Purification of High-Affinity Fab Fragments from Experimental Autoimmune Myasthenia Gravis Rabbits and Their Effect on Isolated Acetylcholine Receptors†

Mirta Mihovilovic and Marino Martinez-Carrion*

ABSTRACT: Immunoglobulins (IgG) were isolated from rabbits suffering from experimentally induced autoimmune myasthenia gravis (EAMG) produced by the injection of acetylcholine receptor (AcChR) isolated from *Torpedo californica*. Fab fragments were prepared from IgG and labeled with fluorescamine to produce fluorescence fragments (Fab_F). Fab_F fragments are immunologically active and show competition with Fab fragments in binding to AcChR. EAMG Fab fragments retain the immunochemical affinity present in EAMG serum as they inhibit formation of immunodiffusion precipitin lines of AcChR against EAMG IgG. Stable complexes of AcChR–Fab_F were isolated by gel filtration of mixtures of AcChR and Fab_F fragments. Fluorescence of the fluorescamine-labeled Fab_F, binding of [^{125}I]-α-bungarotoxin ([^{125}I]-α-Bgt), and sedimentation values were used to determine the molar ratio EAMG Fab_F/AcChR. A maximum of four EAMG Fab fragments bind per mole of AcChR. EAMG Fab–AcChR complexes can also be precipitated by antirabbit IgG goat serum. Incubation of AcChR with a preparation of Fab fragments derived from (EAMG) IgG slows the time-dependent binding of α-Bgt to AcChR. AcChR–Fab complexes isolated by gel filtration also show the same slow α-Bgt binding time dependence. On the other hand, in the presence of Fab fragments, the total amount of α-Bgt that can bind to AcChR decreases only 10–15% after prolonged exposure to α-Bgt. High-affinity Fab–AcChR complexes cannot be broken down by an excess of α-Bgt, nor is α-Bgt displaced from α-Bgt-AcChR complexes by Fab fragments. Chromatographically stable EAMG Fab–AcChR complexes retain most of their ability to bind a cholinergic ligand, propidium. This ligand can also be slowly displaced by α-Bgt. By contrast, in the absence of bound Fab fragments, the displacement of receptor-bound propidium by α-Bgt is instantaneous. It is concluded that in this model for an autoimmune disease most of the antigenic sites in the AcChR molecule are different from those sites responsible for α-Bgt binding and the antigenic sites with high affinity for Fab may partially overlap with a few of the propidium-binding sites.

In patients with myasthenia gravis (MG),[1] a disease which inflicts muscle weakness, AcChR antibodies have been detected (Almon et al., 1974; Almon & Appel, 1975; Lindstrom et al., 1976b). Animals injected with solubilized AcChR preparations show the general symptoms of MG, and this condition is known as EAMG (Lindstrom et al., 1976a,b; Patrick & Lindstrom, 1973).

In both MG patients and EAMG animals fewer toxin-binding sites are detected at the neuromuscular junctions (Engel et al., 1977a,b). The AcChR that can be extracted from those muscles contains bound antibodies. Nevertheless, isolated AcChR can still bind neurotoxins (Lindstrom et al., 1976a). These findings have led to the perception that antibodies in the sera of both MG and EAMG cases are directed primarily against sites other than those responsible for neurotoxin or neurotransmitter binding.

Globulin serum fractions in EAMG animals have high titers of anti-AcChR. These antibodies block the carbamylcholine-induced depolarization of eel electroplaques (Lindstrom et al., 1976b; Sugiyama et al., 1973; Patrick et al., 1973). Furthermore, antibodies against AcChR decrease the receptor response and conductance of postsynaptic membranes (Heinemann et al., 1977). Thus, at present, in MG and EAMG evidence points to both a decrease in AcChR receptor levels and partial inhibition of receptor activity by bound antibodies. However, little is known regarding the effect of anti-AcChR antibodies on the binding parameters of agonists and antagonists to the receptor protein, primarily because no suitable "in vitro" system has been described. Preparations of soluble complexes of AcChR antibody should facilitate the analysis of the effect of bound antibody on the binding of cholinergic ligands to the receptor in solution. Studies carried out with AcChR and total IgG fractions of EAMG serum present two main problems: high concentrations of nonspecific IgG (no more than 2–5% of the IgG populations is anti-AcChR) and the insolubility of the AcChR–IgG complexes. These problems are evident in attempts made to isolate specific antibodies against AcChR from patients suffering from MG (Lefvert & Bergstrom, 1978). Problems associated with the use of IgG can be largely overcome by preparing Fab fragments because they should retain the antigen recognition sites, and, most important from the experimental point of view, they form soluble antigen–Fab complexes. In this work we describe the preparation of high-affinity EAMG Fab–AcChR complexes and some of their properties, including analysis of the binding of α-bungarotoxin and of a cholinergic ligand.

Methods

Purification and Assay of AcChR. AcChR from *T. californica* was purified as previously described (Sator et al., 1979). Preparations showing specific activities of 7.7–8.8 nmol of α-Bgt binding per mg of protein were used.

The concentration of α-Bgt-binding sites was determined by a DEAE-cellulose filter disc assay procedure (Schmidt & Raftery, 1973) using [^{125}I]-α-Bgt prepared from α-Bgt purified

† From the Department of Biochemistry, Virginia Commonwealth University, Medical College of Virginia, Richmond, Virginia 23298. *Received February 8, 1979.* This work was supported by National Science Foundation Research Grant BNS 77-24715. A preliminary report of these findings was presented at the American Society of Biological Chemists Meeting, June 1978, Atlanta, Georgia.

[1] Abbreviations used: AcChR, acetylcholine receptor; α-Bgt, α-bungarotoxin; MG, myasthenia gravis; EAMG, experimentally induced autoimmune myasthenia gravis; $NaDodSO_4$, sodium dodecyl sulfate.

0006-2960/79/0418-4522$01.00/0

from *Bungarus multicinctus* venom (Sigma Chemical Co.) by the procedure of Clark et al. (1972). Protein concentration was determined by the method of Lowry et al. (1951).

Immunization. AcChR (0.1 mg) emulsified with complete Freund's adjuvant was injected subcutaneously into female rabbits, a booster of 0.1 mg of AcChR emulsified with incomplete Freund's adjuvant was given 11–14 days after the first injection, and the animals bled upon development of weakness or a positive immunodifusion test. If additional boosters were required, they were given every 11–15 days. A positive immunodiffusion test is defined as that producing a well-defined precipitive line when a dilution of serum one-fourth or greater is used. These titers are detectable in immunized animals 24–48 h before the onset of paralysis. Microdiffusion experiments were carried out in gels of 0.75% Noble agar, Tes buffer (10 mM, pH 7.3), 0.5% NaCl, 0.01% Triton X-100, and 0.01% NaN_3.

IgG Purification. EAMG IgG and normal rabbit IgG were purified by $(NH_4)_2SO_4$ precipitation (twice with 40% and twice with 33%) followed by DEAE-cellulose chromatography (Campbell et al., 1970). The presence of IgG was determined by its ability to precipitate solubilized AcChR (see below). By this procedure, the final yield of specific anti-AcChR antibody ranged from 95 to 98%. These antibodies consist mainly of IgG molecules as detected by immunoelectrophoresis, $NaDodSO_4$ electrophoresis, and gel filtration. The serum IgG concentration was 9.8 ± 0.5 mg/mL.

IgG Titers. Titers of IgG preparations were obtained through immunoprecipitation of AcChR or the AcChR–[^{125}I]-α-Bgt complex. Two methods were used.

(*a*) *Immunoprecipitation of AcChR.* Aliquots (0.01–0.04 mL) of AcChR preparation (1 to 2 mg/mL) in 10 mM Tris-HCl buffer, pH 7.4, 0.03% Triton X-100, and 0.02% NaN_3 (Tris–Triton buffer) were mixed with increasing volumes of the IgG preparation (10–30 mg/mL) to be tested and brought to a final volume of 0.3–0.4 mL with 0.1 M sodium phosphate buffer, pH 7.4, 0.03% Triton X-100, and 0.02% NaN_3. After incubation for 1 h at room temperature and overnight at 4 °C, the samples were centrifuged and 0.1 mL of the supernates was checked for the presence of AcChR by the DEAE-cellulose filter disc assay. The presence of normal (non-EAMG) rabbit IgG added as carrier did not affect the results.

(*b*) *Immunoprecipitation of AcChR–[^{125}I]-α-Bgt Complex.* AcChR–[^{125}I]-α-Bgt complex was precipitated by EAMG IgG fractions and centrifuged, and the nonprecipitated AcChR–[^{125}I]-α-Bgt complex was measured by the DEAE-cellulose filter disc assay. From the slope of the curve the titer of the IgG preparations was calculated. From the data in Figure 1, a titer of 61.9 μg of AcChR precipitated per mg of IgG (61.9 units/mg of protein) was obtained. Different preparations gave values between 30 and 65 units/mg of protein.

Fab Fragments. Fab fragments were obtained after incubation of the IgG with papain for 3 h at 37 °C. The reaction was stopped by filtration through Sephadex G-75 or G-150, and the Fab fragments were purified by CM-cellulose chromatography (Nisonoff, 1964). $NaDodSO_4$–5% acrylamide gel electrophoresis revealed a single protein band of 45 000 daltons.

Fluorescamine Treatment of Fab Fragments. Aliquots of 0.1 mL of a fluorescamine solution in acetone (3–6 mg/mL) were added every 10–15 min while stirring to a Fab solution (5–15 mg/mL) in 0.1 M sodium borate, pH 9.4–9.5, to a final ratio of 0.5 mg of fluorescamine per 15 mg of protein. After

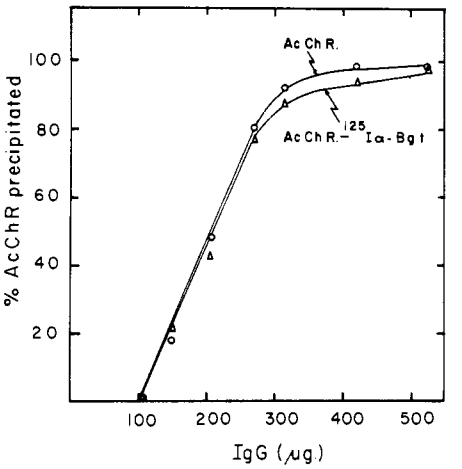

FIGURE 1: Immunoprecipitation of AcChR and α-Bgt–AcChR complexes with rabbit antireceptor IgG. (Δ) [^{125}I]-α-Bgt–AcChR complex precipitated by an EAMG IgG preparation (14.7 μg of AcChR mixed with a 3 times molar excess of [^{125}I]-α-Bgt for 1 h at room temperature before precipitation with EAMG IgG); and (O) AcChR (14.7 μg) precipitated by the same EAMG IgG preparation. AcChR and AcChR–[^{125}I]-α-Bgt were detected in the supernatant of the reaction mixtures after centrifugation of the receptor–IgG complexes.

the last addition, the mixture was stirred for 60 min and applied to a Sephadex G-75 or G-150 column (2.4 × 90 cm) equilibrated with 10 mM sodium phosphate buffer, pH 7.4. All procedures were carried out at room temperature. After dialysis against 10 mM sodium phosphate buffer, pH 7.4, and 0.02% NaN_3, the method described by Handschin & Ritschard (1976) was used to calculate the fluorescamine/Fab ratio. Ratios of 4.75–5.3 were obtained; lower values could be obtained by decreasing the fluorescamine concentration or the pH during the coupling process.

Calculations of EAMG Fab/AcChR Ratio. Titrations of AcChR (0.1 mg) with Fab_F were carried out in Tris–Triton buffer. After incubation for 1 h at room temperature and overnight at 4 °C, the mixtures (2.14–3.7 mL) were passed through Sephadex G-150 (1.5 × 104 cm). AcChR concentration was measured by [^{125}I]-α-Bgt binding, and the fluorescent species were followed by their emission at 480 nm. Fractions showing the presence of the complex were pooled, dialyzed against Tris–Triton buffer, and rechromatographed on a Sepharose 6B column (1.5 × 120 cm) to achieve a complete separation of EAMG Fab_F from the total Fab_F population. M_r values of 45 000 for the Fab fragments and 270 000 for the AcChR (Martinez-Carrion et al., 1975) were used. Emission and excitation spectra of the Fab_F fragments alone and in AcChR–EAMG complexes are identical in 1% $NaDodSO_4$. Furthermore, the presence of 1% $NaDodSO_4$ does not produce changes in the fluorescence intensities of the Fab_F species even though the AcChR–Fab complex dissociates by the treatment as shown by gel filtration on a Sephacryl 200 column (1.0 × 52.0 cm). In all cases the fluorescence intensity of the isolated complexes was compared, after treatment with 1% $NaDodSO_4$, to that of the standard calibration curve of Fab_F fragments in 1% $NaDodSO_4$.

Competition between Fab_F and Fab. Equimolar concentrations of Fab_F and unlabeled Fab were mixed, and the AcChR (0.1 mg) preparation was incubated with such mixtures. The amount of bound Fab_F was followed by fluorescence at 480 nm after passing the mixture through Sephadex G-150 and Sepharose 6B columns.

Fab_F Depleted of the EAMG Fab_F Species. Fab_{F-noMG} fragments were obtained by incubation of the AcChR with

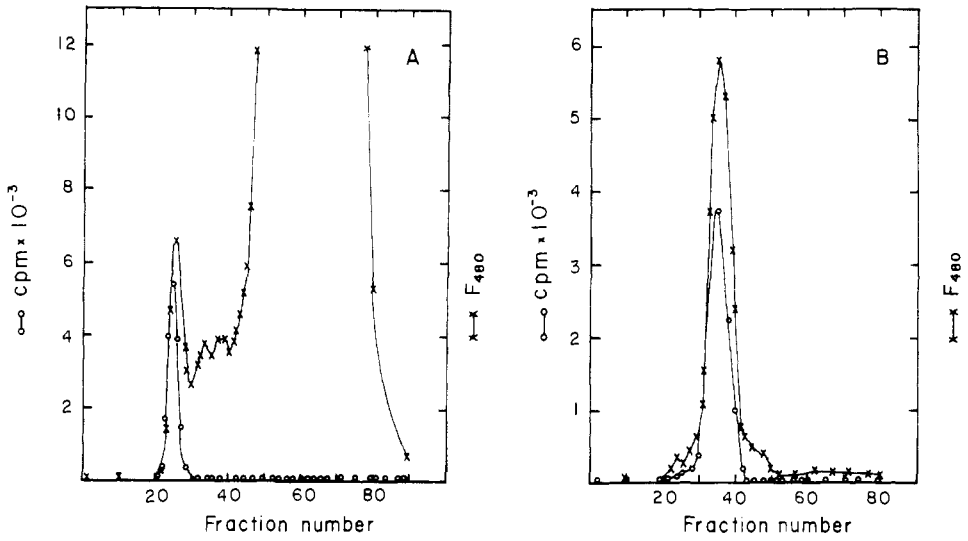

FIGURE 2: Elution profile of AcChR–EAMG Fab_F mixtures. Elution patterns: (A) Sephadex G-150; and (B) Sepharose 6B from a pool of (A) fractions 21–25. (O) [^{125}I]-α-Bgt binding; and (×) fluorescence at 480 nm.

an amount of Fab_F corresponding to 25% of the original IgG titer for 1 h at room temperature and overnight at 4 °C followed by gel filtration through Sepharose 6B. The larger peak of free Fab_F was considered to be Fab_{F-noMG} since in the presence of antirabbit IgG goat serum it could not coprecipitate AcChR.

Titration of the AcChR–Fab_F Complex with Antirabbit IgG Goat Serum. The complex AcChR–Fab_F and a mixture of AcChR and Fab_{F-noMG} in Tris–Triton buffer were incubated with increasing amounts of antirabbit IgG goat serum, and normal goat serum was added in order to give the same absorbance at 280 nm for each sample. After incubation for 1 h at 24 °C and overnight at 4 °C, the samples were centrifuged and 0.1 mL of the supernate was tested for the presence of AcChR by the α-Bgt assay.

Displacement of EAMG Fab_F from the AcChR–Fab_F Complex by α-Bgt. The possible displacement of EAMG Fab by α-Bgt from AcChR–Fab_F complexes was tested by incubation of the complexes (18–72 h) with a 3–30 molar excess of the toxin, subsequent gel filtration through Sephadex G-150 or Sephacryl 200, and monitoring for the appearance of free Fab_F species.

Binding of Propidium. The ability of the AcChR–Fab complex to bind propidium was measured by the fluorescence enhancement of the receptor-bound propidium as previously described (Sator et al., 1977).

Protection of SH Labeling by Fab Fragments. Labeling of the SH groups of AcChR by [^{14}C]-*N*-(3-pyrene)maleimide was carried out as previously described (Sator et al., 1978). Protection by Fab fragments was studied by preincubation of AcChR for 1 h at room temperature and overnight at 0 °C with saturating concentrations of Fab fragments without further purification or isolation of an AcChR–Fab complex.

Results

Purification of EAMG Fab Fragments and Isolation of Fab–AcChR Complexes. EAMG Fab species are a minor fraction of the total Fab fragment preparations obtained from the sera of EAMG-suffering rabbits. These high molecular weight components can represent a major contaminant, on a molar basis, if total Fab is used to titrate AcChR preparations. However, incubation of EAMG Fab preparations with AcChR followed by gel filtration results in the isolation of high-affinity AcChR–Fab complexes. The conditions chosen for the preparation of AcChR–Fab complexes consisted of mixing Fab fragments with solubilized AcChR. Best results for the isolation of AcChR–Fab complexes were obtained when a combination of Sephadex G-150 and Sepharose 6B gel filtrations was used (Figure 2). The protein elution peaks with a constant ratio of fluorescence emission at 480 nm and [^{125}I]-α-Bgt-binding capacity represent AcChR–Fab_F complexes. Such fractions precipitate with antirabbit IgG goat serum (Figure 3). Complexes with a Fab_F/AcChR molar ratio of 1.8–3.6 always precipitate with immune antiserum. However, anti-IgG goat serum never precipitated AcChR activity when the receptor had been incubated with Fab_{F-noMG} (Figure 3). After separation of free Fab_F, the total amount of the tightly bound Fab_F per mole of AcChR was measured. The amount of fluorescence was measured after treatment of the isolated AcChR–Fab_F complexes with 1% $NaDodSO_4$ to correct for possible solvent and binding effects on the fluorescence properties of the Fab_F molecules (see Methods). The values obtained are shown in curve 1 of Figure 4 for an experiment in which the amount of Fab_F was increased at a fixed AcChR concentration. Saturation levels are approached at a value of ~4 mol of Fab_F per mol of AcChR.

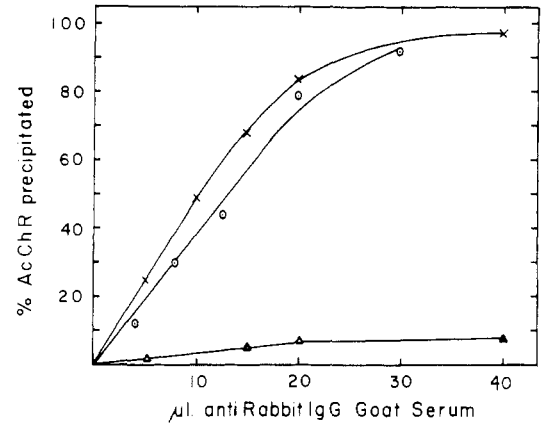

FIGURE 3: Immunoprecipitation of EAMG Fab_F–AcChR complexes with increasing concentrations of antirabbit IgG goat serum: (×) EAMG Fab_F–AcChR complex with Fab/AcChR molar ratio = 2.0 (12 μg of AcChR/mL); (O) EAMG Fab_F–AcChR complex with Fab/AcChR molar ratio = 3.3 (9.4 μg of AcChR/mL); and (Δ) AcChR alone (14 μg/mL) incubated with 14.1 μg/mL Fab produced from non-MG rabbit serum or Fab_{F-noMG}.

Effect of Fluorescamine on the Properties of EAMG Fab

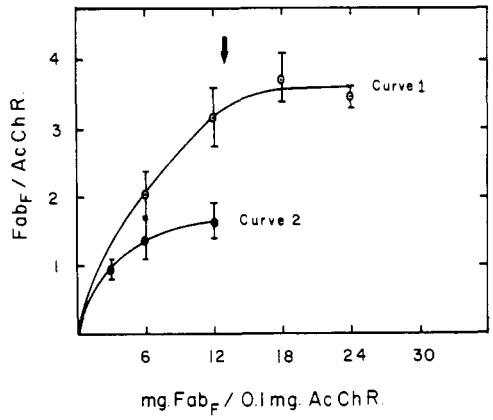

FIGURE 4: Competition between EAMG Fab and EAMG Fab_F for AcChR sites: curve 1, titration of AcChR with Fab_F; and curve 2, same amount of AcChR in the presence of equimolar concentrations of Fab and Fab_F. In both cases, the fluorescence of bound Fab_F was measured after chromatographic separation of the AcChR–Fab_F complex from free Fab_F.

Fragments. Labeling of the Fab fragments with fluorescamine does not appear to affect the EAMG Fab activity. Under conditions close to saturation of the AcChR by the EAMG Fab_F population, competition between Fab_F and Fab fragments shows 50% inhibition of Fab_F binding (Figure 4). Binding was measured after isolation of the complexes by chromatography through Sephadex G-150 and Sepharose 6B.

Determination of Fab Bound by Centrifugation. A different way of determining the amount of Fab_F which can be bound to AcChR makes use of the separation of the Fab_F–AcChR complexes by sucrose gradient centrifugation using Fab fragments, $s_{20,w}$ = 3.8 (Noelken et al., 1965), AcChR, $s_{20,w}$ = 9 (Raftery et al., 1976), and aspartate transaminase, $s_{20,w}$ = 5.7 (Feliss & Martinez-Carrion, 1970) as standard proteins. Two independent runs for an EAMG Fab_F–AcChR complex with a molar ratio of 2.0 Fab_F/AcChR determined by the fluorescamine fluorescence and specifically prepared by adding nonsaturating amounts of Fab_F and subsequent column fractionation showed a component with a sedimentation coefficient $s_{20,w}$ = 11.5. An apparent molecular weight was calculated by utilizing the relationship $M_r = 6\pi\eta_{20,w}Nas_{20,w}/(1 - \bar{\nu}\rho_{20,w})$ (Siegel & Monty, 1966), in which M_r = molecular weight, $\eta_{20,w}$ = viscosity of water at 20 °C, N = Avogardo's number, a = Stokes' radius, $s_{20,w}$ = sedimentation coefficient, $\bar{\nu}$ = partial specific volume, and $\rho_{20,w}$ = density of water at 20 °C.

As Sepharose 6B chromatography gives the same elution profile for the AcChR, free or complexed with EAMG Fab_F, the same Stokes' radius for both species was taken at 72 Å (Sator et al., 1978). On the other hand, partial specific volumes for AcChR, based on the amino acid composition (Raftery et al., 1976), and Fab fragments (Noelken et al., 1965) were reported as 0.735 and 0.738 cm^3/g; therefore, we assumed $\bar{\nu}$ = 0.736 for the complex. These calculations, disregarding the effect of Triton X-100 bound to AcChR, produce a value of 1.76 mol of Fab_F bound per mol of AcChR. Bound Triton X-100 can be accounted for as representing ~0.4 g of bound detergent per g of AcChR (Martinez-Carrion et al., 1975; Sator et al., 1978). If $M_r = M_c/(1 + x)$ (Gibson et al., 1976) related the molecular weight of the receptor (M_r) to the molecular weight of the receptor–detergent complex (M_c) and the number of milligrams of detergent bound per milligram of receptor, x, then the molar ratio of the complex is 2.5 mol of Fab_F bound per mol of AcChR. The sucrose density gradient profiles of AcChR also showed a small peak at 13 S in addition to the 9S component. It probably corresponds to the reported dimeric species (Gibson et al., 1976; Hamilton et al., 1977) obtained under similar conditions.

The reported molecular weights for *T. californica* AcChR and the relationship of the sedimentation coefficient values to specific M_r values are subject to various interpretations. A source of the discrepancies appears to be the corrections used in calcualating the amount of Triton bound to AcChR. These problems have been thoroughly discussed by others (Gibson et al., 1976) and will not be further elaborated upon. Recent findings regarding the amount of detergent bound to AcChR calculated by neutron diffraction methods[2] also agree with our previous results. In any case, our calculations using ultracentrifuge data agree with the stoichiometry values for Fab binding using the fluorescamine chromophore fluorescence values.

Relationship between IgG Sites and α-Bgt Sites. There is some controversy regarding mutual exclusion, in "in vitro" studies, of EAMG antibodies and α-Bgt binding to AcChR and whether the α-Bgt-binding sites are antigenic determinants (Aharonov et al., 1977; Trotter et al., 1977; Zurn & Fulpius, 1977). Thus, both AcChR and AcChR–[^{125}I]-α-Bgt complexes were tested for precipitation with EAMG IgG. The results in Figure 1 show little difference in the total amount of IgG that can bind to AcChR, whether the toxin-binding sites are occupied or free.

Relationship of Fab Sites to IgG Sites. Fab fragment preparations were consistently tested for their ability to block EAMG IgG binding to AcChR. The AcChR–Fab complex was unable to produce precipitin lines after 48-h immunodiffusion tests with EAMG IgG. No precipitin lines formed between AcChR and antibody even when the Fab fragments were added simultaneously to a solution of AcChR in the well of the diffusion plate without prior isolation of the AcChR–Fab complex. Control experiments in which non-EAMG Fab fragments were used instead did not protect against immunodiffusion line formation by EAMG IgG.

Relationship of Fab Sites to α-Bgt Sites. EAMG Fab fragments show significant protection against labeling of the receptor SH groups by *N*-(3-pyrene)maleimide. In isolated AcChR, 11 SH groups are labeled after a 2-h incubation with the SH reagent. In the presence of EAMG Fab fragments, only six SH groups are labeled. Non-EAMG Fab fragments failed to produce the protection. This protection by EAMG fragments contrasts with the poor protective action of α-Bgt against labeling by the same SH compound (Sator et al., 1978).

To follow the relationship between EAMG Fab and the Bgt-binding sites on the AcChR, we performed two sets of experiments. (a) Figure 5 shows that for [^{125}I]-α-Bgt binding to AcChR in the presence of excess immune Fab the time dependence of α-Bgt incorporation is decreased; however, after 18 h of incubation no more than 12% reduction of total [^{125}I]-α-Bgt bound is observed at saturating amounts of Fab (arrows, Figures 4 and 5). Furthermore, (b) similar results are obtained by using isolated AcChR–Fab_F complexes, and incubation with a 3–30 times molar excess of α-Bgt shows no displacement of fluorescent species.

Effect of Bound Fab on Propidium Binding. The AcChR affinity for a variety of ligands appears to indicate the existence of different subsites. One subsite has affinity for prodium (Sator et al., 1977). Prodium is smaller than α-Bgt and has only one positive charge. This property, in principle, refines

[2] A. Karlin, personal communication.

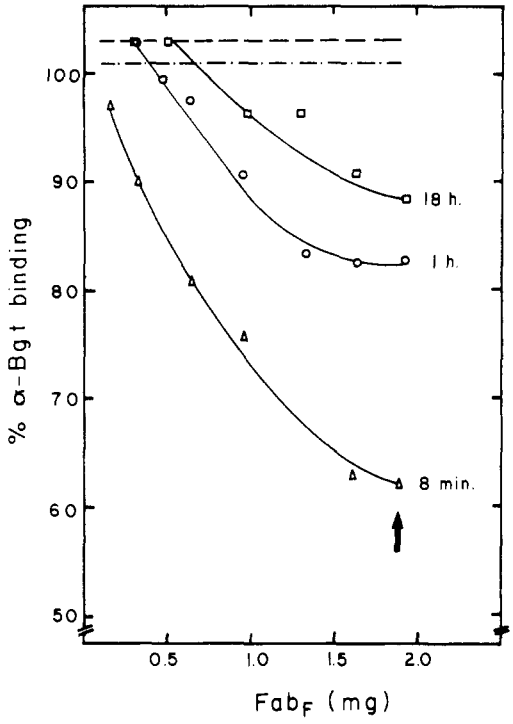

FIGURE 5: Time dependence of [^{125}I]-α-Bgt (3 times molar excess) binding to AcChR in the presence of EAMG Fab fragments: (dashed line) binding measured after 1-h incubation of 14.7 μg of AcChR with [^{125}I]-α-Bgt followed by addition of Fab or Fab$_F$ fragment; (–·–) α-Bgt binding after incubation of 14.7 μg of AcChR with nonimmune rabbit Fab fragments; and (solid lines) α-Bgt binding after incubation of 14.7 μg of AcChR with increasing amounts of a total population of EAMG Fab$_F$ (1 h at room temperature and overnight at 4 °C). The arrow indicates the Fab$_F$ concentration at which saturation of Fab sites occurs. The times indicate the time of exposure of the AcChR–Fab mixtures to α-Bgt before determining the amount of toxin bound.

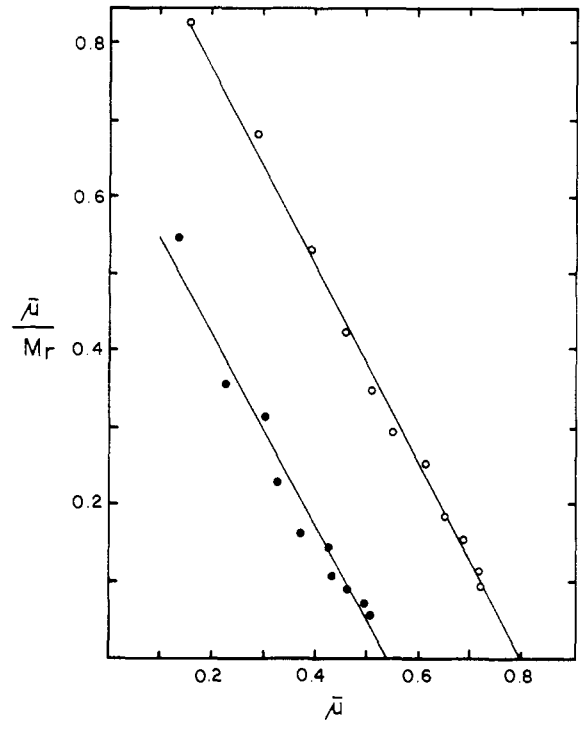

FIGURE 6: Scatchard plot of propidium binding to AcChR (○) and AcChR–Fab complex (●). AcChR concentration was determined by [^{125}I]-α-Bgt binding. Excitation was at 545 nm and emission at 623 nm; $\bar{\mu}$, moles of ligand bound per mole of AcChR.

the degree of selectivity for probing the receptor's surface. A smaller region of the receptor is likely to be the target of the probe rather than the several negative pockets for which the large, and basic, α-Bgt could have affinity. The values obtained for binding of propidium to AcChR and AcChR–Fab complexes showed that the affinity constant (slope) for propidium remains constant (Figure 6) while the number of propidium molecules bound to these preparations is diminished (abscissa intercept). Controls of AcChR treated with Fab$_{noMg}$ and subjected to the same chromatographic procedures do not show a decrease in the amount of propidium bound in similar titrations. The biggest source of error in these titrations, however, is in the quantitation of cholinergic ligand sites in AcChR–Fab complexes. They were estimated on the basis of a time-dependent curve for α-Bgt binding as in Figure 5. Thus, the amount of propidium bound at each point on the Scatchard plot (Figure 6) is calculated on the basis of the number of α-Bgt-binding sites and the independently measured identical fluorescence enhancement values obtained for propidium when bound to AcChR or AcChR–Fab complex.

Binding of propidium, unlike binding of α-Bgt, is instantaneous. Each addition of this fluorescent ligand to Fab–AcChR preparations produces immediate fluorescence enhancements which remain unchanged for over 24 h. On the other hand, the amount of receptor-bound propidium which can be displaced by α-Bgt (Sator et al., 1977) follows the same slow time dependence as for α-Bgt binding (Figure 5) to AcChR–Fab complexes. Such behavior is consistent with mechanisms for propidium displacement in which binding of α-Bgt is the rate-limiting step.

Discussion

Antibodies directed against AcChR have been detected in both MG (human disease) and EAMG (animal model). Though the animal model resembles the human disease in its electrophysiological response (Patrick & Lindstrom, 1973; Seybold et al., 1976; Lambert et al., 1976) and by histological (Engel & Santa, 1971; Engel et al., 1976) and immunological criteria (Lindstrom et al., 1976b), the animals show an acute phase which has no parallel in the human disease[3] (Lindstrom et al., 1976b). However, the chronic phase of EAMG closely resembles human MG (Lindstrom, 1978; Drachman, 1978).

In the impairment of neuromuscular transmission in both MG and in the chronic stage of EAMG, two explanations seem feasible (Drachman, 1978; Lindstrom, 1978): there is a loss of AcChR or inhibition of the AcChR activity. Lower AcChR concentration has been shown in MG and in chronic EAMG, where lysis of the postsynaptic membrane mediated by the C3 component of complement (Sahashi et al., 1978; Engel et al., 1977a,b; Lennon et al., 1978) and other antigenic modulations appear to play roles in the degradation of AcChR in the presence of MG or EAMG sera (Bevan et al., 1977; Appel et al., 1977; Heinemann et al., 1977; Kao & Drachman, 1977). On the other hand, the extent of inhibition of activity of AcChR after binding of antibodies and whether this inhibition is significant to postsynaptic transmission in both EAMG and MG are unresolved questions. As a first appraoch, the development of "in vitro" systems suitable for the study of ligand interaction with solubilized AcChR in the presence of its antibodies should assist in gaining molecular insight for the role of antibody in the impairment of AcChR functions. For reasons of solubility and the lowering of steric hindrance

[3] In our immunization procedures the animals are bled during the chronic phase of the experimental disease. In these animals only 10% of the immunized rabbits (and 1 out of 14 goats where these experiments have been reproduced) developed an acute phase.

effects, EAMG Fab fragments are used instead of the bivalent IgG. These findings can only be interpreted for EAMG for animal models in which the antigen is AcChR from electric fish. Thus, our findings may differ from events in the human disease, MG.

Binding of Fab fragments to isolated AcChR should occur at sites with diverse, juxtaposing and varying degrees of affinity for the antibodies. When a mixed population of Fab fragments prepared from IgG isolated from serum of EAMG rabbits is mixed with AcChR, many types of complexes must be formed. After treatment of the mixture by volume exclusion chromatographic procedures, only tight-binding antigenic sites must remain occupied by Fab. These high-affinity sites appear to be 4/mol of AcChR.

The determination of the number of molecules of Fab bound to AcChR can be affected, at least in part, by the nature of the methods employed. Determination of Fab by the amount of fluorescence assumes a statistical distribution of fluorescamine labeling among all Fab fragments and no selection by the AcChR for either high or low amounts of label in Fab_F fluorescence molecules. Experiments reported in Figure 4 are consistent with our assumption of a statistical distribution of fluorescence label among the Fab_F fragments bound to AcChR. The fact that the fluorescence measurements are always carried out after dissociation of the AcChR–Fab_F complex into Fab_F and AcChR and the fluorescence intensity is compared to standard dilutions of free Fab_F in the same solvent also contributes to minimizing possible sources of error. The possibility that EAMG Fab fragments select among other fragements in the population for a higher or lower fluorescamine labeling is remote.

We propose that in isolated AcChR, antigenic sites of high affinity for antibody remain occupied by EAMG Fab in the presence of the α-neurotoxin ligand. This proposed mechanism is based on the following observations: (a) after 48 h of immunodiffusion, the presence of EAMG Fab fragments considerably inhibits precipitin line formation of AcChR against specific IgG; (b) the presence of α-Bgt only slightly alters the titration pattern of AcChR with IgG (Figure 1); (c) α-Bgt is not capable of displacing Fab_F from AcChR–Fab_F complexes isolated by gel filtration; (d) Fab or Fab_F cannot displace [^{125}I]-α-Bgt preincubated with AcChR; and (e) the affinity of α-Bgt for isolated receptor is very high.

Attempts made to determine the target site(s) of the antibodies directed toward solubilized AcChR have been carried out by others with AcChR–IgG complexes (Almon et al., 1974; Lindstrom, et al., 1976a; Patrick et al., 1973; Sugiyama et al., 1973; Aharonov et al., 1977; Trotter et al., 1977; Lefvert & Bergstrom, 1978), and variable and conflicting degrees of inhibition of α-Bgt or cobratoxin binding have been reported. Those results, however, can be obscured by the fact that they refer to insoluble aggregates in which steric effects of IgG bound to AcChR should be higher than when using the smaller Fab fragments in which intermolecular cross-linking of AcChR by antibody is eliminated. The preparation of AcChR–Fab_F complexes is, in principle, more suitable for the study of the effect of EAMG antibodies on the properties of solubilized AcChR. For this model to be realistic, the Fab fragment must retain the binding ability of the intact IgG molecule. The results obtained for the competition of Fab fragments against IgG in immunodiffusion experiments support such similarities between Fab and IgG.

Binding of α-Bgt to AcChR–EAMG Fab_F complexes in which all Fab species from whole IgG preparations are present (Figure 5) reveals only a change in the time dependence of toxin binding to the receptor, which may be interpreted as little overlap between the antigenic sites and the α-Bgt sites, at least in isolated AcChR preparations. Instead, it appears that the antibody fragments stabilize a conformation of the AcChR for which the α-Bgt has lower affinity. An alternative explanation is that the large α-Bgt molecule has greater difficulty in reaching the target sites on the AcChR surface once the bound Fab fragments block a large portion of the receptor surface. Since we have shown that in our AcChR preparations a considerable amount of detergent is bound to the receptor (Martinez-Carrion et al., 1975; Sator et al., 1978), the latter explanation appears likely. If both the Fab fragments and toxin bind to the detergent-free zones of AcChR, hindrance effects between them are likely. Nevertheless, many of the sites on the receptor surface which are antigenic must be different from the sites for which α-Bgt has affinity. It is of interest that we can obtain similar results in AcChR-rich membrane preparations of *Torpedo* electroplax (M. Mihovilovic and M. Martinez-Carrion, unpublished experiments), which is a more realistic model for a neuromuscular junction surface. The interpretation of separate topographical relationships for Fab fragments and α-Bgt is strengthened by the finding that α-Bgt is a poor protector of SH group modification by *N*-(3-pyrene)maleimide while the EAMG Fab fragments are good protectors (Sator et al., 1978).

The antibodies produced in rabbit during EAMG appear to be a heterogeneous population with respect to their reactivity against AcChR. Only antibodies produced at the latest stages of the disease, just before paralysis sets in, appear to block some of the [^{125}I]-α-Bgt sites (Zurn & Fulpius, 1977). These results produced only 37% inhibition under equimolar concentrations of α-Bgt and AcChR. They do, however, indicate the possible presence of a different type of antibody in serum at this later stage of the disease. Other recent attempts to isolate whole antibodies against AcChR from MG-suffering patients are frought with uncertainty (Lefvert & Bergstrom, 1978). These authors had to cope with the impure state of receptor preparations from human muscle, the tendency of human receptor–neurotoxin complex to dissociate with time, and the fact that assays for ternary antibody–receptor–toxin complexes not only suffer from instability but also may be subject to the solubility and steric hindrance problems mentioned above. Nevertheless, these assays could be interpreted as indicating that in the human disease there are antibodies specific for the receptor's toxin-binding site(s) which are different from those directed against other protons of the receptor molecule. Our antibody preparations are from whole IgG pools of three to five rabbits which are isolated at the onset of paralysis (chronic phase) or 48 h before such symptoms appear. At this time they could contain some antibodies against α-Bgt sites. Since the α-Bgt-binding inhibition is overcome by long exposure to excess α-Bgt, it is unclear whether all the specific antigenic sites (α-Bgt sites) can be reached by α-Bgt with prolonged exposure to the toxin or if our animals have a low concentration of this type of antibody in the Fab pool prepared from their total IgG. Clarification of whether there is generation of selected antibodies against specific binding sites at different stages of the progress of the disease would require a time-dependent study of the α-Bgt binding to the AcChR–Fab complexes for each individual animal at each phase of the disease. Nevertheless, it is of interest that our results obtained with rabbits agree with those obtained with Fab fragments prepared from individual goats bled at the chronic phase of EAMG and thus do not appear to be circumscribed to the rabbit species. Under our ex-

perimental conditions the presence of the total Fab population, which contains both high- and low-affinity Fab molecules, induces in the receptor an α-Bgt-binding behavior identical with that found in experiments in which only purified high-affinity Fab_F–receptor complexes are used.

The results obtained with propidium are consistent with a mechanism in which in our experimental animals there is minor impairment of ligand binding to the AcChR–Fab complex. This is not unexpected since the receptor must have antigenic sites in close proximity to each other. Tight binding can be produced in several of these sites. The presence of Fab can then produce a heterogeneous population of AcChR molecules in which not all Fab molecules are bound to identical surface sites. In this case, some Fab fragments can hinder the propidium site, producing a reduced average number of binding sites such as was observed. Nevertheless, the total amount of propidium bound to the isolated Fab–AcChR complex is significant. Since the affinity constant is essentially unaltered from that measured for free AcChR, a conformational change on the propidium-binding site induced by occupancy of the antigenic sites by Fab fragments does not appear to be the most suitable explanation. Similar results are now being obtained (M. Mihovilovic and M. Martinez-Carrion, unpublished experiments) for the binding of two cholinergic ligands, decamethonium and *d*-tubocurarine, to AcChR–Fab_{MG} complexes.

References

Aharonov, A., Tarreb-Hazdi, R., Silman, I., & Fuchs, S. (1977) *Immunochemistry 14*, 129–137.

Almon, R. R., & Appel, S. H. (1975) *Biochim. Biophys. Acta 393*, 66–77.

Almon, R. R., Andrew, C. G., & Appel, S. H. (1974) *Science 186*, 55–57.

Appel, S. H., Anwyl, R., McAdams, M.-W., & Elias, S. (1977) *Proc. Natl. Acad. Sci. U.S.A.*, 2130–2134.

Bevan, S., Kullberg, R. W., & Heinemann, S. F. (1977) *Nature (London) 267*, 263–265.

Campbell, D. H., Gerney, F. S., Gremer, N. E., & Sussdorf, D. H., Eds. (1970) *Methods in Immunology*, 2nd ed., pp 189–198, W. A. Benajmin, Reading, MA.

Clark, D. G., Wolcott, R. G., & Raftery, M. A. (1972) *Biochem. Biophys. Res. Commun. 48*, 1061–1068.

Drachman, D. B. (1978) *N. Engl. J. Med. 298*, 136–142.

Engel, A. G., & Santa, T. (1971) *Ann. N.Y. Acad. Sci. 183*, 46–63.

Engel, A., Tsujihata, M., Lindstrom, J. M., & Lennon, V. A. (1976) *Ann. N.Y. Acad. Sci. 274*, 60–79.

Engel, A. G., Lindstrom, J. M., Lambert, E. H., & Lennon, V. A. (1977a) *Neurology 27*, 307–315.

Engel, A., Lambert, E., & Howard, G. (1977b) *Mayo Clin. Proc. 52*, 267–280.

Feliss, N., & Martinez-Carrion, M. (1970) *Biochem. Biophys. Res. Commun. 40*, 932–940.

Gibson, R. E., O'Brien, R. D., Edelstein, S. J., & Thompson, W. R. (1976) *Biochemistry 15*, 2377–2383.

Hamilton, S. L., McLaughlin, M., & Karlin, A. (1977) *Biochem. Biophys. Res. Commun. 79*, 692–699.

Handschin, V. E., & Ritschard, W. J. (1976) *Anal. Biochem. 71*, 143–155.

Heinemann, S., Bevan, S., Kullberg, R., Lindstrom, J., & Rice, J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3090–3094.

Kao, I., & Drachman, D. B. (1977) *Science 196*, 527–529.

Lambert, E. H., Lindstrom, J., & Lennon, V. A. (1976) *Ann. N.Y. Acad. Sci. 274*, 300–318.

Lefvert, A. K., & Bergstrom, K. (1978) *Scand. J. Immunol. 8*, 525–533.

Lennon, V. A., Seybold, M. E., Lindstrom, J. M., Cochrane, C., & Ulevitch, R. (1978) *J. Exp. Med. 147*, 973–983.

Lindstrom, J. M. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 2828–2830.

Lindstrom, J. M., Einarson, B., Lennon, V. A., & Seybold, M. E. (1976a) *J. Exp. Med. 144*, 726–738.

Lindstrom, J., Lennon, V., Seybold, M., & Whittingham, S. (1976b) *Ann. N.Y. Acad. Sci. 247*, 254–274.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Martinez-Carrion, M., Sator, V., & Raftery, M. A. (1975) *Biochem. Biophys. Res. Commun. 65*, 129–137.

Nisonoff, A. (1964) *Methods Med. Res. 10*, 134–141.

Noelken, M. E., Nelson, C. A., Burkley, C. E., & Tanford, C. (1965) *J. Biol. Chem. 240*, 218–224.

Patrick, J., & Lindstrom, J. (1973) *Science 180*, 871–872.

Patrick, J., Lindstrom, J., Culp, B., & McMillan, J. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3334–3338

Raftery, M. A., Vandlen, R. L., Leet, T., Moody, T., & Reed, K. (1976) *Cell Membrane Receptors for Viruses, Antigen and Antibodies, Polypeptide Hormones and Small Molecules* (Beers, R. F., Jr., & Bassett, E. G., Eds.) pp 425–433, Raven Press, New York.

Sahashi, K., Engel, A. G., Lindstrom, J. M., Lambert, E. H., & Lennon, V. A. (1978) *J. Neuropathol. Exp. Neurol. 37*, 212–223.

Sator, V., Raftery, M. A., & Martinez-Carrion, M. (1977) *Arch. Biochem. Biophys. 184*, 95–102.

Sator, V., Raftery, M. A., & Martinez-Carrion, M. (1978) *Arch. Biochem. Biophys. 190*, 57–66.

Sator, V., Gonzalez-Ros, J. M., Calvo-Fernandez, P., & Martinez-Carrion, M. (1979) *Biochemistry 18*, 1200–1206.

Schmidt, J., & Raftery, M. A. (1973) *Anal. Biochem. 52*, 349–354.

Seybold, M. E., Lambert, E. H., Lennon, V. A., & Lindstrom, J. (1976) *Ann. N.Y. Acad. Sci. 274*, 275–282.

Siegel, L. M., & Monty, K. J. (1966) *Biochim. Biophys. Acta 112*, 346–362.

Sugiyama, H., Benda, P., Meunier, J. C., & Changeux, J. P. (1973) *FEBS Lett. 35*, 124–128.

Trotter, J. L., Ringel, S. P., Cook, J. D., Engel, W. K., Eldefrawi, M. E., & McFarlin, D. E. (1977) *Neurology 27*, 1120–1124.

Zurn, A. D., & Fulpius, B. W. (1977) *Eur. J. Immunol. 8*, 529–532.