedly extending the limb and assessing the force and rapidity with which it was retracted to the body (Table 2). Rabbit 522 showed slight weakness in the left hindleg 7 days after the first booster injection. Two days

Table 1. Inhibition of binding of *trans*-3,3′-[^{3}H]BisQ to antibody

Inhibitor	IC_{50}, μM	Relative inhibitory concentration
trans-3,3′-BisQ*	0.15	1
3,3′-BisQ amide (5)	0.25	1.7
Methylene stigmine†	0.55	3.7
trans-4,4′-BisQ	0.9	6.0
Decamethonium ion	2	13.3
Acetylcholine	20	133
Succinoylcholine	25	167
Butyltrimethylammonium ion	100	667
Carbamoylcholine	200	1,333
Hexamethonium ion	1,000	6,665
d-Tubocurarine	1,000	6,665

* The *cis* isomer has not been purified. Preliminary studies using a 70:30 *cis/trans* mixture indicate little if any binding of the *cis* isomer.

† *m*-(dimethylcarbamoyloxy)benzyltrimethylammonium bromide, a highly active agonist and homologue of neostigmine (unpublished).

Table 2. Immunization schedule and observations

Date	Procedure	Signs of muscle weakness 522	523
5/18	Primary immunization	—	—
6/8	Bleed and boost	—	—
6/15	Observation	±	—
6/17	Observation	2+	±
6/19	Observation	3+	2+
6/23	Observation	4+	+
6/29	Bleed and boost	4+	+
6/30	Observation	3+	+
7/2	Observation	2+	—
7/6	Observation	±	—
7/9	Observation	—	—
7/31	Bleed and boost	—	—
12/8	Bleed and boost	—	—
12/14	Bleed	—	—
12/23	Bleed	—	—

All booster immunizations were in complete Freund's adjuvant. Rabbit 524 showed no obvious signs of muscle weakness.

later, both hindlegs showed signs of weakness that gradually became more severe and ultimately no retraction was observed after a single extension of the limbs. The signs peaked 15–20 days after the first booster injection. On day 21, a second booster injection was administered. The rabbit improved progressively and appeared normal 8–10 days later.

Rabbit 524 showed no obvious signs of muscular weakness throughout the same immunization schedule.

Rabbit 523 showed signs of weakness in the left hindleg 8 days after the first booster injection. Weakness increased for 2 to 3 days thereafter and then tapered off, although it was noticeable for the next 5 days. After the second booster injection, muscle weakness was not observable. In short, rabbit 523 showed signs that were similar to those of rabbit 522 but less severe.

The animals were also observed for any drooping of the head or ears or tendency to become cyanotic when agitated, but nothing of this nature was seen.

Anti-AcChoR activity of the sera was assayed by complement fixation and by an enzyme immunoassay. Complement fixation data on the activity toward a purified rat receptor preparation (20) of serum (6/23) from rabbit 522, taken 15 days after the first booster injection, when signs of muscle weakness were severe, is shown in Fig. 1*A*. Serum from a patient (K. C.) with myasthenia gravis was studied concurrently. With respect to the rabbit serum, the curves shown are typical of those obtained at excess antibody concentration. Unfortunately, the serum became anticomplementary before additional experiments at higher serum dilutions could be carried out. The data indicate, however, that the anti-AcChoR activity was at least as high as that found in the patient. Anticomplementary activity could be removed by isolation of the IgG portion of the serum by DEAE-cellulose chromatography (22). Conventional complement fixation curves were obtained (Fig. 1*B*) with IgG from serum 6/23 of rabbit 522. We estimate that this titer is about 70% of that of whole serum, presumably because of the absence of IgM. Similar dilution of an IgG fraction prepared from serum taken just prior to the first booster injection of rabbit 522 (serum 6/8) did not fix complement (data not shown).

A more direct and convenient assay of anti-AcChoR activity was by enzyme immunoassay. The binding of a rat receptor preparation (20) by serum 6/29 from rabbit 522, drawn just prior to the second booster injection (i.e., when signs of muscle weakness were still severe) is shown in Fig. 2. Three different

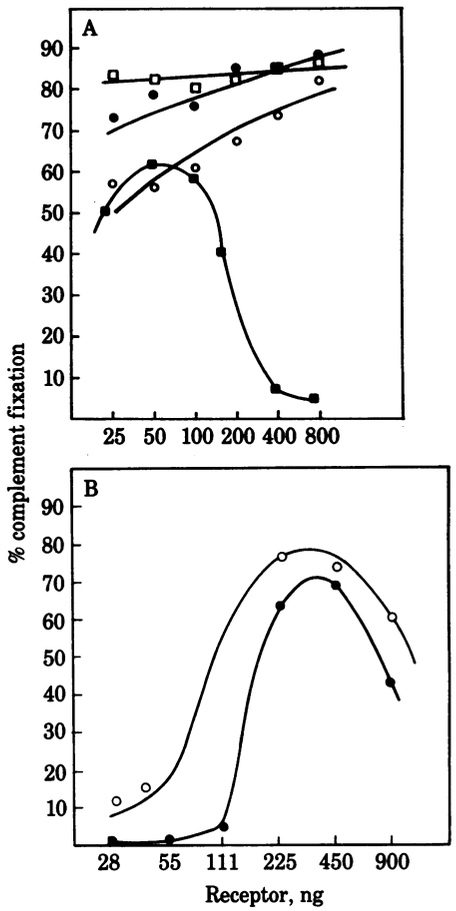

FIG. 1. Complement-fixation assays with rat receptor. (*A*) Serum 6/23 from rabbit 522 diluted 1:25 (□), 1:50 (●), and 1:100 (○) is compared with serum of patient (K. C.) diluted 1:100 (■). (*B*) IgG fraction of serum 6/23 from rabbit 522 diluted 1:8 (○) and 1:10 (●). Original protein concentration of IgG was 0.6 mg/ml; the volume of assay solution used in *B* was 10 times that used in *A*.

sera taken from unimmunized rabbits were also assayed, as was inhibition by soluble receptor and by BisQ. Considerable binding was observed with serum 522-6/29, well above that of unimmunized animals. Binding was inhibited by incubation of the serum with excess soluble rat receptor and by 10 μM BisQ. Binding of *Torpedo* (not shown) and *Electrophorus* purified receptor preparations could also be demonstrated by the enzyme immunoassay (Fig. 3). On the other hand, serum drawn prior to the first booster injection had a low titer, close to that of sera of unimmunized rabbits. (The titer of one of the unimmunized animals is given in Fig. 3).

Sera of the other rabbits were also examined by enzyme immunoassay. Rabbits 523 and 524 showed significant and similar titers after the first booster injection; their titers were 30–40% of that found in rabbit 522. No significant difference in titer between rabbits 523 and 524 could be found (data not shown), despite the fact that signs of muscle weakness were seen in the former and not in the latter.

Finally, the serum of rabbit 522 taken at various times during the immunization protocol was assayed in an attempt to correlate signs of muscle weakness with anti-AcChoR titer (Fig. 4). The titer was highest on 6/29, just prior to the second booster injection, when the rabbit showed signs of severe muscle weakness. (This titer was, within experimental error, the same as that of serum 522-6/23.) Seven days after the second booster injection (serum 522-7/6), the titer decreased to ¼ to ⅓ the earlier

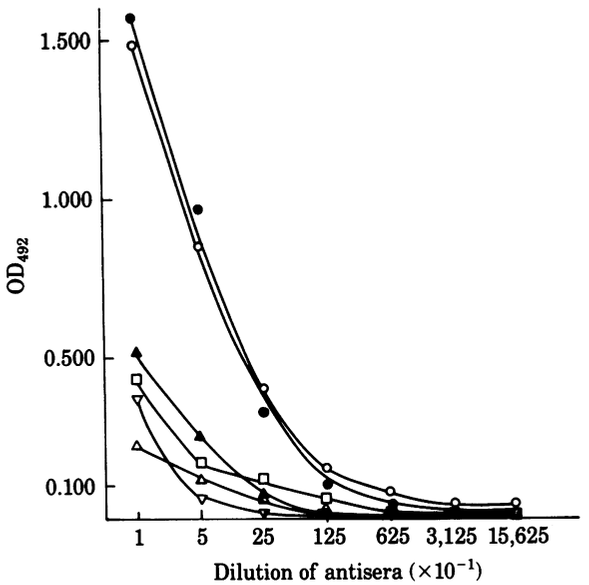

FIG. 2. Enzyme immunoassay with rat receptor. Enzyme label is peroxidase. ● and ○, serum 522-6/29 (two separate runs); □, serum 522-6/29 in the presence of 200 μl of soluble receptor (4 μg/ml); ▲, △, and ▽, sera from three unimmunized rabbits.

value and remained at that level for 25 days (7/31). A third booster injection was given at that time but the animal was not bled for 4 months. The animal appeared to be perfectly healthy during this period. On 12/9/81, a fourth booster injection was administered. Five days later, the titer was extremely low, as low or lower than found in unimmunized animals (compare with Fig. 2) and about the same as that of serum taken just prior to the fourth booster injection (data not shown).

The above booster injections were administered in complete Freund's adjuvant. Rabbit 523 was given a fifth booster injection 1 month after the fourth one but, in this case, anti-BisQ was administered intradermally in saline. Five days later, rabbit 523 was observed to move his hindlegs poorly. Over the next 4 days, he stopped grooming his tail, hindlimbs, and lower back and was able to raise himself only partially on his forelimbs. Two weeks after the saline booster injection, neostigmine (0.4 mg) and atropine (0.1 mg) were administered intramuscularly. Ten minutes later, the rabbit had fasciculations of back muscles and

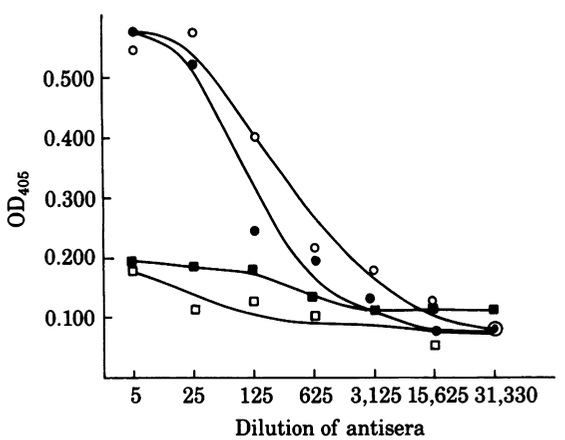

FIG. 3. Enzyme immunoassay. Enzyme label is alkaline phosphatase. ○, Serum 522-6/23 with eel receptor; ●, serum 522-6/23 with rat receptor; □, serum 522-6/8 with eel receptor; ■, normal (unimmunized) rabbit serum with electric eel.

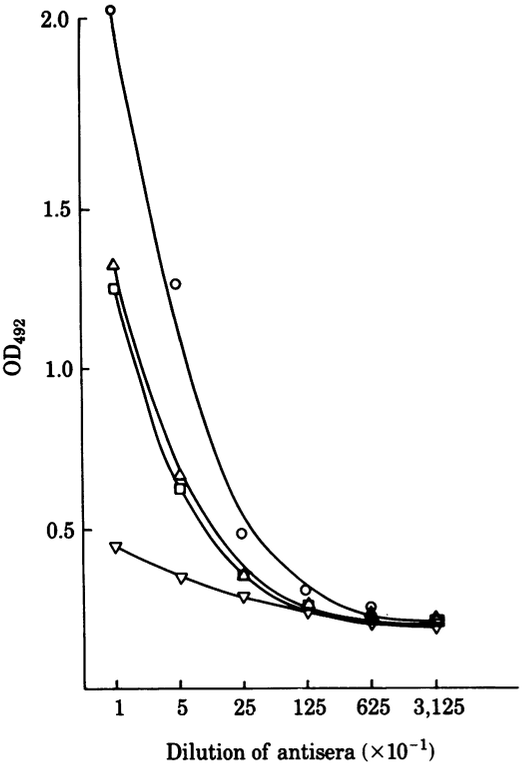

FIG. 4. Change in enzyme immunoassay titer with time. ○, serum 522-6/29; △, serum 522-7/6; □, serum 522-7/31; ▽, serum 522-12/14. The condition of the animals is summarized in Table 2.

perspired heavily behind his neck; 30 min later, the limbs were strong enough to allow him to assume a sitting position and to move about without his forelimbs sliding out from under him. The hindlimbs were stronger against resistance but not enough to allow him to hop. The improvement lasted about 1 hr, after which he gradually became weaker and reverted to his condition before the administration of the drugs.

In another series of immunized rabbits, one rabbit showing generally slow responses and inefficient grooming after the first booster injection showed post-tetanic exhaustion of hindlimb muscles after stimulation of the sciatic nerve at 50 Hz, although no decrement was observed at 3 and 5 Hz.

DISCUSSION

These studies were based on two hypotheses. (*i*) The binding site of an antibody to a potent structurally constrained ligand will resemble the receptor for the ligand with respect to specificity—i.e., anti-ligand ≡ receptor for ligand. (*ii*) Antibodies to the anti-ligand will crossreact with the receptor—i.e., anti-anti-ligand ≡ anti-receptor.

In the BisQ–AcChoR system, these hypotheses have been substantiated. First, the population of antibodies raised to BisQ shows a pattern of specificities similar to that of the AcChoR. The most striking aspect of the antibody specificity was the ability to distinguish between decamethonium ion, an agonist, and hexamethonium ion, an antagonist. This is in agreement with the suggestion (6) that agonists bind to and stabilize the AcChoR when it is in its activated state. The antisera are therefore the "equivalent" of the receptor when the latter is in the activated, rather than the resting, state—i.e., anti-ligand ≡ AcChoR (activated).

With regard to the second hypothesis, immunization of three rabbits with anti-BisQ produced AcChoR-reactive antibodies

in all, as determined by complement fixation and direct binding studies (i.e., by enzyme immunoassays) using eel, *Torpedo*, or rat preparations. The titers were similar to that found in a patient with myasthenia gravis. Moreover, two of the three rabbits showed signs of muscle weakness typical of experimental myasthenia gravis, although milder than that which usually results from immunization with purified receptor preparations (11). The severity of the signs in the two rabbits correlated well with their anti-AcChoR titers. On the other hand, rabbit 524, which had a significant titer, showed no signs of muscle weakness.

Although additional studies are required to confirm it, there is reason to believe that the response to immunization of the rabbits with rabbit anti-BisQ represents a set of anti-idiotypic responses directed at determinants that are near or part of the binding site of the first antibody (although one cannot rule out an additional anti-allotypic response since the rabbits were not deliberately allotypically matched) and that the ability of anti-anti-BisQ to bind the AcChoR is due to determinants on the binding site of the AcChoR that crossreact with determinants on anti-BisQ. The fact that binding to the AcChoR is inhibited by BisQ is in agreement with the earlier suggestion that the antibody resembles the receptor when the latter is bound to an agonist (i.e., in the activated state). An interesting aspect of the response to anti-BisQ is its transient nature in rabbit 522, both with respect to anti-AcChoR titer and to signs of experimental myasthenia gravis. Maximal titer and maximal muscle weakness occurred after the first booster injection and remained high until the second, after which the titer decreased to 25–35% the earlier value and signs of weakness disappeared. Subsequent booster injections actually caused refractoriness; i.e., the anti-AcChoR titer decreased to the range found in unimmunized animals. This kind of behavior could well be caused by an idiotypic response to the anti-anti-BisQ antibody, resulting in suppression of the response (23). More experiments are necessary to support this suggestion and, moreover, to try to enhance, rather than suppress, the response by means of the anti-idiotypic network. On the other hand, severe muscle weakness could be induced in rabbit 523 with anti-BisQ in saline about 6 months after the primary immunization and after several ineffective booster injections in complete Freund's adjuvant.

Our results are in accord with the earlier findings of Sege and Peterson (24) that antibodies raised to anti-insulin antibodies react with insulin receptors. While this work was in progress, similar studies were reported on β-adrenergic receptors (25) and on the chemotactic receptors of the neutrophil (26).

Our findings suggest a new methodology for the study and isolation of receptors. Antibodies to a given receptor can be raised without the necessity of isolating the receptor by raising antibodies to a suitable ligand of the receptor and subsequent immunization with these antibodies. Such receptor-reactive antibodies should be suitable for immunoassay procedures and for immunocytochemistry. Moreover, they should also be applicable to the isolation of receptors on a large scale.

The ability to induce signs of experimental myasthenia gravis has implications concerning the etiology of human autoimmune diseases in which antibodies to receptors are involved; i.e., some of them might result from an anti-idiotypic response to an antibody specific for a ligand of the receptor. For example, in Graves disease, antibodies reactive with thyroid receptor have been found in a great majority of patients (27). At least in some cases, they could have arisen in response to circulating antibodies to thyrotropin. The same kind of phenomena could occur in cases of diabetes in which anti-insulin receptor antibodies have been detected (28).

Note Added in Proof. Two sera from rabbit 522 and one from rabbit 523 have been shown to inhibit the binding of [^{3}H]BisQ to anti-BisQ. Another, taken two days later from rabbit 523, caused enhancement; this one was found to bind [^{3}H]BisQ. Presumably, it contains the next generation of anti-idiotypic antibody directed at the combining site of anti-anti-BisQ.

We thank Dr. R. E. Lovelace for the electromyographic studies and E. Lamme, I. Zilberstein, and S. Ramabhadran for excellent technical assistance. This research was supported by National Institutes of Health Grants NS-15581 and AI-17949 to B.F.E. and NS-17904 to A.S.P., by a grant from the Muscular Dystrophy Association to B.F.E. and a center grant from the Muscular Dystrophy Association, and by Training Grant T32-AI-07161 to the Department of Microbiology from the National Institutes of Health.

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