IMMUNOLOGICAL CHARACTERIZATION OF AN IRREVERSIBLY DENATURED ACETYLCHOLINE RECEPTOR

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1. Introduction

Considerable evidence now exists that myasthenia gravis is an autoimmune disease where acetylcholine receptor (AChR) is a major autoantigen. Cellular and humoral sensitivity to AChR are observed in patients with myasthenia gravis [1—4]. Experimental autoimmune myasthenia gravis (EAMG) induced in several animal species by the injection of purified AChR [5—10] has been demonstrated to be an appropriate model for the human disease. It is still not known which part(s) or antigenic determinant(s) in the AChR molecule are responsible for its myasthenic activity and whether these are overlapping with any site(s) involved in its physiological function. Detailed immunochemical analysis of AChR should throw light on these questions.

AChR is a multisubunit protein molecule [11—14] which seems to dissociate only under denaturing conditions. In this report we describe the preparation of an irreversibly denatured derivative of AChR obtained by complete reduction and carboxymethylation in 6 M guanidine hydrochloride and the immunochemical analysis of this preparation.

2. Materials and methods

AChR was isolated from the electric organ of *Torpedo californica* (Pacific Bio-Marine, Venice, Ca.) and was purified as described elsewhere [14]. Iodinations with ^{125}I of α-bungarotoxin (α-Bgt), AChR and reduced-carboxymethylated AChR (RCM-AChR) were performed by the chloramine-T method [15]. Specific binding of α-Bgt to AChR and RCM-AChR was assayed by measuring the amount of ^{125}I-labeled Bgt which coprecipitates with the receptor in 35% saturated ammonium sulfate [14,16].

2.1. *Preparation of reduced-carboxymethylated AChR (RCM-AChR)*

AChR (0.8 mg/ml) was dialyzed for 3 h against 6 M guanidine–HCl in 0.2 M Tris-buffer, pH 8.5. Reduction was performed with 0.1 M β-mercaptoethanol for 60 min at 37°C under nitrogen. The sulfhydryl groups were blocked by the addition of crystalline iodoacetamide to a final concentration of 0.15 M and maintaining pH 8.2 for 15 min by titration with 2 M Tris-base. RCM-AChR was dialysed against 0.01 M Tris-buffer, pH 7.5, containing 0.1 M NaCl, 10^{-3} M EDTA, 10^{-5} M PMSF, 5×10^{-4} NaN_3 and 0.01% Triton X-100.

2.2. *Immunization*

Rabbits were immunized twice (or three times) each time with 100 μg RCM-AChR (in 1 ml) emulsified with equal vol. complete Freund's adjuvant. Injections were given intradermally at multiple sites at 30 day intervals. For comparison other rabbits were injected once with AChR and if they did not die of EAMG they were given a similar booster injection of AChR after 30 days.

2.3. *Immunological assays*

Sera from the immunized rabbits were analyzed by quantitative precipitin reactions, immunodiffusion, micropassive hemagglutination and radioimmunoassay. For the radioimmunoassay serum dilutions (0.1 ml in 10% normal rabbit serum) were incubated with the radioactive antigen (^{125}I-labeled AChR or ^{125}I-labelled

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RCM-AChR) for 30 min at 37°C. Goat anti-rabbit immunoglobulin serum (0.1–0.2 ml) was added and the tubes were incubated for 30 min at 37°C and then overnight at 4°C. The centrifuged precipitates were washed twice and counted in an autogamma scintillation counter. For inhibition experiments, preincubation of the antiserum (at a dilution that binds ~40% of the radioactive antigen) with different amounts of the inhibitor was performed for 30 min at 37°C and the assay was continued as described above for the binding.

2.3. *Effect of antibodies on ^{125}I-labeled α-Bgt-binding to AChR*

The inhibition by antisera of the binding of toxin to AChR was measured by preincubation of AChR with increasing amounts of serum for 30 min at 37°C before the addition of ^{125}I-labeled α-Bgt and determination of the degree of toxinbinding relative to the binding obtained in the absence of serum.

3. Results

3.1. *Characterization of reduced-carboxymethylated AChR (RCM-AChR)*

Denaturation of AChR was performed by reduction and carboxymethylation in 6 M guanidine–HCl. Amino acid analysis of RCM-AChR demonstrated no significant change in amino acid composition apart from the conversion of all cystines and cysteine residues to *S*-carboxymethyl cysteine. Analytical velocity ultracentrifugation of RCM-AChR revealed one major component of 9.6 S suggesting that the constituent subunits were held together even after reduction and carboxymethylation. Electrophoresis of RCM-AChR on SDS–acrylamide gels [17] gave a similar pattern to that obtained with the unmodified receptor, with a slight difference in the relative colour intensity of the various bands.

RCM-AChR did not show any detectable binding of ^{125}I-labeled α-Bgt.

3.2. *Immunological characterization of RCM-AChR*

Rabbits immunized with RCM-AChR developed antibodies reacting both with the immunizing RCM-AChR and with AChR. Figure 1 shows the cross-reactivity between RCM-AChR and AChR as measured by the quantitative precipitin reaction. Rabbits immunized repeatedly with RCM-AChR do not develop any clinical signs of EAMG although they have high titers of antibodies reacting with AChR. This is in contrast to the onset of the disease in

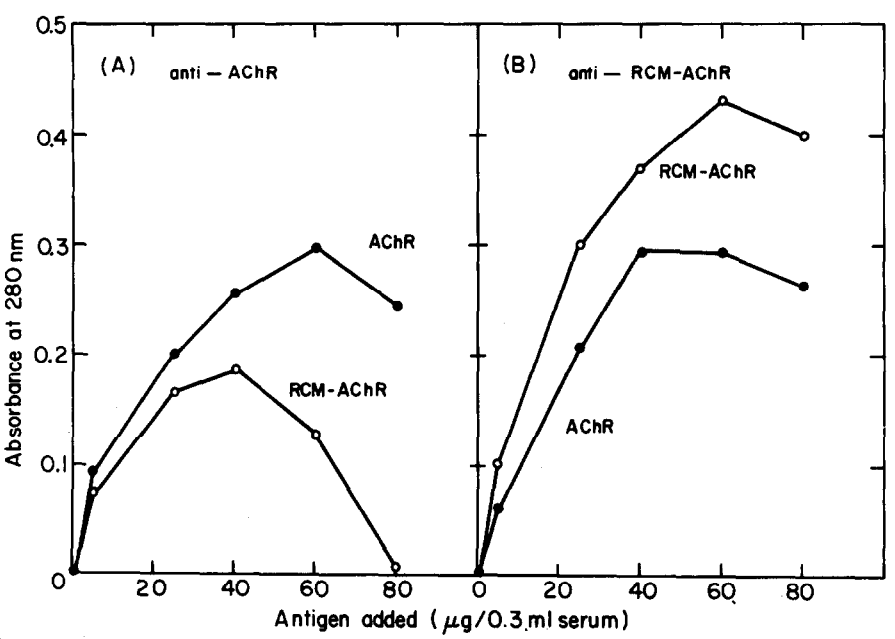

Fig.1. Precipitin reaction of anti-AChR serum (A) and anti-RCM-AChR serum (B) with AChR (•) and RCM-AChR (○).

rabbits usually following one injection (or occasionally two) with AChR.

In addition to the cross-precipitation the cross-reactivity between RCM-AChR and AChR was demonstrated also by micropassive hemagglutination and radioimmunoassay. Anti-AChR and anti-RCM-AChR sera agglutinate AChR-coated formalinized sheep erythrocytes [18] and give similar hemagglutination titers. Both antisera bind ^{125}I-labeled AChR and ^{125}I-labeled RCM-AChR to a similar extent.

The antigenic specificity of the immune response elicited by RCM-AChR is different from that elicited by AChR. Immunodiffusion experiments (fig.2) demonstrate identity between the reaction of anti-RCM-AChR serum with RCM-AChR and AChR indicating that all the antibodies that are precipitable by the homologous RCM-AChR can be precipitated also by AChR. On the other hand, anti-AChR serum shows only a partial cross-reactivity with RCM-AChR (fig.2), suggesting the presence in this antiserum of antibodies against some antigenic determinants which do not exist on the denatured receptor.

The antigenic specificity of RCM-AChR and AChR was determined also by quantitative inhibition of the binding of radioactively labeled AChR and RCM-AChR to the different antisera by the non-labeled antigens. Whereas RCM-AChR and AChR inhibit to the same extent the binding of ^{125}I-labeled AChR to sera of rabbits immunized with RCM-AChR (fig.3, top, right) RCM-AChR is a much weaker inhibitor than the unmodified AChR, of the binding of ^{125}I-labeled AChR to antisera of rabbits injected with AChR (fig.3, top, left). There is no significant difference in the extent of inhibition by RCM-AChR and AChR of the binding of ^{125}I-labeled RCM-AChR to anti-RCM-AChR or anti-AChR sera (fig.3, bottom).

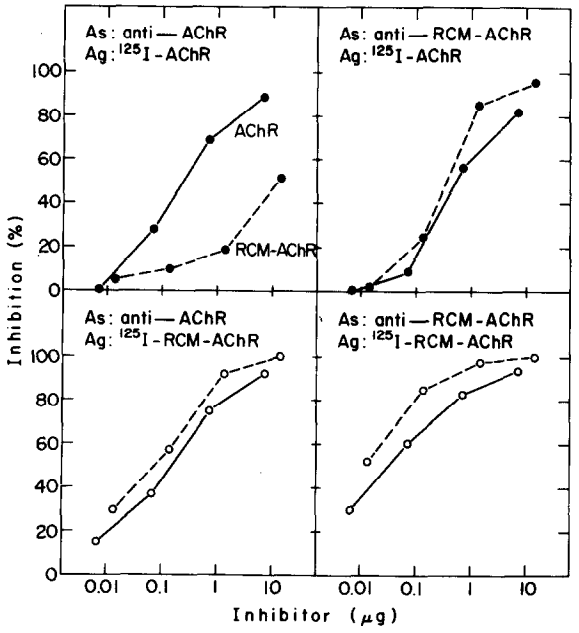

Fig.3. Inhibition of the binding of ^{125}I-labeled AChR (•) and ^{125}I-labeled RCM-AChR (○) to anti-AChR serum (left) and anti-RCM-AChR serum (right) by AChR (——) and RCM-AChR (- - - -).

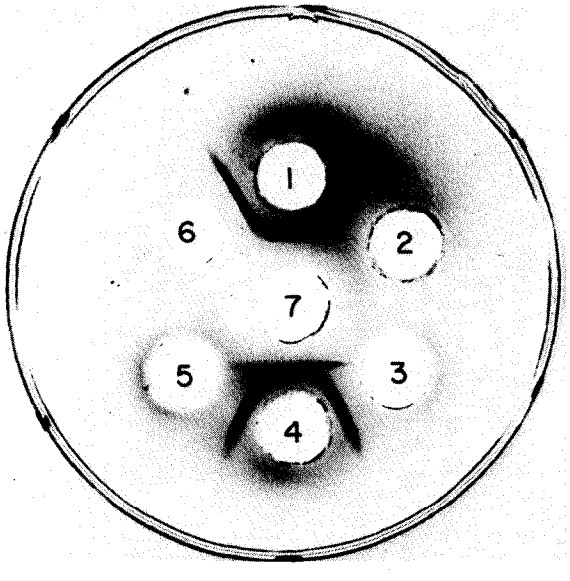

Fig.2. Immunodiffusion of anti-RCM-AChR serum (well 1) and anti-AChR serum (well 4) with RCM-AChR (wells 2, 3, 5) and AChR (wells 6, 7).

3.3. *Effect of antibodies on the binding of bungarotoxin to AChR*

Antibodies to AChR were shown to block AChR physiological activity [19–21] and to inhibit the binding of bungarotoxin to the receptor [14]. Comparison of the effect of antibodies against AChR with that of antibodies against RCM-AChR (fig.4) on the binding of ^{125}I-labeled α-Bgt to AChR shows that the latter antibodies block this binding only to a very limited extent. This differential blocking activity by

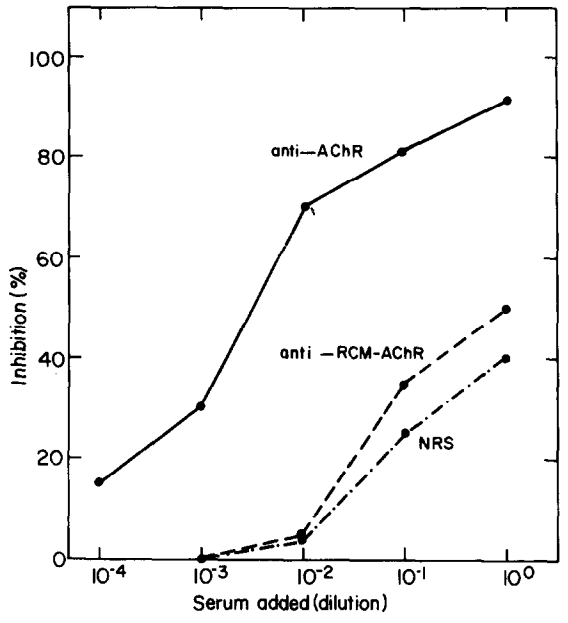

Fig.4. Inhibition of ^{125}I-labeled α-Bgt binding to AChR by anti-AChR serum (——), anti-RCM-AChR serum (- - -) and normal rabbit serum (—·—·—).

the two antisera is compatible with the different antigenic specificities of the two immunogens, resulting from the abolishment of some antigenic determinant(s) of the receptor molecule by the denaturation procedure.

4. Discussion

The molecular structure of AChR is complex. It is therefore desirable to analyse it both biochemically and immunologically on a lower level of complexity.

In attempts to correlate specific structural features of AChR with its unique autoantigenic activity, the immunological properties of the pharmacologically inactive RCM-AChR was studied. RCM-AChR is a good immunogen and elicits upon injection into rabbits high titers of antibodies which cross-react with the intact receptor. However, repeated immunizations with RCM-AChR does not induce any symptoms of EAMG. Similar results were reported by Valderrama et al. [22] and by Lindstrom et al. [23] with SDS-denatured AChR.

The titers of antibodies reacting with AChR in sera of rabbits immunized with RCM-AChR with no signs of EAMG, are at least as high as the antibody titers in myasthenic rabbits injected with intact AChR. Thus a high anti-AChR antibody titer is not by itself a sufficient condition for production of EAMG. In fact, since RCM-AChR does not induce EAMG several booster injections can be given, resulting in an increase of the antibody titers against both RCM-AChR and AChR.

It seems that the major difference between AChR and RCM-AChR leading to their different pathogenicity resides in their different antigenic specificity. The analysis of the antigenic specificities of both systems suggests that some antigenic determinants in the AChR molecule were abolished by the denaturation procedure. However, no additional determinants which were not expressed in the intact molecule became immunopotent after reduction and carboxymethylation.

The different antigenic specificity of antibodies to AChR and RCM-AChR along with their different effect in blocking toxin binding to AChR leads us to propose that the denaturation of AChR destroyed some antigenic determinant(s) which is (are) important for the induction of EAMG, and which may be located closely to the toxin-binding site. We have preliminary results which demonstrate that although RCM-AChR does not induce EAMG, it can be effective in immunosuppression of the disease either by preventing its onset or even by curing it. The cross-reactivity between RCM-AChR and AChR and the non-pathogenicity of RCM-AChR appear to be crucial in governing the immunosuppressive effect of RCM-AChR on EAMG.

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